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INVESTIGATION OF LIPID AND BIODIESEL PRODUCTION FROM Chlorella vulgaris (UTEX 2714) CULTURED IN PHOTO-BIOREACTORS (Spine title: Lipid & Biodiesel production from C. vulgaris grown on dairy effluent)

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INVESTIGATION OF LIPID AND BIODIESEL PRODUCTION FROM *Chlorella vulgaris* (UTEX 2714) CULTURED IN PHOTO-BIOREACTORS

(Spine title: **Lipid & Biodiesel production from *C. vulgaris* grown on dairy effluent**)

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ABSTRACT

The use of microalgae as feed stock for biodiesel production has been under consideration for a number of years. However the process design always had limitations which were associated with the extraction and the harvesting processes. Biodiesel production from microalgae is an important process component in the microalgal biofuel area. In this project, a method for the cultivation of *Chlorella vulgaris* (UTEX 2714) on anaerobically digested dairy farm effluent is proposed. The first part of the work provides a study on the feasibility of microalgae cultivation on pre-treated dairy farm effluent. The microalgae were cultivated using 2% (v/v) and 4% (v/v) CO_2 and two different pre-treatment techniques were employed i.e. using aluminum sulphate and the other using ferric sulphate.

The pre treatment method employing doses of aluminum sulphate and ferric sulphate which provided the best results were 11 g/L and 7 g/L respectively. Triglyceride extractions were carried out using a modified Bligh and Dyer method which yielded triglyceride content to be in the range 17.65 - 22.85 % by weight. The microalgae were capable of removing 80.3 - 83.2 % of the initial NO_3^-, 100 % of the initial NH_4^+, 39.8 - 45.1 % of the initial phosphate and 52.8 - 65.4 % of the initial COD concentrations. The maximum dry weight recorded in these experiments was 2.05 g/L. The post harvesting and concentration resulted in a microalgal slurry with a final dry weight concentration of 15 g/L was obtained. The harvested microalgae cells were then freeze dried for solvent extraction and *Insitu* biodiesel production.

In the second part of the project, the focus was drawn to performing triglyceride extractions and biodiesel production from microalgae using an acid catalyst. For the various microalgae samples

cultivated in the tubular photo bioreactor setup, yield calculations and FAME profiles were developed. A biodiesel yield maximum of 26.67 % by wt. was determined for the microalgae cultivated on dairy farm effluent using a 7 g/L ferric sulphate salt solution as the pre-treatment method. The FAME profile obtained in majority of the microalgae cultivated was mainly composed of C16, C18:0, C18:1, C18:2 fatty acids. This composition obtained for the microalgae very closely resembled the triglyceride composition obtained for canola oil derived biodiesel.

Keywords: Algal biotechnology, microalgae, *Chlorella vulgaris, Chlorella protothecoides,* photobioreactor, production system, environmental, waste-water treatment, triglycerides extraction, nitrogen, phosphate, CO_2 capture, aquaculture, biodiesel, methyl esters, *Insitu* transesterification, cell hydrolysis, second generation biofuel, glucose.

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DEDICATION

This thesis is dedicated to my family for all their love, care and sacrifices made throughout my academic career so far. Thank you, Dad for having faith in me and for always being there for me when I needed advice. Thank you, Mom for providing your continued support. Without your prayers and sacrifices, I could not have fathomed being here today. Thanks to Buneet for being a truly understanding younger brother. Lastly to my late "Darji" who would be a proud grandfather today.

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NOMENCLATURE

FAME	Fatty Acid Methyl Ester
COD	Chemical Oxygen Demand (mg/L)
(v/v)	Volume/Volume
μ	Specific growth rate (d^{-1})
PB1	Photobioreactor 1
PB 2	Photobioreactor 2
PBR	Photobioreactors
TG	Triglycerides
μE	microEinstein (1micro mole of photons)
X	Final Substrate concentration
X_o	Initial Substrate concentration
T	Time (days)
V	Volume (mL or L)
$[NH_4\text{-}N]$	Ammonia Nitrogen Concentration (mg/L)
$[NO_3\text{-}N]$	Nitrate Nitrogen Concentration (mg/L)
$[PO_4^{3-}\text{-}N]$	Phosphate concentration (mg/L0
W	Watt (J/s)

1. GENERAL INTRODUCTION

1.1. Introduction

Considering the increasing worldwide energy demand, photosynthetic microorganisms are being considered as a promising primary resource. One of the daunting challenges faced by today's society is to find a sufficient supply of clean energy to meet the rising energy demand. This is ultimately linked with global energy stability and economic prosperity. Fuels power everything from transportation to manufacturing, and by doing so they represent around 70% of the global energy requirement. It is expected in the near future that with the development of growing economies such as India and China the rise in the consumption of energy will only increase the climate change imbalance (I.E.A. 2007). Therefore it is very crucial to reduce the emissions associated with the burning of fuel.

Biofuels can play an important role in reducing and possibly avoiding the dependence on the fossil fuel currently being relied on. Greenhouse gases emitted to the atmosphere not only cause global warming but also impact the ocean life. The oceans absorb almost 30% of the CO_2 emitted each year therefore if the level of CO_2 in the atmosphere increases the amount of CO_2 dissolved in the oceans will also increase causing the pH to drop significantly. This drop in pH can upset the marine ecosystem and cause irreversible damage to the ocean life (Ormerod, Freund *et al.* 2002). Global warming is a problem which is intimately connected with the various aspects of human life such as economic development and quality of life; this involves tough decisions to be made by the stakeholders for long term strategies. One such step in this direction was taken during the Kyoto protocol in which numerous countries established targets for CO_2 reduction.

1.1.1.Biodiesel

Biodiesel has transformed from a research topic to a fully fledged market commodity. It is composed of simple alkyl esters of fatty acids and is produced by a process known as transesterification. It is produced from oleaginous crops such as rapeseed, palm, soybean and sunflower, by chemically reacting the triglyceride with a short chain alcohol in the presence of a catalyst. The annual production of biodiesel for the US and UK combined was estimated to be 650 million litres in 2001. However the large scale use and development of biodiesel pilot plants are limited by the economic aspects of biodiesel production. This translates to a fuel cost/L for biodiesel to be USD 0.5 /L whereas conventional diesel fuel costs USD 0.35/L. Almost 60-75 % of this biodiesel fuel cost is due to the costs associated with the feedstock (European-Commission 2007). This cost can be lowered if cheaper feedstocks such as non edible oils such as waste grease, animal fats or used frying oils are used. This option is faced with the problem of being short supplied, as there isn't enough quantity of used oil to meet the demand of biodiesel (Canakci and Sanli 2008). Although biofuels are more expensive than fossil fuels their production continues to grow around the world, this however is backed by policy measures and biofuel targets put into place by the country's legislation.

The potential market for biodiesel is beyond what plant oils can meet even after fully pledging 100% of their produced capacity. The plant oil market is also limited by the land requirement for growing these bio energy crops (Scarlat, Dallemand *et al.* 2008). Biodiesel from plant oils can only be advantageous when the crops don't compete with human consumption and are grown on land not deemed as a habitat. Biodiesel, in the recent years has received considerable attention because of its biodegradable, renewable and non toxic

nature. Also no net CO_2 or sulphur emission is associated with the combustion of biodiesel (Xu, Miao *et al.* 2006).

The first generation biofuels which were primarily produced from food crops and oil seeds were limited in their ability to achieve the targets set out in relation to the production capacity and climate change. These concerns have sparked the interest in second generation biofuels produced from non food feedstocks, microalgae being one such example to address this problem. Second generation biofuels such as microalgae derived biodiesel can contribute towards the reduction in the land requirements due to their high energy yields /hectare.

1.2. Microalgal Biodiesel

1.2.1. Microalgae as a feedstock

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms which possess high growth rates and can strive in harsh conditions owing to their structure. The prokaryotic types of microalgae belong to the Cyanobacteria (Cyanophyceae) class and the eukaryotic microalgae, Chlorophyta and diatoms (Bacillariophyta). Microalgae have been studied extensively for more than 50 years with research focussing on the development of an economical and a practical process to achieve the stated objectives. It is estimated that about 50000 species of microalgae exist, out of which 30000 have been analyzed and profiled for biofuel production (Li, Horsman *et al.* 2008). Due to the sparked interest in the microalgal field extensive collection of microalgae strains have been created by pioneer researchers, one such collection is The University of Texas Algal Culture Collection (UTEX), which currently holds 3000 different strains of freshwater algae including representatives of the major algal taxa.

The interest in microalgal triglycerides dates back to 1942 when the large scale cultivation of diatoms to serve as a fat source during World War II was first proposed. The US department of Energy spent $25 million during the years 1978 - 1996 to identify high triglyceride yielding strains to ultimately pave the way for producing microalgal derived fuel. This program was terminated not due to the fact that microalgae derived fuel wasn't feasible but due to the cost ineffectiveness caused by the low price of crude oil. The Aquatic Species program only investigated the autotrophic growth of microalgae (Sheehan, Dunahay *et al.* 1998). This program concluded that production of biodiesel from microalgae was indeed feasible and in order for the technology to achieve the productivities required, intensive research is required, however due to the freeze of funds this program was abruptly discontinued.

Due to the recent increase in the price of crude oil and awareness towards the reduction of greenhouse gases, this interest has been rekindled. Though biodiesel from microalgae isn't ready to compete with fossil fuel based diesel, intensive research is being done to make this alternative source an economically viable one.

1.3. Problem Statement

The current land disposal techniques used to manage animal manure pose a threat of high concentrations of nitrogen and phosphorous leaching into the water bodies and cause eutrophication. This drawback makes the land disposal technique unsustainable. Therefore a typical waste water treatment system includes various unit operations such as anaerobic digestion, nitrification and denitrification to rid the waste stream of any residual nitrogen. Biological treatment such as activated sludge process, trickling filters on the other hand

provide a credible solution to this problem but are only capable of removing a fraction of the nitrogen and phosphorous contained in the waste. The high energy inputs associated with the activated sludge process also holds back its widespread implementation. Microalgae harvesting is another hurdle that must be overcome before they can be utilized as a fuel feedstock. Microalgal cultures possess very low dry cell weight concentration and need to be processed to a concentrated slurry paste that is at least 15 % solids. By utilizing the nutrients within the wastewater, triglyceride rich microalgal biomass can be produced. By doing so the costs associated with developing a growth media are not incurred, also the nutrients end up getting recycled into something much more valuable.

1.4. Research Objectives

The present research investigates the triglycerides and biodiesel production from *Chlorella vulgaris*, cultivated on pre treated dairy farm effluent and Modified Bolds Basal media. The specific objectives of this research project were to:

- Demonstrate the growth of *Chlorella vulgaris* (UTEX 2714) in tubular photo bioreactors
- Demonstrate that anaerobically digested dairy farm effluent is a suitable nutrient source for this species of microalgae
- Optimize the cultivation conditions to maximize the algal biomass and triglyceride production
- Investigate the effectiveness of cellulase enzyme in hydrolyzing the microalgae cell to produce fermentable sugar i.e. glucose

- Devise an effective microalgae harvesting method
- Quantify the total triglycerides weight from lyophilized microalgae samples by performing solvent extraction
- Quantify and profile the (Fatty Acid Methyl Esters) FAMEs produced by *Insitu* transesterification of the microalgae.

1.5. Thesis Overview

The material presented in this study is organized as follows:

- Literature review of work conducted using Chlorophyceae species in relation to its cultivation, waste water remediation, triglyceride extraction and *Insitu* biodiesel production are listed in Section 2.
- The materials and methods are described in Section 3
- Results are presented and discussed in detail in Section 4
- Section 5 covers the main conclusion which can be drawn from this study.
- Section 6 provides the recommendation for future work in this topic.

1.6. Contribution of Thesis

This thesis provides an investigation into the lipid and *Insitu* biodiesel production from microalgae cultivated in tubular photo bioreactors, using anaerobically digested manure effluent as a growth media, triglyceride extraction and production of biodiesel. The key parameters

associated with the growth of the microalgae in the bioreactors will be described. In addition microalgae will be presented as a source of wastewater treatment, biological CO_2 sequestration and for possible use in aquaculture. Also presented is a unique harvesting method which is able to concentrate the dilute microalgae culture to high dry cell weight concentrations. The illustration in Figure 1.1 depicts the model flow diagram for the closed loop microalgal biodiesel production.

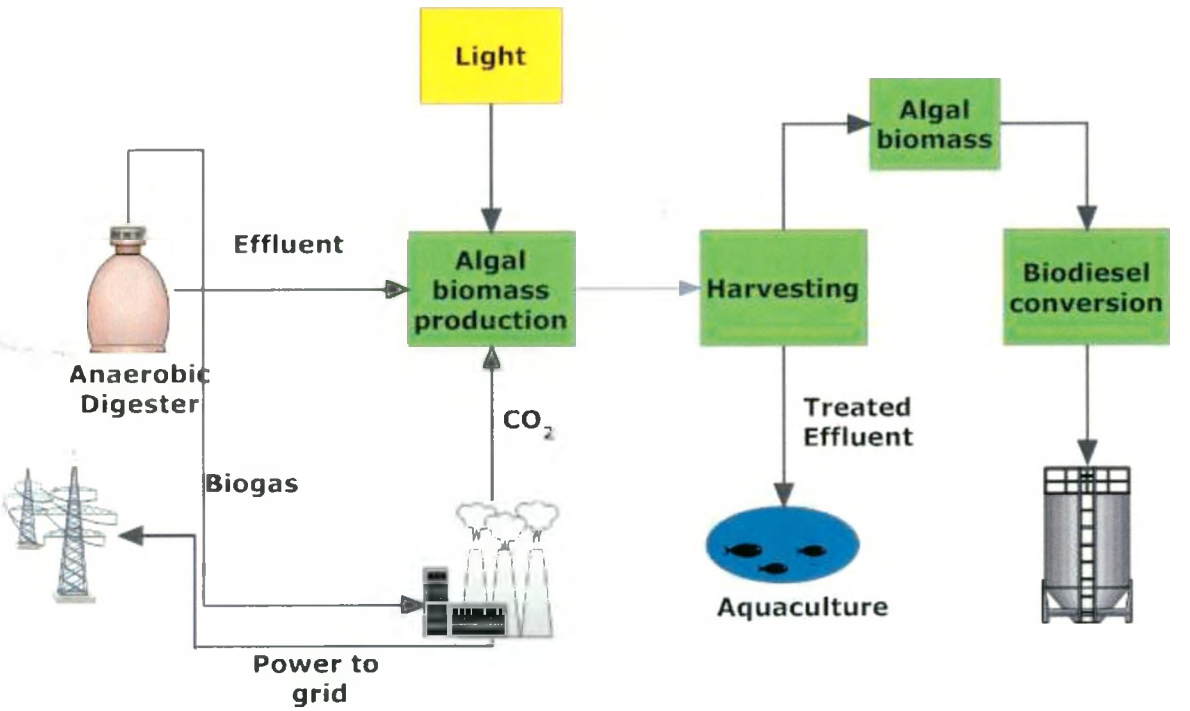


Figure 1.1: Proposed closed loop process of microalgal production

2. LITERATURE REVIEW

2.1. Introduction

Microalgae are single celled photoautotrophic or photo heterotrophic organisms which grow in almost any aquatic environment and use light and CO_2 to generate biomass. Microalgae cells are a type of eukaryotic cell and contain similar internal organelles such as chloroplasts, nucleus etc. Algae range in size from a few micrometers to meters in length. The four most abundant classes of micro algae are diatoms (Bacillariophyceae), green algae (Chlorophyceae), blue-green algae (Cyanophyceae), and golden algae (Chrysophyceae) (Richmond 2004).

This thesis focuses on the green microalgae *Chlorella vulgaris* (UTEX2714). It belongs to the Chlorophyceae genus and is capable of producing triglycerides which are a potential feedstock for biodiesel production. The triglyceride contents of microalgae usually lie in the 20 - 50% range (Table 2.2).

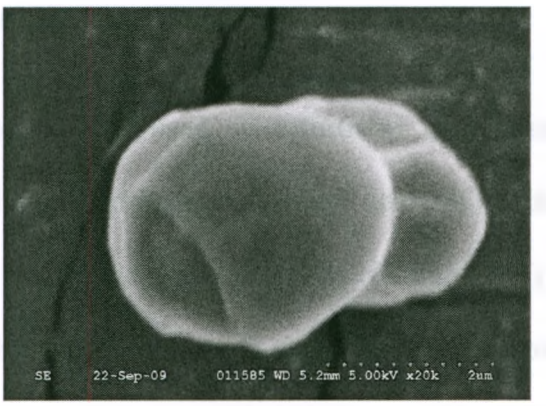


Figure 2.1: SEM image of *Chlorella vulgaris* (x 20000 magnification)

The Figure 2.1 shows a Scanning Electron Microscope image taken at 20000x magnification of the lyophilized *Chlorella vulgaris*. The average size of the dried microalgae determined using the SEM image shown above was found to be 4 - 5 μm in diameter.

2.1.1. Current uses of microalgae

Microalgae posses high triglyceride content, carbohydrate and proteins which makes them good research subjects. Microalgae has applications in the biofuel industry to the food product industry. Microalgae undergo photosynthesis i.e. they are able to take up CO_2 as a nutrient source and can be seen as a means of biological remediation, they possess the ability to uptake nitrogen and phosphorous which are the main causes of eutrophication in water bodies. Microalgae is also rich in potassium, iron, magnesium and calcium (Poulickova and Hasler 2008). It may also contain essential amino acids such as DHA (Chi and Pyle 2007) and EPA (Wen and Chen 2003). Manure grown microalgae can be used as a soil conditioner, fermentation substrate, pharmaceutical products and also food for aqua culturing. In addition to the above mentioned uses, various high added value products can be extracted from microalgae such as fatty acids, pigments (carotenoids) and vitamins (B_{12},C, E) (Del Campo, Garcıa-Gonzalez *et al.* 2007).

2.1.2. Triglyceride metabolism in *Chlorella*

In microalgal cells triglycerides are used as a form of carbon and energy storage. It was determined that microalgal triglycerides contain fatty acids of chain lengths 16 carbons to 22 carbons (Xaborsky 1982). In a study conducted by (Yung 1966) who cultivated *Chlorella pyrenoidosa* under heterotrophic conditions using acetate or glucose with inorganic nitrogen under light limiting conditions. It was concluded that a pH of 7.5 is best for synthesis of triglycerides. This can be explained on the basis of the cells carbon concentrating mechanism, in the presence of more bicarbonate for the synthesis of triglycerides.

Triglyceride accumulation occurs in the cell when the cell growth cycle is inhibited due to unfavourable conditions or the cell division is delayed. Nitrogen deficiency in microalgae leads to greater TAG accumulation in microalgae but this deficiency leads to a detriment in growth rates (Olguina, Galicia *et al.* 2001). The cells tend to accumulate more triglycerides when they are on the verge of cell division.

To maximize triglyceride productivity optimal cell culture conditions to maximize triglyceride accumulation and biomass growth must be implemented. Green microalgae have the bulk of their fatty acids of C16 - C18 chain length, a composition similar to that of vegetable oils. *C. vulgaris* which is a fresh water species has been reported to accumulate up to 40% triglycerides by dry weight when cultivated.

2.1.2.1. Triacylglycerol biosynthesis

Fatty acids produced in the chloroplast are sequentially transferred from CoA to positions 1 and 2 of glycerol-3-phosphate (G3P) this results in the formation of the central metabolite phosphatidic acid (PA) (Ohlrogge and Browse 1995). Dephosphorylation of PA which is catalyzed by a specific phosphatase releases diacylglycerol (DAG). In the final step (Figure 2.2) of TAG synthesis, a third fatty acid is transferred to the vacant position (3) of the DAG, by means of a reaction catalyzed by diacylglycerol acyltransferase, which is unique to TAG biosynthesis.

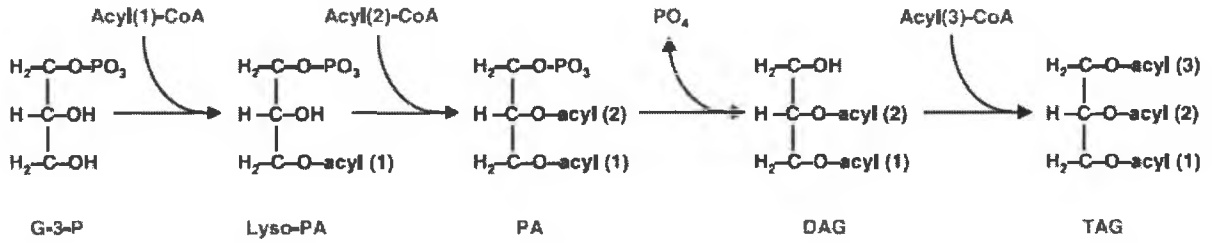


Figure 2.2: Triglyceride synthesis in microalgae (Roessler 1990)

2.1.3. Biodiesel from microalgae

The ability of microalgae species to grow in different environmental conditions impart them an advantage in addition to their ability to harvest the solar energy into valuable products by using only CO_2 and H_2O which contributes in part to the global CO_2 reduction. Microalgae can be grown on arid lands which have been deemed unsuitable for cultivation or in saline water; such is the growth spectra of these microalgae. The biodiesel production yield from microalgae has been reported to be about 20 times higher (Sheehan, Dunahay *et al.* 1998)than their oleaginous counterparts. Although the microalgal oil yield varies across the different species of microalgae, it is generally much greater than vegetable oil crops.

Table 2.1: Comparison of various sources of biodiesel (Sheehan, Dunahay *et al.* 1998)

Crop	Seed oil content (% oil by wt in biomass)	Oil yield (L/ha)	Biodiesel Productivity (kg biodiesel/ ha year)
Corn	44	172	152
Soybean	18	446	373
Canola	41	1,190	995
Jatropha	28	1,892	1582
Coconut	36	2,689	2250
Palm	36	5,950	4977
Microalgae*	30	58,700	49102

*assuming 30% oil content by dry weight

Some microalgae have high oil content which can be induced by optimizing the growth media conditions such as low nitrogen concentration, light intensity etc. The triglyceride content of various microalgae species is shown in Table 2.2 (Teresa, Antonio *et al.* 2010)

These microorganisms when compared to plants possess exceptionally high areal productivity due to their higher photosynthetic efficiency (Chisti 2007). These microorganisms can serve as means to bridge the gap in developing a sustainable energy production system.

Table 2.2: Triglyceride content of microalgae (Teresa, Antonio *et al.* 2010)

Species	Triglyceride content (%)
Scenedesmus obliquus	11–22
Scenedesmus dimorphus	6–7
Chlorella vulgaris	14–40
Chlorella protothecoides	23
Chlorella minutissima	57
Dunaliella salina	14–20
Neochloris oleoabundans	35–65
Spirulina maxima	4–9

Autotrophic cultivation however has its limitation as it requires light, which at higher cell densities becomes one of the factors limiting growth. As the cell density increases the light penetration decreases. Therefore it is very crucial that proper light intensity be incident on the microalgae cultures to ensure good growth.

However the key technical challenges associated with microalgae include selection of a strain which possesses high oil content as well as high growth rate, economical means of harvesting the microalgae and finally extraction and conversion of triglycerides to biodiesel.

2.1.4. Microalgae and waste water remediation

Waste water treatment using microalgae has been researched since the 1950s (Oswald and Gotaas 1957). Phycoremediation can be defined as the use of micro- or macro-algae for the removal of pollutants or to remove residual nutrients from wastewater using algal cultures.

Microalgae offer a low cost process in which they are utilized as bioremediation agents due to their ability to uptake these as nutrients. Microalgae based wastewater treatment processes offer numerous advantages over the activated sludge process which has enormous energy costs associated to aeration and the cost of subsequent sludge processing. Microalgae possess the ability to assimilate carbon as well as inorganic nutrients such as nitrogen and phosphorous from the wastewater for their growth. Therefore microalgae based treatment can serve as a tertiary wastewater treatment form which is an inexpensive alternative to conventional treatment methods. Microalgae cultures are also used in the removal of heavy metals from wastewater which competes with the tradition ion exchange resin technology.

In controlled aquatic systems such as wastewater storage ponds on dairy farms, microalgae can serve as a means of phycoremediation by consuming the Nitrogen and Phosphorous components of the waste.

(Park, Lim *et al.* 2009)examined the growth of three microalgae strains *M. aeruginosa*, *C. vulgaris* and *E. gracilis* in anaerobic digestion effluent. It was noted that the cell population doubled in almost 10 days. There was however a long lag phase observed in *E. Gracilis* and *C. vulgaris* strains. (Órpez, Martínez *et al.* 2009) carried out batch experiments in PBR's using *Botryococcus braunii* as their microalgae strain by cultivating it on secondarily treated sewage from wastewater. The experiments were conducted for a duration of 42 days, at the end of which the *Botryococcus braunii* accumulated 17 % triglycerides.(Aslan and Kapdan 2006) used *C. vulgaris* in batch cultivation and found a decrease of 21.2 mg L^{-1} in the ammonia- nitrogen concentration. However the reduction in the P□$_4$-P concentration was much lower, the culture was able to remove only 7.7 mg L^{-1}.Therefore microalgae can help reduce the eutrophication in the aquatic environment. (Wilkie and Mulbry 2002) studied the removal efficiency of

filamentous algae to treat dairy and swine effluent. A 40-100 % reduction in the initial ammonium was observed, 58-100 % of total initial phosphorous and 33-42 % of the total nitrogen was removed.

2.1.5. CO_2 emissions

Microalgae cultures posses many characteristics which make them ideal candidates for environmental remediation and flue gas treatment. Microalgae possess the ability to remove CO_2 and NO_x during their growth by using these gases as nutrient sources of carbon and nitrogen respectively. Microalgal biomass can contain up to 50% carbon on a dry weight basis all of which is derived from the carbon dioxide solely. The carbon dioxide from the flue gas of power plant can be fed to the microalgae for autotrophic growth where the microalgae can serve as a CO_2 sequestrants for greenhouse gas control (Pulz and Wolfgang 2004).

Thermoelectric plants powered by fossil fuels contribute almost a third of the total CO_2 emissions in the USA or about 7% of the total world emissions alone. Microalgae have been known to grow in CO_2 concentrations in excess of 5% and isolated from lakes or ponds in the vicinity of a coal or oil fired power plant to cultivate at higher CO_2 concentrations (Kadam 2001).

Microalgae possess the ability to uptake CO_2 and fix it using the photosynthesis process. By doing so CO_2 is mitigated and triglyceride rich microalgal biomass is produced. (Zeiler, Heacox *et al.* 1995) demonstrated the use of *Monoruphidium minutum* to utilize flue gas containing high concentrations of carbon dioxide, sulphur and NOx as an energy source to produce microalgal biomass. The simulated flue gas contained 0.015 % NO, 0.02 % SO_2 and 13.6 % CO_2. There was

a drastic pH drop from pH 7.8 to pH 6.4 which was attributed to the high dissolved concentrations of CO_2. The study concluded that nitrogen and phosphorous were the limiting nutrients in the culture growth. Isolated *Chlorella* Strains from hot springs showed tolerance and fixation potential at temperatures up to 42 °C and at an elevated CO_2 concentration 40% CO_2 (Sakai, Sakamoto *et al.* 1995). Similar studies were conducted in this research which looked at the CO_2 consumption in the twin PBR setup.

The potential impact microalgae can have on large scale fuel oil production is justified by the short generation time, high doubling time and high oil content when compared to traditional crops.

All of these benefits and the synergistic effect of algae production and waste water treatment make this approach a viable one in the near future.

2.2. Factors Affecting triglyceride accumulation

The amounts of triglyceride that can be accumulated are strain/species specific and are ultimately controlled by the genetic makeup of the species. When the microalgae culture is exposed to a chemical stimulus, the energy storage response is triggered. These stimuli can be in the form of nutrient starvation, temperature and pH.

(Hsieh and Wu 2009) studied the effects of Urea (N) limitation on triglyceride productivity. *Chlorella sp.* was cultivated in batch mode using 320 μmol photon m^{-2} s^{-1} in a media containing different concentrations of Urea as a nitrogen source. It was found that after 6 days of cultivation the culture with the lowest Urea concentration had the highest triglyceride content. However this came as a trade off to the biomass concentration, which was the lowest in the case of the lowest Urea concentration batch. This result is consistent with the work carried out *(Illman, Scragg et*

al. 2000) on *Neochloris oleoabundans* and *Chlorella sp.* ,where a 2-fold increase in TG content was observed at low nitrogen concentrations.

(Converti, Alessandro *et al.* 2009) studied the effects of temperature and nitrogen on the triglyceride content of *Nannochloropsis oculata* and *Chlorella vulgaris*. A direct negative correlation was observed between the TG content and temperature (outside of the optimum range). An increase of temperature from 20 - 25 °C increased the TG content from 7.9 % to 15 %, whereas a decrease in TG content from 14.7 % to 6 % was observed when the culture temperature was increased from 25 to 30 °C. (Converti, Alessandro *et al.* 2009)also observed the same phenomenon as (Illman, Scragg et al. 2000) , as the TG content increased from 7.9 % to 15 % when a 75 % reduction in the nitrate concentration was applied.

2.3. Advantages of using microalgae for biodiesel production

Microalgae can be considered as micro factories capable of uptaking carbon dioxide from the atmosphere and converting it into a high value liquid form of energy. Microalgae are easy to cultivate, require the most basic nutrients and can grow on water unsuitable for human consumption. Microalgae reproduce using photosynthesis by converting the solar energy into chemical energy. Their growth rates can be accelerated by the addition of nutrients and sparging of air through the culture volume. These species can also adapt themselves to thrive in a variety of growth conditions. Also species indigenous to a particular environmental can be isolated and mass cultivated accordingly. When compared to conventional energy crops and plants which serve as a source for biodiesel, microalgae require much less cultivation area. Therefore the

competition for crops for human consumption and cultivable land is greatly reduced when considering microalgae.

Therefore microalgae can also be a feedstock for fuels such as biodiesel, ethanol, methane and hydrogen. The biodiesel derived from microalgae does not contain any sulphur and burns cleaner as there is a reduction in the particulate matter, CO and SO_x emissions associated with it.

Microalgae possess the ability to grow under harsh conditions and also their low requirements of nutrients makes them suitable for cultivation in non cultivable land and use wastewater effluent as a growth media. This eliminates the need for freshwater and the wastewater can be recycled after all the nutrients have been up taken by the microalgae. Wastewater removal using microalgae works on the fundamental that algae are capable of uptaking the NH_4^+, NO_3^-, PO_4^{3-} as nutrients. Microalgae are capable of removing CO_2 from flue gases by fixating the carbon dioxide and storing the energy as carbohydrates within the cell. The microalgal biomass left post oil extraction can be used as a livestock feed, organic fertilizer or as a feedstock for producing ethanol or methane. Because of the wide variety of high value by-products which can be derived from microalgae, these microorganisms can have a huge impact on industrial applications such as pharmaceuticals, cosmetics , nutrition supplements and Greenhouse Gas capture.

2.4. Microalgae based biodiesel process

When compared to traditional biodiesel feedstocks, microalgae are not significantly different. They also require proper cultivation, harvesting, processing to maximize the biodiesel production efficiency.

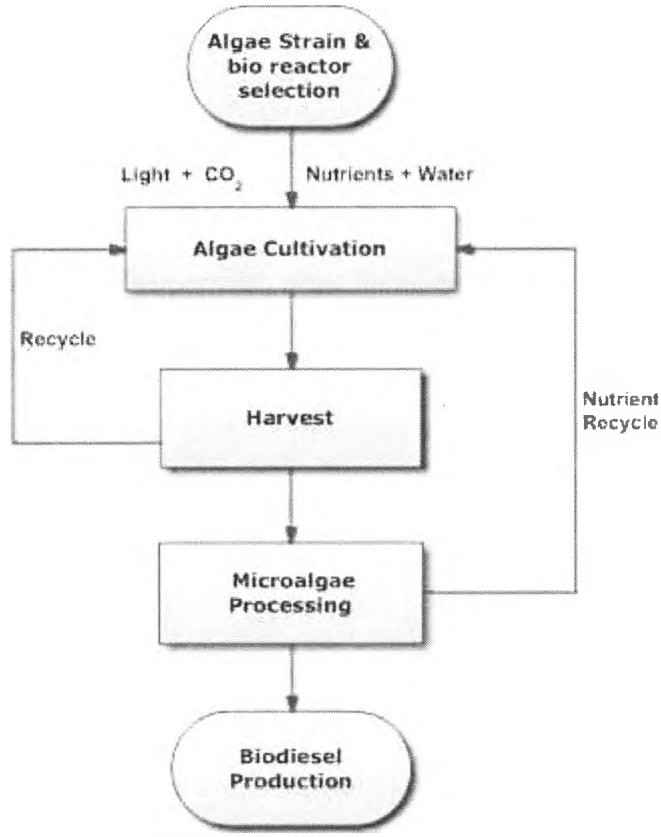


Figure 2.3: Schematic of the microalgal biodiesel production stages

Figure 2.3 shows the schematic representation of the various stages involved in the algal biodiesel production. The process starts with the selection of the Microalgal strain based on the desired objective which is followed by its cultivation. The cultivation stage requires the addition of nutrients to the growth media if any required, light source and CO_2 for aeration. The cultivation stage is followed by the harvesting step in which the microalgae culture can be recycle to serve as an innoculum for future experiments.

Due to the large volumes associated with microalgae cultivation it is important to reduce the volume by applying various processing techniques such as dewatering, filtering or drying. This processing leads to a finished microalgae product which can be used for biodiesel production.

2.4.1. Microalgae strain and bioreactor selection

One of the main contributing factors which effects the economic viability of this process is the implementation of a cultivation reactor system which is cheap to build and yet scalable. In regards to the species selection proper care must be taken to meet the nutrient requirements and cultivation conditions which may affect its growth. The photo bioreactor must be able to mimic the conditions in which the microalgae will be exposed to. Also it should be able to supply the optimal dose of nutrients and light to each individual microalgae cell even when scaled up.

2.4.2. Microalgae cultivation

Microalgae cultivation can be done in various modes: autotrophic, heterotrophic and mixotrophic. Autotrophic cultivation involves using light as the sole energy source during which it gets converted to chemical energy by photosynthesis. Heterotrophic cultivation requires the addition of organic compounds which can be used as a carbon source. In a mixotrophic mode of cultivation the microalgae can use a mix of different sources of energy and carbon i.e. Perform photosynthesis as well as uptake organic carbon and CO_2. When cultivated in a batch mode of operation the microalgae growth curve has the following phases (Figure 2.4):

- Lag phase
- Exponential phase (the maximum growth rate phase)

- Stationary phase
- Death phase

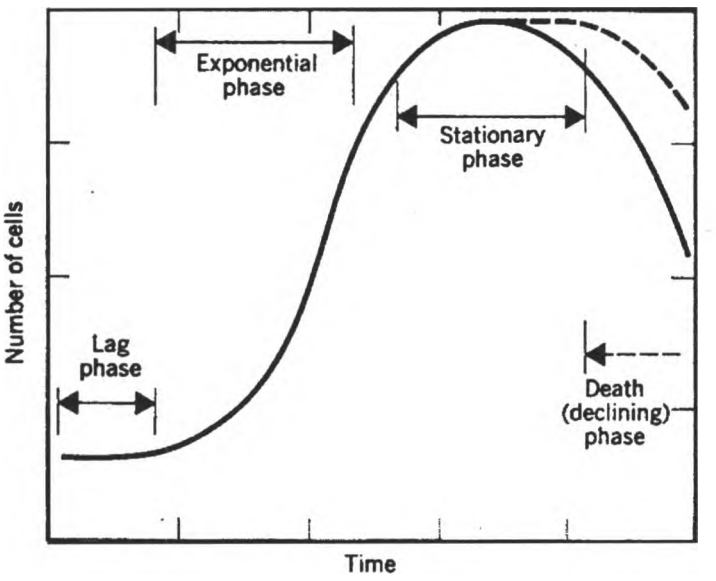


Figure 2.4: Batch culture cultivation growth profile (Richmond 2004)

The major hurdle in large scale microalgae cultivation is the inability to cultivate one selective species; other unwanted species can take over the entire culture volume in some cases due to competitive conditions.

Various factors affect the microalgal growth such as nutrient concentration, CO_2, pH, temperature, light intensity, pathogen contamination, mixing, reactor depth and harvesting frequency.

2.4.3. Microalgae harvesting and concentration

The harvesting and concentration step is another step accounts for almost 30% of the total microalgae production process cost (Grima, Belarbi *et al.* 2003). The cost of processing a large

volume of dilute algae culture to produce a highly concentrated slurry makes the harvesting and concentration process economically challenging. This requires removal of huge quantities of water. There are several mechanical, chemical and biological ways of tackling this problem. Sedimentation, centrifugation, chemical flocculation and filter press are a few approaches which have been applied. Sedimentation is a slow process and needs some sort of external energy to be applied if a quick separation is desired. Flocculation utilizes chemicals which are capable of aggregating the microalgae cells. The flocculation stage if included post cultivation can ease the load on the secondary harvesting stage being applied i.e. Sedimentation, centrifugation or filtration. Filter presses on the other hand can be applied to concentrate large volumes of culture however this system can work for larger microalgae such as *S. Platensis* but proves ineffective when working with small microalgae such as *Dunaliella* or Chlorella (Grima, Belarbi *et al.* 2003).

Ultrafiltration is capable of providing a fully continuous mode of operation with an ultrafiltration membrane housed in a vessel with fresh microalgae culture constantly being filtered through the hollow membrane filters (Figure 2.5) and filtered media being collected in a separate vessel.

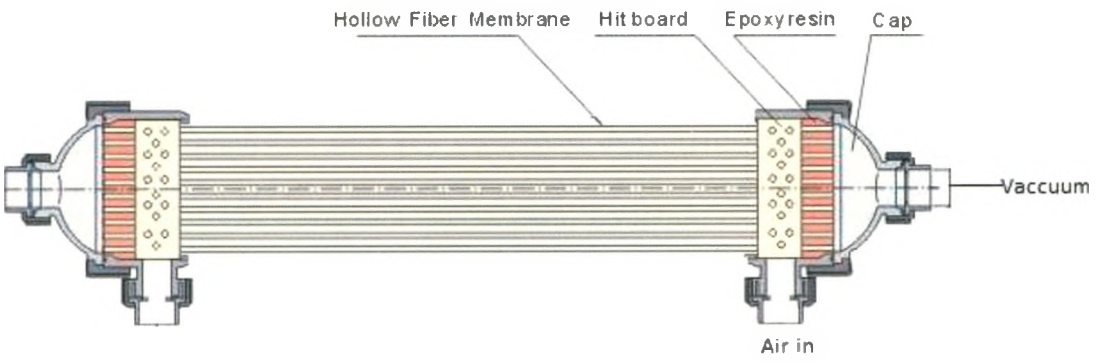


Figure 2.5: Schematic of an ultrafiltration membrane (Water 2009)

The costs associated with the process of recovering the cells from the culture solution are crucial in determining the economic feasibility of the process. The growth media cost, harvesting and the extraction stage are the main contributors in increasing the costs associated with the cultivation of microalgae. Most of the harvesting methods currently being employed still have economic and technical drawback such as high energy costs, scale up issues or flocculant toxicity.

2.4.4. Microalgae Processing

In order for microalgae to be a workable feedstock it must be processed after it has been concentrated to about 15% dry weight. The shelf life of microalgae is reduced if stored too long post harvesting. Dehydrating the microalgae can increase the shelf life and not cause a physicochemical change in the properties of the microalgae culture. There are different techniques by which microalgae can be dried: spray drying, sun drying and freeze drying. Sun drying is the least effective method of the three as the sheer volume culture makes this process ineffective. Following the drying process the microalgae needs to be disrupted to make further processing easier. This is achieved by applications such as high pressure cell homogenizers, ultrasonication, bead milling, autoclaving or freeze drying.

Freeze drying accomplishes two key objectives dehydration and cell disruption in one step, making the need for cell disruption unnecessary. Lyophilisation is advantageous in terms of the physical characteristics due to the fine powder when compared to the flaky texture obtained when sun drying etc.

2.4.5. Acid catalyzed Biodiesel production reaction mechanism

Transesterification is a reaction which involves the reaction of triglycerides with a short chain alcohol (methanol) in the presence of a catalyst to produce the corresponding fatty acid methyl esters. Acid catalysts give very high yields in alkyl esters, but the reactions are slow, requiring temperatures above 70 °C and more than 3 hours to reach complete conversion (Freedman, Pryde *et al.* 1984). The mechanism of the reaction is as follows. The protonation of the carbonyl group of the ester leads to the formation of a carbocation (II) which undergoes a nucleophilic reaction to produce a tetrahedral intermediate (III) to form the new ester (IV) and the reaction is carried out by the regeneration of the catalyst H^+. This mechanism is shown in detail in Figure 2.6

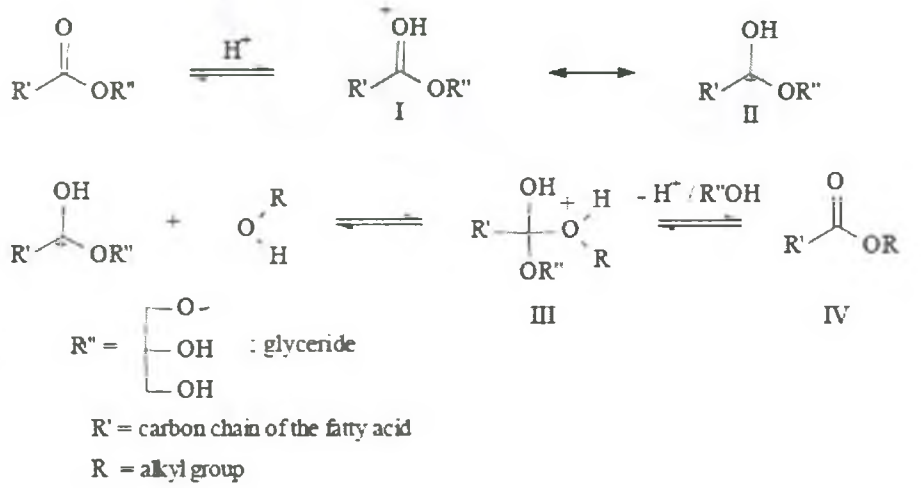


Figure 2.6: Acid catalyzed transesterification reaction mechanism (Freedman, Pryde *et al.* 1984)

2.5. Microalgae Culturing

Different options exist for the cultivation of microalgae. This can be done in open systems such as lakes, ponds or in closed systems such as photobioreactors (PBR's). Each system has its advantages and disadvantages. Open ponds are one of the simplest systems for mass cultivation of microalgae. This system is usually a pond about 1 foot deep in which microalgae can be

cultured in conditions identical to the natural environment. A baffle wheel provides the circulation and mixing of the microalgal cells and nutrients. The pond is divided into channels (Figure 2.7) through which the flow is guided to maximize the residence time and circulation. These systems cost less to build and operate when compared to closed systems. However there are drawback such as water evaporation, unwanted contamination by other competing species and the maintenance of optimal culture conditions.

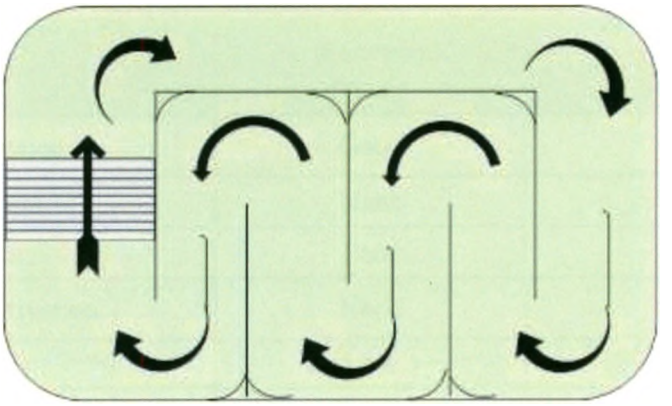


Figure 2.7: Raceway design schematic

Closed photo bioreactors offer better control over contamination and evaporation. The productivity obtained in tubular PBR's is almost 13 times that obtained in a traditional raceway pond (Chisti 2007). These reactors can be configured in either a vertical fashion or horizontally. Tubular PBR's in general provide better control over pH, temperature, aeration, CO_2, have lower evaporation rates and allow the microalgae culture to reach higher densities.

Another option which exists for microalgae cultivation is the use of stirred tank reactors. The main advantage associated with this design is the uniform mixing which is achieved. However

due to the continuous agitation of the culture, the shear stress applied can greatly inhibit the growth. The light distribution is greatly reduced as the diameter of the reactor is increased.

Tubular photo bioreactors can be used for monoculture cultivation and are most scalable and suitable for large scale production. The turbulence in the photo bioreactors provides the optimum balance of continuous cycling between the light zones and dark zones (Lee, Ding *et al.* 1995).

Table 2.3: Comparison between raceway ponds and tubular PBRs (Richmond 2004)

System	Raceway ponds	Tubular photo bioreactors
Light transmission	Good	Excellent
Temperature control	None	Excellent
Gas transfer	Poor	Low–high
Monoculture cultivation	None	Achievable
Sterility	Low	High
Volumetric productivity	High	Low

2.6. Factors affecting Microalgal growth

2.6.1. Light intensity

Light intensity is most important limiting factor for the cultivation of microalgae in photo bioreactors or outdoor ponds. Due to the autotrophic nature of microalgae it is crucial to optimize the light intensity reaching the cells. Light penetration is inversely proportional to cell density and reactor depth (Chen 1996). It is observed that at high cell concentrations the opacity of the culture dramatically increases causing less light to pass through. As the light intensity per cell decreases with increase in cell culture density, the cells begin to cycle between the illuminated and the dark regions in the photo bioreactor (Eriksen, Poulsen *et al.* 1998).

From literature it can be seen that cultures of *C. vulgaris* in photo bioreactors are usually irradiated with about 50-300 μ Einsteins/ (m^2 s) of light (Chiu, Kao *et al.* 2008) and (Yun, Park *et al.* 1996). Studies have also been conducted which report the effect that a high light irradiance of 1000-1500 μE/ (m^2 s) on the growth of cultures of *C. vulgaris* (De-Bashan, Trejo *et al.* 2008). In regards to light intensity (Kaewpintong 2004) concluded that the cell density and specific growth rate are both increased with an increase in light intensity, however beyond a certain threshold value light can become toxic and thereby inhibiting the growth.

2.6.2. Temperature

Another major factor which affects the microalgal growth is temperature. If the optimum temperature of the microalgal culture is exceeded by a mere 2 - 4 °C, it can result in the loss of the microalgal culture volume (Moheimani 2005). According to (Chisti 2007) the optimum temperature for most microalgae is between 20-30 °C.

2.6.3. Mixing

Mixing plays an important role in microalgae cultivation as it has a synergistic effect when combined with light intensity. Mixing in microalgae cultivation vessels is preferred through aeration as mechanical mixing using impellers may cause cell damage. Aeration allows circulation of cells continuously from dark zones to the lit zones. Too much mixing turbulence and shear caused by bubble explosion can be detrimental to the condition of the microalgae culture (Richmond 2004).

2.6.4. Concentration of CO_2 supplied

In a study published in *Engineering Life Sciences* (Chiu, Kao *et al.* 2008) reported an increase in the biomass production and triglyceride accumulation with an increase in the CO_2 concentration for cultivation of *Nannochloropsis oculata*. Similar conclusions were drawn by (Morais and Costa 2007) for *Scenedesmus obliquus* and *Chlorella kessleri* cultures which were isolated from a thermoelectric plant in Brazil. (Kaewpintong 2004) carried out studies to better understand the effect of aeration and light intensity. It was concluded from this study that aeration plays a crucial role in maintaining a homogenous culture condition thereby preventing unwanted high concentration pockets of microalgae from developing and the creation of nutrient gradients. In the study conducted by (Chiu, Kao *et al.* 2008) to see the toxic effects of supplying 15-20% (v/v) CO_2 concentration on the microalgae growth. It was noticed that the growth of *Chlorella vulgaris* was inhibited to a certain extent. This inhibition can be overcome by gradually increasing the CO_2 concentrations at the subculture level, to build up the resistance to higher CO_2 concentrations for large scale cultivation.

2.6.5. pH conditions

There is a natural buffering system which exists between the H^+ and CO_2 species. The equilibrium reaction is shown in equation below.

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

Equation 2.1: Carbon dioxide equilibrium in water (Ormerod, Freund et al. 2002)

During photosynthesis the CO_2 supplied is utilized causing a shift to the left which results in an increase in the medium pH. *Chlorella vulgaris* is highly sensitive to a highly alkaline pH

environment as this may cause the irreversible damage to the cytoplasm. (Mayo 1997) carried out work that shows that *Chlorella vulgaris* can tolerate acidic pH as low as 3.0.

2.6.6. Nutrient supply

To achieve successful growth of microalgae in a culture, a growth media which meets the nutrient requirements of the species is required. The absolute necessary nutrients include carbon, phosphorous, nitrogen and magnesium (Richmond 2004). Other elements such as Iron and manganese are also required. In addition to these, trace elements such as zinc, copper and boron are also essential.

(Illman, Scragg et al. 2000) investigated the effect of nitrogen deficiency on five different *Chlorella* strains and observed a significant increase in the triglyceride content. Another study conducted by the NREL concluded that under nutrient starvation the oil production remains high, leading to an accumulation of oil in the cells. These higher content of triglyceride in the cells are more than offset by lower rates of cell growth (Sheehan, Dunahay *et al.* 1998).

3. MATERIALS AND METHODS

3.1. Microalgae strain

The strain selected in this research was obtained from University of Texas, Austin, USA. *Chlorella vulgaris* (UTEX 2714) was isolated from a secondary effluent of a wastewater treatment stabilization pond near Santafe de Bogota, Colombia. This species was chosen as it can withstand temperatures up to 26 ^{o}C and has the capability of accumulating triglycerides in addition to the waste water remediation qualities.

3.2. Growth media

The microalgae subculture was maintained in a Modified Bold's Basal Media as shown in Table 3.1. This media was the mixture of macro and micro nutrients supplied to the microalgae for different batch cultures. During the batch cultivation experiments carried out using the dairy farm effluent, the growth media was replaced with the treated effluent media. However the subculture added to the batch cultivation experiment in this case was the subculture maintained on the Modified Bold's Basal media.

Table 3.1: Modified Bold's Basal media recipe

Component	Stock Solution (g/L dH_2O)	Quantity Used (ml)
Macronutrients		
$NaNO_3$	25	10
$CaCl_2 \cdot 2H_2O$	2.5	10
$MgSO_4 \cdot 7H_2O$	7.5	10
K_2HPO_4	7.5	10
KH_2PO_4	17.5	10
NaCl	2.5	10
Alkaline EDTA Solution		1
Na_2EDTA (EDTA)	63.9	
KOH	552.5 ml of 1M KOH	
Acidified Iron Solution		1
$FeSO_4 \cdot 7H_2O$	4.98	
H_2SO_4		1
Boron Solution		1
H_3BO_3	11.42	
Trace Metals Solution		1
$ZnSO_4 \cdot 7H_2O$	8.82	
$MnCl_2 \cdot 4H_2O$	1.44	
$CuSO_4 \cdot 5H_2O$	1.57	

For the experimental studies carried out using the anaerobically digested manure collected from Stanton Farms, Illderton, Ontario. The manure effluent was mixed with an aluminum sulphate pre-treatment solution. Once the two solutions were completely mixed and allowed to settle, there was a clear separation as the larger heavy solids moved to the bottom, lighter particles moved to the top and the middle layer was drawn off (Figure 3.1) and filtered through a hollow fibre ultrafiltration membrane. At any given time 8L of subculture were maintained in flasks with continuous aeration and natural illumination. For all the experiments a 12 h dark cycle was maintained. All experiments were carried out room temperature (20 - 23 ^{o}C)

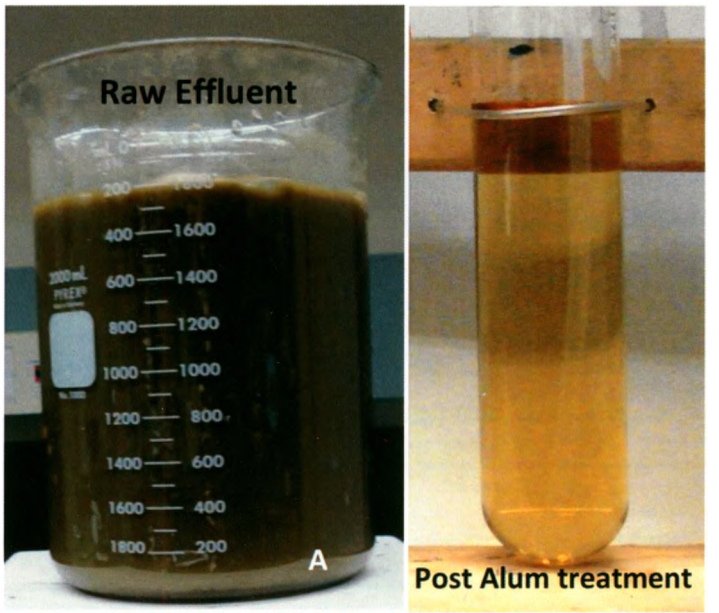


Figure 3.1: Raw dairy farm effluent vs. Alum treated effluent

The Figure 3.1 above shows the raw effluent as obtained from Stanton Farms vs. pre treated effluent. The efficiency of the treatment method is clearly visible, as the aluminum sulphate was able to precipitate out all of the colloidal and fibrous solids which could later interfere with the light transmittance for cultivation.

3.3. Photo bioreactor description and operating protocol

The microalgae were cultivated in acrylic photo bioreactors (Figure 3.2) 2.18 m high with an inside diameter of 0.1 m and a wall thickness of 0.01×10^{-2} m. The stainless steel air sparger was located at a height of 7.62×10^{-2} m above the bottom of the photo bioreactor. The O.D. and I.D. of the sparger were 0.09 m and 0.06 m respectively with aeration holes measuring 0.15×10^{-2} m in diameter. The units displayed in the figure below are in inches.

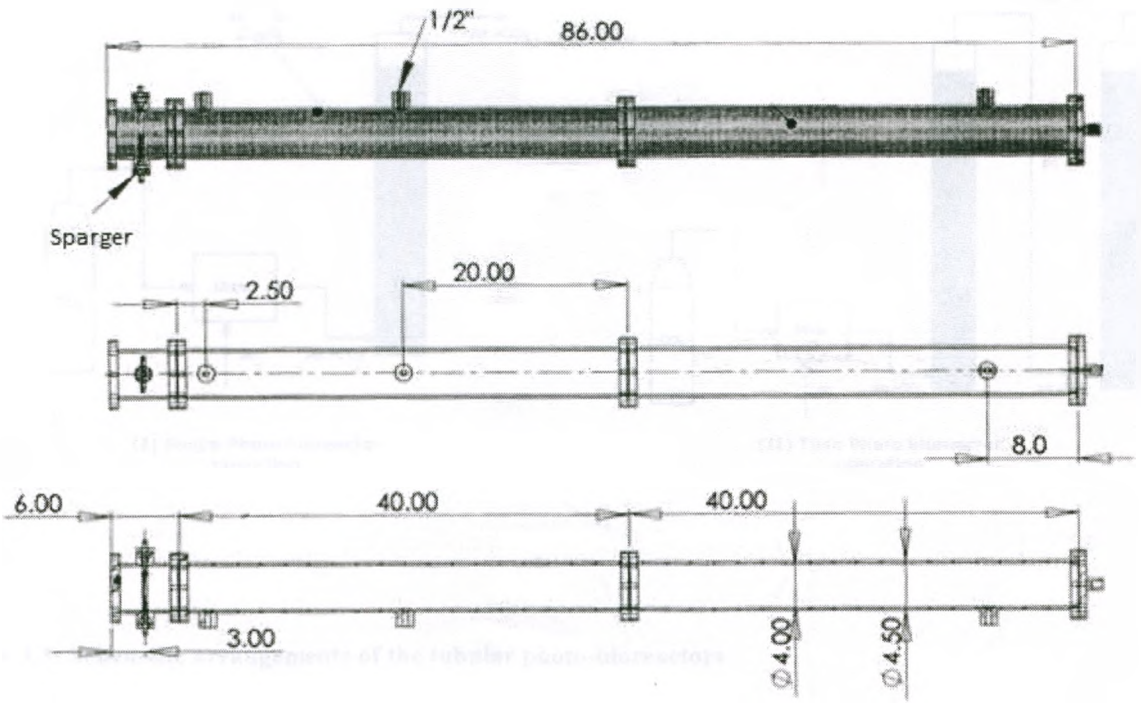


Figure 3.2: Photo bioreactor design

The configuration shown above was arranged in a vertical arrangement for microalgae cultivation.

The schematic shown in Figure 3.3 demonstrates the employed photo bioreactor design configurations for the *Chlorella vulgaris* cultivation. The first (I) figure in the schematic shows the single tubular photobioreactor configuration whereas the second (II) configuration shows the twin photo bioreactor arrangement. In this arrangement the CO_2 / air % (v/v) either 2 % or 4 % was fed through a loop system arrangement, with the exhaust CO_2/air mix from photobioreactor 1 (PB1) being fed through the bottom of the photobioreactor 2 (PB2).

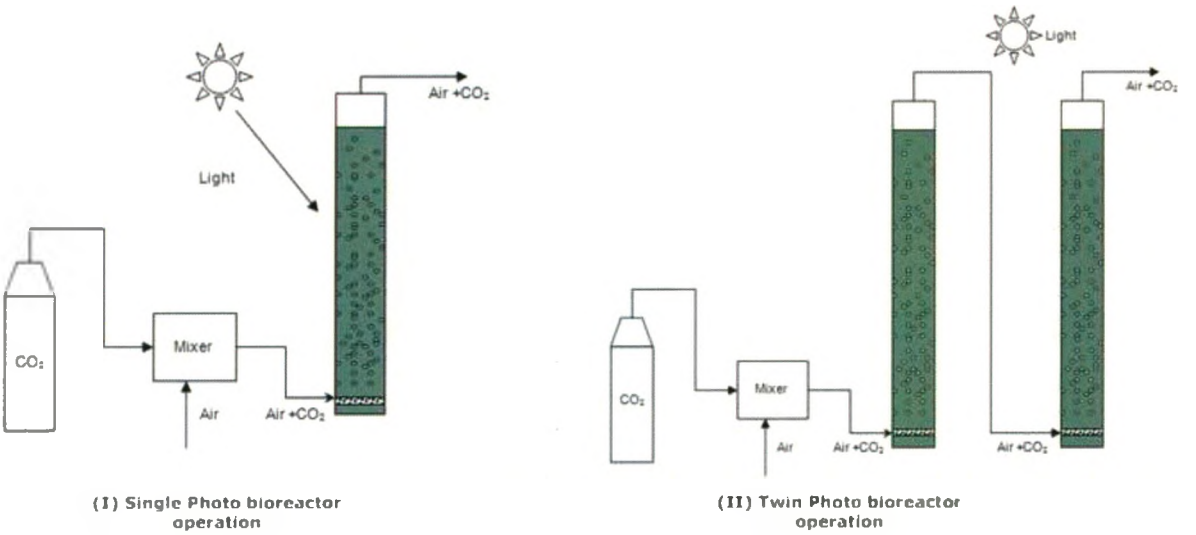


Figure 3.3: Schematic arrangements of the tubular photo-bioreactors

This arrangement demonstrates the ease with which the single photo bioreactor setup can be expanded to include more cultivation volume either for cultivation in batch mode or continuous mode. Due to this design simplicity and effectiveness, the system can be scaled up by adding more tubular photo bioreactors in series.

Figure 3.4: Tubular photo bioreactor in operation

The Figure 3.4 shows the sparger and lighting setup for the photo bioreactor in the lab. Based on the operating volume for each batch the subculture volume was always 30% of the total working volume of the reactor with the rest being made up by freshly prepared growth media i.e. Modified Bolds Media or pre treated dairy farm effluent.

3.3.1. Aeration

Laboratory experiments were carried out at 2 % (v/v) and 4 % (v/v) CO_2 concentrations by means of an Omega Instruments rotameter (Omega Engineering, USA). This stream of CO_2/air was then bubbled through the bottom of the PBR by means of a sparger. The schematic shown in

(Figure 3.3) shows the air CO_2 mixing loop in which the gas outlet of PB1 feeds the CO_2/Air to inlet of PB1.

3.3.2. Lighting

The PBRs were placed at a distance of 20'' from the window wall. Illumination for the reactors was provided by natural lighting during the months April-November. However, for the months December-March, illumination was supplemented by attaching 4 of the 40W Cool White^{+} fluorescent tube lamps (Philips, USA) vertically at a distance of 25 cm from the outer wall of the PBR's. Figure 3.5 shows the top view of the PBR arrangement. Light intensity measurements were conducted using an EPP 2000, BLACK-*C*omet UV-VIS Spectrophotometer (StellarNet Inc., Florida, USA) with a wavelength range of 190 – 900 nm to determine the overall light intensity being incident on the photo bioreactors. In total 8 readings were taken at positions 1, 2 and (1, 2) covering all possible light incident angles as labelled in (Figure 3.5) to provide an accurate measurement of the photon flux being incident on the photo bioreactor. The spectrophotometer provided an output of individual irradiance values (W/m^2) being emitted by the light source being tested for a wavelength range of 300 nm-700 nm. The area under this curve was integrated to provide a final light intensity value in μ Einsteins/m^2s or μ mole photons/ m^2s. The calculations for which are shown in Appendices.

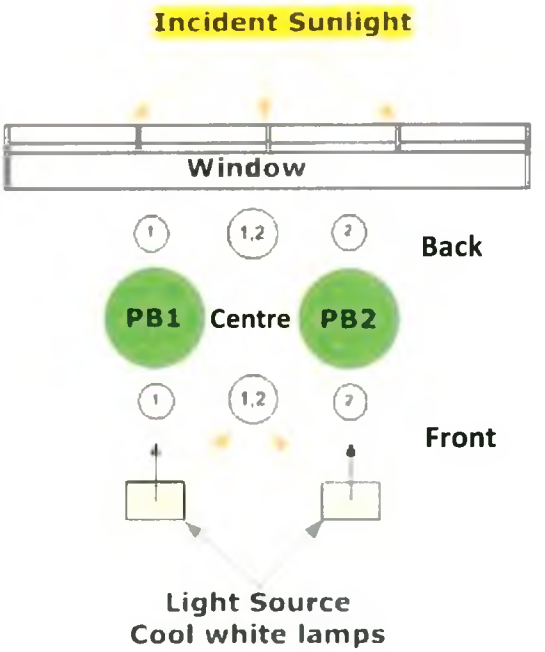


Figure 3.5: Top view of the twin photo bioreactor arrangement

Light intensity calculations were done at PB1 at point 1 facing the fluorescent lamps, point 1 facing away from the window, at point 1 facing towards the window. Similar measurements and calculations were done at the same positions for PB2. Two more common set of reading were taken at point (1, 2) facing towards the fluorescent lamps and the other with the light sensor facing towards the window.

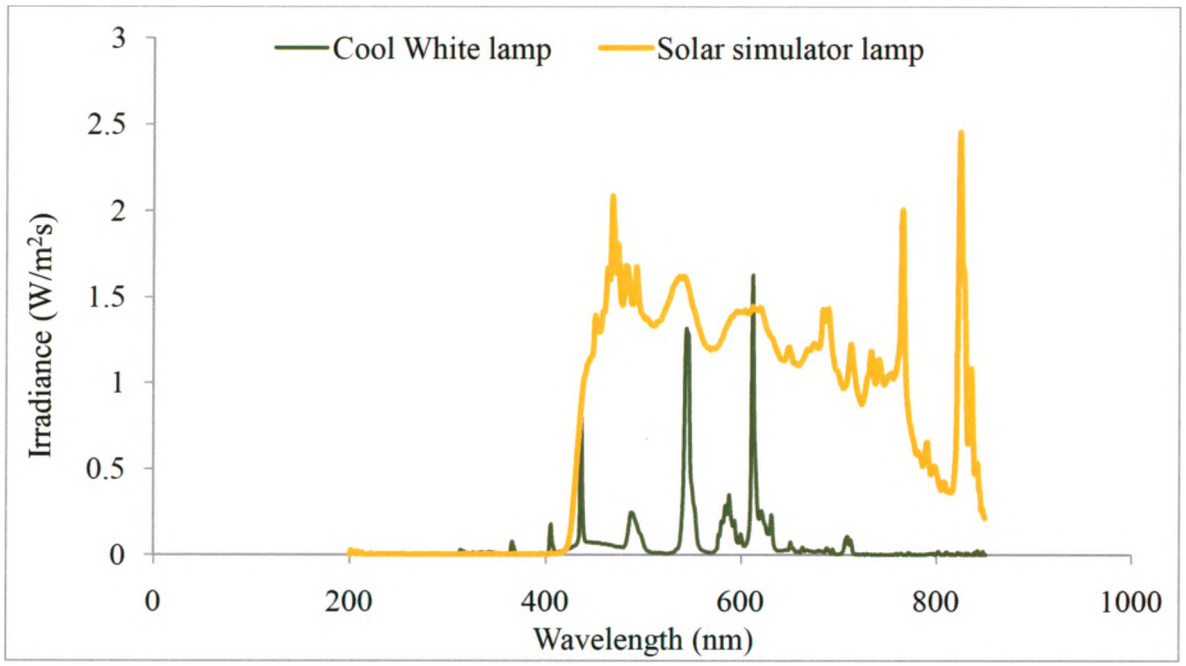


Figure 3.6 : Energy spectrum for sunlight and cool white lamp

As can be seen in Figure 3.6 the fluorescent lamps were able to provide similar emission spectra to the solar spectra. Particularly it can be seen that the lamp is strong in the blue area of the spectrum, which is the wavelength well suited for growth of autotrophs.

In addition to the constant light intensity provided by the fluorescent lamps, daily incident light readings were monitored using a Dual range Light meter (VWR International, USA) with a 0-50000 lux range.

3.4. Determination of cell number and dry cell weight

For determining the daily cell counts and dry weight, 20 ml of the microalgae culture volume was withdrawn from the PBRs and cell number counts were performed on the microalgae culture using a hemocytometer (Figure A.3) and a microscope.

The biomass concentration was measured using optical density which was measured at a wavelength of at 600 nm using a CARY 50 Bio UV-visible spectrophotometer (Varian, Inc., USA).The sample was carefully diluted using distilled water so as to maintain the absorbance below 1.0 for accurate results.

For Dry cell weight analysis, 10 ml of the microalgae suspension previously collected was vacuum filtered through a pre-weighed 45 μm filter paper (VWR, USA). The filter paper was then placed in an oven maintained at 80 °C for 12 h to remove any residual moisture and reweighed. The resulting filtrate was collected in a test tube for further nutrient analysis.

3.5. pH measurement and control

For all experiments carried out the pH was measured using a Beckman Coulter 340 pH meter (Beckman Coulter Inc., CA, USA). The optimal pH of the culture system was 6.8. The pH was adjusted by the addition of 1M HCl or 1M KOH.

3.6. Total Chlorophyll

The total chlorophyll concentration was determined by using a method used by (Junqueira, Bedendo *et al.* 2004). To the dried microalgae 2.5 ml of methanol were added and the samples were heated at 40 °C for 5 min in a water bath (Thermo Electron, USA). The sample was allowed

to cool down and optical density measurements at OD_{662} and OD_{642} were done in the CARY 50 Bio UV-visible spectrophotometer (Varian, Inc., USA). The chlorophyll concentration was measured using the following equation

$$\text{Chlorophyll (mg/L)} = 20.2^* OD_{642} + 8.02\ OD_{662}$$

Equation 3.1: Total chlorophyll measurement (Junqueira, Bedendo *et al.* 2004)

3.7. CO_2 analysis

For measuring the CO_2 concentration, a 2 L gas sample was collected in a 3 Litre Gas Sampling Bag (SKC Inc. PA, USA) from the photo bioreactor inlet and outlet ports. The CO_2 concentration in the inlet and outlet gas was analyzed by means of a HeySep D column (30 m x 2 mm ID x 2 μm) and their concentrations were analyzed with a thermal conductive detector (TCD) in a gas chromatography (Shimadzu, GC 2014). 1 ml of the gas sample was injected. Ultra high purity helium was used as the carrier gas at a 30 ml/min flow rate, the total run time was 13.6 min. The initial temperature of the oven was held at 35 °C for 6 min and then ramped up to 200°C with a 25 °C/min rate to be held there for 1min. The injector port was maintained at 200°C and the detector port was set at 250 °C. The retention time for CO_2 was determined to be 11.95 min.

3.8. Nutrient analysis

A DR 2800 Spectrophotometer was used to measure NO_3-N, NH_3-N, PO_4^{3-} and COD in the culturing media with time. For COD measurement a DRB 200 heating instrument was used after initial mixing and dissolving the reagent with sample in the vial.

3.8.1. NO_3 –N Measurement

A reagent blank measurement was conducted using nitrate free water as the test sample. This reagent blank value was later subtracted from the sample values to give the true value of N content.

Each time 1 ml sample was added in Test 'N Tube vial for the measurement purpose. Since the media was in the range of 0-30 mg/L no further dilution of the sample was required. 1 ml sample was added in the sampling test tube and the contents of the NitraVer X Reagent B Powder Pillow (chromotropic acid) were added to the vial. The vial was held in a vertical position with the cap pointing up; the vial was mixed by carefully inverting the vial a few times to allow complete interaction of the reagents and the sample. The nitrate in the sample reacts with chromotropic acid under strongly acidic conditions for 5 minutes to yield a yellow product in the presence of nitrogen with a maximum absorbance at 410 nm.

The reaction (Figure 3.7) is shown below; two moles of nitrate react with one mole of chromotropic acid to form a yellow reaction product.

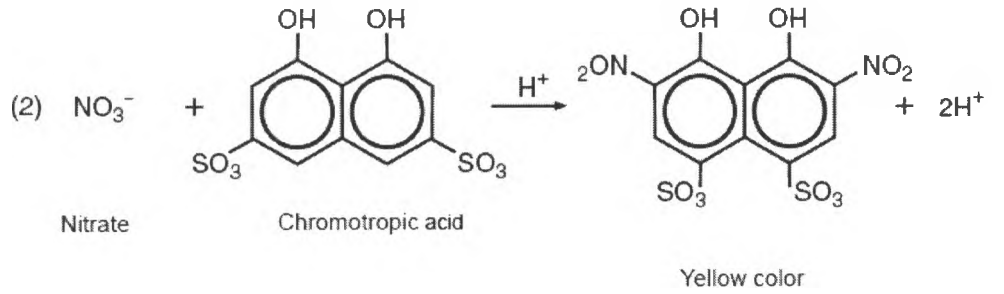


Figure 3.7: Nitrate analysis reaction

3.8.2. NH_3 –N Measurement

NH_3 –N in the samples was measured using AmVer™ Reagent Set for Nitrogen, Ammonia, High Range Test 'N Tube Vial (0-50 mg/L). Since the initial concentration of NH_3 was higher than the detection range of the test solution, the media was diluted 10 times.

Initially 0.1 ml of deionized water (ammonia-free) was used in place of the sample for carrying out the blank run correction.

To 0.1 ml of sample in a sampling test tube one Ammonia Salicylate Reagent Powder Pillow was added and one Ammonia Cyanurate Reagent powder pillow was also added to the tube. The tube was then shaken thoroughly and allowed to sit for a 20 minutes reaction period before taking the reading. The sample was tested in the 655 nm range in DR 2800 Spectrophotometer.

3.8.3. PO_4^{3-} Measurement

High Range Reactive Phosphorus Test 'N Tube Vial (0-100 mg/L) was used to measure reactive phosphate in the media. The media P concentration was outside the detection range and hence was diluted twice. To incorporate the blank reagent correction, 5.0 mL of deionized water was added to a measurement tube for the blank run.

To a high range Reactive Phosphorus Test 'N Tube 5.0 ml of media sample was added and gently shaken vertically to mix completely and allowed to sit for seven minutes to react before sampling. Orthophosphate reacts with the molybdate in an acid medium to produce a mixed phosphate/molybdate complex. In the presence of vanadium, a yellow molybdo-vanado-phosphoric acid product is formed. The intensity of the yellow color is proportional to the phosphate concentration. The results were measured at 420 nm.

3.8.4. COD Measurement

Dichromate COD Method is the most widely used Chemical Oxygen Demand (COD) method in the world. TNT plus COD Low Range (3-150 mg/L) vial tests with automatic method detection was used to measured COD by using a DR 2800 spectrophotometer. A silver salt is added as a catalyst to promote oxidation of resistant organic compounds. Mercuric sulphate is also included to inhibit the interference from the oxidation of chloride ions by dichromate. Initially sample was in higher range than test range need to dilute five times to get accurate results. 2 ml of deionized water was added to the measurement tube for the blank run. 2ml sample was added to the other measurement tube. Initially both vials were put into the DRB 200 heating instrument at 150^0 C for two hours to react completely. After 20 minutes had elapsed the sample was allowed to cool down to 120 ^oC before stopping the DRB 200 instrument. Both tube vials (blank and sample)

were shaken several times to allow cooling down to room temperature to get accurate data. The sample was finally measured in the DR 2800 spectrophotometer at 420nm absorbance.

In another independent study, the microalgae culture volume was cultivated on dairy farm effluent pre-treated using different doses of alum as a pre-treatment method. Two simultaneous experiments were carried out using two different CO_2 concentrations i.e. 2 % (v/v) and 4 % (v/v) respectively. The microalgae was harvested by means of centrifugation and lyophilized subsequently for total triglyceride content measurement.

3.9. Heterotrophic cultivation

Chlorella protothecoides (UTEX 249) was cultivated heterotrophically in 4 flasks containing supplemental glucose and peptone (Table 3.2)

Table 3.2: Concentrations of media supplement added

Flask	Conc. (g/L)	Media Supplement
1	0.27	Glucose
2	0.27	Peptone
3	0.55	Peptone
4	0.55	Glucose

Cultivation was done in autoclaved 1L Erlenmeyer flask through which only air was supplied for the duration of the cultivation. Natural illumination in the lab was the only source of light energy.

3.10. Harvesting

The microalgae culture was allowed to grow in the PBRs till the time a stationary phase was reached and the run was stopped when the culture had transitioned into the death phase. The reactor volume was collected in 5 gallon containers, after which they would be further processed. A final sample was taken to measure the final culture dry weight and residual nutrient concentrations.

3.10.1. Centrifugation

The microalgae culture volume from the photo bioreactors was harvested by centrifugation at 5000 RPM for 10 minutes (Sorvall RC6*plus,* Thermo Electron, USA). The microalgal suspension from each of the photo bioreactors was concentrated down to $1/10^{th}$ of its original volume. The microalgae biomass pellets obtained from the multiple centrifugation experiments were combined after being washed with distilled water twice.

3.10.2. Ultrafiltration

In addition to the centrifugation technique for harvesting and concentrating the microalgae culture, ultrafiltration was employed. An Ultrafiltration membrane model: Zenon ZW1 (GE WATER, USA) was used as explained in Figure 2.5.

According to the manufacturer the module operates in an ''outside-in'' configuration, where a negative pressure provided by the vacuum pump induces a flow of water from outside to the inside of the membrane fibres thereby allowing only the growth media to be filter through and to be recovered separately in a vacuum flask. This led to an 80 - 90% recovery of the post growth media.

The resulting microalgal slurry obtained by applying the ultrafiltration and centrifugation techniques was shell frozen (Figure 3.8) using an acetone and dry ice bath. Once the slurry was frozen solid it was freeze dried for a period of 24 h using a ModulyoD freeze dryer (Thermo Scientific, USA) to obtain the lyophilized microalgal powder as shown in Figure 3.8.

Figure 3.8: Shell freezing technique and the lyophilized UTEX 2714

The microalgae powder obtained after freeze drying was used as the subsequent feedstock for triglyceride extraction and *Insitu* biodiesel conversion.

3.11. Triglyceride Extraction

Once the microalgae had been lyophilized, a known gram weight of the sample was quantified for TG's using a modified Bligh and Dyer method (Bligh and Dyer 1959). All extractions were carried out using a solvent final volume ratio of 1: 1: 0.9 for Chloroform: Methanol: Water. The extractions were carried out in 50 ml test tubes following the procedure steps shown in Figure 3.9 below.

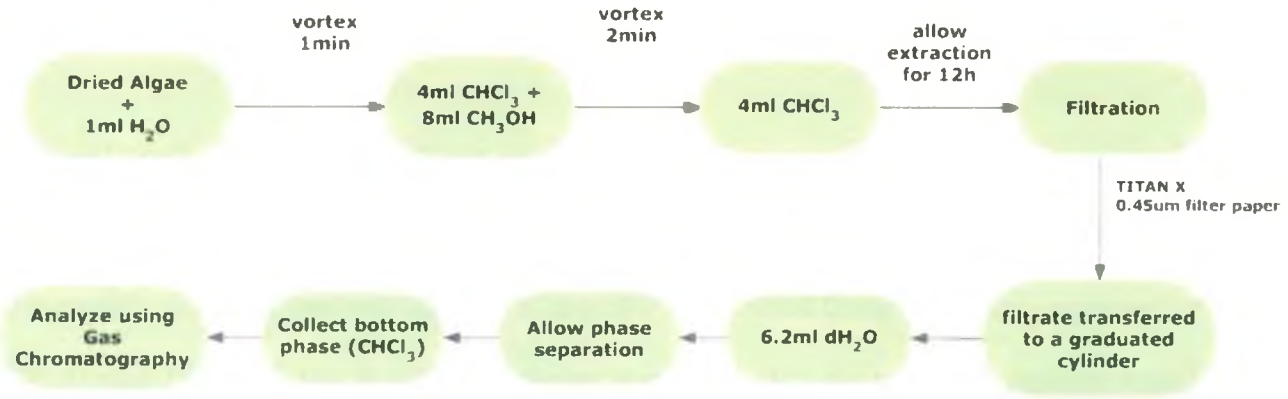


Figure 3.9: Bligh and Dyer extraction of triglycerides (Bligh and Dyer 1959)

As outlined in Figure 3.9, for every extraction run, a microalgae sample which had been previously freeze dried was used. To this sample 1ml of distilled water was added to form a slurry mixture by vortexing it at high speed for one minute. To this microalgae slurry paste, 4ml of HPLC grade methanol and 4 ml of chloroform was added and the sample was vortexed for 2 min to allow solvent interaction with the microalgae. To this mixture 4 ml of chloroform was added and vortexed again for 2 minutes to homogenize the microalgae with the solvents to ensure efficient extraction. The resulting microalgae/solvent suspension was allowed to extract for a period of 12 h after which it was filtered through a Sun Sri PTFE 0.45µm filter paper. The filtrate was then transferred into a graduated cylinder and 6.2 ml of distilled water was added to create a biphasic system to develop separate chloroform and aqueous methanol layers. This ensured triglyceride migration to the bottom phase (chloroform). The bottom layer was then carefully siphoned out by applying slight positive pressure through the pipette and centrifuged to remove any traces of methanol or water. The extract was stored in sealed vials pending gas chromatography analysis.

3.12. *Insitu* biodiesel production from *Chlorella vulgaris*

Biodiesel production was carried out using lyophilized microalgae powder. For most of the biodiesel extractions 5 g of lyophilized biomass was used unless stated otherwise. The biodiesel transesterification reaction was carried out in a 500 ml jacketed Erlenmeyer flask equipped with a 24/40 neck (Figure 3.10). A reflux condenser was attached to the top of the reaction vessel to prevent loss of any solvents. The reaction temperature was set at 70 °C for the course of the reaction which was allowed to run for 5 hours total. The reaction temperature was maintained by means of a Neslab RTE-110 Water Bath / Circulator (Thermo Electron, USA).

Figure 3.10: Transesterification reaction vessel design

The reactor vessel was first allowed to reach the desired reaction temperature. 5 g of lyophilized microalgae lyophilized previously was added to the vessel directly. Following this 40 ml of HPLC grade methanol and 20 mL of chloroform solvent were added separately. The reaction timer was started once 3 mL of sulphuric acid was added to the reaction volume. The reaction

vessel contents were mixed by means of a magnetic stirrer bar at the lowest setting on the stirrer plate. The volatile solvents were recirculated over the course of the reaction due to the reflux condenser. Once the reaction time had elapsed, 4 ml of dH_2O was slowly added to the reaction volume to end the reaction. The water bath circulation was also stopped and the reaction vessel contents were allowed to cool down to room temperature before being transferred into centrifuge vials. The reaction mix was centrifuged in 15ml centrifuge vials using a table top centrifuge for 15 min at 3000 RPM. Post centrifugation the supernatant was collected in a separatory funnel and 6ml of dH_2O was added to create a biphasic system. The bottom layer was transferred into a 20 ml screw cap vial, pending further GC analysis.

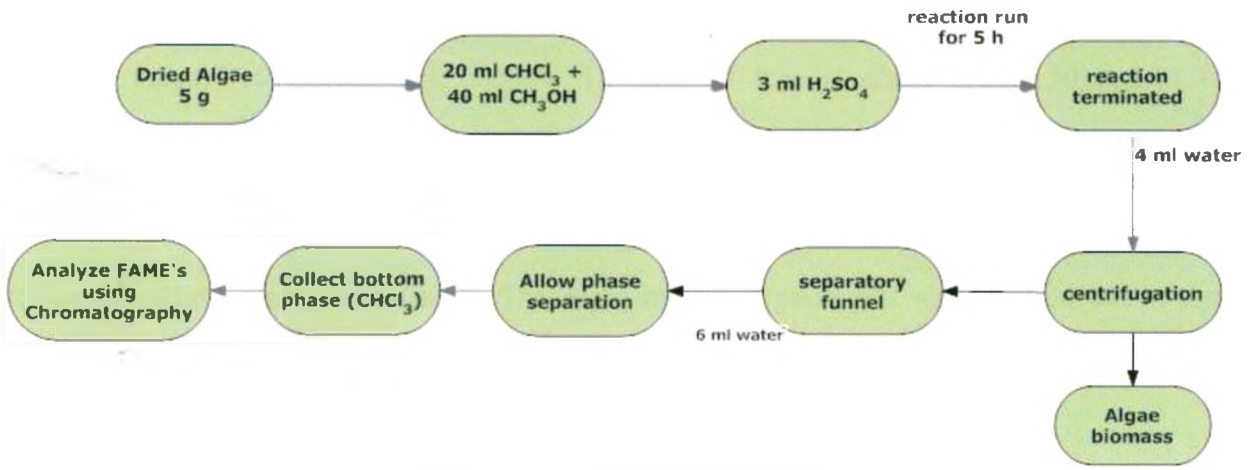


Figure 3.11: In situ transesterification flow diagram

The Figure 3.11 above outlines the main steps involved in the production of FAME from microalgae that were followed in this research.

3.13. Hydrolysis of the microalgae using cellulase for the production of glucose

Three sets of experimental studies were carried out using dry algae as the substrate. Each of the three studies were conducted using six Erlenmeyer shake flasks in a sodium acetate buffer of 50 mM strength and pH 4.7. A stock solution of a 250 mM buffer was prepared by dissolving 5.15 ml of glacial acetic acid water and 21.78 g of sodium acetate tri-hydrate in 800 ml of distilled at room temperature. Once the $NaAc.3H_2O$ was fully dissolved the solution was brought up to the 1L mark and stored at room temperature for later use. Cellulase (EC 3.2.1.4) (Sigma Aldrich, Mississauga, Canada) enzyme from *Aspergillus niger* was used in this hydrolysis study.

The aim of this study was to study the effect of Cellulase enzyme on degrading the cell wall of *Chlorella vulgaris* to fermentable sugars. To each of the six shake flasks 50 mg of dry lyophilized microalgae were added and suspended in 30 ml of the 50 mM Sodium acetate buffer. In each of the six shake flasks, the effect of enzyme loading was investigated by adding increasing cellulase enzyme dosages. The experimental experiments were carried out at three different temperature i.e. 30 °C, 35 °C and 40 °C at 200 RPM for carrying out the hydrolysis.

3.14. Gas Chromatography analysis

The quantification for triglycerides, FAME concentrations were carried out as follows.

3.14.1. Triglycerides

For the analysis of triglycerides Triolein standards were prepared in different concentrations ranging from 20 mg/ml to 2.5 mg/ml through serial dilution in Chloroform and 1µL of this was injected into an Agilent 6890 GC equipped with a Flame Ionization Detector using a Zebron ZB-

wax plus column (30 m x 0.25 mm I.D. x 0.25 μm) (Phenomenex Inc., USA). The injector and detector temperatures were maintained at 250 °C and a 50:1 inlet split ratio was employed. The oven was maintained at 60 °C for 2 min, after which the temperature was ramped up to 240°C at a rate of 13 °C/min and held there for 13 min. The total run time was 30.92 min with helium carrier gas flow rate set point being 2 ml/min. Total triglyceride content and yield of triglyceride were estimated as their weight relative to weight of microalgal biomass.

3.14.2. FAME analysis

The fatty acid composition of the biodiesel produced was done by comparing the retention times of Methyl Linoleate, Palmitate, Myristate, Stearate, Oleate, Arachidate and Behenate. These were quantified by comparing the peak area of different stock concentrations of these pure standards (Sigma Aldrich, MO, USA) through serial dilution in $CHCl_3$. The GC operation procedure was same as described previously in Section 3.14 . All injections were carried out in triplicates and the average of the three experiments obtained was used for further calculations.

3.15. HPLC analysis of glucose

The concentrations of glucose were determined using HPLC (Agilent 1200, refractive index detector, UV-Vis detector, quaternary pump and column thermostat) equipped with ion exchange column (Aminex HPX-87H, 300 mm x 7.8 mm, Mississauga, ON). The samples were eluted isocratically using 9 mM sulphuric acid as mobile phase at a flow rate of 0.6 ml/min and a column temperature of 65°C. All samples were diluted appropriately with the mobile phase and filtered through a 0.45μm filter (VWR International, USA). The injection volume was maintained at 25 μL for all injections. The calibration curve for glucose was constructed using different concentrations of glucose and noting their corresponding peak areas.

4. RESULTS AND DISCUSSION

4.1. Single PBR cultivation using modified Bolds Basal Media

The single photo bioreactor cultivation experiments were carried out using the arrangement shown in Figure 3.3. The microalgae culture volume in these experiments was 7.6 L, the growth parameters measured for these experiments are shown in Figure 4.1 and Figure 4.2. A detailed summary of the total lipid yield and specific growth rates achieved are provided in Table 4.1. The growth media used in these experiments was the Modified Bold's media and the microalgae cultures were cultivated using 2 % (v/v) and 4 % (v/v) CO_2.

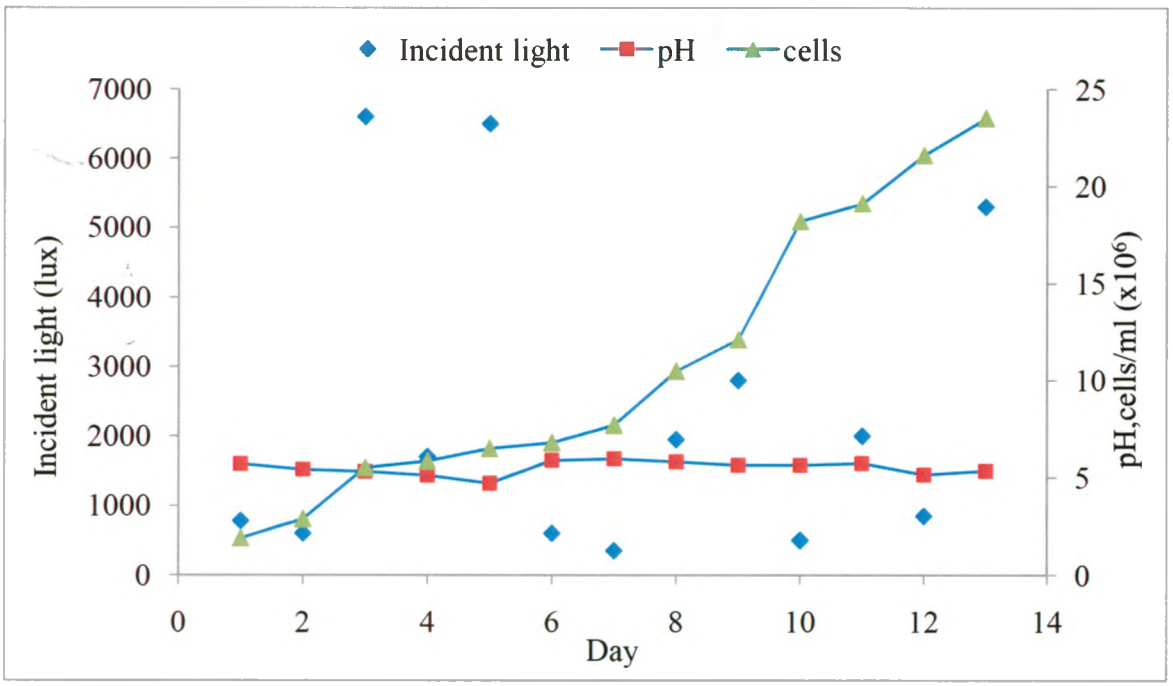


Figure 4.1: Growth parameters during the Bolds Media experiment, 2% (v/v) CO_2

The total PBR working volume in this experiment was 7.58 L. The subculture volume used in this experiment was 2.53 L with a microalgae cell count of 5.24 x 10^6 cells/ml. The photo bioreactor was illuminated with an average incident light intensity of 2348 lux. During the course of this experiment 2% (v/v) CO_2 was supplied to the photo bioreactor. The pH during the course of this run was left unadjusted, the average value was 5.5. The experiment was terminated at the end of Day 13 to perform triglyceride analysis on the microalgae. To assess the effect of CO_2 concentration on the growth of the microalgae, a cultivation experiment using 4% CO_2 (v/v) was conducted.

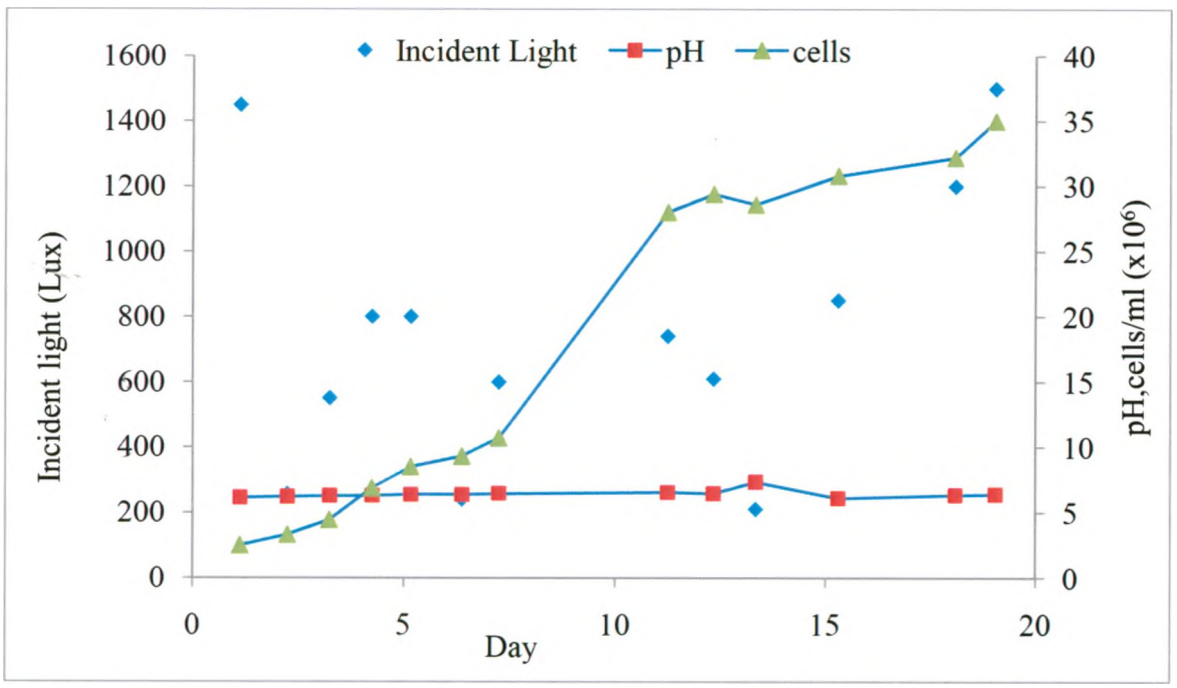


Figure 4.2: Growth parameters during the Bold's media, 4% (v/v) CO_2

The average light intensity during the (Figure 4.2) experiment was measured to be 755 lux. However this was offset by providing 4% (v/v) CO_2 to the microalgae which translated into higher cell growth and significantly higher triglyceride content. The 2% (v/v) CO_2 run yielded 2

grams of microalgae and 2.7 grams for the 4% (v/v) CO_2 run, after lyophilisation with a total chlorophyll concentration of 13.07 mg/L and 11.96 mg/L respectively. Lipid analysis was carried out in triplicates following the method outlined in Figure 3.9. The total lipid content values shown in Table 4.1 are representative of the average of these three experiments.

Table 4.1: Growth parameters and triglyceride content for Modified Bolds Media grown microalgae

Parameters	2% (v/v) CO_2	4% (v/v) CO_2
Cell Concentration (cells/ml)	$24x10^6$	$35x10^6$
Biomass Concentration (g/L)	0.29	0.39
Specific growth rate (μ)d^{-1}	0.21	0.18
T (^oC)	21.3	20.7
pH	5.5	6.41
Average incident light (lux)	2348	755
Total triglyceride content	17.65%	22.84%

The microalgae obtained by an independent study by cultivating the microalgae on dairy farm effluent pre-treated using 4.4 g/L aluminum sulphate solution was also analyzed for the total triglycerides content following the method outlined in Figure 3.9. The 2% (v/v) CO_2 and 4% (v/v) CO_2 experiments yielded 3.1 and 4 grams of dry microalgae respectively. The results for which are outlined in Table 4.2. The specific growth rates were also calculated.

These experiments provided an insight into the nutrient rich composition suitable for the cultivation of microalgae. These experiments provided an insight into the effect of varied alum doses on the growth of *Chlorella vulgaris*. However, light intensity had an average value of 800

lux over the 22 day course of the culture cycle during the 4 % CO_2 experiment. The lipid content in the 2 % CO_2 batch was found to contain ~23 % lipids whereas the 4 % run contained 18 % lipids. The observed specific growth rate had a higher value (Table 4.2) for the 4% run when compared to the 2 % run. Also the chlorophyll content in the 4 % CO_2 run was calculated to be 17.54 mg/L.

Table 4.2: Growth parameters and triglyceride content for dairy farm effluent grown microalgae

Parameters	2% CO_2	4% CO_2
Cell Concentration	138.5 x10^6 cells/ml	462x10^6 cells/ml
Biomass(g/L)	1.57	1.97
Specific growth rate (μ)d^{-1}	0.17	0.23
Alum Dosage (g/L)	4.4	4.4
Average Incident light (lux)	2000	800
pH	7.2	7.21
Total triglyceride Content	22.88%	18.27%

The individual specific growth rates for the different pre-treatment alum doses are shown in Table 4.2. Due to the high specific growth rate in the 4% (v/v) batch, the culture was able to attain higher biomass concentrations but at the expense of a lower triglyceride content. These results are in conjunction with literature that states there is a trade off between cell growth and triglyceride accumulation.

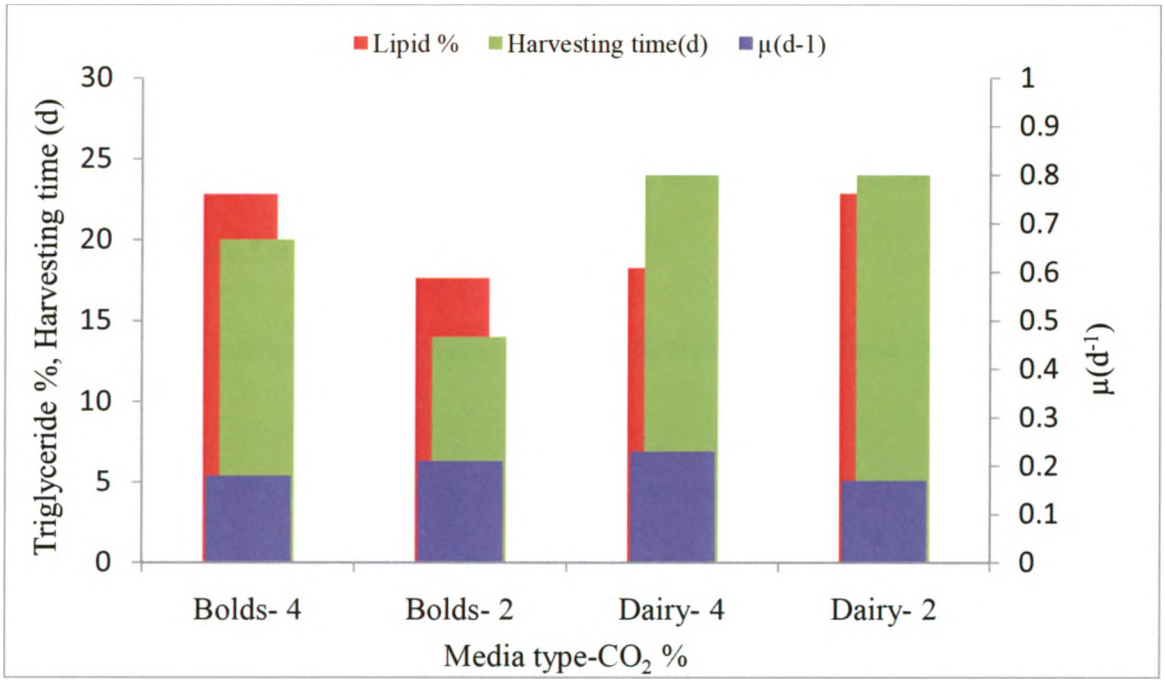


Figure 4.3: Triglyceride content vs. different media conditions

It is hypothesized that at higher concentrations of CO_2, the excess CO_2 gets converted to its CO_3^{2-} form. On the contrary, if the CO_2 gas supply is scarce, the microalgae will utilize carbonate to maintain its growth. If the microalgae uses CO_2 (aq.) in its bicarbonate form due to the lack of CO_2 in the gas supply, this will result in increase of pH.

4.2. Twin PBR configuration

The following experiments were conducted during using the twin photo bioreactor configuration as shown in Figure 3.3.

4.2.1. PB1 cultivation using ferric sulphate pre treated dairy farm effluent media

The media pre-treatment used in this experiment was using 12.27 g/L ferric sulphate solution. A total working volume for 14.15 L for each of the PBRs was used with an air flow rate of 2.26 L/min using 4% CO_2. A subculture volume of 4.6 L was used for both photo bioreactors with a microalgal cell count of 179.4 x 10^6 and 222 x 10^6 cells/ml for PB1 and PB2 respectively. During the course of the cultivation experiment, the pH, optical density, incident light, dry weight, CO_2, Nitrate, Ammonia, Phosphate and COD were all monitored. At the end of the run the microalgae biomass was concentrated using the Zenon Ultrafiltration membrane. By doing this 90% of the treated effluent was recovered from each of the PBRs. *Insitu* transesterification was carried out on these microalgae to profile the biodiesel produced and provided a yield by wt% of 26.67 % for PB1 and 18.21 % for PB2.

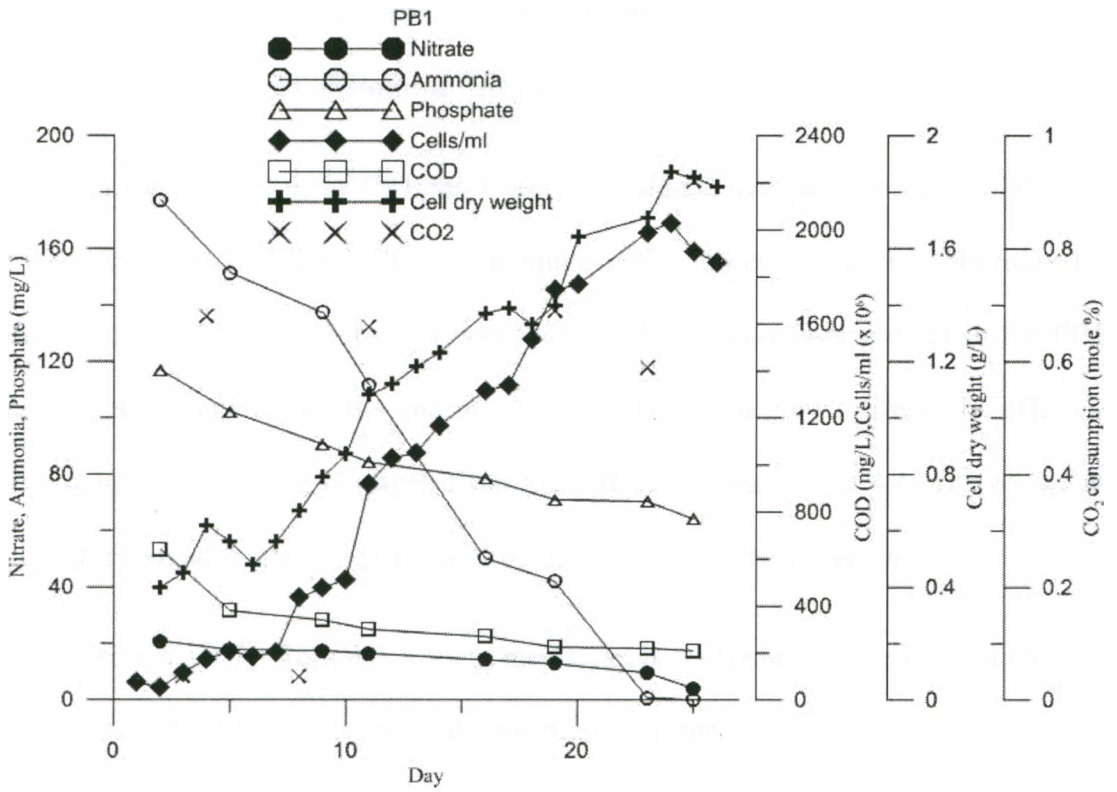


Figure 4.4: Growth Profile and nutrient consumption in PB1

This culture exhibited a slight lag phase which lasted 7 days; during this stage the CO_2 consumption (Figure 4.4) was negligible and the nitrate consumption was 80%. This could be due to the fact the cells were adapting to the change in the effluent media treatment methodology. Also the CO_2 consumption during the lag phase was negligible for the duration of the lag phase. During the exponential growth phase, there was almost a 10-fold increase in the CO_2 consumption (mole %). The pH had very little fluctuation during the course of the run with an average value of 6.35. The specific growth rate for this batch of microalgae culture was calculated to be 0.13 d^{-1}.

A maximum dry weight concentration of 1.8 g/L was achieved during the course of this experiment. Due to the very high absorbance of the microalgae culture at this dry weight concentration the light transmittance through the culture was greatly reduced.

The synergistic consumption of CO_2, nitrogen and phosphorous translated into a high cell culture density and dry weight of biomass being achieved.

As seen in Figure 4.4, in PB1 the *Chlorella vulgaris* was able to uptake 80 % of the nitrate within 25 days. This is reflected in the spike in the cell count numbers after day 11 after which the almost 50 % of the nitrate was consumed. The ammonia-N present in the PB1 media was consumed completely by the end of 25 days. This again goes to show the effectiveness of the microalgae in uptaking Nitrogen from the effluent media Figure 4.4. The phosphate and COD concentration also saw a significant drop of 45 % and 67 % respectively.

The nitrogen uptake from the nutrient media can be explained on the basis of the thermodynamic stability of the different types of nitrate present in the effluent media. For eukaryotic algae the only forms of inorganic nitrogen which they can directly assimilate are nitrate, nitrite and ammonium. Out of these three sources, nitrate is the most thermodynamically stable form therefore it does not require any reduction before it can be taken up by the microalgae (Lourenco, Barbarino *et al.* 2002). The fixing of nitrate requires its conversion to ammonium which is carried out with the help of two enzymes Nitrate Reductase and Nitrite Reductase. The Nitrate Reductase enzyme is located in the cytosol of the cell whereas Nitrite Reductase is located in the chloroplasts of the eukaryotic algae (Barsanti and Gualtieri 2006). The reduction is carried out by means of the electron flow which is generated during the photosynthetic process.

$$NO_3^- + 8e^- + 10H^+ \rightarrow NH_4^+ + 3H_2O$$

Equation 4.1: Overall reduction stoichiometry

The ammonia produced by the overall reduction reaction in Equation 4.1 is assimilated by another enzyme called glutamine synthetase to produce ADP (Barsanti and Gualtieri 2006), which is later converted interchangeably to ATP by ATP synthases.

4.2.2. PB2 cultivation using ferric sulphate pre treated dairy farm effluent media

In PB2 the total microalgae culture volume was 14.15 L. The growth media used in PB2 was pre treated using a 7 g/L ferric sulphate solution.

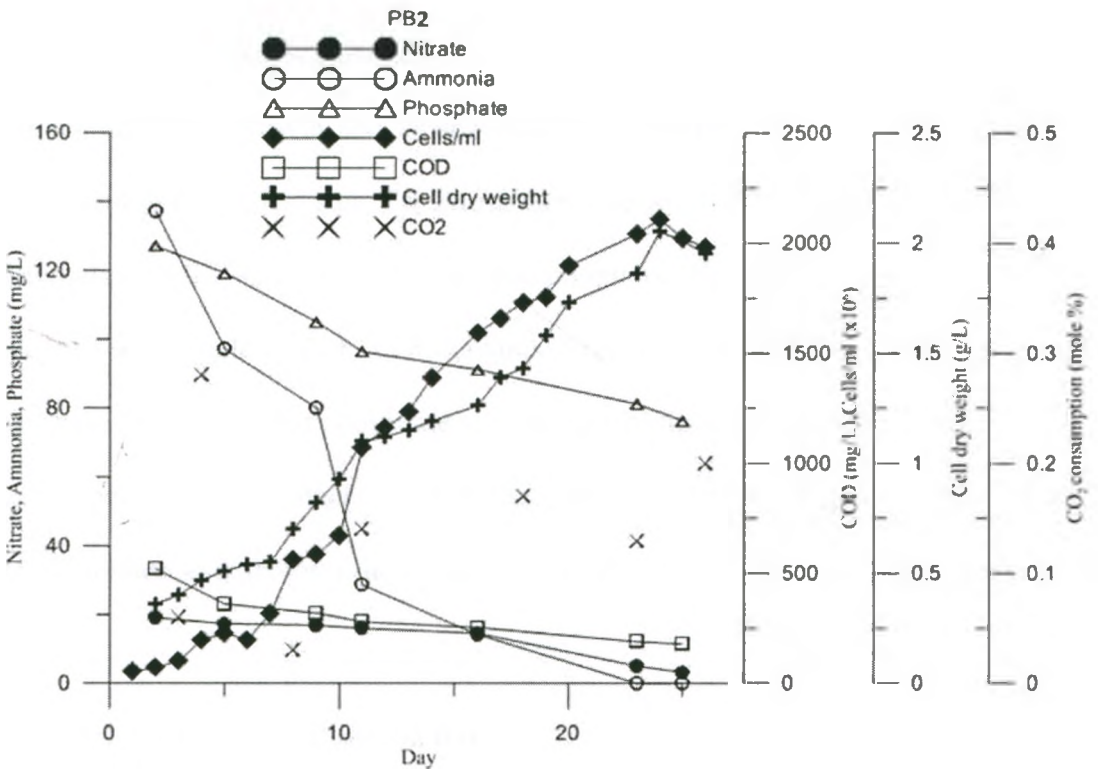


Figure 4.5: Growth Profile and nutrient consumption in PB2

A similar growth profile (Figure 4.5) was achieved in PB2 as well with a maximum dry weight concentration of 2.05 g/L at the end of Day 23.The CO_2 consumption was lower in this PBR This run saw a higher consumption of nitrate when compared to PB1 in which the ammonia

consumption was higher. The specific growth rate was higher in this batch experiment when compared to the PB1 run. The specific growth rate was determined to be 0.15 d^{-1}.

In PB2 the *Chlorella vulgaris* was able to uptake only 22% of the nitrate and 100% of the ammonia-N present within 25 days. This is reflected in the spike in the cell count numbers after day 16 after which the almost 50 % of the ammonia-N was consumed. This again goes to show the effectiveness of the microalgae in uptaking Nitrogen from the effluent media Figure 4.5. The phosphate and COD concentration also saw a significant drop of 40% and 65% respectively.

In addition to the nitrogen source uptake, phosphorous is believed to be of significant relevance for the algal cell growth and its subsequent metabolism. It is a component of the cell's DNA, RNA and ATP (Adenosine Tri Phosphate).The synthesis of ATP in the mitochondria of the microalgae cell is carried out using photophophorylation, which serves as the energy source for the cell. One molecule of ATP contains three phosphate groups, and it is produced by ATP synthase from inorganic phosphate and adenosine diphosphate (ADP). Any limitation caused due to the phosphorous concentration in the media can have a negative impact on the triglyceride accumulation and photosynthetic operation of the algal cell (Barsanti and Gualtieri 2006).

4.2.3. Total incident photon flux

The total incident photon flux light intensity for PB1 was calculated to be 188.98 $\mu E/m^2s$ whereas for PB2 it was calculated to be 156.19 $\mu E/m^2s$, the calculations for which are shown in Section A.1. Photon flux measurements. In PB1 94% of the incident light from the fluorescent lamps was absorbed by the microalgae culture. Whereas in PB2 92.8% of the light incident on

the PBR by the fluorescent lamps was absorbed. The transmittance of light decreases as the culture density and cell population increases.

The average incident light during this experiment was measured to be 5563 lux and 4888 lux for PB1 and PB2 respectively.

4.2.4. FAME profile and yield

The *Insitu* transesterification was carried out on 5.09 g of PB1 and 5.05g of PB2, dried microalgae using the same steps outlined in Figure 3.11. The biodiesel wt. % yield was calculated to be 26.67 % for PB1 and 18.21 % for PB2. The % composition obtained for each of the experiments is shown in Figure 4.6 below.

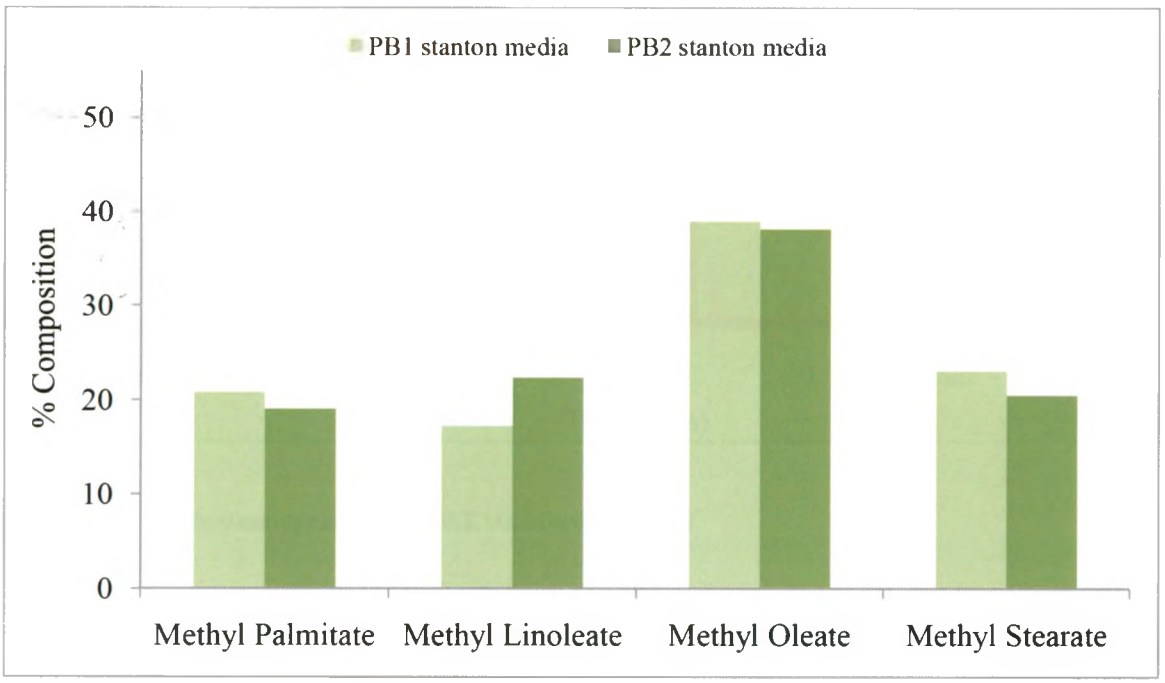


Figure 4.6: FAME profile for PB1 and PB2, Stanton media grown microalgae

The fatty acid composition obtained for the two simultaneously carried out experiments were in close confirmation to each other. The PB1 microalgae yielded 8% more biodiesel. This could be

explained on the basis of the nutrients supplied to each of the twin PBRs in Figure 4.4and Figure 4.5. PB1 was supplied with 8 % more Nitrate concentration which it could uptake from NO_3, whereas ammonia-N concentration was 20% less. However the culture in PB1 consumed 80.4 % of the NO_3-N when compared to 83.2 % in PB2. Both of the microalgae cultures were in addition to the NO_3-N, able to consume 100 % of the NH_4-N with starting concentrations being 177 mg/L and 137 mg/L in PB1 and PB2 respectively. The phosphate consumption in PB1 was 52.8 mg/L whereas the consumption in PB2 was 50.8 mg/L.

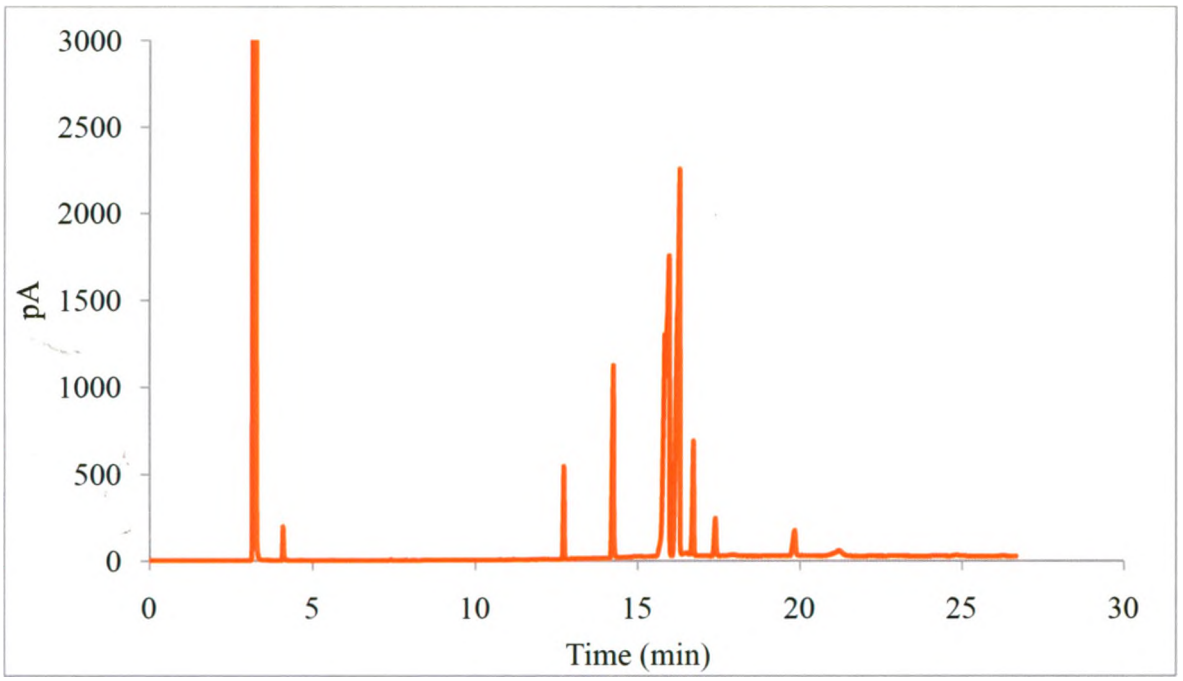


Figure 4.7: Gas chromatogram for FAME standard C14-C22

The figure above shows the gas chromatogram profile achieved for the C14-C22 FAME standard which characterizes the FAME composition as a reference standard for comparison to the microalgal prepared FAME extract, which is shown in Figure 4.8.

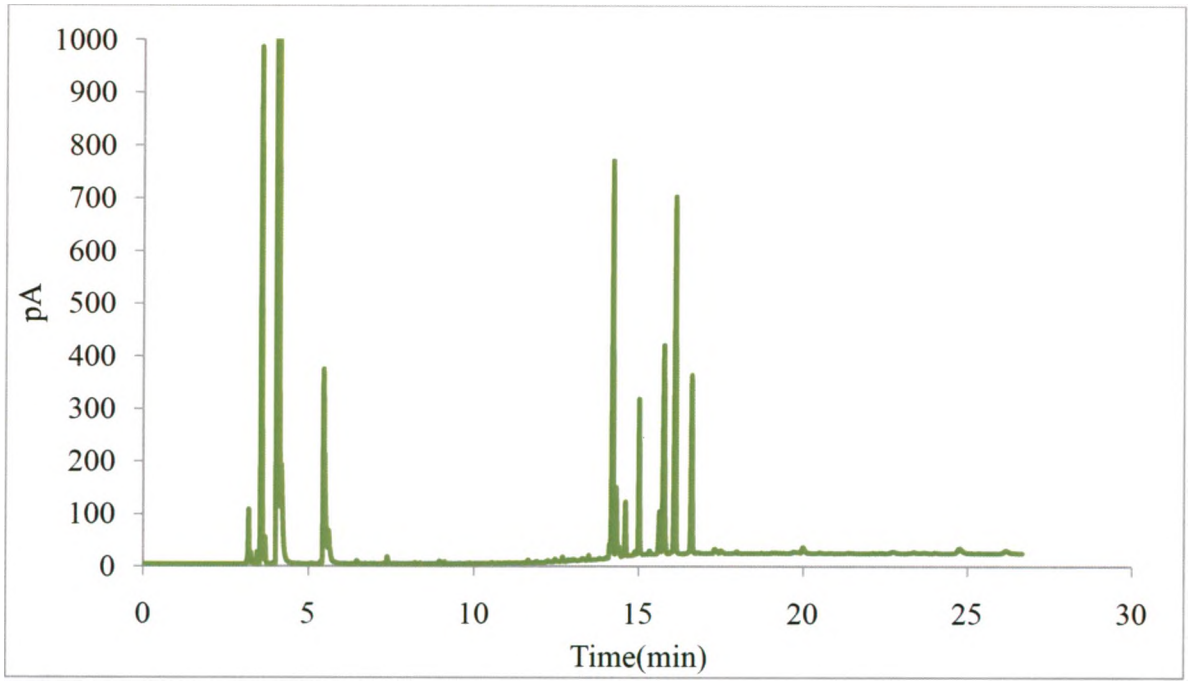


Figure 4.8: Gas chromatogram for microalgal FAME

The microalgal FAME composition was found to contain in majority C16-C18 chain length fatty acid methyl esters based on the comparison of the retention time of the various standard FAME species.

4.3. Twin PBR cultivation on aluminum sulphate pre treated dairy farm effluent media

The reactor configuration as shown in Figure 3.3 was used in this experiment. The media pre-treatment method employed in these experiments were 15 g/L and 11 g/L aluminum sulphate solution in PB1 and PB2 respectively. A total working volume for 15.6 L for PB2 was used with an air flow rate of 2.26 L/min using 4% CO_2 whereas in PB1, 13.6 L of working volume was employed using the same aeration flow rate. The subculture volume of 3.6 L used in PB2 had an initial microalgae cell count of 140 x 10^6 cells/ml. The pH, optical density, incident light, dry

weight, CO_2, Nitrate, Ammonia, Phosphate and COD were all monitored during the course of this batch cultivation experiment in PB2. The microalgae culture in PB2 was profiled for FAME composition to provide yield by wt. %.

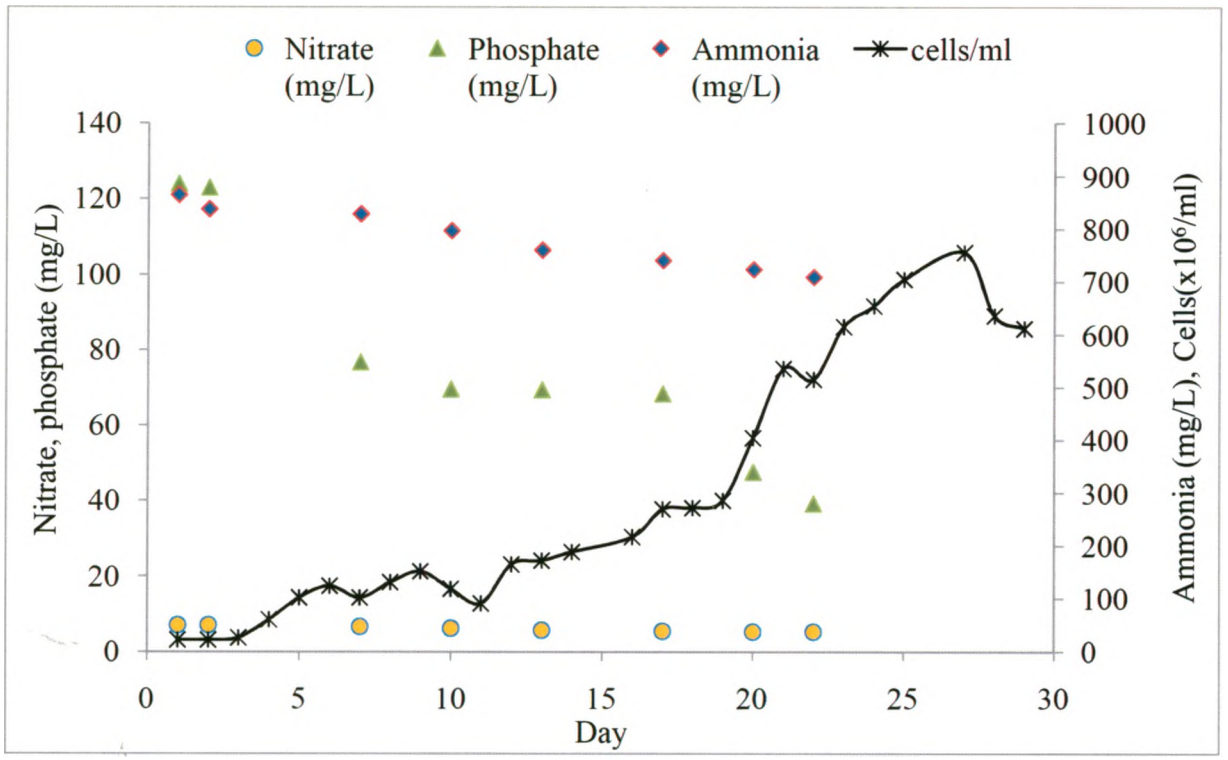


Figure 4.9: Growth and nutrient consumption profile for PB2

In PB2 the *Chlorella vulgaris* was able to uptake 25 % of the nitrate within 29 days. The ammonia-N present in the PB1 media saw a reduction of only 18 % during the course of the culturing. The phosphate concentration also saw a consumption of 68.3 %. This low consumption of the Nitrogen and Phosphorous translated into a low yield of the FAME obtained. During this experiment a specific growth rate of 0.15 d^{-1} was determined.

The alum dosage in PB1 i.e. 15 g/L proved toxic to the microalgae culture and the microalgae growth was negatively impacted to the extent that the cells were bleached within 12 days and

began to die the growth profile of which is shown in the Appendices, Figure A.4. This experiment pinpointed the upper limit threshold for the alum concentration when pre treating the effluent. However, the microalgae culture during this experiment was able to reach a in PB2 the microalgae culture obtained a maximum of 1.6 g/L at the end of Day 27.

The average incident light during this experiment was measured to be 1877 lux and 2564 lux for PB1 and PB2 respectively. After 14 days the PB1 culture perished due to the toxic aluminum sulphate concentrations present.

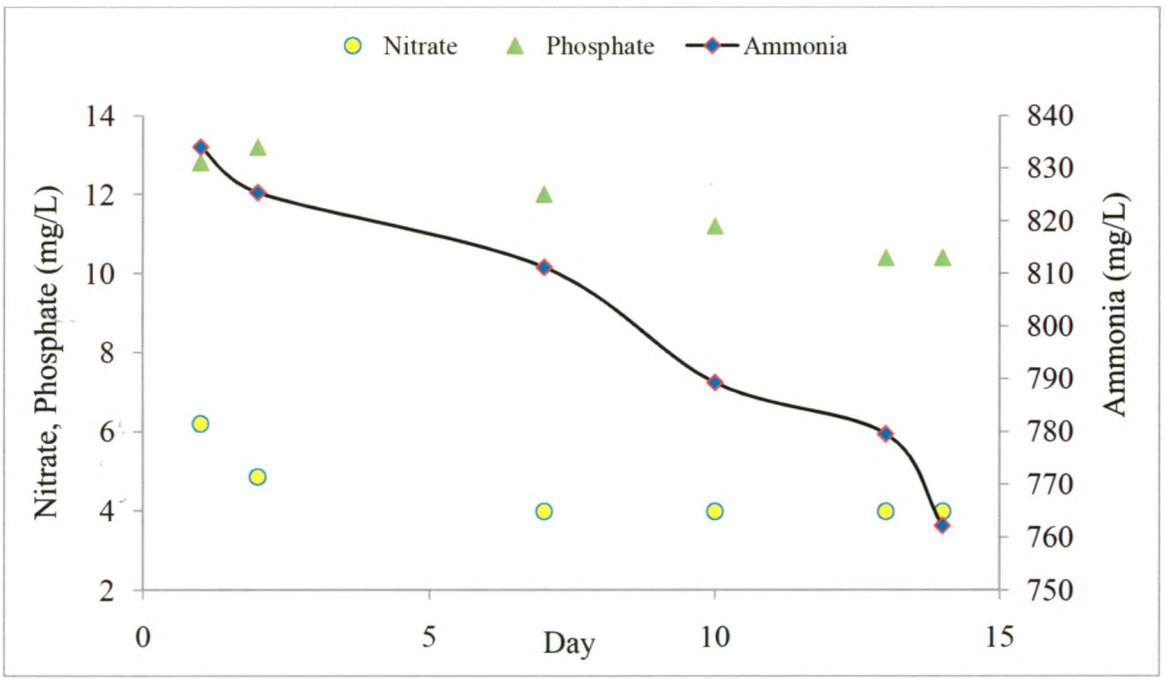


Figure 4.10: Nutrient consumption profile for PB1

During the short 15 day cultivation time before the culture in PB1 perished, the *Chlorella vulgaris* was able to uptake 36% of the initial nitrate in 14 days Figure 4.10. The ammonia-N present in the PB1 media saw a consumption of only 8.7 %. The phosphate concentration also saw a consumption of only 18.75 %. Nutrient consumption was drastically reduced in this

experiment as the excess alum inhibited the growth of microalgae. This was reflected in the low value of the specific growth rate, which was calculated to be 0.009 d^{-1}.

4.3.1. FAME profile and yield

The *Insitu* transesterification was carried out on 4.93 g of dried microalgae from PB2 using the same steps outlined in Figure 3.11. The biodiesel wt. % yield was calculated to be 13.04 %. The % composition obtained from performing GC analysis is shown in Figure 4.11.

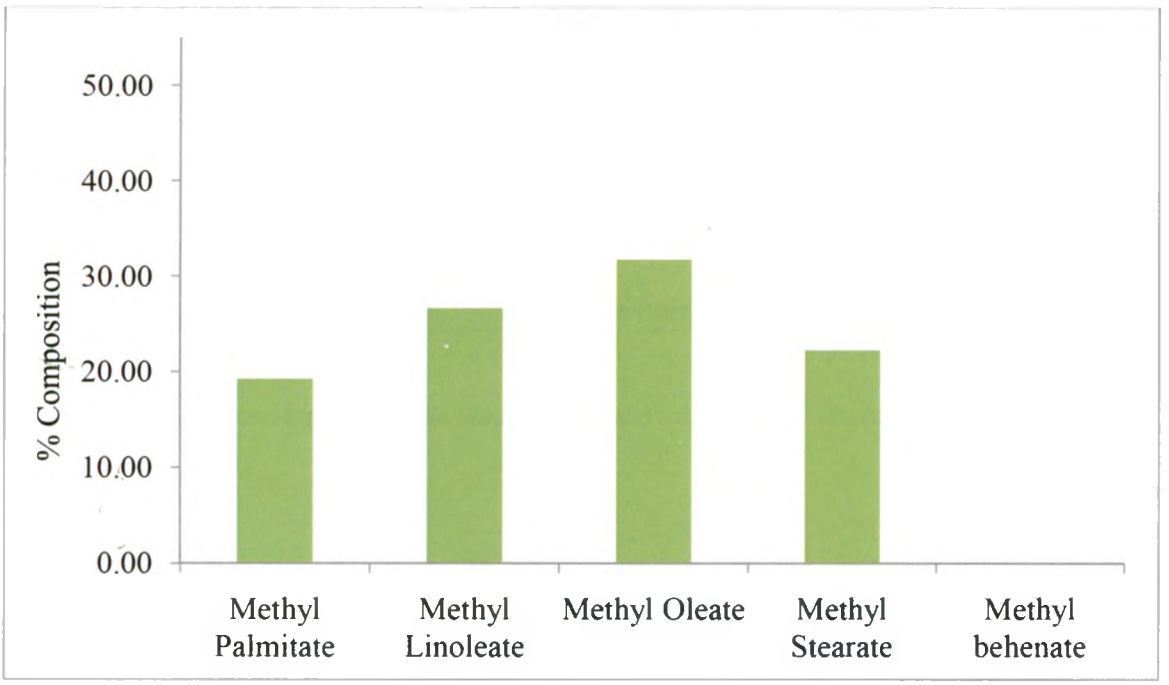


Figure 4.11: FAME profile for PB2

Although the FAME yield obtained was 13.04 %. This culture was able to accumulate C18 chain length FAME, Oleate (C18:1), Linoleate (C18:2) and Stearate (C18:0).

The high nitrogen contained in dairy manure wastewater, can inhibit the algal lipid/fatty acid synthesis (Liliana Rodolfi, Zittelli *et al.* 2008). This is possibly the reason why the overall algal

fatty acid content obtained in this work was still relatively low. The low lipid content was compensated by the faster growth and higher biomass accumulation in the nitrogen rich medium.

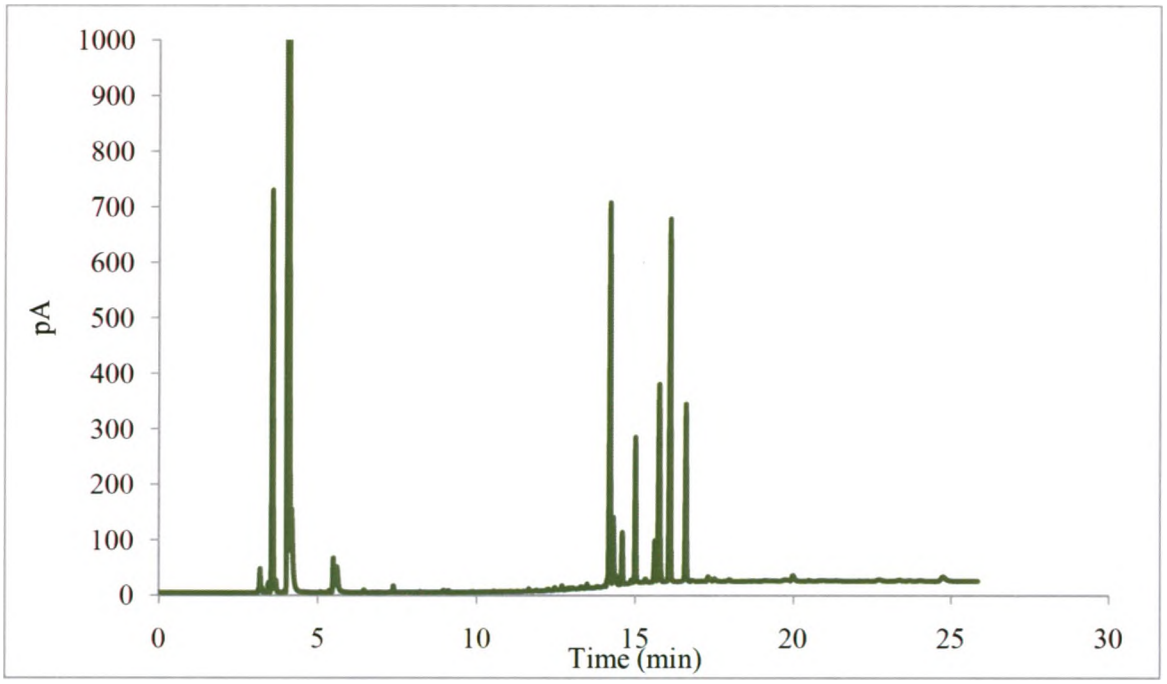


Figure 4.12: Gas chromatogram profile obtained for microalgal FAME extract

The Figure 4.12 above shows the FAME profile obtained from the injection of the washed FAME biodiesel obtained from carrying out the *Insitu* transesterification.

4.4. Single photo bioreactor cultivation on modified Bolds Basal Media

The nitrate and phosphate concentration were simulated in the synthetic Bolds Basal Media to simulate the nutrient content of the dairy farm effluent for the microalgae growth media being used in photo bioreactor PB1. This strategy allowed for a comparison run to assess and validate the effectiveness of the dairy farm effluent as a replacement growth media.

The total reactor working volume in this experiment was 11.5L which was inoculated with 2 L of the subculture volume with an initial cell concentration of 405 x 10^6 cells/ml. The initial concentration for the Nitrate-N was 182.35 mg/L whereas Phosphate-P was 162.6 mg/L.

The nutrient consumption as well as the growth profile is shown in Figure 4.13.

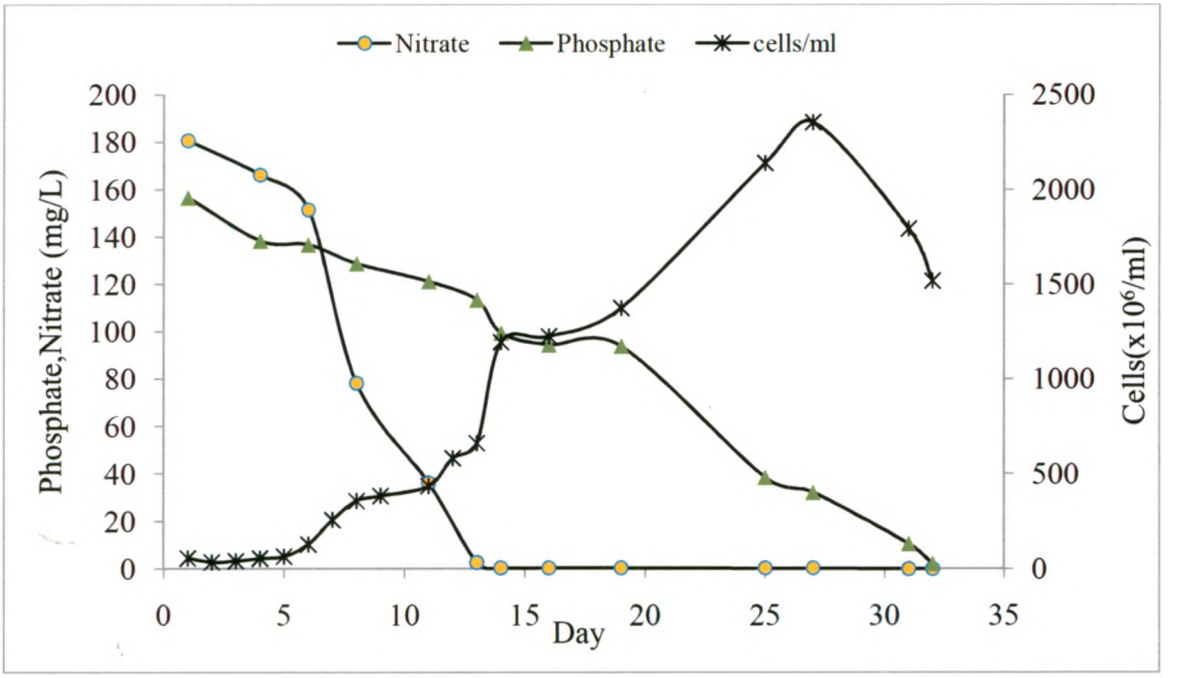


Figure 4.13: Growth and nutrient consumption profile

The microalgae were able to consume 100 % of the nitrate and 98 % of the initial phosphate concentration supplied over a period of 29 days. The total chlorophyll concentration in the harvested microalgae was 8.44 mg/L. The specific growth rate in this experiment was calculated to be 0.10 d^{-1}.

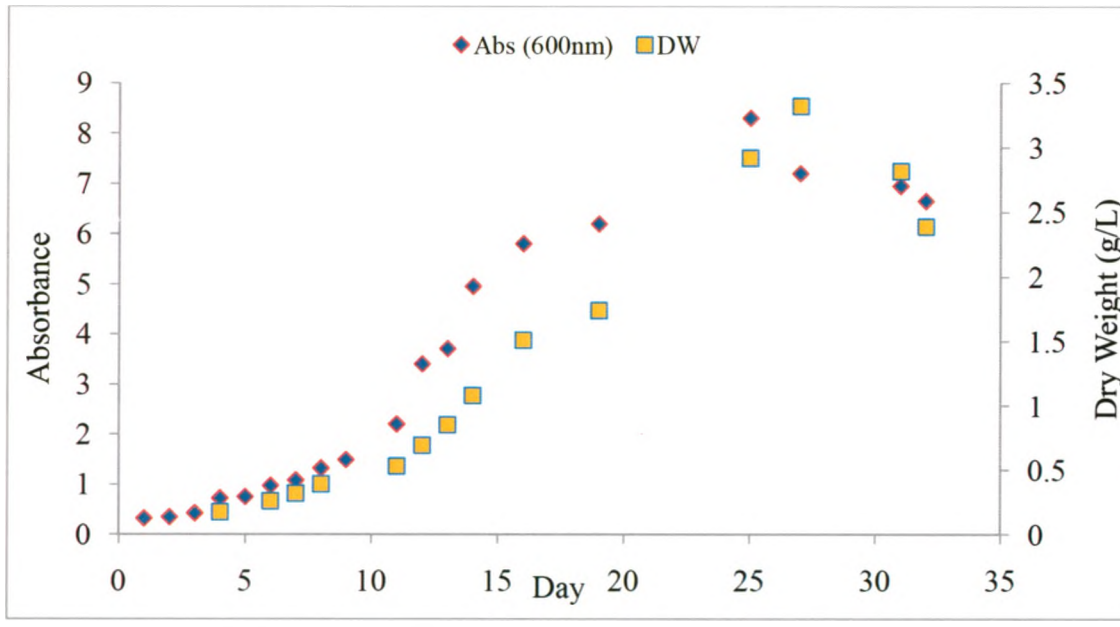


Figure 4.14: Dry weight and optical density

The high dose of Nitrogen and Phosphorous supplied and consumed led to a maximum dry weight of 3.25 g/L being achieved in just three weeks. This experiment had an average incident light value of 3980 lux (Figure 4.15) which, due to its high value had a synergistic effect on the growth.

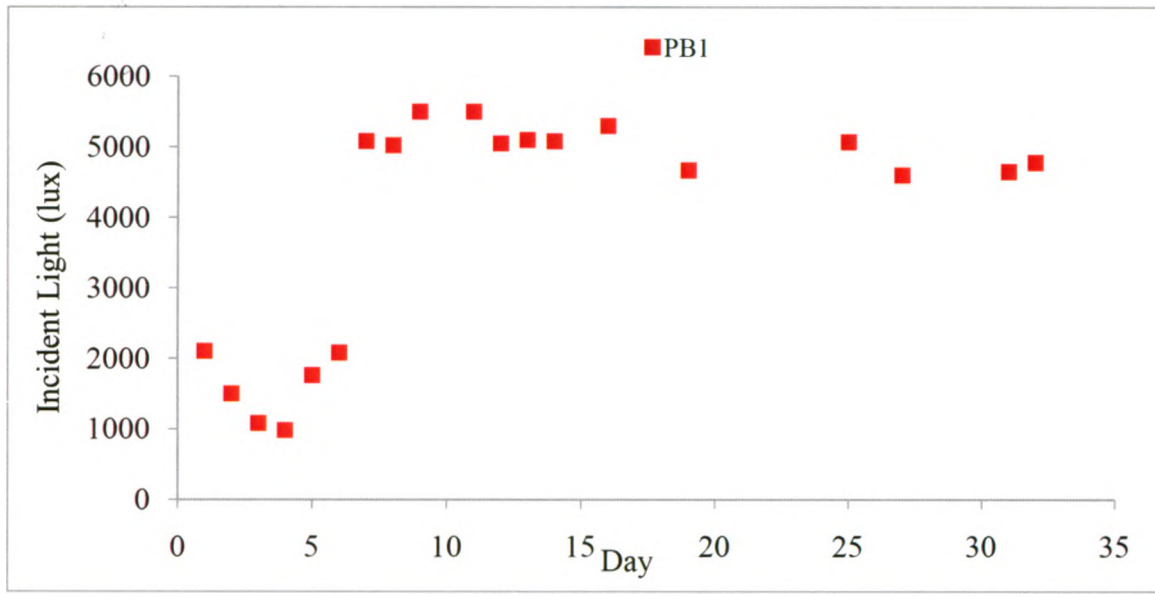


Figure 4.15: Incident light on PB1

Insitu transesterification on 4.8 g of lyophilized microalgae provided a biodiesel wt. % yield of 19.70 %.The FAME composition of the biodiesel prepared from the microalgae obtained is shown in Figure 4.16 . It can be thus seen that the triglycerides in microalgae are composed of C16:1, C18:0 C18:1, C18:2 and trace amounts of C22:0. This is very similar to the fatty acid composition of canola oil (Council 2008). Due to the operation of the photo bioreactor under nitrate limiting conditions in this experiment the FAME yield was significantly higher when compared to other bolds media experiments conducted.

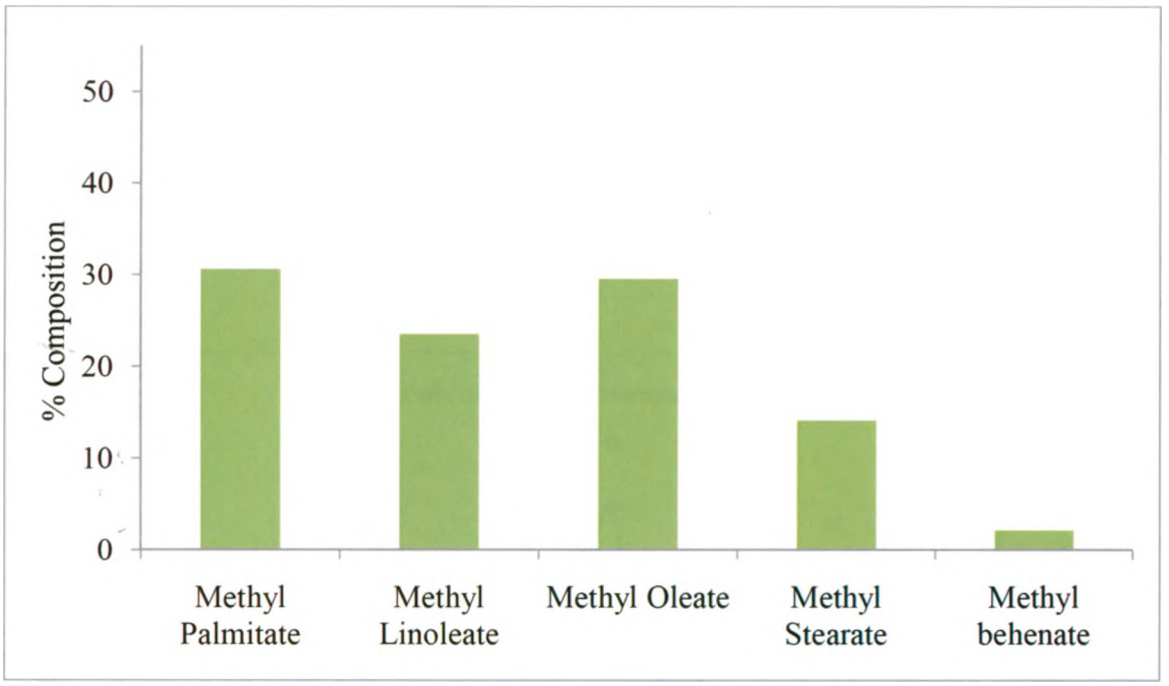


Figure 4.16: FAME profile for PB1, bolds media cultivation

The extent of the nutrient limited cultivation time and the complete uptake of the N and P from the Bolds Media directly translated into a high dry weight concentration and FAME content being achieved.

The FAME composition saw an increase in the synthesis of Methyl Palmitate (C16 chain length) at the expense of the Methyl Stearate (C18:0). There was trace amounts of C22 chain length, Methyl Behenate also produced.

4.5. Single PBR cultivation using Modified Bolds Media

The following experiment was conducted using 4% CO_2 with a total reactor working volume of 15 L in PB2. The initial Nitrate, ammonia and phosphate concentrations in this experiment were 35.87 mg/L, 1082.85 mg/L and 134.80 mg/L respectively. A subculture volume of 4 L was used as the innoculum with a microalgal cell concentration of 405 x 10^6 cells/ml. The biodiesel yield by wt. % was estimated to be 9.3 % with a composition that is shown in Figure 4.19.

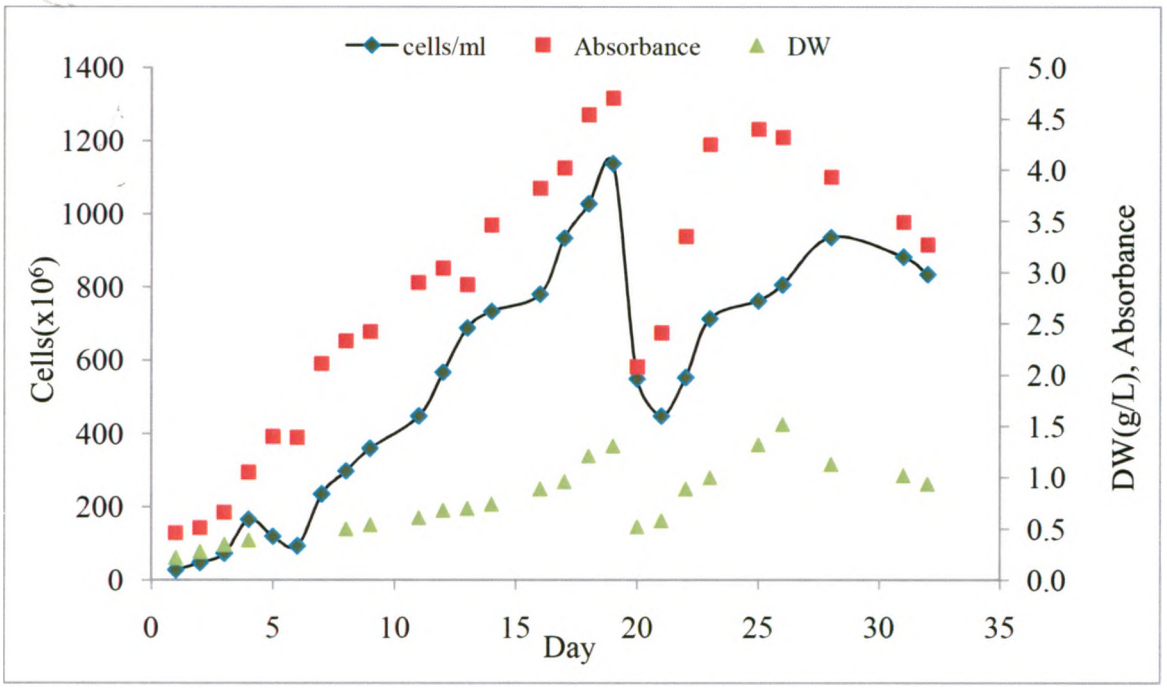


Figure 4.17: Cell growth and dry weight profile

The specific growth rate was estimated to have a value of 0.12 d^{-1}. The rate of nutrient consumption was exceptionally high during the first 15 days which translated into a very high cell culture density and dry weight during this period.

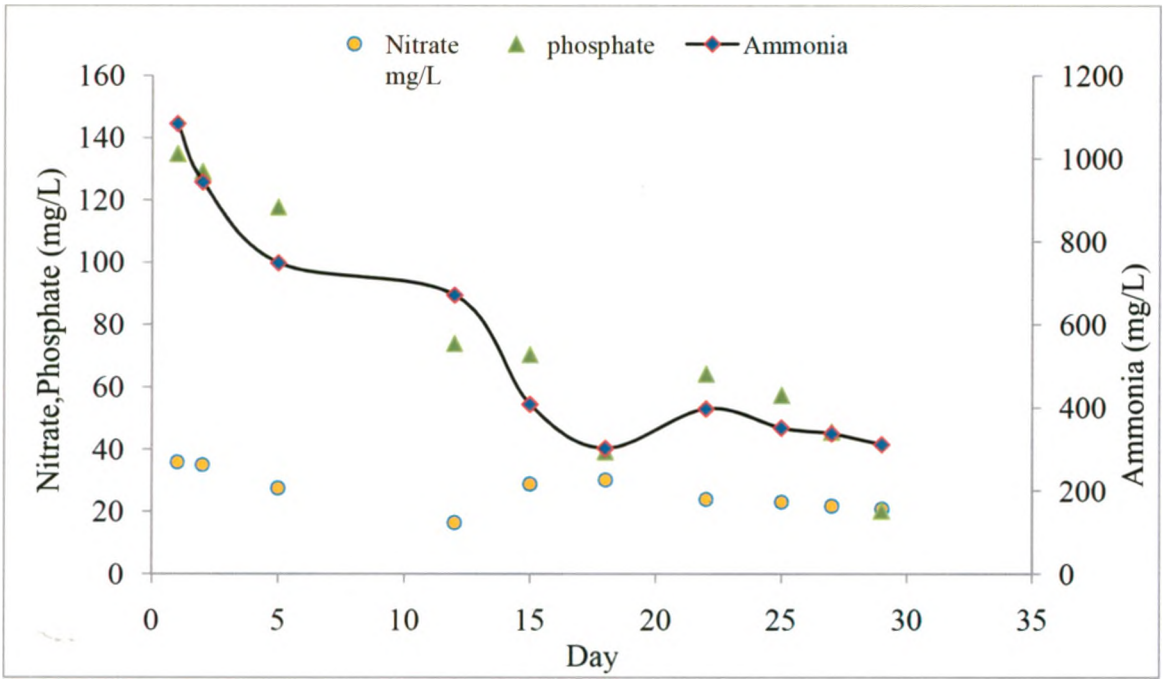


Figure 4.18: Nutrient consumption profile

The culture was able to uptake almost 80 % of the initial ammonia concentration over the course of the cultivation run. This uptake preference agrees with the nitrogen uptake mechanism preference for the thermodynamic stable form of N, which in this case was the Ammonia-N.

The average value for incident light during the course of this experiment was 4927 Lux which is well above the lower threshold limit. The average pH during the course of this run was 5.93 which can have detrimental effects on the microalgae culture, when compared to the other experiments carried out in Modified Bolds Media.

Although the culture saw exceptional growth and nutrient uptake over the first 15 days, there was however a loss of the microalgae culture volume overnight on Day 18. This culture volume loss was compensated by adding fresh Bolds Media to the Photo bioreactor. Following the addition of the media the microalgae culture reached a dry weight of 1.5 g/L.

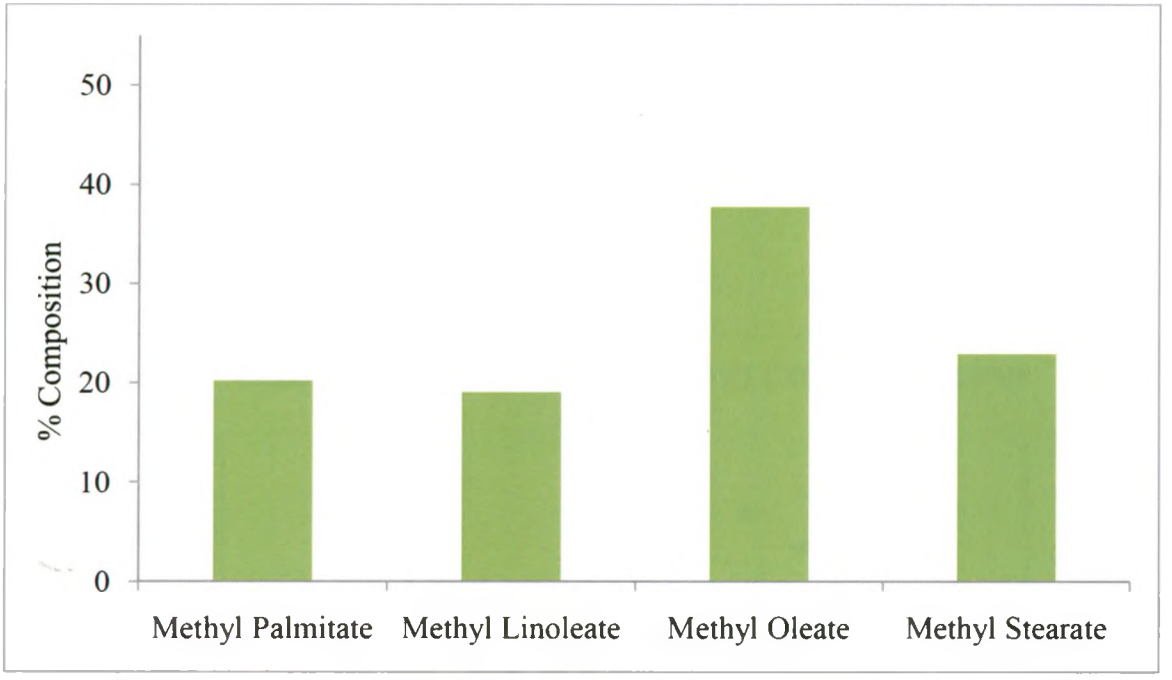
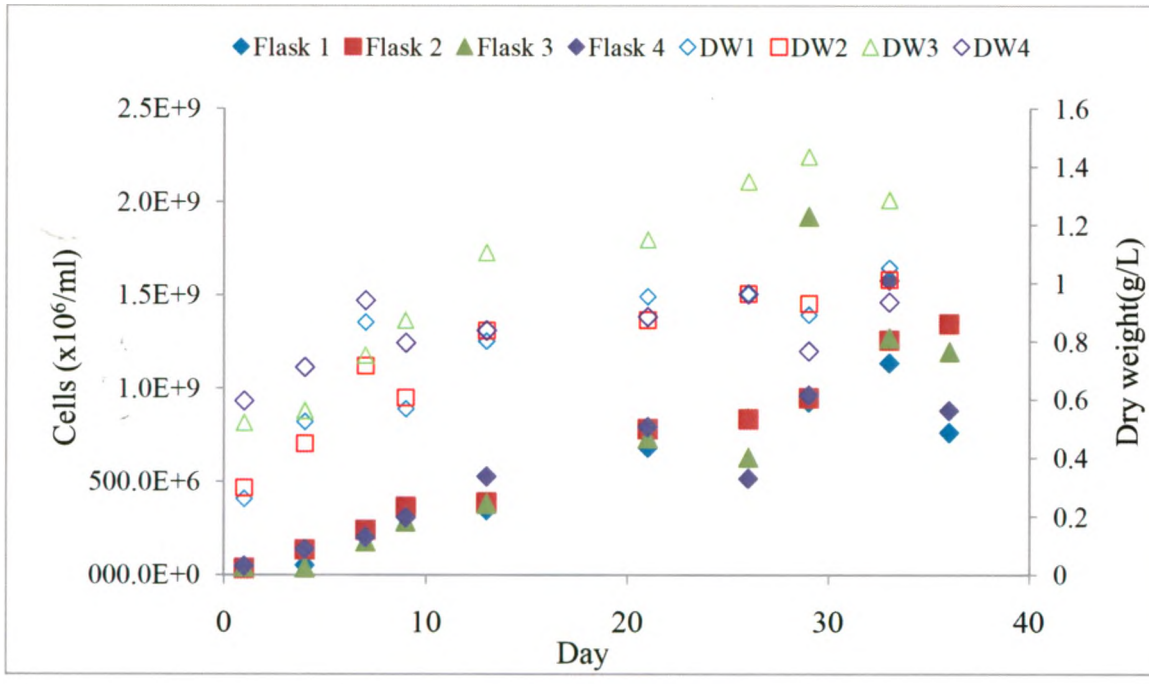


Figure 4.19: FAME profile for PB1 bolds media

The biodiesel yield was the lowest attained in this experiment. This could be explained on a basis of multiple factors. Factor one being that during the course of the PBR operation majority of the cell culture was lost due to foaming caused overnight after Day 15 and secondly due to the low value of pH during the course of the experiment

4.6. Heterotrophic Cultivation

In these set of experiments the microalgae were cultivated on glucose and peptone supplemented media as specified in Table 3.2.The initial microalgae cell concentration in each of the four flasks were approximately 40 x 10^6 cells/ml. The total chlorophyll concentrations in the four flasks were determined to be 7.36 mg/L, 12.03 mg/L,11.96 mg/L and 12.03 mg/L respectively for Flasks 1 - 4. The specific growth rate observed in the Glucose supplemented flask had an average value of 0.08 d^{-1}, whereas in the peptone supplemented culture flask the growth rate had an average value of 0.095 d^{-1}.



*DW denotes dry weight

Figure 4.20: Heterotrophic growth profile

The cell density achieved in these experiments was similar to the dairy farm effluent with comparable dry weights of microalgae obtained during the course of this culturing.

Table 4.3: Specific growth rates and dry weights for heterotrophic growth

Flask	μ (d^{-1})	Dry weight (g/L)
1	0.08	1.05
2	0.10	1.01
3	0.09	1.29
4	0.08	0.94

Insitu transesterification was carried out on lyophilized microalgae from Flasks 1 and 3 to provide a FAME profile for heterotrophic cultivation. For flask 1 *Insitu* transesterification was conducted using 0.21 g of dry microalgae and for Flask 3 using 0.31 g of dried Microalgal powder.

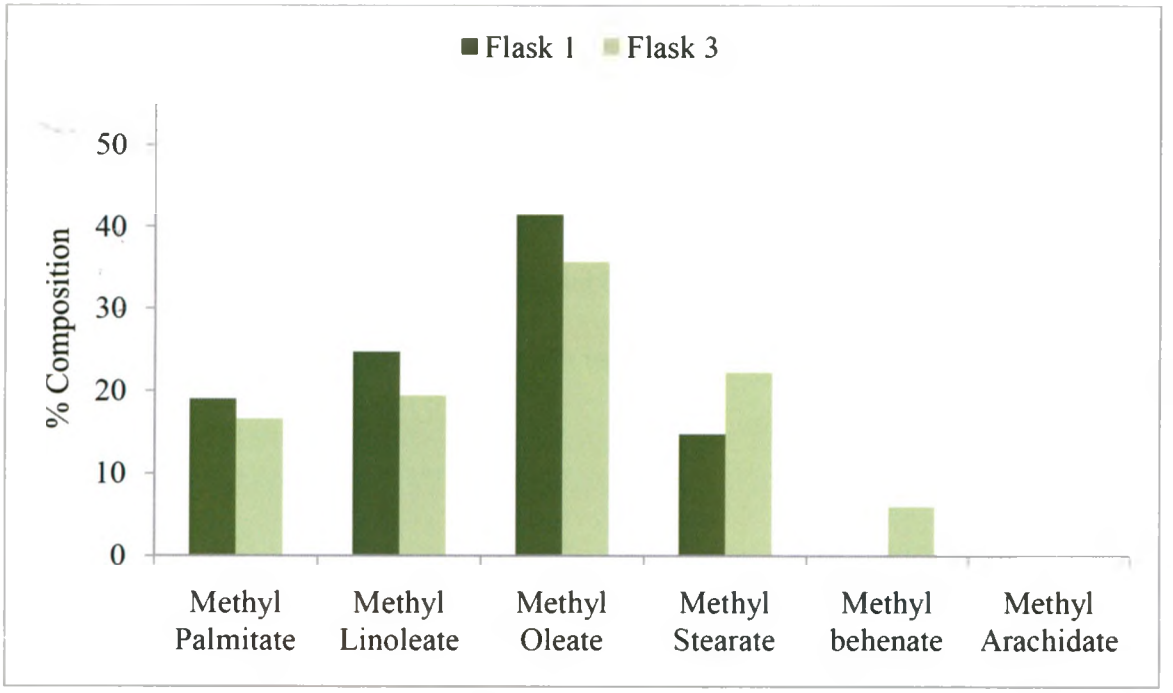


Figure 4.21: FAME profile of heterotrophically grown microalgae

Biodiesel yield by wt. % was calculated to be 17.1 % for microalgae grown on glucose as the carbon source whereas there was 21.2 % biodiesel yield obtained for microalgae cultivated using peptone. These findings were in close relationship to the data presented in Table 2.2. The FAME profile composition for the glucose and peptone grown microalgae had close resemblance. The peptone cultivated microalgae had trace amounts of the C22:0 chain length fatty acid in addition to the C16 - C18 chain length fatty acids.

4.7. *Insitu* transesterification of canola oil

To compare and validate the efficiency of transesterification, canola oil was used as a substrate in similar gram weights as the microalgae. It should be noted that canola oil is 100 % triglycerides whose fatty acid profile is well researched. This reaction served as a means of direct comparison of the microalgal FAME profile to that of Canola oil derived FAME. The reaction was carried out in the same reaction vessel set up as shown in Figure 3.10 using 5.06 g of canola oil. The FAME composition profile is shown in Figure 4.22.

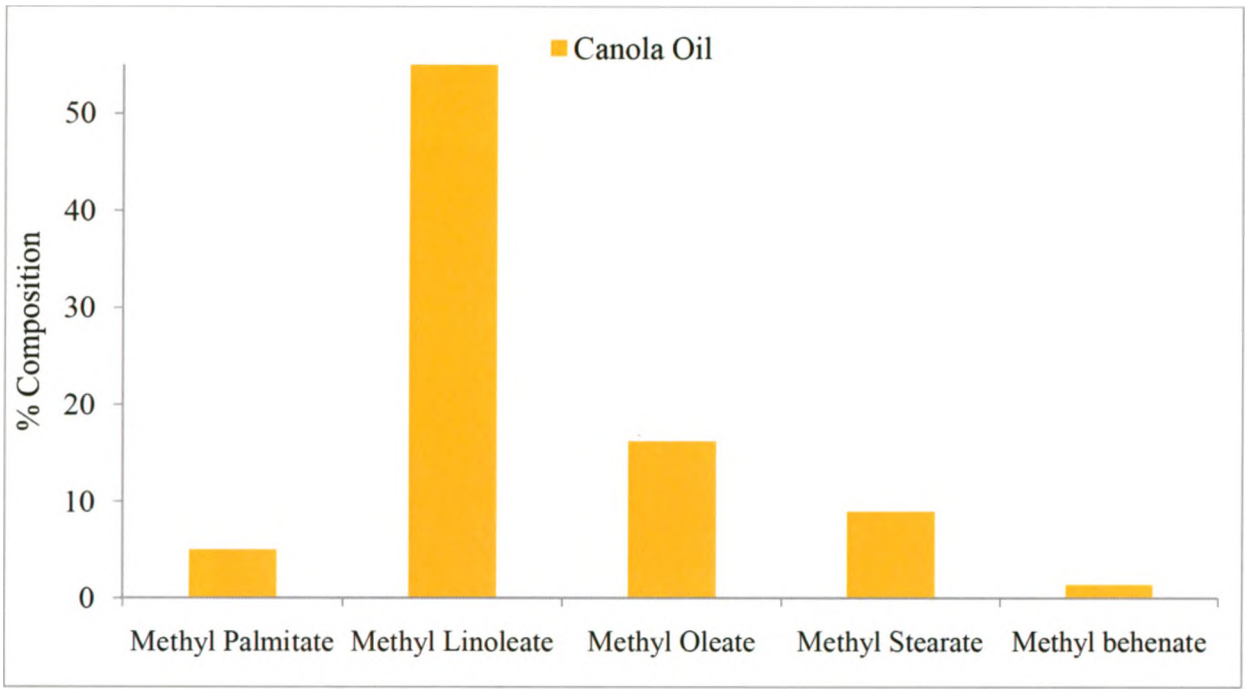


Figure 4.22: Biodiesel composition prepared from canola oil

The biodiesel yield achieved in 5 hours was 51.87 %. The FAME profile quantification obtained using the calibration curves prepared is very similar to the FAME profile as reported by (Nucheckprep 2009).

4.8. Hydrolysis of *Chlorella vulgaris* using cellulase enzyme

As specified in Section 3.13, the hydrolysis of the *Chlorella vulgaris* cell by cellulase was investigated by carrying out hydrolysis using six different enzyme concentrations at 30, 35 and 40 °C. From the results obtained it was concluded that a temperature between 35 °C and 40 °C was best for carrying out the hydrolysis.

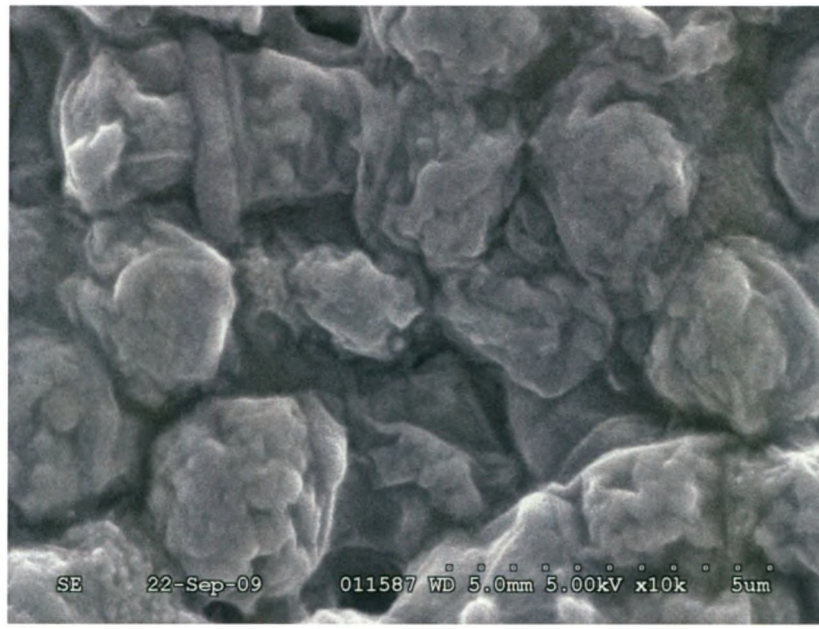


Figure 4.23: Post enzymatic hydrolysis *Chlorella vulgaris*

The degrading effect of cellulase on the microalgae cells can be seen in the SEM image shown in Figure 4.23 above, when compared to Figure 2.1 .

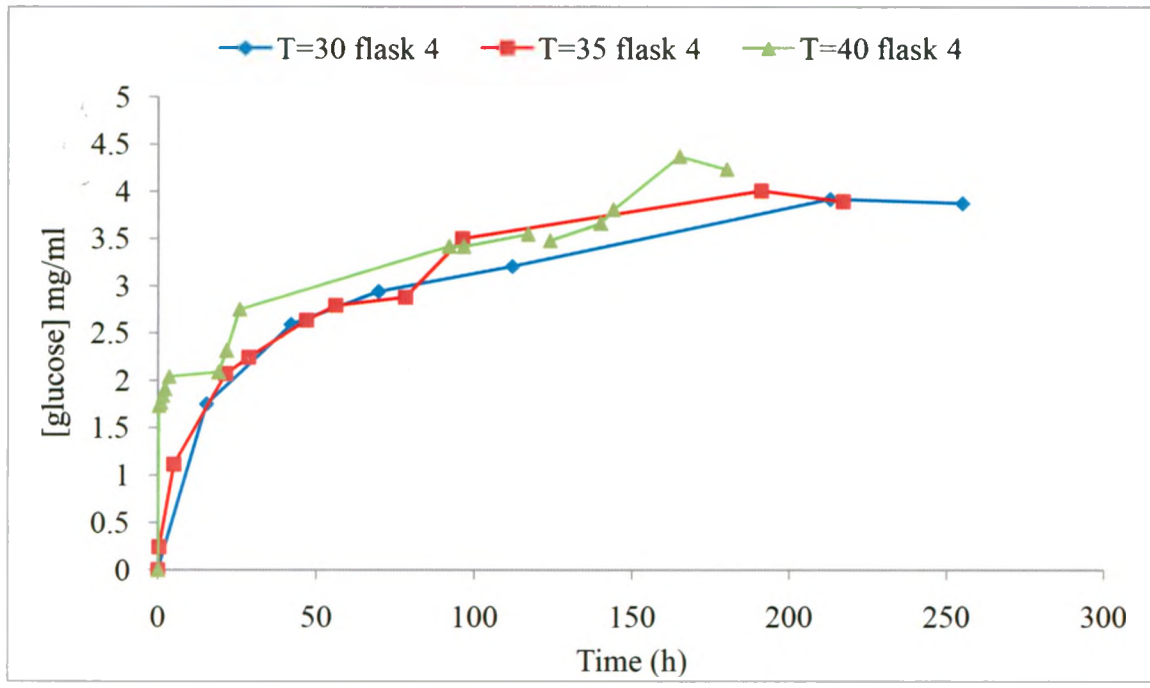


Figure 4.24: Time course profile for glucose production in Flask 4

The shaker flasks were terminated when the production of glucose reached a plateau as seen in the figure above. It can be seen that all three flasks were able to reach approximately the same concentration of glucose being produced for once particular enzyme loading. The yields were calculated on a (by weight) basis of microalgae used which was kept at a constant 55 ± 2 mg. Similar trends in production of glucose were seen for the other flasks as well, data shown in Section A.2.

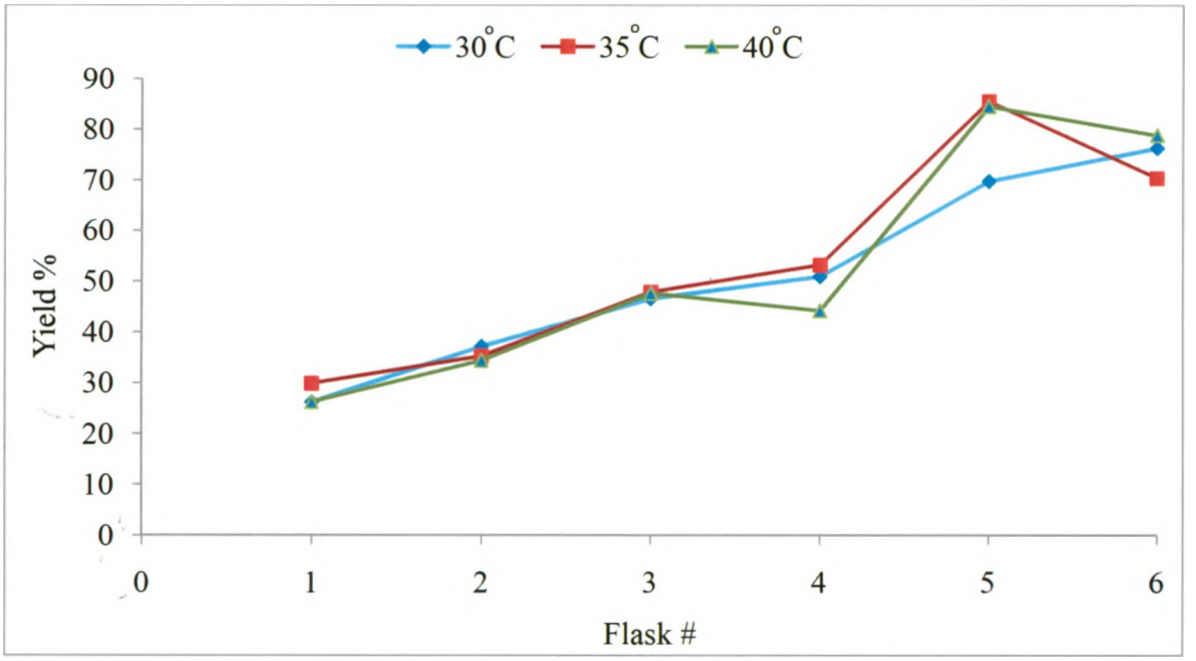


Figure 4.25: Yield profile obtained for different enzyme loadings and temperatures

From the results summarized in the figure above it can be concluded that the desired temperature for carrying out the hydrolysis lies between 35 - 40 °C. There was no direct correlation observed with temperature however the enzyme loading had a linear relationship to the % yield obtained in each of the three runs.

5. Conclusions

The work presented in this thesis leads to an understanding into the use of microalgal biomass as a biodiesel feedstock. The plant and algal photosynthesis process is what drives the biological carbon cycle. The entire process from growth to downstream processing of the microalgae was looked at. The work conducted in regards to the cultivation provide proof that the microalgae *C. vulgaris* (UTEX 2714) is capable of growing on anaerobically digested dairy farm effluent. The microalgal biomass production is a bottleneck in making the process of triglyceride conversion to biodiesel, economical.

The results also show the capability of this microalgae to be cultivated using concentrations of CO_2 as high as 4%. The growth of *C. vulgaris* showed various growth phases such as lag, exponential, linear, stationary and declining phases. The addition of EDTA served as a chelating agent in the growth media and makes the necessary nutrient metals readily available to the microalgae when using modified bolds media. Also large scale microalgae cultivation potential was shown in twin photo bioreactor configurations with a total of 32 L operating volume. The microalgae cultivation times varied from 14 days to 29 days during which the potential for microalgae as a waste water remediation and a triglyceride source was demonstrated. The microalgae were capable of removing 80.3 - 83.2 % of the NO_3^--N, 100 % of the NH_4^+ - Nitrogen, 39.8 - 45.1 % of the initial phosphate and 52.8 - 65.4 % of the initial COD. The final concentrations in each of the photo bioreactors were reduced from an initial value of 20.8 mg/L, 177.1 mg/L, 117 mg/L and 640 mg/L to 4.1 mg/L, 0 mg/L, 64.2 mg/L and 210 mg/L for NO_3^- N, NH_4^+ -Nitrogen, Phosphate and COD.

The microalgae culture was also able to consume 1% (mole %) of the CO_2 supplied to it. These remediation benefits were achieved in addition to the production of microalgal biomass which contained 9.3 % to 26.7 % biodiesel (dry weight). The biodiesel FAME profile which was obtained was similar to that of canola oil. The major fatty acid methyl esters present in the microalgal biodiesel were Methyl Palmitate, Linoleate, Oleate, and Stearate.

The issues due to light limitation were exemplified in the culturing of UTEX 2714 in these experiments. The clarifying efficiency of the pre treatment solution is a main factor in controlling the amount of light transmitted through the culture. A direct relationship was observed with the nitrate consumption and cell growth. However the culture that were cultivated in nitrogen deficiency had better biodiesel yield which translates into better triglyceride accumulation. Acid catalyzed *Insitu* processing method can be employed in industrial applications as well. This is due to the fact that unlike base catalyzed transesterification, there is no soap formation even if the microalgae contains higher moisture content than the freeze dried matter.

6. Recommendations for Future Work

In summary, the results presented in this thesis demonstrate the fact that UTEX 2714 can be grown on anaerobically digested dairy farm effluent. The properties of this microalgae makes it an ideal candidate for waste water remediation. This is done by stripping the digested effluent off of the N, P content. However, further improvement needs to be made in designing a pre-treatment process that is continuous and least energy intensive as possible.

Although the microalgae growth and harvesting stages were streamlined for continuous operation in this work, the extraction and conversion step still need further intensive research for the possible development of a continuous system capable of extracting triglycerides and separation of biomass in one step.

The production of glucose from this microalgae demonstrates the potential application of microalgae as a feedstock for bio ethanol or bio butanol fermentation. Although the Cellulase enzyme employed in this study was obtained from Sigma Aldrich, USA; the fungus *Aspergillus niger* can be cultivated on the dairy farm manure to produce the enzyme. This would greatly offset the costs associated with this process.

The fact that this research used freeze dried microalgae with less than 1% moisture content for all downstream extraction experiments , would not be economically feasible in a full scale industrial process setting. Therefore there is a need for a drying method which is scalable.

Further study is needed to develop various strategies (such as nitrogen starvation) to improve the algal fatty acid content while maintaining a high biomass yield.

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A. APPENDICES

A.1. Photon flux measurements

These calculations were carried out for wavelengths 400 nm to 700 nm. Sample calculation for a single wavelength is shown below.

N_A= Avogadro number

h= Planck's constant

λ (nm) = 400

Irradiance (W/m^2) or J/m^2 sec = $2.91x10^{-3}$

Energy of photon (J) = $\frac{hc}{\lambda} = \frac{6.634x1^{-34}*3x10^8}{400x1^{-9}} = 4.97x10^{-19}$

Number of photons = $\frac{Irradiance}{Energy\ of\ photon} = 5.85x10^{15}$

Moles of photons = $\frac{Number\ of\ photons}{N_A} = 9.71x10^{-9}$

Once the moles of photons were estimated for each wavelength, Simpsons rule was applied to estimate the moles of photons obtained under the spectra (μ Einsteins/m^2s).

A.2. Glucose production from microalgae

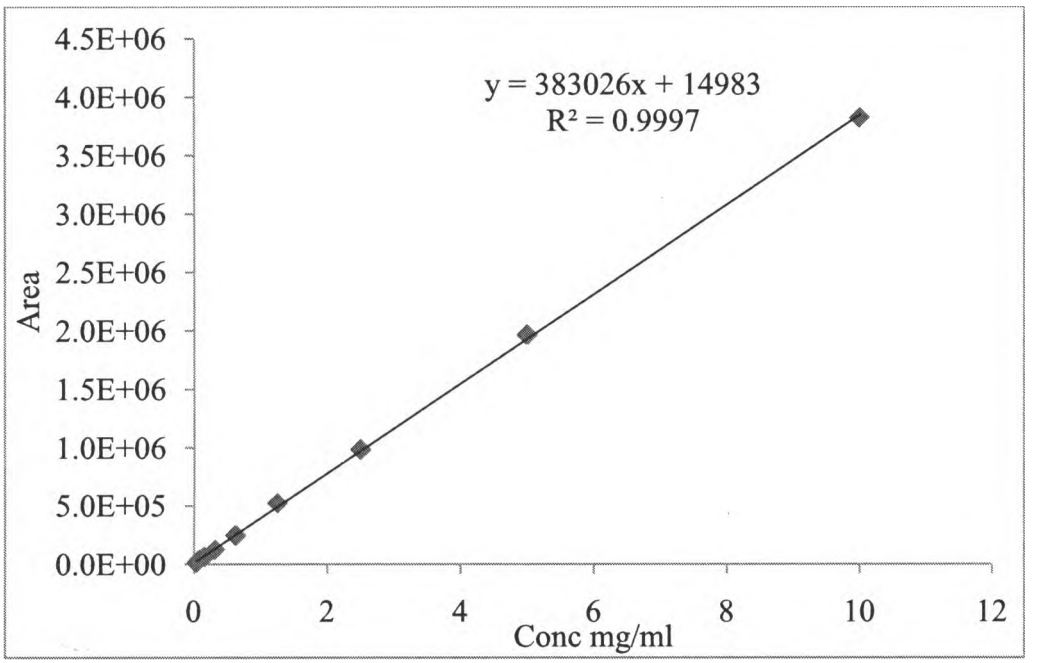


Figure A.1: Glucose calibration curve obtained using Aminex 87H column.

Samples were analyzed for glucose by high performance liquid chromatography using an Aminex HPX-87H column at 65 °C. The mobile phase was 9 mM H_2SO_4 at flow rate of 0.6 ml/min.

Table A1: Raw data for HPLC calibration

C (mg/ml)	Average Area
10	3.82E+06
5	1.97E+06
2.5	9.83E+05
1.25	5.23E+05
0.62	2.47E+05
0.31	1.23E+05
0.15	6.28E+04
0.078	3.81E+04
0.039	1.45E+04

A.2.1. Weights of microalgae and enzyme units used in each of the six shaker flasks

Table A2: Case I @ 30°C

Flask	Algae (mg)	Cellulase (units)
1	68.5	27.15
2	61.3	38.4
3	62.8	48.4
4	61.3	57.65
5	60.4	68.3
6	62.6	78.05

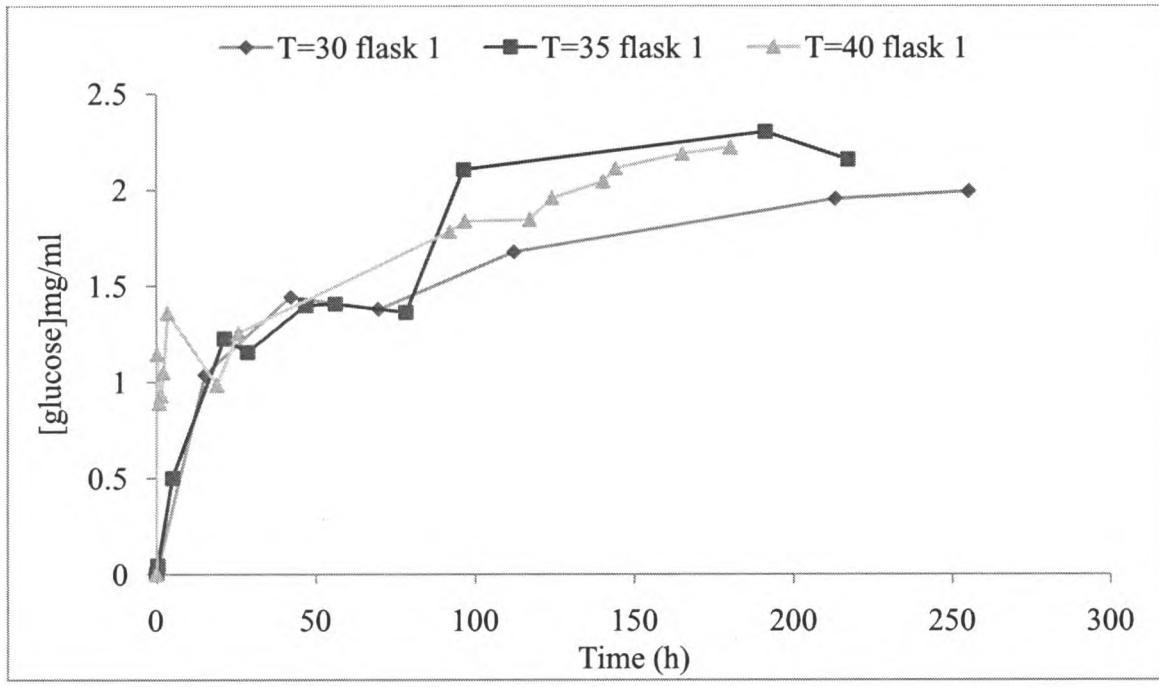
Table A3: Case II: 35°C

Flask	Algae (mg)	cellulase (units)
1	54.2	29.95
2	54.3	36.4
3	57.2	46.25
4	54.9	57.1
5	55.6	65.85
6	55.4	76.15

Table A4: Case III : 40°C

Flask	Algae (mg)	cellulase (units)
1	53.9	28.45
2	51.3	36.45
3	53.3	45.4
4	54.4	55.15
5	53.1	66.05
6	55.7	76.05

A.2.2. Time course concentration profiles for Flasks 1-6



FigureA2: Flask 1 concentration profile for different temperatures

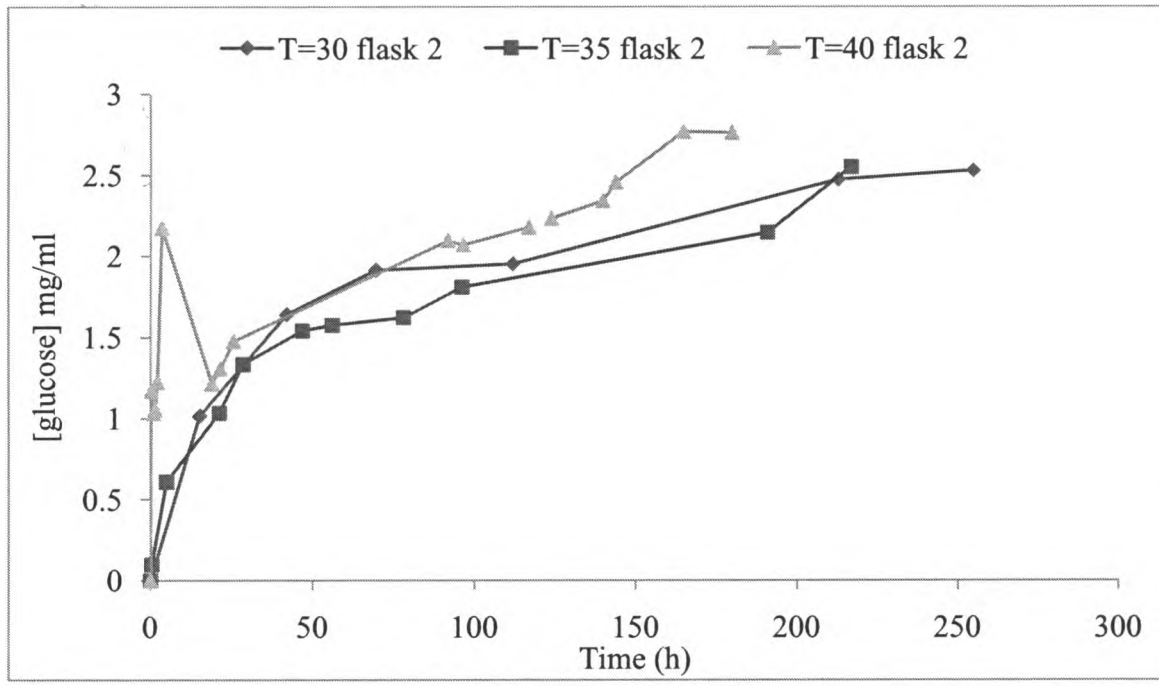


Figure A3: Flask 2 concentration profile for different temperatures

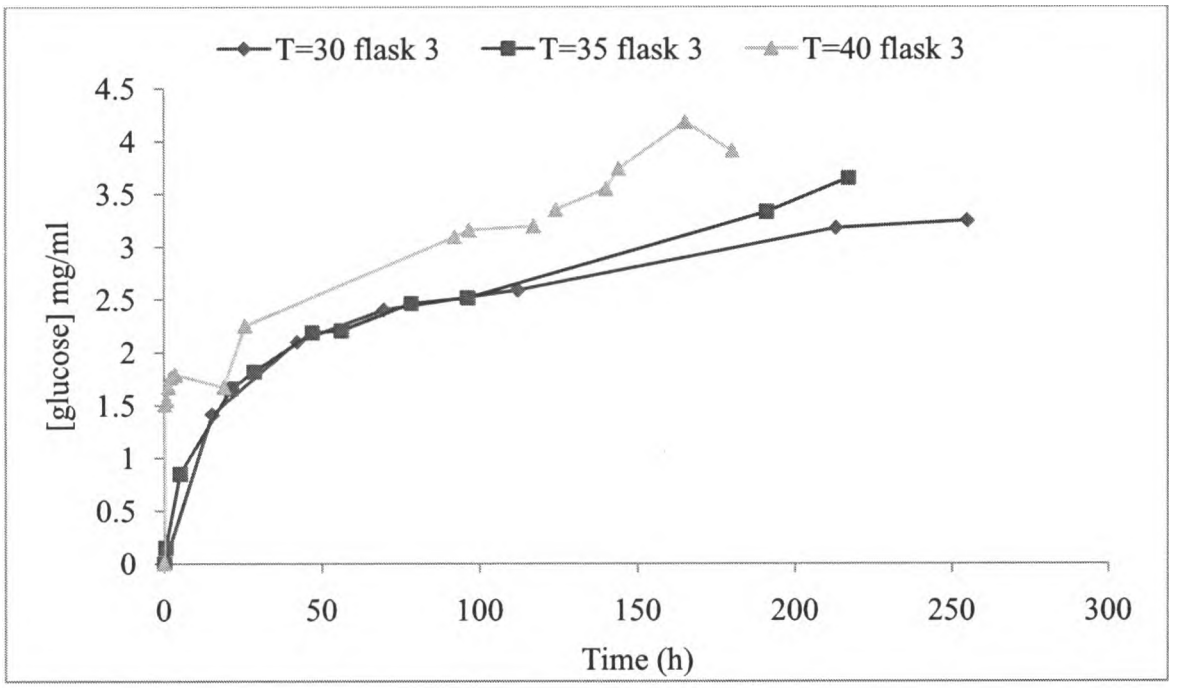


Figure A4: Flask 3 concentration profile for different temperatures

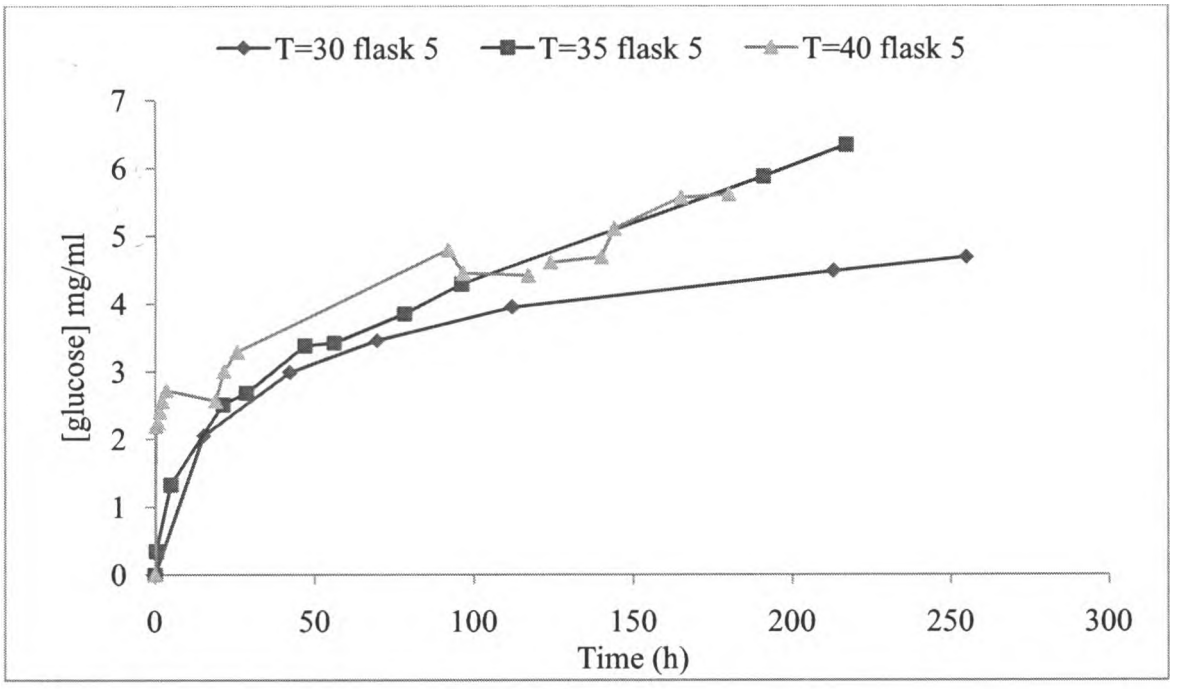


Figure A5: Flask 5 concentration profile for different temperatures

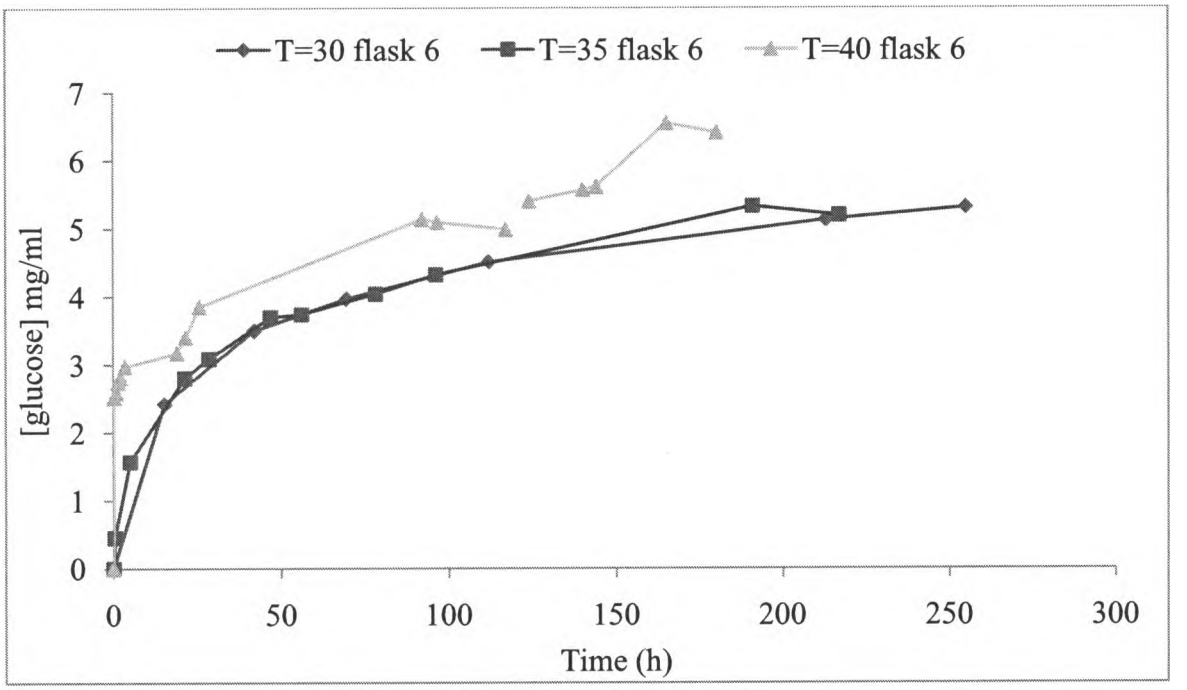


Figure A6: Flask 6 concentration profile for different temperatures

A.2.3. Concentration profile data

The concentration profile data for each of the six flasks in all three cases plotted above is shown below

Case I: 30 °C

Table A5: Flask 1

time	Conc.
0	0
15.3	1.03
42.1	1.44
69.6	1.37
112	1.67
213	1.95
255	1.99

Table A6: Flask 2

time	Conc.
0	0
15.3	1.01
42.1	1.64
69.6	1.91
112	1.95
213	2.47
255	2.52

Table A7: Flask 3

time	Conc.
0	0
15.3	1.41
42.1	2.10
69.6	2.40
112	2.58
213	3.17
255	3.24

Table A8:Flask 4

time	Conc.
0	0
15.3	1.75
42.1	2.59
69.6	2.94
112	3.21
213	3.92
255	3.87

Table A9: Flask 5

time	Conc.
0	0
15.3	2.05
42.1	2.99

69.6	3.45
112	3.95
213	4.47
255	4.68

Table A10: Flask 6

time	Conc.
0	0
15.3	2.42
42.1	3.49
69.6	3.96
112	4.51
213	5.12
255	5.30

Case II: 35°C

Table A11: Flask 1

Time	Conc.
0	0
0.4	0.046
5	0.50
21.3	1.23
28.6	1.15
46.9	1.39
56.1	1.41
78.2	1.36
96.2	2.11
191	2.30
217	2.15

Table A12: Flask 2

Time	Conc.
0	0.00
0.4	0.10
5	0.61
21.3	1.03
28.6	1.33
46.9	1.54
56.1	1.58
78.2	1.62
96.2	1.81
191	2.15
217	2.55

Table A13: Flask 3

Time	Conc.
0	0.00
0.4	0.15
5	0.85
21.3	1.66
28.6	1.82
46.9	2.19
56.1	2.21
78.2	2.46
96.2	2.52
191	3.33
217	3.65

Table A14: Flask 4

Time	Conc.
0	0.00
0.4	0.24
5	1.11
21.3	2.07
28.6	2.25
46.9	2.64
56.1	2.79

78.2	2.88
96.2	3.50
191	4.01
217	3.89

Table A15: Flask 5

Time	Conc.
0	0.00
0.4	0.35
5	1.33
21.3	2.51
28.6	2.68
46.9	3.38
56.1	3.42
78.2	3.85
96.2	4.29
191	5.88
217	6.34

Table A16: Flask 6

time	Conc.
0	0
0.4	0.45
5	1.56
21.3	2.79
28.6	3.08
46.9	3.69
56.1	3.73
78.2	4.07
96.2	4.32
191	5.32
217	5.19

Case III: 40°C

Table A17: Flask 1

time	Conc (mg/ml)
0	0
0.25	1.14
0.9	0.89
1.5	0.93
2.2	1.04
3.5	1.35
19	0.98
25.7	1.25
92	1.78
96.6	1.83
117	1.84
124	1.95
140	2.04
144	2.11
165	2.18
180	2.21

Table A18: Flask 2

time	conc
0	0.00
0.25	1.17
0.9	1.03
1.5	1.05
2.2	1.22
3.5	2.17
19	1.21
21.6	1.30
25.7	1.48
92	2.10
96.6	2.07
117	2.18
124	2.23
140	2.34
144	2.45

165	2.77
180	2.761084

Table A19: Flask 3

time	Conc.
0	0.00
0.25	1.51
0.9	1.55
1.5	1.67
2.2	1.77
3.5	1.79
19	1.67
25.7	2.25
92	3.10
96.6	3.16
117	3.20
124	3.35
140	3.55
144	3.74
165	4.18
180	3.91

Table A20: Flask 4

time	Conc.
0	0
0.25	1.73
0.9	1.77
1.5	1.84
2.2	1.91
3.5	2.04
19	2.09
21.6	2.32
25.7	2.75
92	3.42
96.6	3.41
117	3.55
124	3.48

140	3.66
144	3.81
165	4.37
180	4.23

Table A21: Flask 5

time	Conc.
0	0
0.25	2.20
0.9	2.25
1.5	2.41
2.2	2.56
3.5	2.72
19	2.57
21.6	3.00
25.7	3.29
92	4.79
96.6	4.45
117	4.41
124	4.61
140	4.69
144	5.11
165	5.56
180	5.61

Table A22: Flask 6

time	conc.
0	0
0.25	2.52
0.9	2.59
1.5	2.74
2.2	2.81
3.5	2.97
19	3.17
21.6	3.40
25.7	3.85
92	5.14
96.6	5.09

117	4.99
124	5.40
140	5.57
144	5.61
165	6.55
180	6.41

A.3. Triglyceride Analysis

The calibration curve for triolein is shown in Figure A.2. The curve is based on the average value of the triplicate injections which were carried out.

Equation A.1: Triolein standard calibration equation

$$Y=0.0087x-5.9396$$

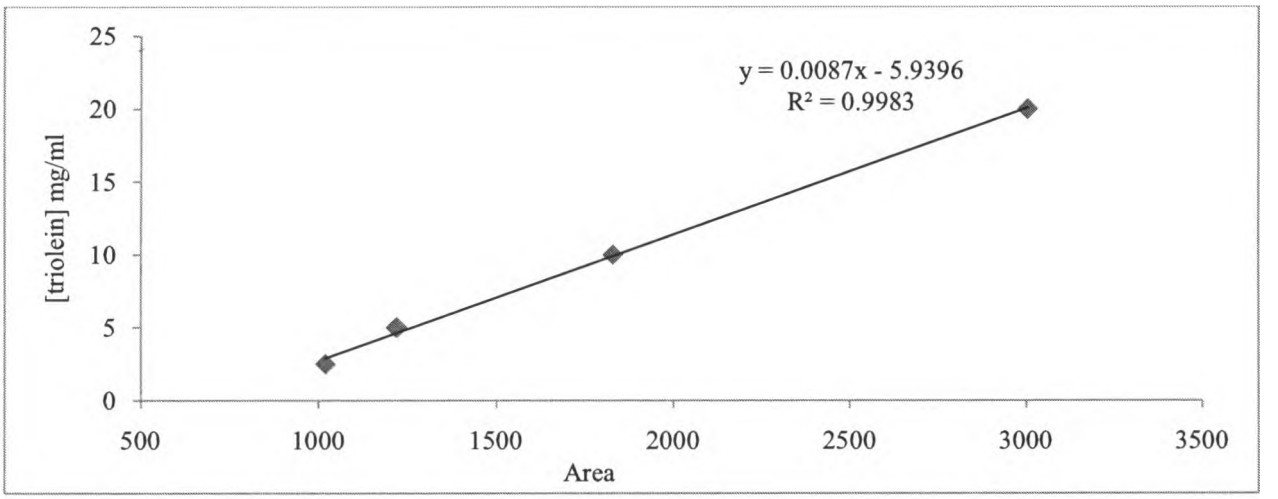


Figure A.2: Triolein calibration curve

A.4. Sample Calculations for the specific growth rate

The following calculation are presented for the specific growth rates in Figure 4.1.

T=13days; X_o=1.9x10^6cells ;X=24 x10^6cells

X/X_o=12.63

$Ln(X/X_o)$ =2.54

→μ=2.54/12

= 0.21d^{-1}

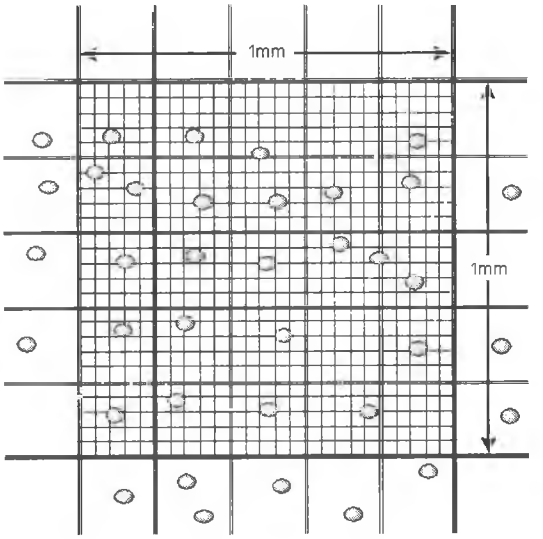


Figure A.3: Hemocytometer grid for cell counts

Source: http://www.di.uq.edu.au/sparq/images/grid.jpg

Triglyceride analysis Data PBR

Peak area obtained was converted to provide the corresponding concentration by using Equation A.1.

Algae, mg	Sample label	Peak Area	Conc. mg/ml	V_{CHCl3}	Conc. mg	Yield %	Average yield %
463.9	Sample B1	1935.85	10.90	6.2	67.59	14.57	17.66
451.4	Sample B2	2262.9	13.75	6.2	85.24	18.88	
421.6	Sample B3	2207.9	13.27	6.2	82.27	19.51	

Calculation for specific growth rate for Figure 4.2.

T=19days; X_o=2.5x10^6cells; X=35 x10^6cells

X/X_o=32.71

$Ln(X/X_o)$ =3.48

→μ=3.48/19

= 0.18d^{-1}

Triglyceride Analysis Data PBR

Peak area obtained was converted to provide the corresponding concentration by using Equation A.1.

Algae, mg	Sample label	Peak Area	Conc. mg/ml	V_{CHCl3}	Conc. mg	Yield %	Average yield %
300.5	Sample B10	1700	8.85	7	61.95	20.62	22.85
300.2	Sample B11	1918.8	10.75	7	75.28	25.08	

Pre treated effluent Tubes data

Peak area obtained was converted to provide the corresponding concentration by using Equation A.1.

Triglyceride analysis Data, tubes 2%CO_2

Algae, mg	Sample label	Peak Area	Conc. mg/ml	V_{CHCl3}	Conc. mg	Yield %	Average yield %
162	Sample B4	1260	5.02	6.00	30.13	18.60	22.88
147	Sample B5	1370.62	5.98	6.00	35.91	24.43	
162	Sample B6	1477.71	6.92	6.00	41.50	25.62	

Sample Calculation for specific growth rate for 2% CO_2 tube experiment.

2%CO_2 tube 1

T=22days; X_o=2.78x10^6cells; X=172x10^6cells

X/X_o=64.03

$Ln(X/X_o)$ =4.15

→μ=4.15/22

= 0.18d^{-1}

Sample Calculation for specific growth rate for 4% tube.

4%CO_2 tube 1

T=22days; X_o=2x10^6cells; X=864x10^6cells

X/X_o=432

$Ln(X/X_o)$ =6.06

→μ=6.06/22

= 0.27d^{-1}

ICP Analysis of metal concentration

Table A.1: ICP analysis on tubes 1 and 5

All the following values are reported in ppm.

Elements	Tube 1- 5.25g/L Alum	Tube 5 - 3.5g/L Alum	Tube 1 Final	Tube 5 Final	Detection Limit
Al	11.3	0.52	0.67	0.63	**0.04**
B	1.2	0.92	1.9	0.93	**0.02**
Ca	131	87.5	71.6	65.7	**0.001**
Cr	0.05	0.04	0.05	0.03	**0.01**
Cu	0.19	0.08	0.19	0.08	**0.01**
Fe	0.42	0.07	0.15	0.03	**0.01**
Ir	0.04	ND	0.03	ND	**0.02**
K	351	365	274	304	**0.2**
Mg	115	111	60.5	80.9	**0.001**
Mn	0.74	0.39	0.39	0.25	**0.002**
Ni	0.04	0.04	0.04	0.03	**0.01**
Zn	0.53	0.08	1.6	1.1	**0.01**

A.5. Biodiesel Calibration curves

Table A.2: Calibration curve equations used for biodiesel characterization

Species	mX	c
Methyl palmitate	0.204	-620.16
Methyl linoleate	0.165	-565.7
Methyl oleate	0.327	-2591
Methyl stearate	0.194	-378
Methyl behenate	0.261	-186.8

The graphs presented below represent the average of three injections/sample.

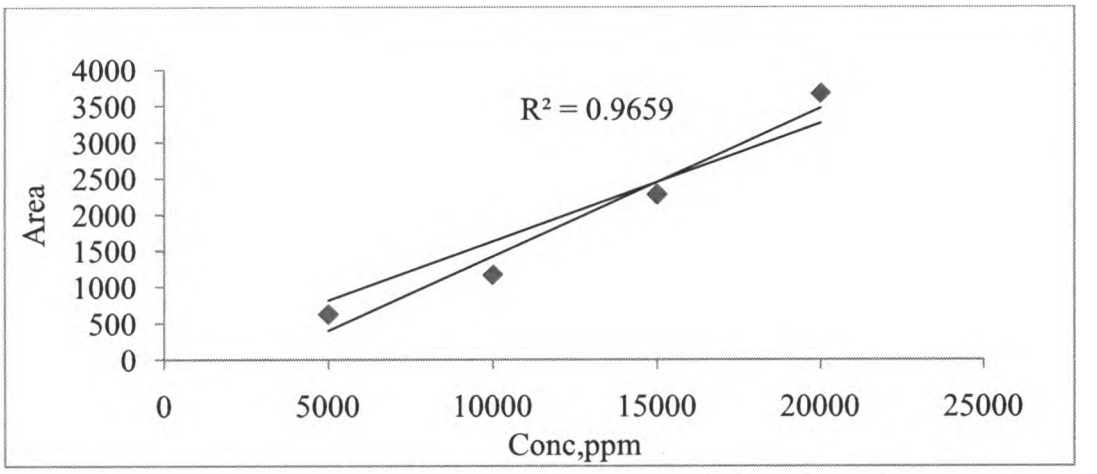


Figure A7: Methyl Palmitate calibration curve

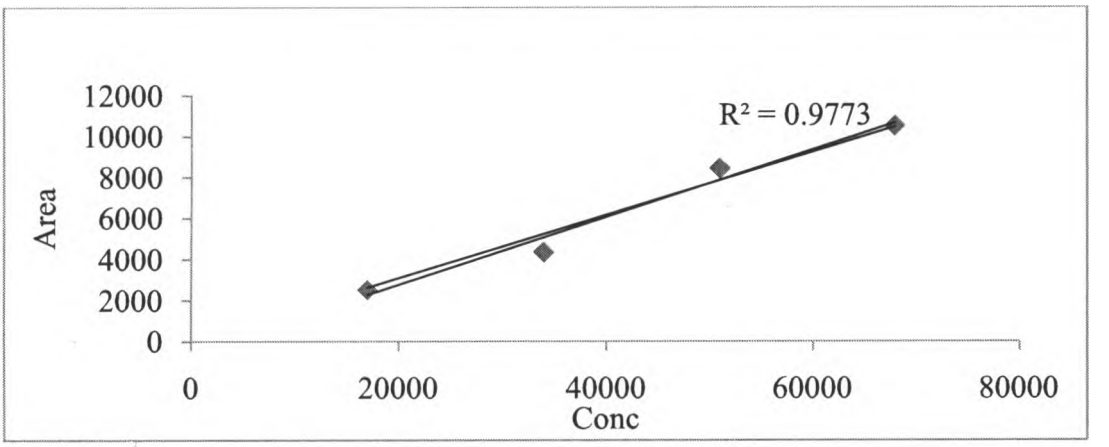


Figure A8 : Methyl Linoleate calibration curve

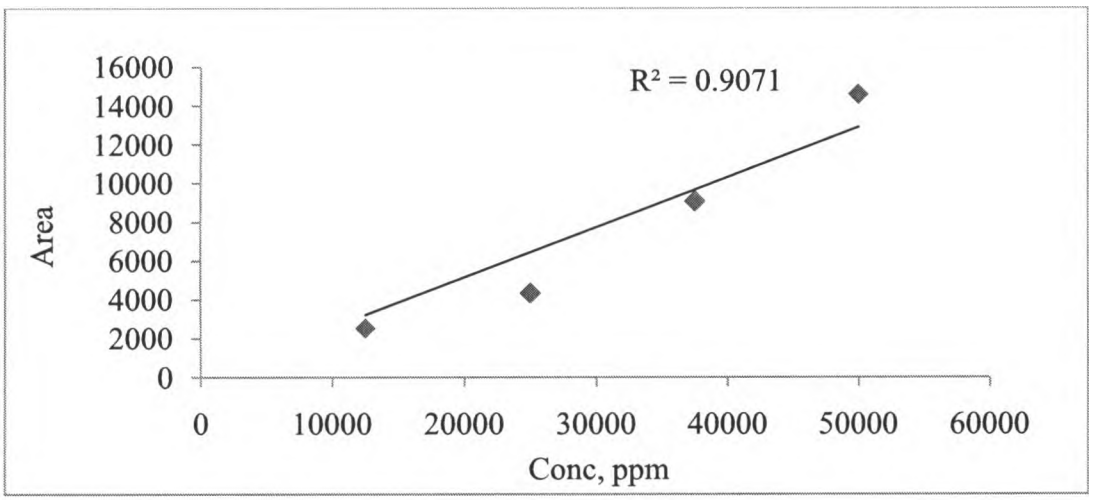


Figure A9 :Methyl Oleate calibration curve

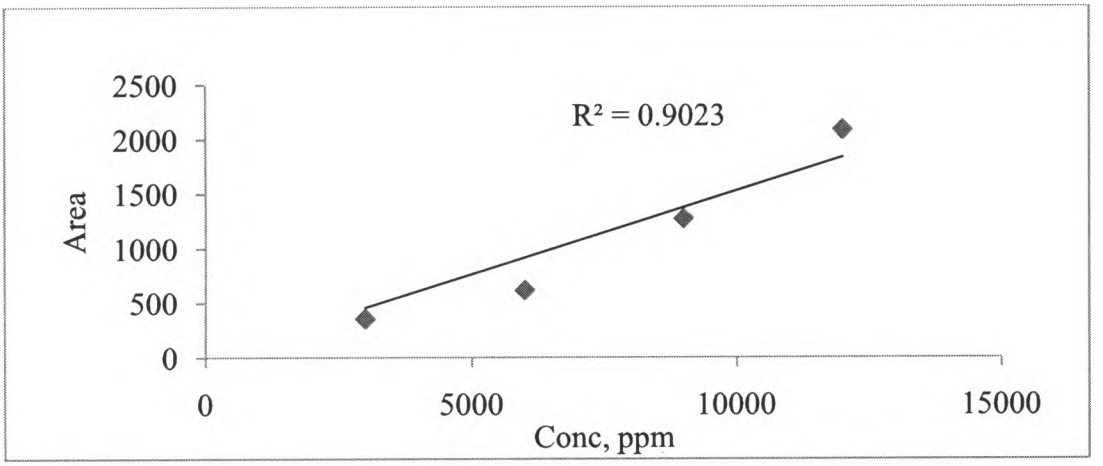


Figure A10 : Methyl Stearate calibration curve

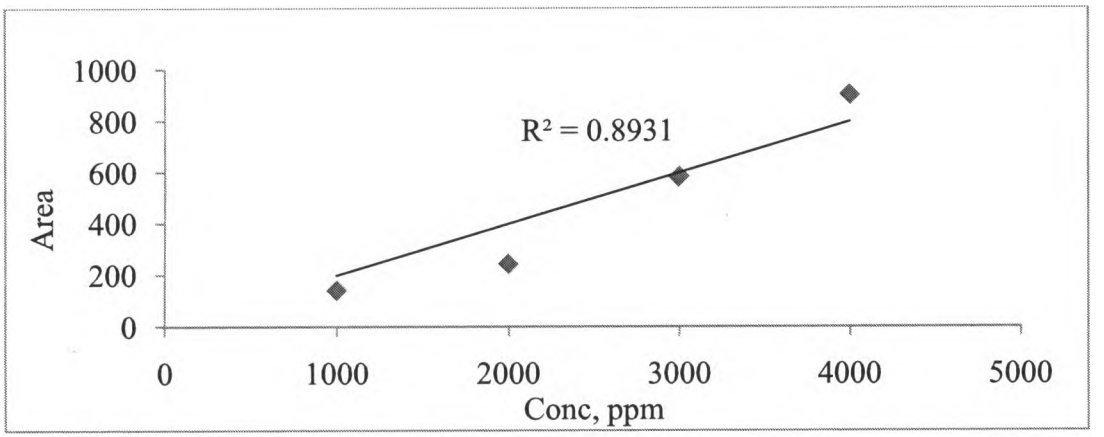


Figure A11: Methyl Behenate calibration curve

Raw Data data for PBR experiment in Section 4.2.1 and Section 4.2.2

Table A.3: Data for PB1

Day	Nitrate mg/L	Ammonia mg/L	Phosphate mg/L	COD mg/L	CO_2 mole%
2	20.81	177.14	117.00	640.00	0.04
5	17.82	151.42	102.20	380.00	0.68
9	17.37	137.14	90.60	340.00	0.04
11	16.45	111.42	84.20	300.00	0.66
16	14.17	50.30	78.60	270.00	0.69
19	12.8	42.14	71	225	0.59
23	9.6	0.71	70.4	220	0.92
25	4.08	0	64.2	210	

Table A.4: Data for PB2

Day	Nitrate mg/L	Ammonia mg/L	Phosphate mg/L	COD mg/L	CO_2 mole%
2	19.04	137.00	127.00	520.00	0.06
5	17.37	97.15	119.20	360.00	0.28
9	16.91	80.00	105.00	320.00	0.03
11	16.00	28.57	96.40	280.00	0.14
16	14.62	14.28	91.20	255.00	0.17
23	5.02	0	81.2	190	0.13
25	3.2	0	76.4	180	0.20

FAME profile data PB1

Species	Area 1	Area 2	Area 3	average	Conc. (mg/L)
palmitate	211.088	288.93	289.23	263.08	4329.62
linoleate	261.23	279.67	279.54	273.48	5085.94
oleate	217.848	257.74	259.47	245.02	8672.84
stearate	510.8	536.76	534.93	527.49	4667.51

Total conc. = 22756 ppm

Volume of extract=4.04 ml

Yield wt% algae=18.21 %

FAME profile data PB2

Species	Area 1	Area 2	Area 3	average	Conc. (mg/L)
palmitate	379.161	329.565	369.6	359.44	6009.83
linoleate	196.63	190.93	198.14	195.23	4973.42
oleate	299.96	326.06	300.55	308.85	11239.75
stearate	604.93	674.44	620.09	633.15	6652.32

Total conc. = 28875.33ppm

Volume of extract=4.72ml

Yield wt% algae=26.67%

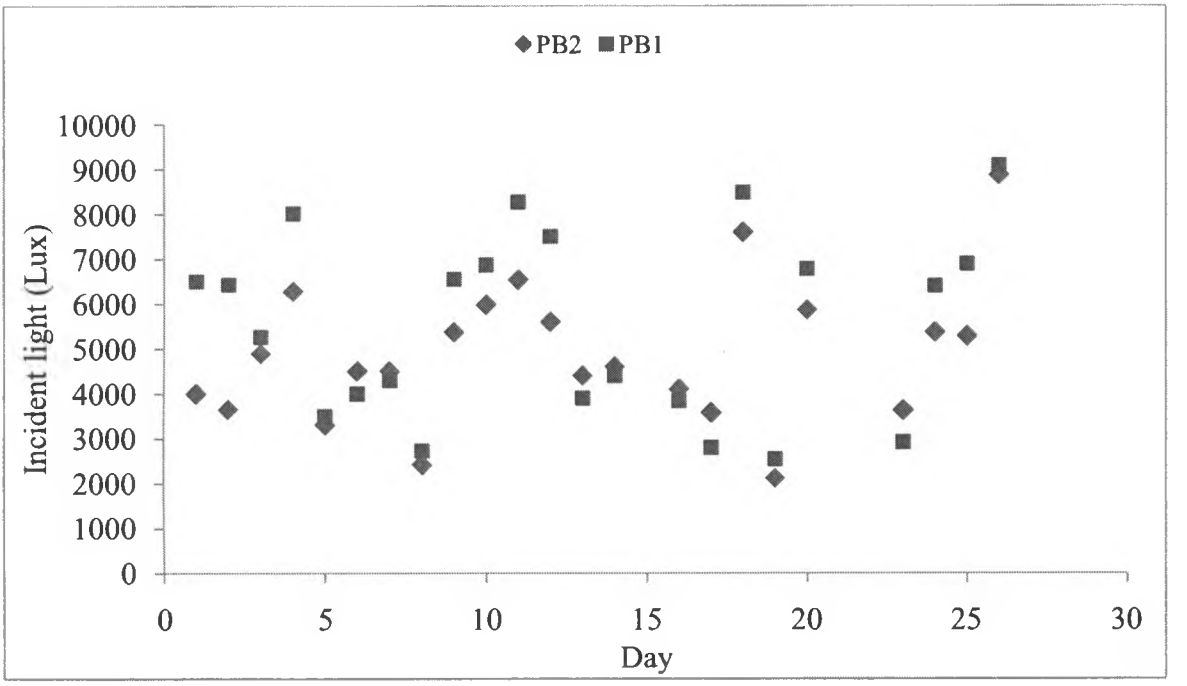


Figure A12: Incident light intensity on PB1 and PB2 as seen in section 4.1

Raw Data for PBR experiments in Section4.3

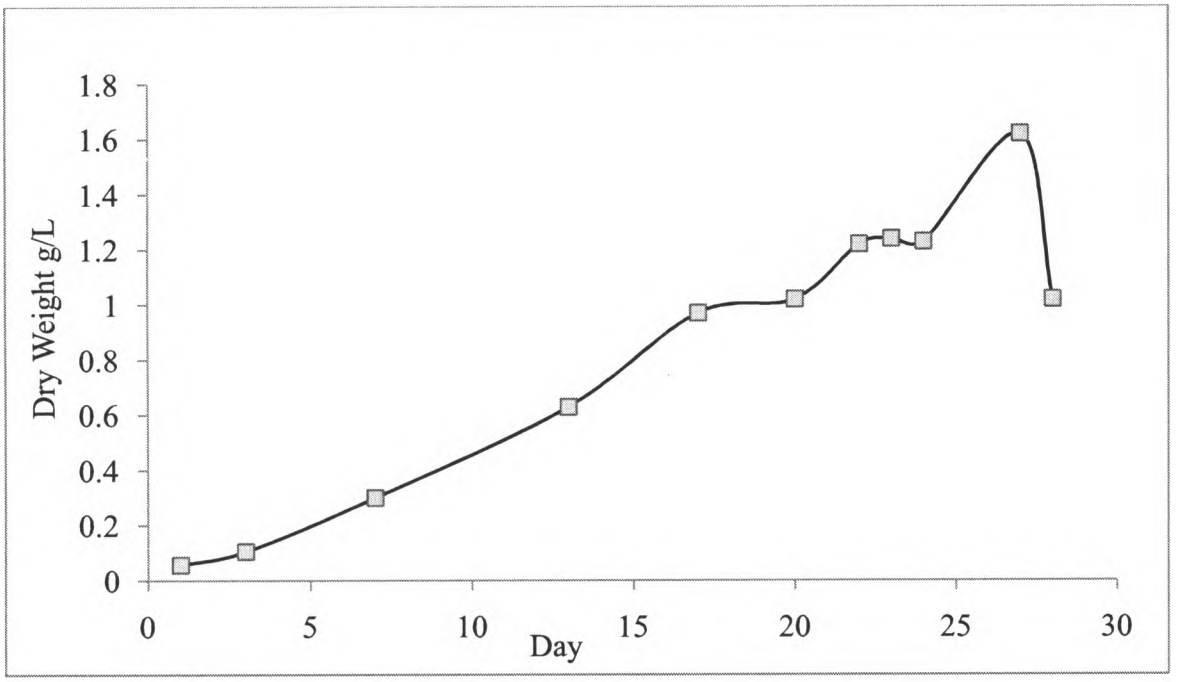


Figure A13: Dry weight profile for PB2

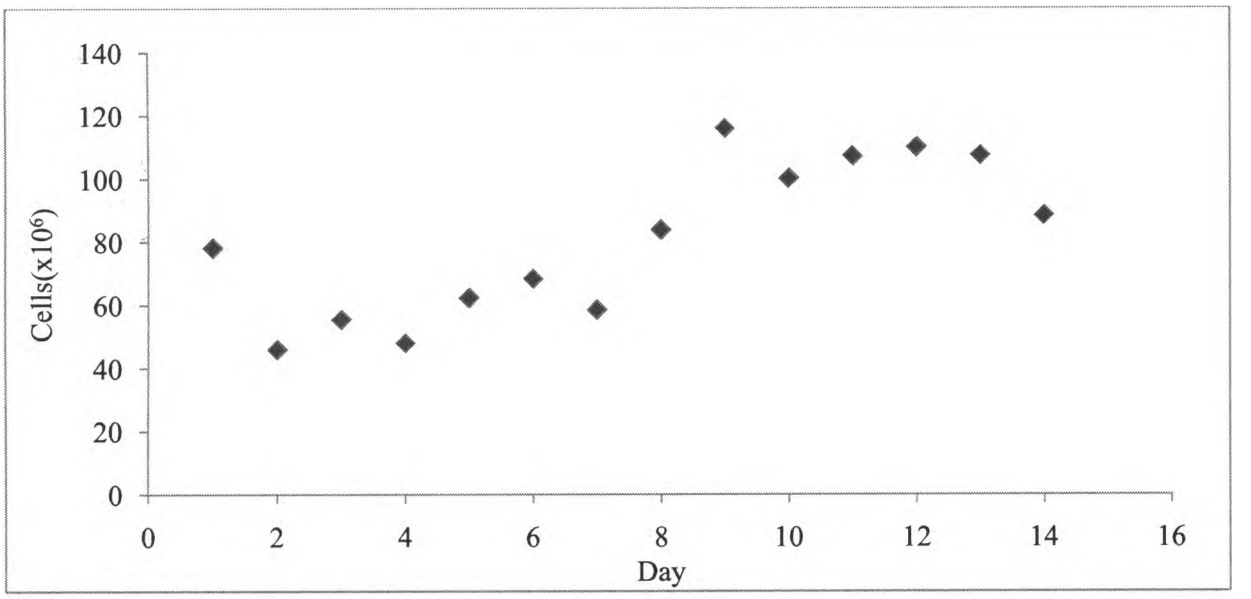


Figure A.4: Growth profile of alum overdose experiment PB1

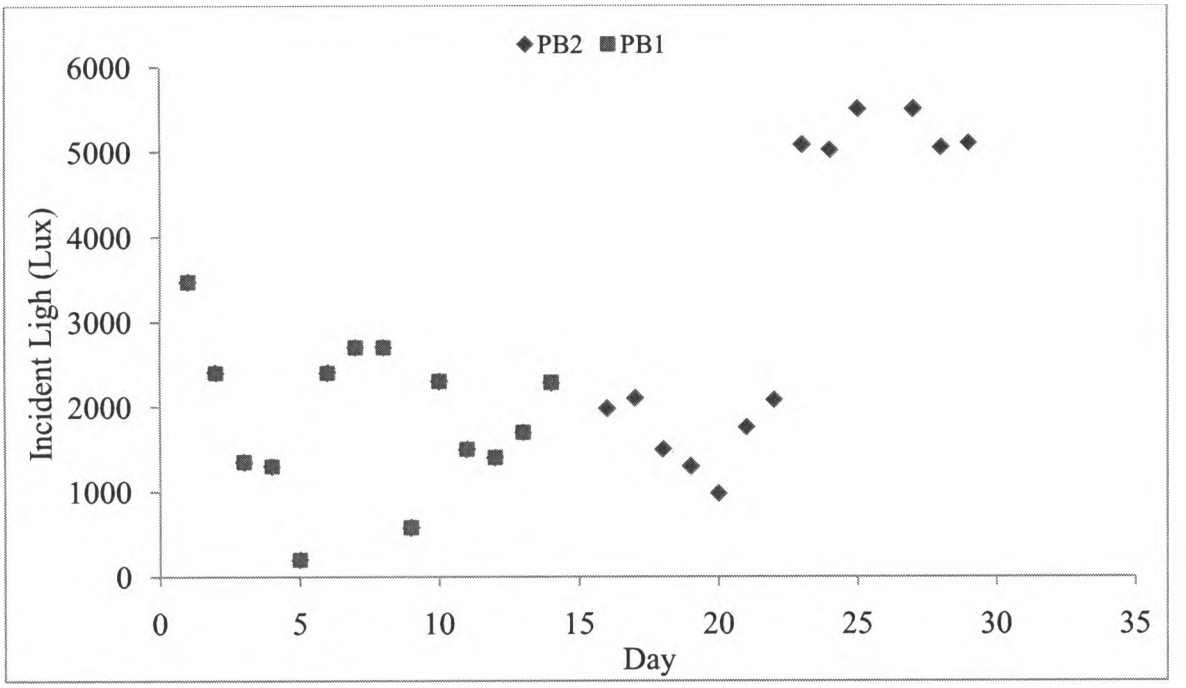


Figure A14: Light intensity measurements for PB1 and PB2 section 4.3

Table A.5: Nutrient concentrations for PB1

Day	Nitrate (mg/L)	Ammonia (mg/L)	Phosphate (mg/L)
1	6.20	834.00	12.80
2	4.86	825.31	13.20
7	3.98	811.13	12.00
10	3.98	789.28	11.20
13	3.98	779.57	10.40
14	3.98	762.12	10.40

Table A.6: Nutrient concentrations for PB2

Day	Nitrate (mg/L)	Ammonia (mg/L)	Phosphate (mg/L)
1	7.08	864.45	124.00
2	7.08	837.25	123.00
7	6.63	828.13	76.80
10	6.20	796.57	69.60
13	5.75	760.13	69.40
17	5.49	740.71	68.4
20	5.31	723.71	47.6
22	5.31	709.14	39.2

FAME profile data PB2

Species	Area 1	Area 2	average	Conc(mg/L)
palmitate	2195.93	2474.96	2335.44	14488.25
linoleate	1193.14	1345.32	1269.23	11120.81
oleate	1882.49	2073.39	1977.94	13972.29
stearate	873.02	964.04	918.53	6683.15
behenate	73.75	81.58	77.66	1013.27

Total conc. = 47277.76ppm

Volume of extract=20ml

Yield wt% algae=19.69%

Raw Data for PBR experiment in Section 4.4

Table A.7: Nutrient concentrations PB1

Day	Nitrate (mg/L)	Phosphate (mg/L)
1	180.68	156.60
4	166.23	138.4
6	151.45	136.8
8	78.2	128.8
11	36.2	121.2
13	2.66	113.4
14	0.44	99.4
16	0.44	94.4
19	0.39	93.8
25	0.22	38.4
27	0.22	32.1
31	0	10.4
32	0	2.1

FAME profile raw data

Species	Area 1	Area 2	Average	Conc. (mg/L)
palmitate	579.8857	713.08917	646.48	6209.06
linoleate	243.68	326.78	285.23	5408.97
oleate	663.0576	847.93604	755.49	10233.94
stearate	882.6996	1148.58	1015.64	7183.71

Total conc. =29035.67ppm

Volume of extract=22ml

Yield wt% algae=13.04%

Raw Data for PBR experiment in Section 4.5

Table A.8:PB2 nutrient concentrations

Day	Nitrate mg/L	Ammonia mg/L	Phosphate mg/L
1.00	35.87	1082.85	134.80
2	34.98	942.85	129.2
5	27.45	748.6	117.8
12	16.38	671	74
15	28.78	408	70.4
18	30.11	302	39.2
22	23.9	397	64.2
25	23.02	351	57.4
27	21.7	337.15	45.6
29	20.81	311.43	20.21

FAME profile raw data

Species	Area 1	Area 2	Area 3	average	Conc(mg/L)
palmitate	336.154	378.13	269.92	328.068	4648.18
linoleate	154.8	154.19	160.74	156.5766667	4377.43
oleate	192.05	256.36	282.99	243.8	8669.11
stearate	502.59	673.85	755.32	643.92	5267.63

Total conc. = 22962.35ppm
Volume of extract=6.7ml
Yield wt% algae=9.3%

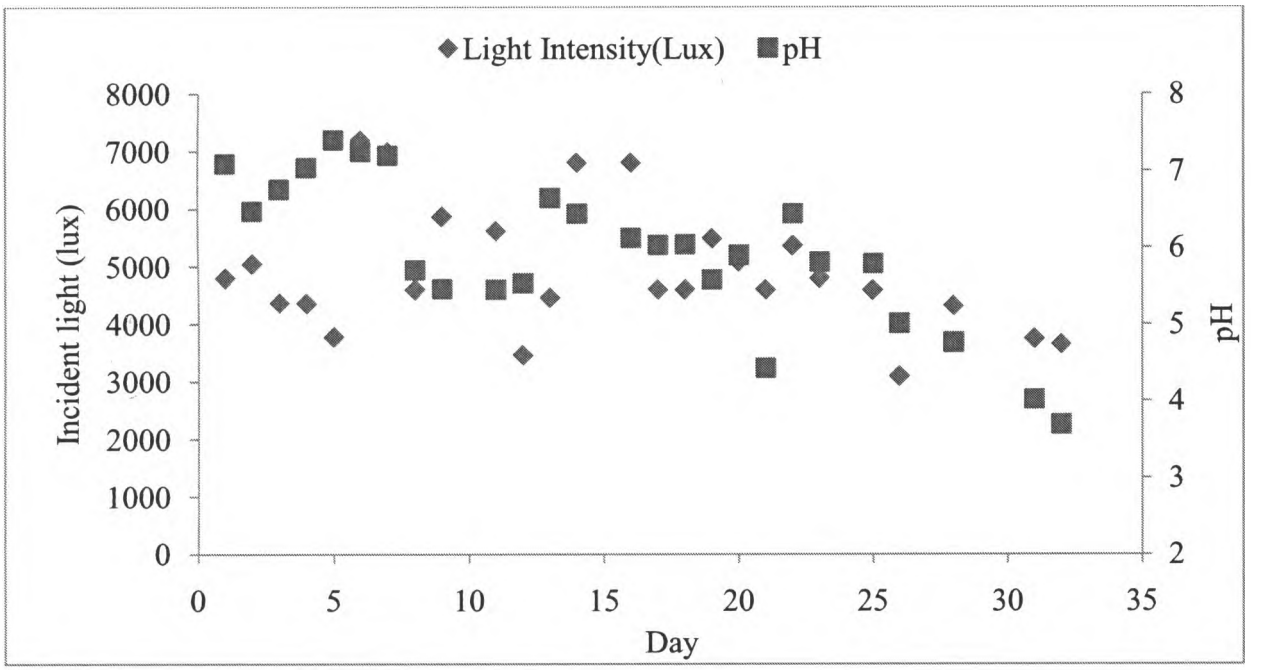


Figure A15: Incident light and pH profile for PB2 section 4.5

Heterotrophic cultivation Section 4.6

FAME profile raw data for Flask 1

Species	Area 1	Area 2	average	Conc. (mg/L)
palmitate	192.25	231.99	212.12	4079.80
linoleate	328.53	287.98	308.25	5296.70
oleate	323.54	289.46	306.5	8860.86
stearate	250.39	223.1	236.74	3168.79

FAME profile raw data for Flask 3

Species	Area 1	Area 2	average	Conc. (mg/L)
palmitate	227.06	332.35	279.705	4411.10
linoleate	298.73	272.45	285.59	5159.33
oleate	527.92	486.63	507.27	9474.85
stearate	810.72	725.16	767.94	5906.91
behenate	208.66	250.87	229.76	1596.03

The chloroform volumes were adjusted accordingly to 2.4ml and 1.7ml respectively in accordance with the dry weight and solvent ratio.

Raw data for Canola oil transesterification in Section 4.7

Table: FAME profile raw data for canola oil study

Species	Area 1	Area 2	average	Conc. (mg/L)
palmitate	854.6917	574.81	714.75	6543.68
oleate	4358.295	4216.09	4287.19	21034.24
linoleate	13929.4	13835.5	13882.45	87564.55
stearate	1896.369	1880.25	1888.31	11682.02
arachidate	278.8049	274.65	276.73	1804.82
behenate	88.2067	83.52	85.86	1044.70

Light measurement data

Calculated using (Figure 3.6 : Energy spectrum for sunlight and cool white lamp) by performing Simpsons rule integration of the area under each curve.

Photo bioreactor 1

Front Photon Flux : 110μE/m^2s

Back Photon Flux: 5.9μE/ m^2s

Window photon Flux: 51.8μE/ m^2s

Photo bioreactor 2

Front Photon Flux: 80μE/ m^2s

Back Photon Flux: 5.7μE/ m^2s

Window photon Flux: 49μE/ m^2s

Photo bioreactor (1, 2) centre

Centre Photon Flux: 49.1μE/ m^2s

Window photon flux: 5.27μE/ m^2s