DURBAN UNIVERSITY of TECHNOLOGY

# Screening for Indigenous Algae and Optimisation of Algal Lipid Yields for Biodiesel Production

Submitted in fulfilment of the requirements of the degree of Master of Technology: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology

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BSc Hons: Microbiology

May 2011

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## Declaration

# Screening for Indigenous Algae and Optimisation of Algal Lipid Yields for Biodiesel Production

**Ismail Rawat**

I hereby declare that the dissertation represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

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**2011**

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I, _____ (full name of student) and, _____ (full name of supervisor) do hereby declare that in respect of the following dissertation:

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   (a) no other similar dissertation exists;
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# Approval

I hereby approve the final submission of the following dissertation.

_____

**Prof. F. Bux**
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This _____ day of _____, 2011, at the Durban University of Technology.

*** SUBMISSION APPROVED FOR EXAMINATION**

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## Abstract

The depletion of global energy supplies coupled with an ever increasing need for energy and the effects of global warming have warranted the search for alternate renewable sources of fuel such as biodiesel. First generation biofuels are not sustainable enough to meet long term global energy requirements and more recently there has been concern expressed as to the potential negative implication of crop based biofuels in the form of negative energy balances and potentially no greenhouse gas benefit due to land utilisation not being taken into account. Microalgae have shown great promise as a sustainable alternative to first generation biofuels. They have faster growth rates, have greater photosynthetic efficiencies, require minimal nutrients and are capable of growth in saline waters which are unsuitable for agriculture. Microalgae utilise a large fraction of solar energy and have the potential to produce 45 to 220 times higher amounts of triglycerides than terrestrial plants. The use of microalgae for biodiesel production requires strain selection, optimisation and viability testing to ascertain the most appropriate organism for large scale cultivation. This study focuses on bioprospecting for indigenous lipid producing microalgae, screening, selection and optimisation of growth and lipid yields with respect to nutrient limitation. Further we have ascertained the sustainability of a selected species of microalgae in open pond system. *Chlorella* sp. and *Scenedesmus* sp. were found to be dominant amongst the isolates. Strains we selected and underwent media selection and growth and lipid optimisation trials. BG11 media was selected as the most appropriate media for the growth of the selected *Chlorella* and *Scenedesmus* strains. Little variation in growth was observed for both cultures ten days into cultivation under varying nitrate concentrations. Phosphate optimum was shown to be 0.032g/l for *Scenedesmus* sp and 0.04g/l for *Chlorella* sp. Best lipid yield determined during exponential growth was achieved in cultures with 0.3g/L to 0.6g/L nitrate and phosphate as per BG11 medium. pH optimisation showed that cultures may be adapted to growth at higher pH over time. The optimum pH range for growth was determined to be narrow and was found to be between pH 10 and pH 11. *Chlorella* sp. was shown to be sustainable as a dominant culture in open pond system. Open pond systems however are prone to contamination by other species of microalgae within weeks of inoculation.

## Acknowledgements

**I would like to acknowledge the following people:**

- My parents, for their unwavering faith, support and encouragement.

- Prof F. Bux, for his advice, assistance and encouragement during the course of this project. His commitment to research excellence ensured that my project conformed to the highest standards.

- Staff and students at the Institute for Water and Wastewater Technology, Durban University of Technology for their analytical support and guidance.

- The National Research Foundation and Durban University of Technology for funding.

# Preface

Aspects of the work covered in the following dissertation can be found in the following accepted publication and conference presentation:

**Rawat, I., Kumar, R. R., Mutanda, T. & Bux, F. (2010).** Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. *Applied Energy* **doi:10.1016/j.apenergy.2010.11.025**

**Rawat, I. & Bux, F. (2009)** Sustainability of Chlorella sp. in open pond system. Poster presentation: Leading Edge Technology Conference, Singapore, June 2009.

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# Chapter 1: Introduction and Literature Review

## 1.1 Introduction

Due to the depletion of fossil fuel deposits and the effects of global warming, it has become necessary for the world to seek renewable sources of fuel. Diesel is one such fuel that is heavily relied on. Biodiesel is produced from oil, thus plant oils can be substituted for crude oil in its production. Biodiesel is a mono-alkyl ester produced by the transesterification of triglycerides or free fatty acids with short chain alcohols and has the ability to be used in conventional diesel engines with little or no modification (Du et al., 2008). First generation biofuels have been around for over a century (Campbell et al., 2011). Biodiesel is currently produced from soybean oil in the United States (Chisti, 2007). Other sources of commercial biodiesel include canola oil, animal fat, palm oil, corn oil, waste cooking oil, and jatropha oil. The use of plant oils for fuel production is however, highly controversial and requires resources such as arable land. This may not be available in large enough quantities to meet fuel requirements of a designated area and will greatly affect food security (Lee, 2011). Currently 1% of the arable land available globally is used for crop based (first generation) biofuels. This is sufficient only to meet 1% the requirement of the global requirement (Brennan & Owende, 2010). Complete reliance on first generation biofuels is thus not feasible due to food security among other considerations. There is also some debate as to whether the energy balance is favourable (Campbell et al., 2011). Microalgae represent a promising new source of oil feedstock for fuel manufacture.

Environmental considerations largely favour the use of biodiesel rather than petro-diesel. Global warming and soil pollution are amongst these concerns. Global warming is to a large extent caused by the burning of fossil fuels. The $CO_2$ released from power plants which would ordinarily be released into the atmosphere may be collected and used positively by photosynthetic microalgae as they are capable of fixing $CO_2$ to produce energy and chemical compounds upon exposure to sunlight with no net contribution to atmospheric $CO_2$ levels (Braun, 1996; Miyamoto, 1997). Biodiesel has been experimentally shown to be less eco-toxic than petro-diesel. In studies conducted by Lapinskiene et al. (2006), it was shown that diesel fuel at concentrations greater than 3% (w/w) is toxic to soil microorganisms. Biodiesel however, is

non toxic at total soil saturation. Biodiesel contributes no net carbon dioxide or sulphur and overall less gaseous pollutants to the atmosphere than petro-diesel (Han Xu et al., 2006; Huber et al., 2006; Williams & Laurens, 2010). With growing concern for the environment, these factors play an important role in the acceptability of biodiesel. Algal biodiesel has been shown to be potentially more environmental friendly than crop based biodiesel as there is currently concern over the green house gas balance as indirect land use change has not been taken into account and thus there is no green house gas benefits from the use of first generation biofuels (Dornburg et al., 2010; Campbell et al., 2011).

The technology for sustainable production of biofuels from microalgae exists but requires improvement in order to make the process economically viable (Brennan & Owende, 2010). The first step in this process is selection of an appropriate species for biodiesel production (Ahmad et al., 2011). The species selected must meet the requirements for large scale cultivation in open pond systems. Selection of high lipid producers is paramount to success. However, this is just one of the considerations to be taken into account. Eukaryotic microalgae are preferred to prokaryotes as they have been shown to store more lipids (Williams & Laurens, 2010). The species selected should have some competitive advantage such as the ability to grow in selective environments (high nutrient or alkaline environment amongst others) to enable successful culturing at large scale (Borowitzka, 1999; Brennan & Owende, 2010). The ability to adapt to various changes in conditions needs to be considered as a factor in selection as temperature fluctuations and diurnal cycles are very difficult to control in open systems (Brennan & Owende, 2010). The strain of choice should preferentially be isolated from area close to the site of production. This allows for reduction in the time required for acclimatisation to climatic conditions. The strain should be able to be produced in open system to make the process economically viable. Phototrophic cultivation is the lowest costing type of cultivation as the energy and carbon sources used are available in the form of sunlight and atmospheric carbon dioxide respectively, as well as being able to be produced in open systems (Chen et al., 2011a). The purpose of this research project was to bioprospect for hyper lipid producing indigenous microalgae in KwaZulu-Natal. Therefore focus was targeted more towards isolation screening and optimisation of growth and lipid yields. Investigation of the physiological aspects of selected strains will be the focus of future research.

## 1.2 Aims and Objectives

### 1.2.1 Aim

Isolation and characterisation of microalgae from aquatic environments that have the potential for lipid production.

### 1.2.2 Objectives

- Screening of aquatic environments for microalgae and purification of isolated cultures
- Selection of algal lipid producers
- Optimisation of conditions to increase lipid yields at laboratory scale
- Operation of a raceway pond to determine viability of open system culturing

## 1.2 Literature Review

### 1.2.1 Crop based biodiesel vs. microalgae

More than 95% of first generation biodiesel sources are edible oils. First generation biofuels have a great impact on food security and the potential to drive up the cost of food crops such as Soybean thus also making biodiesel production more expensive. Second generation biofuels such as Jatropha oil, waste cooking oil and animal fats do not affect food security and have significant advantages of first generation oil crops. Sustainability of second generation biofuels is not favourable. Moreover production of crop derived biofuels gives rise to challenges such as poor cold flow properties and free fatty acids contained in animal fats give rise to production difficulties and may constitute a bio-safety hazard (Ahmad et al., 2011). Microalgal oil is regarded as third generation biofuels source. Microalgal productivities can be twenty times that of oilseed crops on a per hectare basis and is thus the more viable alternative is the use of oil produced by microalgae (Antoni et al., 2007; Chisti, 2007; Ahmad et al., 2011; Feng et al., 2011). Microalgae have faster growth rates than plants and are capable of growth in saline waters which are unsuitable for agriculture (Huber et al., 2006). Microalgae have greater photosynthetic efficiencies thereby utilising a larger fraction of solar energy, whilst requiring

minimal nutrients for growth (Gordon & Polle, 2007). The lipid content of microalgae, on a dry cellular weight basis varies between 20 and 40 % however lipid content as high as 85 % have been reported for certain microalgal strains (Hanna & Ma, 1999; Luque et al., 2010). Microalgae have the potential to produce 25 to 220 times higher triglycerides levels than terrestrial plants (Huber et al., 2006; Ahmad et al., 2011) and can be readily converted to biodiesel by transesterification (Hanna & Ma, 1999). As compared to biomass such as trees and crops, microalgal oil is more economical in that, transportation costs are relatively low. Microalgae have the added advantage of not effecting food security (Ahmad et al., 2011).

### 1.2.2 Microalgae

Microalgae are classified as polyphyletic autotrophic or heterotrophic organisms ranging in 2 - 200µm in size (Mutanda et al., 2010). Autotrophic microalgae may be described as chlorophyll-*a* containing plants that lack root, embryos, vascular systems, stems and leaves (Brennan & Owende, 2010; Singh et al., 2011b). They are an extremely diverse group of organisms. Approximately 30000 species have been described. This is estimated to be less than 10% of the total number of species worldwide (Benemann, 2008). Microalgae exist in a variety of forms from unicellular, multi-cellular and to fairly complex forms. Microalgae have the ability to grow in a variety of environments from damp places to water bodies. Like higher plants, microalgae require sunlight, carbon dioxide and water for growth via photosynthesis (Williams & Laurens, 2010; Singh et al., 2011b). Microalgae contribute between 40 and 50% of the oxygen in the atmosphere. Microalgal biomass may have diverse biofuels potential in that biomass can be used for several different types of renewable biofuels including methane, biocrude oil and biodiesel (Brennan & Owende, 2010).

The composition of algal biomass varies greatly between strain and type of algae. Macroalgae generally do not produce lipids and consequently have little value to the production of biodiesel. They can however be used for the production of alternate fuels as they produce natural sugars and other carbohydrates. Conversely microalgae are seen as an ideal source of biodiesel as they have high photosynthetic efficiency and the ability to produce high quantities of lipids (Braun,

1996; Miyamoto, 1997). Chisti, (2007) estimated the molecular formula of microalgal biomass as $CO_{0.48}H_{1.83}N_{0.11}P$. Selection of microalgal species should be dependent on the desired route to the production of alternate fuels. Production of biodiesel from renewable and sustainable source thus leans favourably towards the use of microalgae due the high lipid content and production capability (Singh et al., 2011b). The production of microalgae actively fixes atmospheric carbon dioxide (Chisti, 2008). Moreover microalgae can be grown in environments unsuitable for the production of agricultural crops and farming. Microalgae also have the ability to grow in water that is unsuitable for use on agriculturally produced crops (Costa & Morais, 2011). As freshwater becomes an increasingly scarce commodity, the use of alternate water sources is hugely beneficial. Uptake of nutrients from the growth solution serves to improve the water quality and has been used as a tertiary wastewater treatment step (Costa & Morais, 2011).

### 1.2.3 Advantages and challenges associated with microalgal biofuels production.

Microalgae are capable of year round production unlike oilseed crops. Microalgae require less freshwater for cultivation than terrestrial plants due to production in aqueous solution. Microalgal cultivation can occur on non-arable land, in brackish water thus reducing strain on resources required for the production of food crops whilst reducing other environmental effects. The rapid growth rate of microalgae and high lipid storage capacity, far outcompete terrestrial crops. Microalgal biodiesel is environmentally advantageous in that it is a carbon neutral fuel. Microalgal growth actively utilizes 1.83kg of $CO_2$ for every 1kg dry biomass produced (Chisti, 2008). Growth of microalgal can effectively remove phosphates and nitrates from wastewater, thus making it an ideal substrate for the cultivation of microalgae for biofuels production whilst acting as a tertiary treatment for wastewater. Some microalgae produce valuable by-products in the form of high value proteins and pigments (Brennan & Owende, 2010). Large scale production of microalgal poses a number of challenges. Strain selection is of paramount importance to the successful culturing of microalgae at large scale (Chisti, 2007). Ideally the species of choice should balance requirements for biofuels and production of value added co-products. This is generally a difficult task in that bioprospecting of a large number of species is required. Production systems need to be tailored towards attaining high photosynthetic activities. Contamination by undesirable algae, bacteria and grazers is often difficult or costly to control at

large scale. There is also a requirement for development of techniques for prevention of losses by $CO_2$ diffusion and evaporation. There is a severe lack of data available with regard to large scale production due to the very few commercial production operations (Brennan & Owende, 2010).

### 1.2.4 Large-Scale Cultivation of Microalgae

For the industrial cultivation of microalgae in the quantities required to make biodiesel from microalgal lipids viable, certain criteria must be met. These include capital and operating costs analysis, maintaining temperature control, assessing water requirements (source, recycle, chemistries and evaporation issues), determining $CO_2$ availability and delivery methods, providing necessary microalgal nutrients, land availability, land cost and examining the environmental impacts (Borowitzka, 1999; Chisti, 2007). Paramount to commercial success is the selection of microalgal species for high-level oil production and good growth rates. High lipid content alone does not necessarily translate to high productivity (Benemann, 2008). Acquiring such strains would involve screening microalgal from culture collections and species from nature for the best productivity characteristics. Characteristics that require investigation include growth rate, oil content and fatty acid profile, robustness and resistance to invasion, or metabolically engineering microalgal strains for enhanced lipid productivity (Chisti, 2007). In this regard isolation and identification of lipid producing microalgal species are of importance as related species likely give oil with similar constituents. Morphological identification is a rapid method identifying microalgae to some degree. However the extent of the identification is more often than not, limited to the algal structure as seen under a light microscope. Electron microscopy reveals further detail showing greater diversity (Simon et al., 2000). Molecular methods for identification such as the use of probes, Fluorescent *in situ* hybridisation and dot blot techniques may prove invaluable in the search for better oil producers.

Screening for oil producers among the isolates is a vital part in the optimisation of biodiesel production. In order to gain high oil yields it is necessary to ascertain the amount of oil, if any is produced under normal conditions. This is likely to allow maximum overproduction under stress. Yield optimisation is very important from the economic feasibility point of view. The viability of

a species of microalgae for use in the production of biodiesel is often dependant on the yield. The proportion of TAG in the total lipid of healthy and actively dividing cells is usually low, however during stationary phase certain species can have elevated proportions. Stationary phase may be induced by limiting one or more variables that control growth, e.g. nitrogen, phosphorus or silicon limitation (Mansour et al., 2005). It is generally accepted that the ability of microalgae to adapt to environmental conditions is reflected in an exceptional variety of lipid patterns as well as with their ability to synthesize a number of unusual compounds (Guschina & Harwood, 2006). This is likely the reason that microalgae overproduce fatty acids when subjected to stressful conditions. It is thus possible to enhance lipid content by imposing nitrogen starvation or other stress factors. Lipid fractions as high as 70 to 85% on a dry weight basis have been reported (Chisti, 2007). Such high lipid contents exceed that of most terrestrial plants (Miyamoto, 1997).

Other nutrient deficiencies such as silicon may lead to an increase in cellular lipid content. Lipid contents of the diatom *Navioua pelliculosa* increased by about 60% during a 14 hour silicon starvation period (Miyamoto, 1997). The switch from carbohydrate accumulation to lipid accumulation in these diatoms occurs very rapidly, though mechanisms involved are not yet understood. This makes the use of diatoms for production of algal lipids has great potential (Miyamoto, 1997). During early stages of growth, green microalgae produced relatively large amounts of polar lipids and polyunsaturated $C_{16}$ and $C_{18}$ fatty acids. On approaching the stationary phase of growth however, the dominant lipids produced by microalgae are neutral, and consist primarily of saturated 18:1 and 16:0 fatty acids. In the case of blue-green algae, the lipid and fatty acid composition show relatively little change during the growth cycle. Light enhances the formation of polyunsaturated $C_{16}$ and $C_{18}$ fatty acids as well as mono- and di-galactosyl-diglycerides, sphingolipids and phosphoglycerides in *Euglena gracils* and *Chlorella vulgaris*. Low temperatures increase the synthesis of polyunsaturated $C_{18}$ fatty acids by *Monochrysis lutheri*, and cause changes in the fatty acid composition of *Dunaliella salina* (Miyamoto, 1997).

Stresses induced by changes in salt concentration of the aquatic environment have been shown to affect the quantity of lipid contained within microalgal cells. The intracellular lipids and triacylglycerides content of saltwater microalgal species grown in 1.0M NaCl were markedly higher (67% and 56% lipid) than those in the culture with 0.5M NaCl (60% and 40% lipid). NaCl concentration higher than 1.0M inhibits biomass production however below 1.0M has no marked effect on biomass. *Dunaliella* cells were reported to secrete glycerol in response to increase of NaCl concentration (Takagi et al., 2006).

Characterisation of lipids is required as different species of microalgae produce different types of fatty acids. Some fatty acids are more suitable for transesterification to biodiesel than others. Most microalgal oils are rich in polyunsaturated fatty acids with four or more double bonds. The problem associated with this degree of polyunsaturation is, these fatty acids and fatty acid methyl esters (FAME) are susceptible to oxidation during storage, thus reducing their acceptability for use in biodiesel (Chisti, 2007).

Raceway ponds (Figure 1) and photobioreactors (Figure 2) are the methods used for culturing large volumes of microalgae (Grima et al., 2003). Each of these methods has its merits and the deciding factor as to which best suits the production of the desired strain is dependent on the initial capital outlay as well as the strain to be cultured. Both configurations have their own challenges (Benemann, 2008). Raceway ponds are open circular ponds that are typically not deeper than 300mm and have a paddle wheel to constantly circulate the biomass and prevent sedimentation. The depth is limited by the fact that light can only penetrate approximately 300mm of water. Use of a pond with greater depth does not increase efficiency. Circulation of the biomass also aids in the reduction of the shading effect by constant mixing. In this respect baffles aids in mixing by giving turbulent flow. One of the chief benefits associated with the use of raceway ponds is that the cost is significantly lower than that of photobioreactors. This may however be offset over a period of time as photobioreactors give better productivity. Raceway ponds however do not allow the levels control that photobioreactors do. Light is not artificially supplied and thus the photoperiod and light intensity cannot be controlled. Temperature cannot

easily be controlled. These fluctuate seasonally and therefore are climate dependant. Loss of water due to evaporation may be significant. Raceway ponds are prone to contamination by other microalgae as well as microorganisms such as rotifers that consume microalgae. A method used to slow the rate of contamination and allow control of conditions such as temperature and limit evaporation is to enclose the raceway within a greenhouse. This however significantly increases the cost (Chisti, 2007). Large amounts of biomass are required to improve the techno-economics of biodiesel production. All large-scale microalgae production systems used presently are open systems, however only a small number of species have been successfully grown (Borowitzka, 1999).

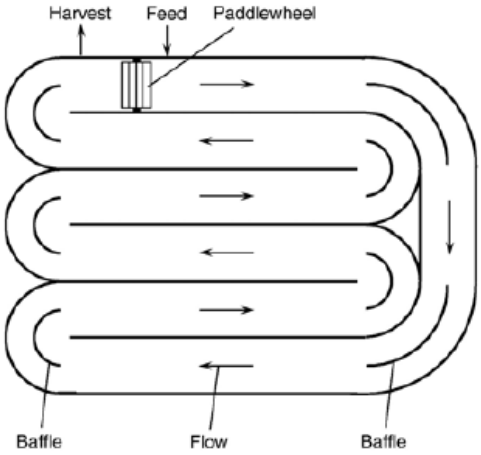


Figure 1: Schematic of a raceway pond with paddlewheel to prevent biomass settling (Chisti, 2007)

Photobioreactors allow control of most of the abiotic factors. Light intensity and photoperiod may be controlled dependant on the light source. If artificial light is used as the light source, control is possible, this does however add to the cost of production (Borowitzka, 1999; Chisti, 2007). Natural light is cost effective and allows the extension photoperiods by the use of artificial light (Chisti, 2007). All other parameters can be controlled. Photobioreactors allow the ability to grow monocultures. The increased cost of setup and running of photobioreactors is due to the need for degassing of the growth medium (Figure 2). Oxygen will accumulate to toxic levels within the media and slow growth considerably. The factor that may make the greatest

contribution to the choice of a photobioreactor over a raceway pond is that the productivity in photobioreactors is considerably higher. A cost to production ratio is helpful in the decision as to which configuration is more viable (Chisti, 2007). Life cycle analysis is an important factor in the determination of the system of choice. The net energy return from photobioreactors is less than that of raceway ponds thus making raceway ponds more economical (Campbell et al., 2011; Collet et al., 2011). The advantages and disadvantages of open ponds and photobioreactors have been summarised by Brennan & Owende, (2010) in Table 1.

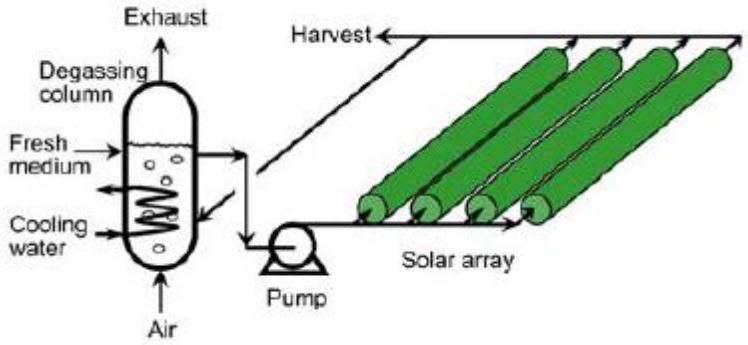


Figure 2: Industrial tubular photobioreactor configuration including degassing step (Chisti, 2007).

Table 1: Summary of advantages and disadvantages of photobioreactors and open ponds (Brennan & Owende, 2010)

| Production system | Advantages | Limitations |
|---|---|---|
| Raceway pond | Relatively cheap<br>Easy to clean<br>Utilises non-agricultural land<br>Low energy inputs<br>Easy maintenance<br>Good for mass cultivation | Poor biomass productivity<br>Large area of land required<br>Limited to a few strains of algae<br>Poor mixing, light and $CO_2$ utilisation<br>Cultures are easily contaminated<br>Difficulty in growing algal cultures for long periods |
| Tubular photobioreactor | Large illumination surface area<br>Suitable for outdoor cultures<br>Relatively cheap<br>Good biomass productivities | Some degree of wall growth<br>Fouling<br>Requires large land space<br>Gradients of pH, dissolved oxygen and $CO_2$ along the tubes |
| Flat plate photobioreactor | High biomass productivities<br>Easy to sterilise<br>Low oxygen build-up<br>Readily tempered<br>Good light path<br>Relatively cheap<br>Easy to clean up<br>Good for immobilization of algae<br>Large illumination surface area<br>Suitable for outdoor cultures | Scale-up require many compartments and support materials<br>Difficult temperature control<br>Small degree of hydrodynamic stress<br>Some degree of wall growth |
| Column photobioreactor | Compact<br>High mass transfer<br>Low energy consumption<br>Good mixing with low shear stress<br>Easy to sterilize<br>High potentials for scalability<br>Readily tempered<br>Good for immobilization of algae<br>Reduced photoinhibition and photo oxidation | Small illumination area<br>Expensive compared to open ponds<br>Shear stress<br>Sophisticated construction<br>Decrease of illumination surface area upon scale-up |

### 1.2.5 Harvesting strategies

Effective wastewater treatment and the production of biomass for biofuels require separation of biomass from water. The selection of harvesting method is of great importance to the economics of biofuels production as harvesting can make up 20-30% of the total cost of production. Selection of the harvesting method depends strongly upon the characteristics of the culture grown (Brennan & Owende, 2010). Microalgae that are suitable for the remediation of wastewater and production of biofuels tend to be unicellular forms of low density. This makes economical biomass harvesting difficult and the cost of biomass recovery significant (Chisti, 2007; Griffiths & Harrison, 2009; Brennan & Owende, 2010; Pittman et al., 2010; Park et al., 2011). Methods of biomass recovery include filtration, centrifugation, sedimentation and flocculation and floatation (Mutanda et al., 2010).

Continuous centrifugation is the preferred method for biomass separation as it is rapid, efficient and universal (Mutanda et al., 2010; Chen et al., 2011a). This is however not economically feasible for large scale harvesting due to its process being highly energy intensive (Pittman et al., 2010). Gravity sedimentation is a common method of harvesting biomass. The process is rudimentary but works for various types of microalgae and is highly energy efficient. Efficacy of gravity sedimentation is strongly influenced by the density and radius of algal cells (Brennan & Owende, 2010). The process may be enhanced by the use of lamella separators and sedimentation tanks. Filtration is another method commonly used. Both sedimentation and filtration may be used in conjunction with flocculation. Flocculation is used to enhance the settling characteristics by increasing particle density of culture that may be unsuccessfully separated due to low particle density (Chen et al., 2011a).

Conventional flocculation works by the mechanism of dispersion of charge. Microorganisms are generally negatively charged. The addition of metal salts act to displace the charge and allow aggregation of the organisms thus allowing more efficient sedimentation or filtration to occur. Alum is generally used in conventional wastewater treatment and can suitably flocculate algal biomass. However it may impede oil extraction depending on the dominant algal strain being

cultivated (Grima et al., 2003). Flocculation may also be achieved by the use of cationic polymers or the addition of alkali substances to increase the pH (Brennan & Owende, 2010). Use of cationic polymers is expensive and will likely adversely affect techno-economics. Autoflocculation is the spontaneous aggregation of particles, resulting in sedimentation of the microalgae. This may occur, or can be induced by limitation of carbon or certain abiotic factors (Pittman et al., 2010). Flocculation is a process in which dispersed particles are aggregated together to form large particles for settling. The mechanism of autoflocculation may be as a result of precipitation of carbonate salts with algal cells in elevated pH that occurs as a consequence of photosynthetic $CO_2$ consumption with microalgae. Autoflocculation may be induced by pH adjustment with NaOH (Chen et al., 2011a).

Filtration is a method commonly used for solid-liquid separation. Vacuum filtration is effective in the recovery of larger microalgae (greater than 70µm) when used in combination with a filter aid. Smaller microalgae however require the use of membrane microfiltration or ultra-filtration for effective harvesting (Brennan & Owende, 2010). These methods tend to be costly, energy intensive and require frequent membrane replacements (as a result of fouling) and pumping of the biomass (Pittman et al., 2010).

Immobilisation of the microalgal culture provides an efficient alternative for biomass retrieval. Immobilisation is the artificial attachment or encapsulation in alginates or similar substances. This may only be suitable for biomass production, if a high flow rate is used to prevent the decrease in growth rate. This type of setup has been shown to take as much nutrients as that suspended microalgae. Immobilised biomass can be used for biofuel conversion by thermal or fermentative means. Lipid extraction efficiency and effects of the immobilisation on lipid is yet to be determined (Pittman et al., 2010).

Floatation of algal biomass has shown promise in terms of harvesting smaller and unicellular microalgae in laboratory scale trials. Ozonation-dispersed floatation and floatation employing a

method of creating charged bubbles (Cheng et al., 2010). The mechanism of action is interaction with the negatively charged hydrophilic surfaces of algal cells. It is to be noted that the trial was carried out with washed cells (Cheng et al., 2010). Ozonation-dispersed flotation of cells grown in open pond culture may prove challenging due to contamination. Lipid content of *C.vulgaris*, harvested by ozonation-dispersed flotation showed an increase from 31% to 55% in the flotation stage according to Cheng et al., (2010). An additional benefit of the use of ozone is its ability to cause cell lysis. Lysis of cells release biopolymers that act as coagulating agents thus enabling more effective separation. Algal cell lysis may also serve to enhance the extraction of lipid. A disadvantage of ozonation-dispersed flotation is that it is an expensive process. The effectiveness of dissolved air flotation may be increased by reduction of the negative charge carried by the air bubbles. This may be achieved by the addition of a cationic surfactant or other chemicals to give a net positive charge (Cheng et al., 2011). Floatation may be aided by the fact that certain microalgal strains have the propensity to float as the microalgal oil content increases. The process of flotation is beneficial in that it is fast compared to the process of sedimentation, however efficiency may be poor in shallow ponds such as raceway ponds commonly used for large scale cultivation (Singh et al., 2011b).

### 1.2.6 Lipid extraction methods

Extraction of microalgal lipid is central to the production of biodiesel from microalgae. Lipid extraction can be performed by chemical methods in the form of solvent extractions, physical methods or a combination of the two. Extraction methods used should be fast, effective and non-damaging to lipids extracted and easily scaled-up (Medina et al., 1998). Extraction using a modified Bligh & Dyer, (1959) method is the most commonly used approach (Mutanda et al., 2010). The choice of solvent for lipid extraction, as with harvesting, will depend on the type of microalgae cultivated. Other preferred characteristics of the solvents are that, they should be inexpensive, volatile, non-toxic and non-polar and poor extractors of other cellular components. Lipids have different types of associations which need to be disrupted for effective extraction. Hydrophobic interactions in non-polar/neutral lipids are disrupted by non-polar organic solvents; hydrogen bonding in polar lipids is disrupted by polar organic solvents such as alcohols. Change

in pH towards more alkaline is useful for the disruption of stronger ionic forces that may be present (Medina et al., 1998).

Pre-treatment of samples may be required for oil extraction of certain types of biomass. This is generally not necessary for extraction from wet biomass, as solvents generally rupture the cells. When cell disruption is required, it may be accomplished by sonication, homogenisation, grinding, bead beating or freezing (Medina et al., 1998). Other methods for cell disruption include autoclaving, osmotic shock, microwaving and freeze drying (Mutanda et al., 2010). The selected method of cell disruption will be determined by the type of biomass, state of biomass and scale that needs to be used.

Direct esterification, simultaneous extraction and transesterification of microalgal fatty acids can be performed on both wet and dry biomass making it a versatile method of biofuels production. The process is multistep and requires a combination of solvent extraction, ultra-sonication, heating at high pressure (3.5 atm.), filtration, density separation of liquids and solvent and oil recovery by evaporation to dryness (Belarbi et al., 2000).

### 1.2.7 Lipid identification methods

Microalgae storage lipids differ from strain to strain and even within a single culture under different growth conditions. It is necessary to identify lipids as the lipid fraction will dictate the properties of the biodiesel produced. Lipid qualification and quantification can be carried out by several means including Nile Red fluorescence microscopy, Nile Red spectrofluorometry, Fourier transform infrared micro-spectroscopy (FTIR), Thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC) or gas chromatography (GC) or any chromatography with mass spectrometry (Medina et al., 1998; Mutanda et al., 2010). Nile Red microscopy is used primarily to ascertain presence of lipid vesicles within cells as an initial screen for lipid accumulation and as a semi-quantitative method for lipid storage. Nile Red spectrofluorometry can also be used as a semi-quantitative method of lipid content determination. These methods

however give no indication as to the lipid fraction and type of lipid present. FTIR can be used for determination and quantification of lipid and carbohydrate storage and is an efficient tool for monitoring of lipid. Microalgal lipid profiling is generally done by gas chromatography with flame ionization detector (CG-FID) and is carried out using the methylated ester form of the lipid (Mutanda et al., 2010). HPLC is a technique not commonly used, however as most fatty acids do not absorb UV at 254nm (the wavelength commonly used in HPLC instruments) and requires conjugation of unsaturated fatty acids or acids containing aromatic moieties that allow easy detection (Medina et al., 1998).

### 1.2.8 Transesterification

Raw microalgal oil is high in viscosity and therefore requires conversion to lower molecular weight constituents in the form of fatty acid alkyl esters. Transesterification is the process of converting raw microalgal lipid (triacylglycerols/free fatty acids) to give renewable, non-toxic and biodegradable biodiesel (Figure 3). The reaction is reversible and thus requires the supply of excess alcohol to maintain equilibrium shift towards the product and improve reaction rate (Singh & Singh, 2010). Transesterification is a reaction of the parent oil with a short chain alcohol, usually methanol, in the presence of a catalyst. Products of the reaction are fatty acid methyl esters (FAME) and glycerol (Mutanda et al., 2010). Catalysts that take part in the reaction are acids, bases or enzymes. Base catalysis is a faster reaction but is limited by the free fatty acids content. Free fatty acids contents in the region of 20% to 50% are responsible for saponification during base catalysed transesterification. Saponification is responsible for consumption of the base catalyst as well as making downstream recovery difficult (Hanna & Ma, 1999). Acid catalysis is suitable for transesterification of oils containing high levels of free fatty acids (Antoni et al., 2007). The reaction however is slow. Speeding up the acid catalysed reaction requires an increase in temperature and pressure making it prohibitively expensive at large scale (Mutanda et al., 2010). The presence of moisture in the reaction is also responsible for saponification and reagents must therefore by dry (Singh & Singh, 2010).

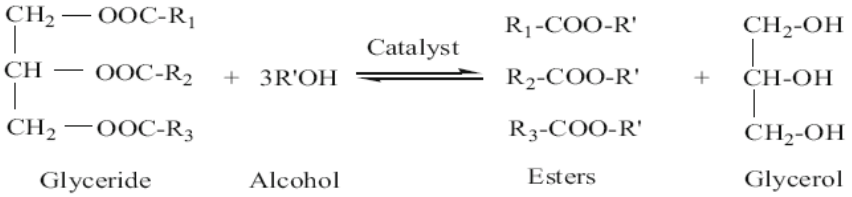


Figure 3: Transesterification of triacylglycerols with alcohol in the presence of acid, base or enzyme catalyst to give Fatty acid methyl esters and glycerol (Du et al., 2008; Mutanda et al., 2010).

Chemical catalysed transesterification has disadvantages in that the process is energy intensive, the catalyst needs to be removed from the product, alkaline water from washing requires remediation, water and free fatty acids result in loss of product due to saponification and recovery of glycerol is difficult (Benemann, 2008). Enzymatic catalysed or biocatalysed esterification is a viable method for parent oils containing high levels of free fatty acids as they can also be converted to alkyl esters (Figure 4). Other benefits include moderate reaction conditions thus less energy intensive, lower alcohol to oil ratio requires for production and easier product recovery (Benemann, 2008). For large scale production this may not be economically viable due to high enzyme production costs and the enzyme catalysed reaction does not run to completeness (Antoni et al., 2007; Benemann, 2008).

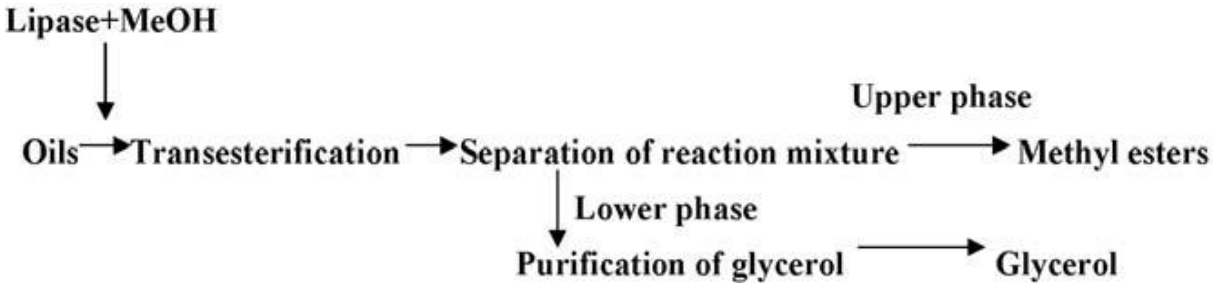


Figure 4: Flow diagram of enzyme mediated alcoholysis for FAME production (Singh & Singh, 2010).

Enzyme catalysis takes place in the form of immobilised lipase, whole cell catalyst and liquid lipase mediated. Immobilised lipase catalysis as the name suggests, employs suitable immobilised extracellular lipases as opposed to free lipases due to increased stability and the potential for repeated use without the requirement of complex separation. Glycerol produced during the alcoholysis is insoluble in oil and readily adheres to the immobilised lipase surface thus diminishing enzyme activity. Removal of glycerol can be a complex process and may impede the continuity of larger scale operations (Benemann, 2008).

Whole cell biocatalysis utilises intracellular lipases and is seen as a method of reducing lipase costs by negating the need for isolation, purification and immobilisation required in conventional immobilised extracellular lipases. Whole cells can be immobilised during cultivation of the organism and stabilised to allow prolonged use as a biocatalyst. Whole cell biocatalysts however, are prone to mass transfer resistance and may require pre-treatments to overcome this. Further research is required into the use of whole cell biocatalysts engineered to overproduce intracellular lipase for biodiesel. The reduction in cost given by this technology is a promising prospect for industrial biodiesel production (Benemann, 2008). Liquid lipase mediated catalysis is a promising prospect due to ease of preparation and thus lower cost. Liquid lipase mediated catalysis is conducted in a water containing system thus aiding recovery of the enzyme. Further research needs to be carried out on feedstocks used for production as well as enzyme recovery (Benemann, 2008).

The use of oxide nanoparticles as catalysts for esterification of triacylglycerols from soybean oil has been tested for their efficiency as catalysts and have shown efficiency of up to 89% conversion. Metal oxide nanoparticles can have acidic or basic properties depending on the metal. This coupled with their large surface areas make them potentially suitable for use as heterogeneous catalysts for alcoholysis of long chained fatty acids. These particles are less toxic than conventional catalysts used. Much research is still required to determine the impact of this technology on the production of biodiesel (Tran et al., 2010).

### 1.2.9 Thermal conversions of biomass to biofuels

The thermal conversion of biomass to biofuels has come to the forefront strongly as an attractive feedstock for liquid fuel production as it is renewable, sustainable, environmentally friendly, is no threat to food security and has economic potential due to diminishing levels of fossil fuels (Demirbas, 2008). Thermal decomposition of algal biomass can yield different types of energy fuels depending on temperature used for conversion as given in Fig 5 (Brennan & Owende, 2010).

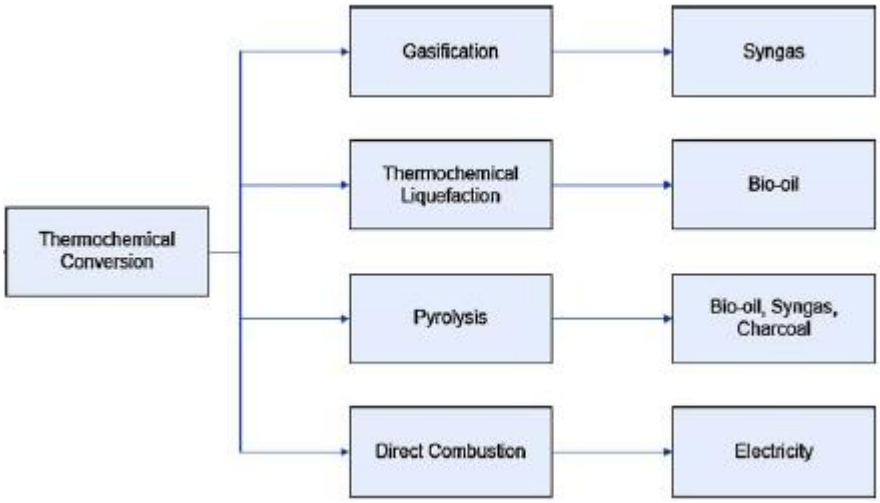


Figure 5: Potential energy products derived by thermal decomposition of microalgal biomass (adapted from Brennan & Owende (2010).

Gasification produces syngas (mixture of combustible gases) by partial oxidation of biomass at high temperature (800 - 1000°C). Biomass is reacted with oxygen and water in the form of steam to produce a mixture of carbon dioxide, hydrogen gas, nitrogen and methane (Brennan & Owende, 2010). Syngas can be burned directly or used as fuel for diesel or gas turbine engines (Mutanda et al., 2010). Syngas produced by gasification is advantageous in that it can be produced from a wide variety of biomass (Brennan & Owende, 2010).

Thermo-chemical liquefaction of biomass is used for conversion of wet biomass to biocrude oil at low temperature (300-350°C) and high pressure (50-200atm) in the presence of a catalyst. Hydrothermal liquefaction occurs by decomposition of biomass into smaller molecules with high energy by utilizing high water activity in sub-critical conditions (Ross et al., 2010). Liquefaction of microalgae have resulted in production of 30-65% dry weight of oil depending on species used (Brennan & Owende, 2010; Mutanda et al., 2010; Ross et al., 2010). The major benefit of thermochemical liquefaction is the ability to use wet biomass. Reactors are however expensive due to their complexity (Brennan & Owende, 2010).

Pyrolysis is the thermal conversion of one substance into another, in presence or absence of a catalyst and the absence of air or oxygen. Pyrolysis of biomass produces charcoal, condensable organic liquids, acetic acid, acetone, methanol and non-condensable gaseous products by a simple, effective, zero waste and pollution free process (Demirbas, 2002; Brennan & Owende, 2010; Demirbas, 2010). Slow pyrolysis (350-700°C) produces high charcoal content. Fast pyrolysis of microalgal biomass conventionally produces 60-75% wt. of liquid bio-oil, 15-25% wt. solid charcoal and 10-20% wt. non condensable gases (Mutanda et al., 2010). Flash pyrolysis uses moderate temperature (500°C) with short vapour residence time for the conversion to liquid biofuels with conversion ratio of up to 95.5% (Brennan & Owende, 2010). Pyrolysis of algal biomass has given promising results and has been shown to produce higher quality bio-oil than lignocellulosic compounds (Brennan & Owende, 2010). Lipid containing biomass has been shown to produce higher heat balances and bio-oil yields (Ross et al., 2010).

The process of direct combustion is burning of biomass in the presence of air for the conversion of stored energy into hot gases usually in a furnace, boiler or steam turbine at temperatures above 800°C. These gases cannot be stored and must be used immediately. The conversion efficiency of biomass to energy is more favourable than that of direct combustion of coal. A disadvantage of direct combustion is the requirement for biomass containing low amounts of water (<50%) giving the requirement for drying and other pre-treatments that may affect the energy balance. The cost of pre-treatment of biomass however makes it less viable than coal. The overall

efficiency of the process may be improved by co-combustion of biomass with coal. There is currently little evidence of the viability of the combustion of algal biomass due to very limited data thus further research is required (Brennan & Owende, 2010).

### 1.2.10 Challenges associated with Algal Biodiesel

Some of the challenges with respect to the use of biodiesel as fuel are that it is susceptible to bacterial oxidation and may cause corrosive within storage tanks (Antoni et al., 2007). Production of biodiesel can be an energy intensive process. Large amounts of glycerol are produced as a by-product and will likely flood the market thus driving prices down thus giving the requirement for other avenues of use. Methanol used in the transesterification process is currently derived from crude sources. These challenges can be overcome by implementation of measures such as designing of tanks for biodiesel storage. High energy input may be overcome by use of the biorefinery concept. Glycerol produced in large quantities could be used to make higher value products or to benefit the community in the form of soap and candles in 3rd world countries. An effective use of glycerol is as a fermentation stock to produce methane as part of the biorefinery concept. Methanol used for transesterification is currently derived from crude sources. Biodiesel has the potential to be a 100% biological fuel in the future (Antoni et al., 2007).

### 1.2.11 Worldwide Biofuels goals and perspectives

Many industrialised nations have already established the need for alternate fuels and have set targets accordingly. The European Union intends using biofuels to replace 5.75% of transportation fuels by 2010 and 10% by 2020. Germany has an ongoing biofuels program and in 2005 had produced 2.5 million tons of crop biodiesel. Spain and France have endeavoured to produce both biodiesel and bioethanol. Australia has targeted 1% fuel replacement by biofuels by 2010. Africa has enormous production potential that is yet to be exploited. Efforts are however underway. Brazil is a major producer of bioethanol and plans to produce 2 billion litres of biodiesel per annum by 2020. The U.S had capacity to produce 18 billion litres bioethanol in 2006 and intends to produce 27.8 billion litres of biofuel by 2015. Canada intended the

replacement of 3.5 % of fuels by biofuels by 2010. Many countries worldwide incentivise the production and use of biofuels in the form of tax breaks (Antoni et al., 2007; Bloyd, 2007). Biodiesel produced by these nations however is limited to crop based biodiesel. Algal biodiesel has the added benefit sequestering carbon dioxide and being a carbon neutral fuel, thus allowing carbon credit accumulation (Chisti, 2007).

# Chapter 2: Bioprospecting for indigenous microalgae

## 2.1 Introduction

Bioprospecting is a vital step in the collection of microalgal strains and the isolation of potential hyper-lipid producers (Ahmad et al., 2011). The selection of indigenous microalgae is vital to the success of large scale culturing of microalgae especially for the production of biofuels (Figure 6). These organisms are specific to certain environments and prevailing climatic conditions (Brennan & Owende, 2010; Mutanda et al., 2010; Williams & Laurens, 2010). The diversity of microalgae is key to the success of producing biodiesel from microalgae in that there are reported to be between 1 and 10 million algal species (Mutanda et al., 2010). These species, especially microalgae occur in diverse environments including freshwater, brackish, marine and hyper-saline (Brennan & Owende, 2010).

The first reported case of isolation of microalgae and purification to axenic culture was achieved by Beijerinck in 1890, who isolated *Chlorella* sp. and *Scenedesmus* sp. (Preisig & Anderson, 1995). Successful isolation of microalgae requires a number of steps to be undertaken: 1) understanding the natural environmental conditions, 2) elimination of contaminants, 3) continued sub-culturing (Anderson & Kawachi, 1995). Understanding of environmental conditions allows mimicking of those environments to encourage growth. The simplest way of accomplishing this is measurement of pH, temperature, etc. and the use of culture enrichment for growth before purification. Some knowledge of the taxonomic group being targeted is also useful to encourage *in vitro* growth. Addition of essential nutrients should occur as soon as possible after sample collection. This will help prevent culture death that may occur due to change in conditions. Collection of samples during warmer months has been shown to be more adaptable to change in temperature. Contaminants in the form of bacteria, algal grazers and undesirable cultures may be eliminated by a combination of one or more techniques. Filtering of samples at collection with a 100µm sieve is useful for the removal of debris and some zooplankton. Serial dilution, streaking on agar plates and the use single cell isolation techniques are extremely useful for elimination of contaminants. Continued sub-culturing allows determination of supply of essential nutrients.

Culture death after a number of sub-cultures indicates the lack of an essential nutrient or by accumulation of metabolic waste in the culturing environment (Anderson & Kawachi, 1995).

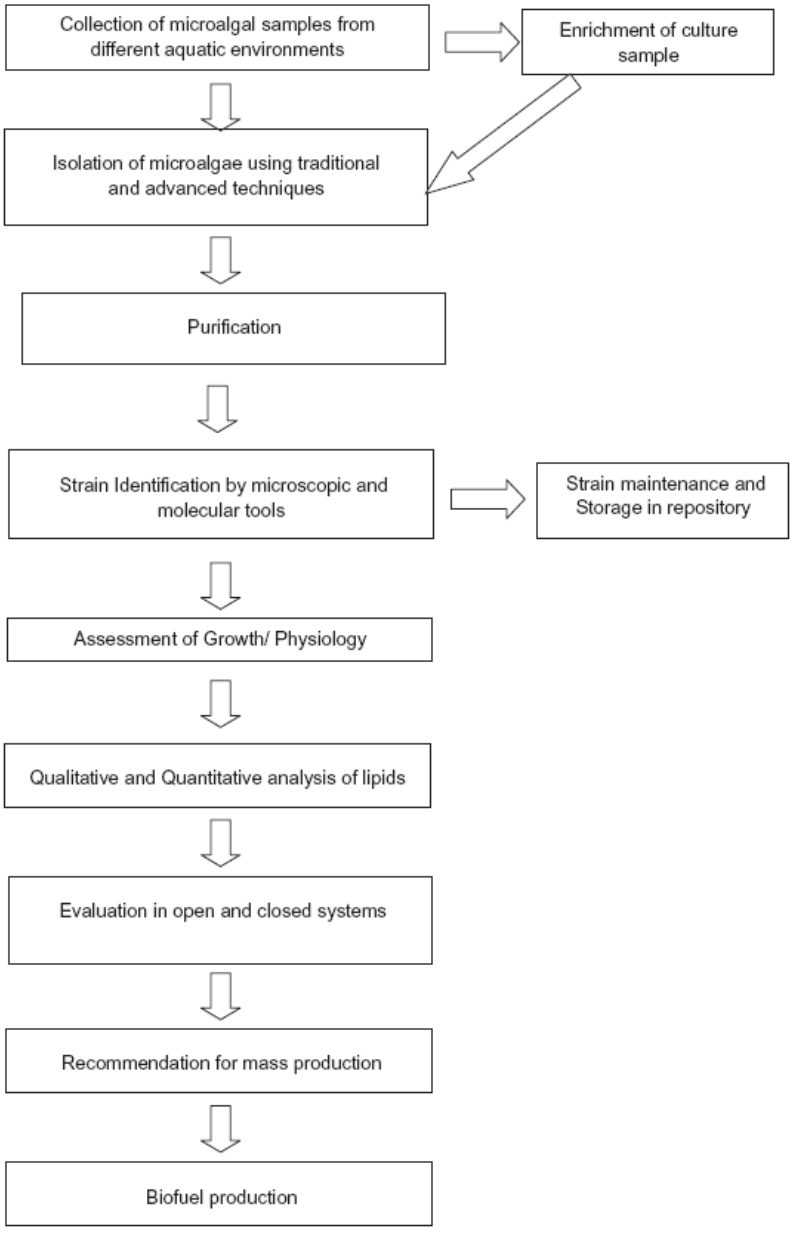


Figure 6: Schematic representation of procedure for bioprospecting through to biodiesel production (Mutanda et al., 2010).

Enrichment of cultures and media used for isolation in the form of agar plates or broth should not contain high concentrations of nutrients. Nutrient rich media may cause culture shock and result in death brought about by nutrient toxicity (Watanabe, 2005). Care should be taken to avoid excessive bacterial growth that may be supported by addition of yeast extract and other components. Overgrowth of bacteria can cause death of the microalgae by inducing anoxic conditions or causing culture toxicity (Anderson & Kawachi, 1995).

Purification of microalgae can be achieved by a number of methods. Popular methods include single-cell isolation by micropipette, isolation using agar plates, atomised cell spray, serial dilution, gravimetric separation and advanced techniques such as micromanipulation and flow cytometry (Mutanda et al., 2010). Each of the methods has advantages and limitations. Single cell isolation allows picking up a single cell but can lead to cell damage by excessive handling. Moreover the process requires a skilled technician for production of the micropipettes to the appropriate size and the ability to make very fine movements. Isolation by agar was a preferred method for coccoid cells and is accomplished by streaking of cultures onto agar plates and purification by re-streaking of single colonies onto agar plates or into broth. Atomised cell spray is accomplished by applying a fine spray of liquid cell suspension over an agar plate using sterile air under pressure. The technique requires a sterile environment to prevent contamination by airborne bacteria. Dilution employs the common microbiological serial dilution technique to obtain a single cell in a small volume. The method is however based on probability and thus may be time consuming and give variable results (Anderson & Kawachi, 1995). Gravimetric separation employs gentle centrifugation of samples in order to separate larger cells from smaller cells. The method is a preliminary technique as it almost never results in isolation of single cells (Anderson & Kawachi, 1995; Mutanda et al., 2010). Micromanipulation is an advancement of isolation of single cells by micropipette. Micromanipulators are commercially available and are primarily used for *in vitro* fertilisation. The micromanipulation technique employs a stereo microscope and micromanipulator to allow fine movement of a capillary pipette allowing removal of single cells from plates or solution. It does however require expertise and experience and is thought to be an ideal technique for single cell isolation. Flow cytometry uses Fluorescence-activated cell sorting in to order electronically separate cells by one or more cell

characteristics. The method is a rapid technique but requires skilled personnel and costly equipment. The objective of this aspect of the study was to isolate and purify producing microalgal for their potential use as biodiesel feedstock.

## 2.2 Materials and Methods

### 2.2.1 Selection of aquatic environments

Aquatic environments are often rich in microalgal diversity. These environments were selected on their potential to harbour strains that are unique to the variation in environmental conditions. Various ponds, lakes, rivers, streams, estuaries were selected. These water bodies often contain a rich diversity in plant and animal life, both aquatic and terrestrial. Richer biodiversities generally imply the presence of efficient nutrient cycling systems and thus ideal environments for algal proliferation. Ponds and lakes are generally slow moving, allowing growth of microalgae that would otherwise be damaged by shear forces caused by rapid movement of water. Rivers, streams and estuaries were selected on the basis of having areas of slower moving water and having well developed ecosystems. Water bodies found in natural environments such as natural gardens, parks and conservancy areas were preferentially selected. These included maturation ponds of wastewater treatment plants due to high nutrient content.

### 2.2.2 Sampling protocol

Samples were collected from various sites in KwaZulu-Natal. Predetermined water bodies were sampled preferentially where evidence of chlorophyll containing microorganisms was present. Samples were collected using the grab sample technique. Glass bottles were immersed into the water-body concerned and samples withdrawn. Scrapings of submersed rocks and loose sediments were also sampled using metal spatulas. Water temperature and pH were measured on site as far as possible and recorded. Type of water body and details of surrounding environment were also recorded.

### 2.2.3 Storage and processing of samples

All samples were returned to the lab in the shortest possible time where they were enriched with modified Bolds Basal Medium (BBM) (Appendix A) and Spirulina Medium (SP) (Appendix B). Media for growth of marine cultures were supplemented with 30g/l NaCl. BBM is considered to general broad spectrum medium for freshwater (Mutanda et al., 2010). SP medium was selected on the basis of containing a very similar macronutrient composition to BBM with the addition of a carbon source in the form of sodium carbonate and sodium hydrogen carbonate. All samples were grown at ambient temperature exposed to natural light.

### 2.2.4 Purification of cultures

Purification of enriched samples was attempted by the use of Nikon Narishige micromanipulator used in conjunction with a Nikon Eclipse TS100 stereo-microscope with dark phase optics and maximum magnification of 400X. Capillary micropipettes (id= 20µm) were designed specifically for this purpose and manufactured for us by Tygerberg Reproductive Technologies. Commercially available capillary micropipettes could not be obtained with the required internal diameter. Enriched cultures were drawn in a single line across the surface of an agar plate and single cells were removed and placed on a demarcated area of the plate free from sample. This area was subsequently removed from the plate by excising using a sterile scalpel and introduced into 15ml tubes containing 2ml media.

Failing isolation using micromanipulation the agar plate isolation method was employed. Samples were spread in 0.1ml aliquots onto the appropriate media containing agar plates. Following growth, colonies were selected based on differing morphology and were streaked onto agar using the T-streak method for purification. Agar plates were parafilmed to prevent drying out of the culture medium and incubated under ambient conditions and natural light. Single colonies were subsequently re-streaked on the basis of purity carried out by light microscopy using a Nikon Eclipse 80i microscope.

### 2.2.5 Culture maintenance

Pure cultures were maintained on AF6 agar (Appendix C) and sub-cultured every 4 to 6 weeks. Working stock was produced by sub-culturing of cultures into AF6 broth.

## 2.3 Results

Samples were collected from various environments in KwaZulu Natal. Sample designations were assigned randomly. Sampling environments targeted were those showing potential for the growth of microalgae as wild types with medium to high potential for nutrient cycling. Of the 55 samples collected, 39 cultures grew under *in vitro* conditions (Table 2). Growth on SPM was predominant with 28 cultures growing *in vitro*. 5 cultures grew on BBM media and 6 grew on both BBM and SPM. The original samples were found to have a pH between 6.75 and 8.48. Sample IP2 was taken from a constructed office park pond. This pond was uncovered but did not show strong evidence of the presence of microalgae. Further the pond showed no evidence of having growth of other plant or bird life. Sample JP was taken from a park. The pond showed strong evidence of pollution and possible anoxic conditions due to what appeared to be a thin film of oil on the surface.

Table 2: List of samples, growth of culture in enriched media and isolates from indigenous sampling sites in KwaZulu Natal

| No. | Sample Designation | Sampling site | Sample type | pH | Growth on Media | Isolate Designation |
|---|---|---|---|---|---|---|
| 1 | 1 | Durban Botanic gardens | Fish Pond | 7.53 | SPM | 1 |
| 2 | 2 | Durban Botanic gardens | pond | 7.23 | SPM | 2 |
| 3 | 3 | Durban Botanic gardens | pond | 8.48 | SPM | 3 |
| 4 | 4 | Durban Botanic gardens | Lake | 7.09 | SPM | 4.1,4.2 |
| 5 | 5 | Reactor Room, DUT | Disused Reactor | 7.72 | SPM | 5 |
| 6 | HP | Hollingwood Pond, PMB | disused pool | 8.07 | SPM | HP |
| 7 | SP1 | Randles road park, Durban | pond | 7.31 | SPM | SP1 |
| 8 | SP2 | Randles road park, durban | pond | 7.88 | SPM | SP2 |
| 9 | SP3 | Randles road park, durban | pond | 7.6 | SPM | SP3 |
| 10 | MP | Mitchell Park, Durban | pond | 8.25 | SPM | MP |
| 11 | VP | Virginia Bush Nature reserve, Durban | Pond | 7.58 | SPM | VP |
| 12 | BM | Beachwood Mangroves, Durban | estuary | 7.49 | BBM | BM |
| 13 | K1 | Kingsburgh WWTP, Kingsburgh | Maturation pond | 6.78 | SPM | K1.1,K1.2 |
| 14 | K2 | Kingsburgh WWTP, Kingsburgh | disused clarifier | 6.86 | SPM | K2 |
| 15 | K3 | Kingsburgh WWTP, Kingsburgh | disused raceway | 6.95 | SPM | K3 |
| 16 | JT | Japanese gardens, Amanzimtoti | pond | 6.68 | SPM | JT 1.1, JT1.2 |
| 17 | IP1 | constructed pond, sezela | pond | 7.03 | SPM | IP 1 |
| 18 | IP2 | constructed pond, sezela | pond | 7.16 | | |
| 19 | BR | Black Rocks, Scottsburgh | pond | 7.58 | SPM | BR1.1, BR1.2 |
| 20 | TR | Klein Manzimtoti river, Amanzimtoti | river | 7.61 | SPM | TR 1.1 |
| 21 | TR2 | Klein Manzimtoti river, Amanzimtoti | estuary | 7.61 | SPM | - |
| 22 | JP | Japanese gardens, Amanzimtoti | pond | 6.86 | - | - |
| 23 | UR | Umkomaas estuary, Umkomaas | estuary | 8.12 | - | - |
| 24 | SB | Scottsburgh beach, Scottsburg | rock pool | 7.99 | SPM | SB 1.1 |
| 25 | MB | Mziyazi river lagoon, Scottsburg | estuary | 7.95 | - | - |
| 26 | RL | Rocky Bay Lagoon, Scottsburgh | estuary | 7.57 | SPM | RL 1.1 |
| 27 | BRL | Black Rocks lagoon, Scottsburgh | estuary | 7.54 | SPM | BRL 1.1 |
| 28 | LR | Lovu River, N2 near scottsburgh | river | 7.3 | SPM | LR 1 |
| 29 | U1 | UKZN PMB Botanic Gardens | Pond | 7.54 | BBM | U 1.1, U 1.2 |
| 30 | U2 | UKZN PMB Botanic Gardens | stream | 7.41 | SPM | U2.1, U2.2 |
| 31 | U3 | UKZN PMB | Pond | 7.43 | - | - |
| 32 | U4 | UKZN PMB | Pond | 7.39 | - | - |
| 33 | U5 | UKZN Westville | Pond | 6.97 | SPM | U 5.1 |
| 34 | U6 | UKZN Westville | Pond | 7.04 | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| 35 | ME | Mount Edgecombe Golf Course, Durban | pond | 7.76 | BBM | ME 1.1, ME 1.2 |
| 36 | ME2 | Mount Edgecombe Golf Course, Durban | pond | 7.61 | - | - |
| 37 | A1 | Japanese gardens, Riverside, durban | pond | 8.47 | SPM | A1.1 |
| 38 | A2 | Maydon Warf road, Durban | canal | 7.89 | SPM | A2.1 |
| 39 | A3 | Charlies Croft Marina (43) | Marine | 8.14 | - | - |
| 40 | A4 | Charlies Croft Marina | Marine | 8.27 | - | - |
| 41 | A5 | Charlies Croft Marina (21) | Marine | 8.17 | - | - |
| 42 | A6 | Wilsons Wharf Marina (21)b | Marine | 8.08 | SPM | A6.1 |
| 43 | A7 | Wilsons Wharf Yatch Basin, Durban | Marine | 8.08 | - | - |
| 44 | A8 | Blue Lagoon , Durban | estuary | 7.69 | - | - |
| 45 | A9 | Mangroves River , Durban | Brackish | 7.86 | - | - |
| 46 | A10 | Golf Road River, PMB | river | 7.94 | BBM/SPM | A10.1 |
| 47 | A11 | Kingfisher lake, Botanic gardens, Pietermaritzburg | lake | 7.94 | BBM/SPM | A11.1, A11.2 |
| 48 | A12 | Durban University of Technology fountain, Durban | Fountain | 8.01 | SPM | A12.1 |
| 49 | A13 | Camps Drift, Pietermaritzburg | river | 7.91 | BBM | A 13.1 |
| 50 | A14 | Botanic Gardens pond, Pietermaritzburg | pond | 8.04 | BBM/SPM | A14.1, A14.2 |
| 51 | A15 | Wetland Pond, Botanic gardens, Pmb | Pond | 7.99 | BBM/SPM | A 15.1 |
| 52 | A16 | River, Botanic gardens, Pietermaritzburg | river | 8.03 | BBM/SPM | A 16.1 |
| 53 | A17 | Maturation pond 2, Northern Works WWTP, Durban | Maturation Pond | 6.75 | BBM/SPM | A17.1 |
| 54 | A18 | Maturation pond , Northern Works WWTP, Durban | Maturation Pond | 7.05 | - | - |
| 55 | A19 | Clarifier, Northern Works WWTP | Clarifier | 6.9 | BBM | A 19.1 |

Samples UR, MB, A8 were taken from estuaries, near the mouth thus coming under influence from ocean currents similar to sample TR2. Sample U3, U4 and U6 were taken from constructed ponds in areas with human traffic and limited sunlight. Sample A18 was taken from a maturation pond that was overgrown by water plants to the degree of showing evidence of death of fish and birds. Only one of the marine samples (A6) showed growth. This sample was taken from the marina under a walkway close to an area of the shore where there was little evidence of water movement.

Micromanipulation as a technique for isolation of microalgae yielded variable results. It was found to be nearly impossible to select single cells from liquid culture droplets placed into clean petri dishes. Even the finest movement of the needle (micropipette) caused movement of the

microalgal cells within suspension. Furthermore insertion of the needle into the drop caused algal cells to move towards and accumulate around the tip of the needle. This caused undesirable species to enter the tip of the needle giving the requirement of removal of the needle from the drop of liquid and removal of the undesirables from the needle before repetition of the process. The use of spreadplates proved to be more successful however upon selecting a single cell, the needle often became clogged by the agar due to pressure required to pick up the cell. The lack of fluid reduced capillary action and therefore physical pressure and suction was required to take up the selected cell. Unclogging of the needle often required more force than could be supplied by the pneumatic system and required removal of the needle from the manipulator. This generally led to loss of the cell and damage to the needle tip in some cases. The best results were yielded by placing a single line of algal suspension on the surface of an agar plate using a Pasteur pipette. This allowed sufficient liquid to be present to achieve capillary action for the selection of coccoid cells by capillary action. Larger cells (less than 20µm) could be sucked into the needle using vacuum. Flagellate cells however presented a challenge of sometimes being lodged in the tip of the needle which may cause cell damage.

Table 3: Growth of algal cells isolated from mixed cultures by micromanipulation

| | **Cell size 5 to 10µm** | | **Cell size 11 to 20µm** | |
|---|---|---|---|---|
| **Cell no** | **Growth** | **Purity** | **Growth** | **Purity** |
| **1** | - | N/A | - | N/A |
| **5** | - | N/A | - | N/A |
| **10** | - | N/A | - | N/A |
| **20** | - | N/A | + | + |
| **40** | - | N/A | + | + |
| **60** | + | - | + | + |
| **80** | + | - | + | + |
| **100** | + | - | + | + |
| **200** | + | - | + | + |

**+** : positive result for growth or purity

**-** : negative result for growth or purity

N/A: Not applicable

The method worked well for separation of larger microalgae with distinct characteristics. A minimum of 20 cells are required for successful culturing (Table 3). Smaller coccoid microalgae however proved to be more difficult to separate. Picking up single cells of smaller coccoid microalgae is easier to accomplish as they easily move into the needle by capillary action. It is however extremely difficult to distinguish between different types of coccoid cells. The maximum magnification is limited to 400X which is insufficient for morphological distinction between cell type that share a similar size, making it extremely difficult to obtain pure cultures (Table 3). Moreover a higher number of cells are required for successful culturing. Cultures that are larger than 20µm require larger needles. These are difficult to use in a micromanipulator as the tip of the needle blocks much of the field of view. They are also prone to attachment of smaller cells to the outer surface of the needle. The majority of the samples were therefore isolated by the agar plate isolation technique.

## 2.4 Discussion

Microalgae are still considered a largely untapped resource (Singh et al., 2011b). Bioprospecting is a method of obtaining the best performing microalgal strains as they occur naturally. Table 2 gives an indication of the vast array of environments from which it is possible to isolate microalgae. The nutritional compositions of aquatic environments strongly dictate the type of microalgae that grow in such environments (Mutanda et al., 2010). All of the water bodies that yielded cultures (Table 2) shared similar characteristics in that these are situated in areas rich in foliage, had some area of its surface that was shaded by trees, and generally had some bird and animal life as part of ecosystem. The aforementioned ecosystems show great potential for nutrient cycling.

Micromanipulation in literature was suggested to be a promising technique for purification of microalgae (Mutanda et al., 2010). The method however has its limitations as shown by table 3. Selection of large numbers of cells requires long periods of time when the technique is used by a skilled operator. This micromanipulator is a very sensitive piece of equipment that requires a stable vibration free surface for operation. This makes movement of the micromanipulator into a

laminar flow unit impossible. This limitation is likely to be the one of the most restrictive to the success of the technique. Due to the open conditions in which isolation occurs, the technique is extremely prone to airborne contamination. This could be potentially overcome by the use of antibiotics in the culture media. This may however impact growth of the microalgae and requires further investigation (Anderson & Kawachi, 1995).

Isolation of microalgae is the first step towards production of biofuels from microalgae. This is followed by one of the most important steps, culture selection. Optimisation of conditions for the production of desired products is a very important step to ensure feasibility of producing microalgal biofuels. This can potentially be achieved by stimulated evolution or the selection of wild type microalgae that are already adapted to the growth conditions of an area. Thus bioprospecting for indigenous microalgae aids in the optimisation and may further reduce the requirement for additional nutrients and lessen the requirement for directed evolution. Marine microalgal species have been shown to produce higher levels of phospholipids than triacylglycerols (TAGs) (Singh et al., 2011b). These types of lipid are unsuitable for the production of biodiesel via transesterification, thus we have focused on bioprospecting for lipid producing microalgae from freshwater and brackish environments. Despite the huge diversity of naturally occurring lipid producing microalgae, very few species have been shown to have commercial significance including *Chlorella, Spirulina, Dunaleila* and *Haematococcus* spp. These species however are cultivated for high value products in the form of pigments such as beta carotene, proteins for human consumption and neutrocueticals (Singh et al., 2011b).

# Chapter 3: Selection of Algal strains

## 3.1 Introduction

The criterion for selection of superior lipid producers should not simply be microalgae that produce the largest amount of lipid but the strain with the highest lipid productivity (Benemann, 2008). Productivity is a measure of the amount of lipid produced taking into account the growth rate of the alga concerned. As lipid is formed as a storage product there is a general inverse relationship between lipid production and biomass yield. Microalgal lipid consists of primarily triglycerides but also contain fractions of isoprenoids, phospholipids, glycolipids and hydrocarbons. They contain more oxygen and are more viscous than crude oil (Johnson & Sprague, 1987). Desirable characteristics of species are 1) production of biofuels and valuable co-products, 2) high photosynthetic efficiency, 3) high oil productivity, 4) potential for favourable energy balance taking into account energy required for growth and processing (Chisti, 2007; Brennan & Owende, 2010). The primary target towards the goal of finding a strain that meet the desirable characteristics for biodiesel production from microalgae is selection of a high lipid producing strain.

Pre-screening of various cultures by a rapid method is vital to the process of selection. Conventional lipid analysis techniques require large sample volumes and are time and labour intensive processes making rapid screening difficult (Chen et al., 2011b). Pre-screening determines the potential of the culture to produce lipid. These cultures are then further screened for high growth rate and subsequently optimisation of growth conditions and lipid yield. Cultures showing evidence of higher quantities of lipid are preferentially selected. It must however be considered that cultures accumulate lipid due to nutrient stress. Selection of cultures with little to no nutrient stress is likely to yield better results during optimisation. Nile Red staining offers a rapid method for the detection of neutral lipid (Kimura et al., 2004).

Nile Blue is a non fluorescent dye used for histochemical detection of lipid, having the property of staining acid lipids blue and neutral lipids red. Nile Blue however binds to fatty acid to form a

soaps. Nile Red forms a component of Nile Blue dye and thus selectively stains neutral lipid. Benz(a)pyrene is a fluorescent dye that is also used in the detection of lipid. Fluorescence relative to that of Nile Red is significantly lower although lipid droplets can be clearly seen. Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) is an intensely fluorescent dye (figure 7) that with properties that allow for its use as a vital stain for intracellular lipid droplets. Nile Red is poorly soluble in water but readily soluble in a variety of organic solvents. The excitation and emission wavelengths of Nile Red can vary over a range of 60nm depending on the hydrophobicity of the solvent. Fluorescent intensity of the dye at the appropriate wavelength is the same for all solvents.

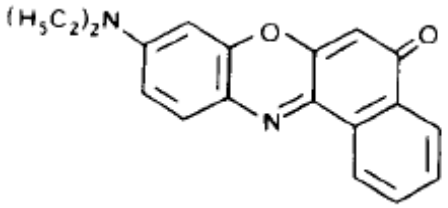


Figure 7: Chemical structure of hydrophobic, intensely fluorescent Nile Red dye

The property of selective fluorescence is partly due to fluorescent quenching by the dye in aqueous solution. Aqueous quenching thus allows only the hydrophobic cell constituents (lipid) to fluoresce intensely (Greenspan et al., 1985; Kimura et al., 2004). Cellular lipids may fluoresce but to a lesser extent than neutral lipid and may appear an orange colour (Greenspan et al., 1985). The objective of this aspect of the work was the selection of lipid producers on the basis of oil presence and growth kinetics for further optimisation.

## 3.2 Materials and Methods

### 3.2.1 Pre-screening

Nile red staining was used as a method to screen for lipids. Nile red of concentration 250mg/L will be prepared by the addition of 25mg Nile red powder to 100ml acetone. This staining solution was stored at 4°C in an amber bottle. Algal cultures were stained at a ratio of Nile Red: culture medium at 2:1000 (v/v) and allowed to develop for 3 to 5 minutes in the absence of light before viewing as the dye is prone to fading, which begins after 5 mins (Kimura et al., 2004). Slides were prepared as wet mounts and viewed using 450-490nm excitation filter under fluorescence using a Zeiss Axiophot epifluorescence microscope with Zeiss Axiolab 8.0 software. Centrifugation was employed as required to concentrate the samples with low biomass concentrations. Biomass was observed as a red colour and neutral lipid as bright to golden yellow (Greenspan et al., 1985; Kimura et al., 2004).

### 3.2.2 Growth kinetics

Modified AF6 media (Appendix C) was found to support growth of all cultures cultured in either BBM or SPM and was thus used as a general purpose growth media. AF6 broth (Appendix C) was used for preliminary investigations. Cultures were grown at ambient temperature and constant illumination provided by Sylvania Gro-Lux (Germany) fluorescent tubes at 150µmol/$m^2$s. The trials were carried out in triplicate. Determination of growth rate was done spectrophotometrically using a Merck Pharos 300 Spectroquant using a 10mm quartz cuvette. Optimal wavelength determination was done by using a wavelength scan from UV to near infrared wavelengths and an optimal wavelength of 680nm was determined for the cultures the results ranged from 677nm to 684nm. The wavelength of 680nm was confirmed by literature (Lee et al., 1998; Qin, 2005). Growth rate (K) and generation time (G) was calculated by the equations 1 and 2 (Qin, 2005).

$$K = \frac{log(\frac{OD_f}{OD_i})}{T} \times 3.322$$

**ODf : final optical density**
**ODi: initial optical density**
**T: time in days**

Equation 1: Calculation of growth (K) rate for microalgae using optical densities

$$G = \frac{0.301}{K}$$

Equation 2: Calculation of generation time (G) in days

### 3.2.3 Identification

Microalgae identification was performed using morphological taxonomic keys found in The freshwater algae of the British Isles (John et al., 2002) and How to know freshwater algae (Prescott, 1970).

## 3.3 Results

Pre-screening yielded 15 cultures that produced lipid. These cultures are detailed in Table 4. These lipid droplets were seen in varying degrees. All of the oil producing cultures showed growth to varying degrees under the same cultural conditions. Cultures A11.2 and BM showed significant amounts of lipid as seen in Figures 8 and 9 respectively. The micrographs (Figures 8

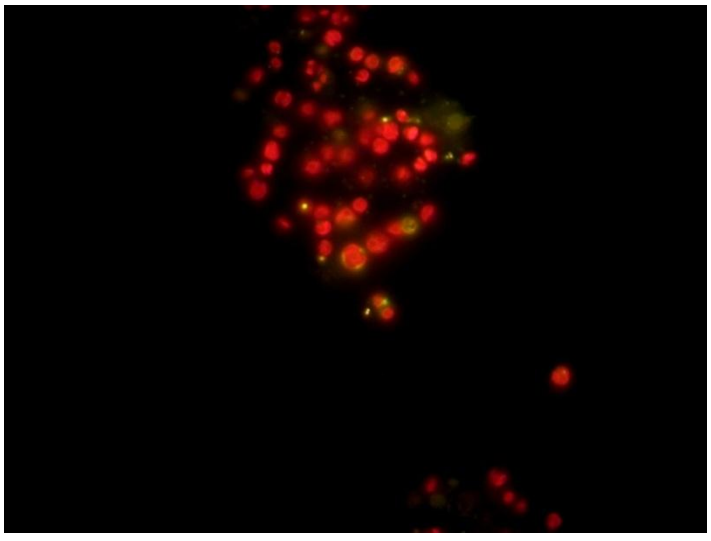

Figure 8: Nile Red micrograph taken using epifluorescence of culture A11.2

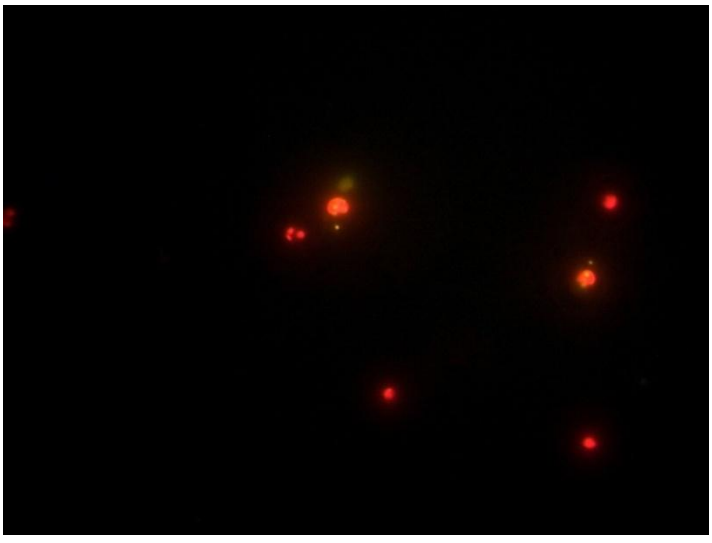

Figure 9: Nile Red micrograph taken using epifluorescence of culture BM

and 9) depict algal cell as red and lipid droplets as a golden yellow making them clearly distinguishable. The qualities of micrographs are sometimes variable due to use of a colour camera for capturing of images. Colour cameras have the disadvantage of absorbing part of the fluorescence as it passes through the lens. Obtaining micrographs with good definition and colour saturation sometimes requires removal of excess noise and white balance adjustment. Nile Red fading caused by light quenching was overcome by exposure of the stained colour to light and especially fluorescence for as short a period as possible. Micrographs were taken after development for 3 to 5 minutes to aid in the prevention fading.

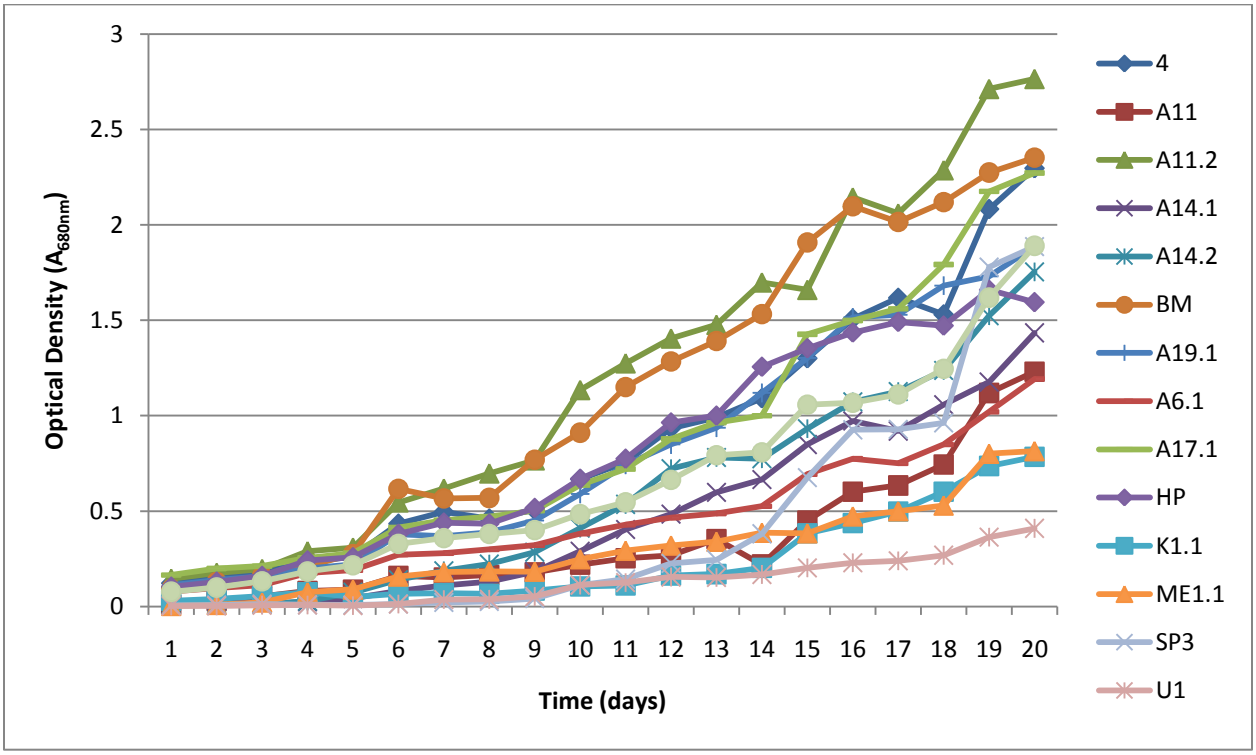


Figure 10: Growth rates of oil producing cultures in AF6 media grown at lab scale using ambient temperature

Oil producing strains were cultured to ascertain growth rates and generation time (Table 4) for the determination of the most appropriate cultures for optimisation. The growth conditions were chosen on the basis of feasibility for culturing a large number of samples simultaneously. Standard deviations are omitted from Figure 10 due to the depiction of many samples which

exhibited overlap during the period of growth. Cultures A11.2 and BM showed the highest growth reaching culture densities of 2.7654 and 2.352 respectively (Figure 10). All cultures showed a similar trend, in that, growth rates were low for the first 5 days of cultivation. From days 6 to 9, all isolated showed an increase in growth but still did not achieve the maximum growth rate. This was achieved from days 10 to 16 of cultivation period before a significant decline in growth rate.

Table 4: Tentative identification of oil producers isolated showing maximum density achieved, growth rates (K) and generation time (G) under lab conditions

| Name | Tentative Identification | Final OD (680nm) | Growth Rate (K) | Generation Time (hrs) |
|---|---|---|---|---|
| A11.2 | *Scenedesmus* sp. | 2.7645 | 0.2126 | 33.98 |
| BM | *Chlorella* sp. | 2.352 | 0.2346 | 30.79 |
| A17.1 | *Navicula* sp. | 2.271 | 0.1891 | 38.20 |
| 4 | *Chlorella* sp. | 2.296 | 0.2114 | 34.17 |
| A19.1 | *Chlorella* sp. | 1.887 | 0.2034 | 35.52 |
| A14.2 | *Chlorella* sp. | 1.7535 | 0.3435 | 21.03 |
| HP | *Chlorella* sp. | 1.5945 | 0.1969 | 36.68 |
| SP3 | *Scenedesmus* sp. | 1.8855 | 0.4037 | 17.89 |
| U2.1 | *Chlorella* sp. | 1.89 | 0.2304 | 31.35 |
| A14.1 | *Chlorococum* sp. | 1.434 | 0.3393 | 21.29 |
| A11.1 | *Scenedesmus* sp. | 1.23 | 0.3471 | 20.81 |
| A6.1 | *Chlorella* sp. | 1.1925 | 0.1967 | 36.72 |
| ME1.1 | *Scenedesmus* sp. | 0.8145 | 0.3931 | 18.38 |
| K1.1 | *Scenedesmus* sp. | 0.7845 | 0.2342 | 30.84 |
| U1 | *Chlorella* sp. | 0.4095 | 0.3678 | 19.64 |

From Table 4 it can be seen that of the 15 oil producing cultures, 14 fall into the category of green microalgae and 1 (*Navicula* sp.) was found to be a diatom (Figure 12). *Chlorella* sp. (Figure 11) was found to be 8 of the oil producers, 5 *Scenedesmus* sp. (Figure 14) and 1 *Chlorococum* sp. (Figure 13) were amongst the isolates. All of the species are known to be oil producers from literature (Chisti, 2007; Illman et al., 2000; Singh et al., 2011).

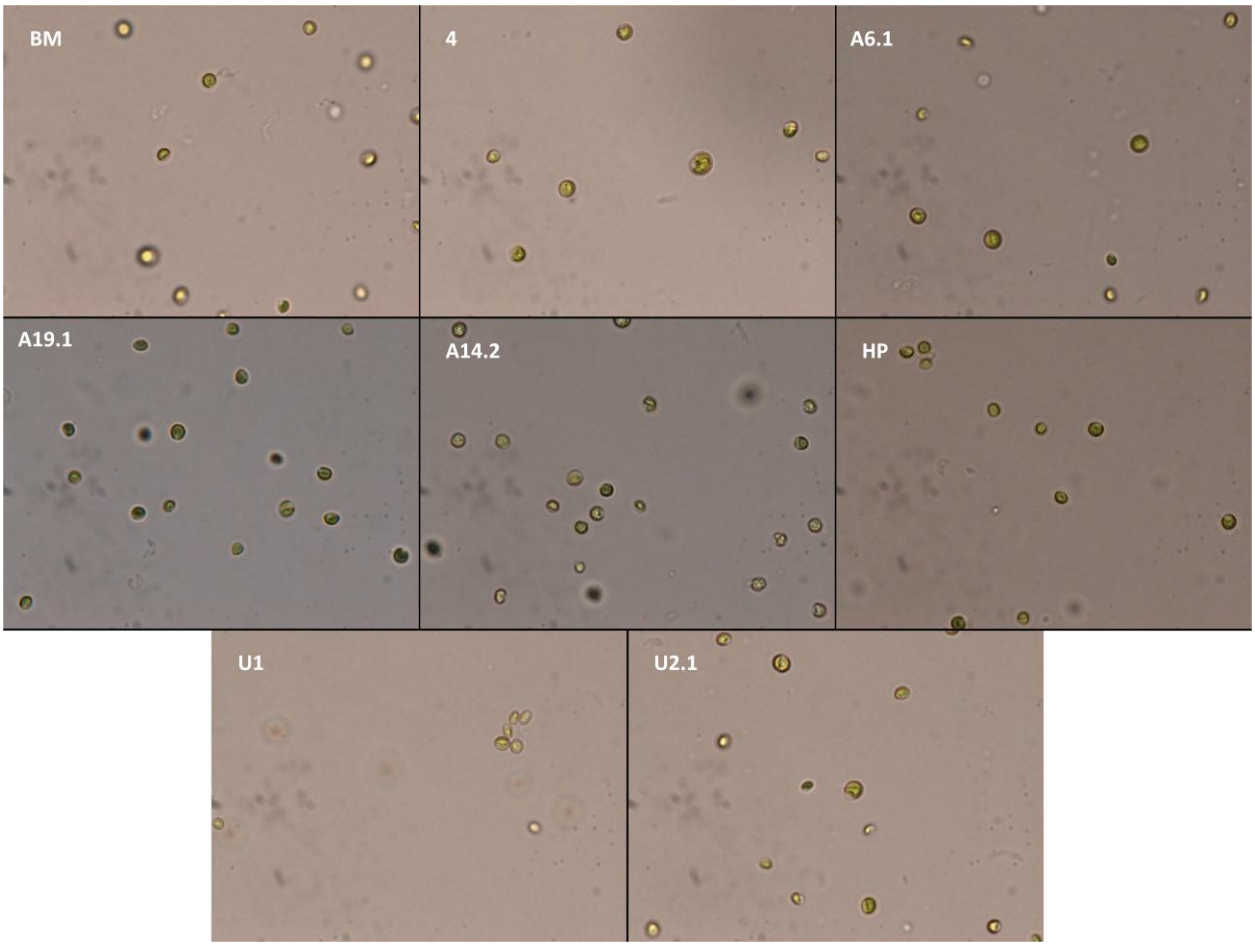


Figure 11: Light micrographs of oil producing *Chlorella* sp. isolated from various environments

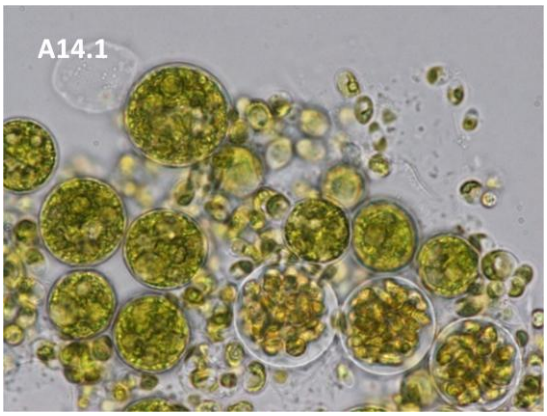


Figure 12: Light micrographs of oil producing Chlorella sp. isolated from Pmb Botanic gardens pond

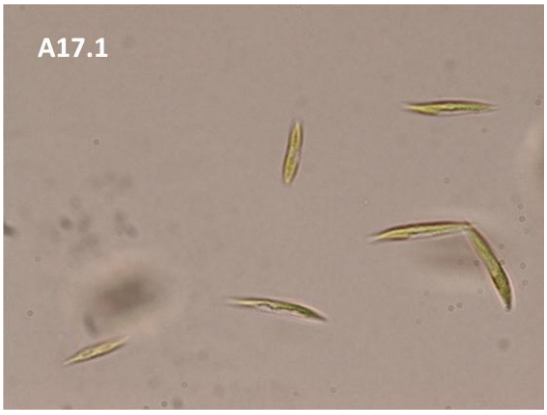


Figure 13: Light micrographs of oil producing *Navicula* sp. isolated from a maturation pond

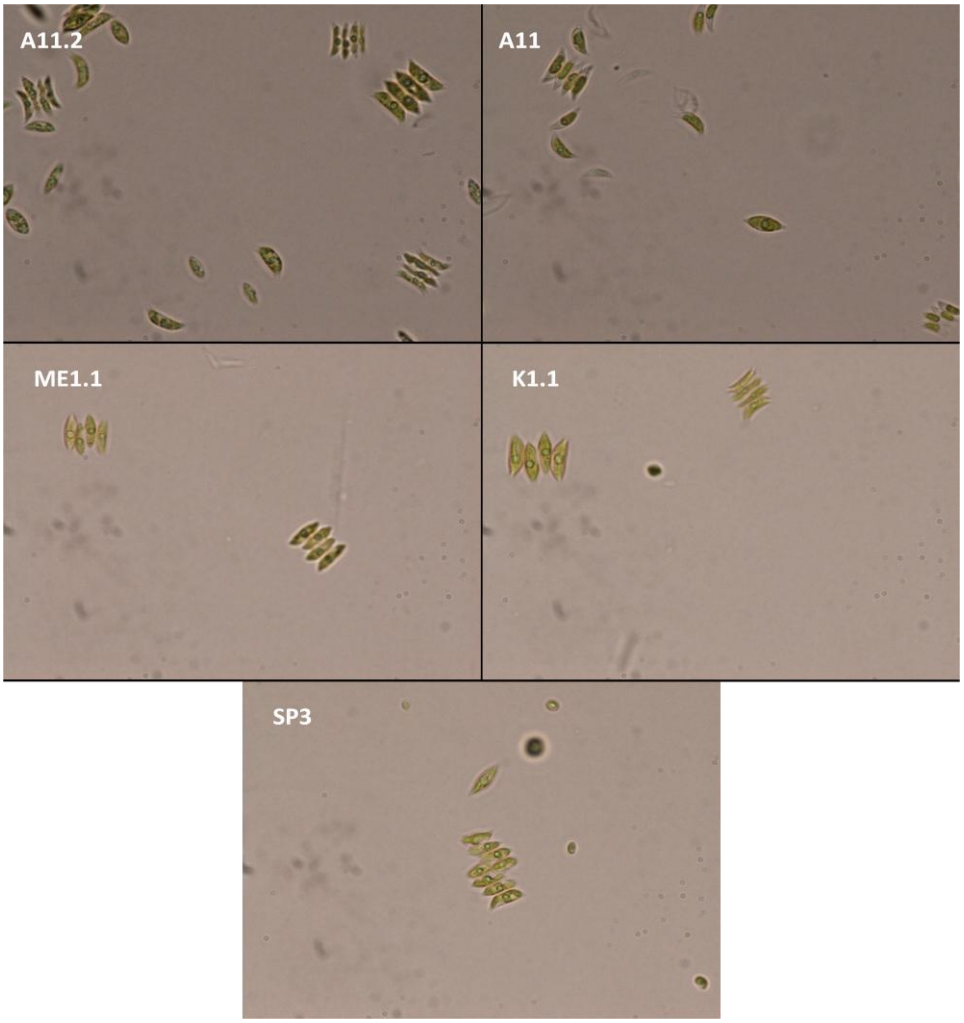


Figure 14: Light micrographs of oil producing *Scenedesmus* sp. isolated from various environments

## 3.4 Discussion

Oil producers isolated from the various sampling environments showed the best producers of lipid to be predominantly *Scenedesmus* and *Chlorella* sp (Table 4). Preliminary screening for the production of oil showed that most of the oil producers (Table 2) were isolated from stagnant ponds or very slow moving streams. 46% of the oil producing samples was isolated from botanic gardens ponds. These strains can then be further optimised for improved lipid efficiency by adaptation of the cultural conditions and/or genetic engineering (Singh et al., 2011a). Modification of metabolic pathways for growth and lipid synthesis can be obtained in certain strains by the modification of a single nutrient concentration. Certain strains of *Chlorella*

*vulgaris* may be modified the simple increase in iron concentration. Table 5 shows various strains of microalgae that produce lipid in moderate quantity thus giving the basis for increased lipid production by optimisation.

Table 5: Various strains of microalgae showing protein, carbohydrate, lipids and nucleic acid content on percentage dry matter basis (Singh et al., 2011b).

| | Protein | Carbohydrate | Lipids | Nucleic acid |
|---|---|---|---|---|
| Freshwater algal species | | | | |
| *Scenedesmus obliquus* | 50–56 | 10–17 | 12–14 | 3–6 |
| *Scenedesmus quadricauda* | 47 | – | 1.9 | – |
| *Scenedesmus dimorphus* | 8–18 | 21–52 | 16–40 | – |
| *Chlamydomonas rheinhardii* | 48 | 17 | 21 | – |
| *Chlorella vulgaris* | 51–58 | 12–17 | 14–22 | 4–5 |
| *Chlorella pyrenoidosa* | 57 | 26 | 2 | – |
| *Spirogyra sp.* | 6–20 | 33–64 | 11–21 | – |
| *Euglena gracilis* | 39–61 | 14–18 | 14–20 | – |
| *Spirulina platensis* | 46–63 | 8–14 | 4–9 | 2–5 |
| *Spirulina maxima* | 60–71 | 13–16 | 6–7 | 3–4.5 |
| *Anabaena cylindrica* | 43–56 | 25–30 | 4–7 | – |
| Marine algal species | | | | |
| *Dunaliella bioculata* | 49 | 4 | 8 | – |
| *Dunaliella salina* | 57 | 32 | 6 | – |
| *Prymnesium parvum* | 28–45 | 25–33 | 22–38 | 1–2 |
| *Tetraselmis maculata* | 52 | 15 | 3 | – |
| *Porphyridium cruentum* | 28–39 | 40–57 | 9–14 | – |
| *Synechoccus sp.* | 63 | 15 | 11 | 5 |

All cultures showed only a small increase in biomass density from days 1 to 5, this period corresponds to the lag phase in which cells undergo metabolic changes whereby they adapt to the cultural conditions. Microalgae employ *de novo* synthesis and recycling of lipids for rapid adaptation to new environments (Williams & Laurens, 2010). Subsequent to day 5 all cultures showed steady growth changes in various periods of growth (Figure 10). No evidence of culture entering stationary phase is likely due to the nutrients still being available in quantities sufficient enough to sustain growth. Generation times for the various strains ranged from 17.9 hours for the fastest growing isolated culture to 38.19 hours for the slowest (Table 4). Microalgae are known to have rapid growth rates with certain cultures having the capacity to double in 24 hrs with some cultures doubling as fast as 3 to 4 hours (Chisti, 2007; Liu et al., 2011). When considering the growth rates achieved, it must be noted that the growth rates were calculated over a long

cultivation period. This brings in variability due to nutrient depletion and decrease in light intensity. The cultures that showed higher growth rates were generally low in density. Increased culture density results in a decrease in light intensity due to the shading effect. Furthermore cultures require different nutrient levels for optimum growth (see Chapter 4). Cultures high in density will consume nutrients at a more rapid rate than cultures with lower densities. Despite cultures being of the same genus, variability was observed in growth rates. Latala et al.(1991) tested *Chlorella* sp. in batch culture and despite both species belong the same genus, certain interactions may be species specific.

Isolation of indigenous *Chlorella* sp. was a promising result as *Chlorella* sp. is known as lipid producers (Pruvost et al., 2011). Several *Scenedesmus* species have also been shown to produce lipid in similar quantities to *Chlorella* sp. (Table 5). *Chlorella* sp. is generally high in C16:0 fatty acids. C16:0 fatty acids are suitable for conversion to biodiesel. Cheng et al., (2010) showed that certain fatty acids produced by *Chlorella* sp. could be increased from 31% to 55%. *Scenedesmus* sp. has shown excellent potential for $CO_2$ sequestration and production of lipid (Cheng et al., 2011). *Chlorella* sp. has been found to accumulate between 28% and 36% lipid content per gram of dry cell weight (Anderson & Kawachi, 1995; Chisti, 2007; Chen et al., 2011a). *Scenedesmus* sp. is reported to accumulate 21.1% lipid content per gram of dry cell weight (Chen et al., 2011a; Singh et al., 2011b). Both *Chlorella* sp. and *Scenedesmus* sp. have potential for good biomass productivity and excellent carbon dioxide uptake rates (Brennan & Owende, 2010).

The ultimate aim of the study is isolation of high lipid yielding algal species for the production of biodiesel. Being a low value product, the logical choice for industrial production is that of an open pond system. Since high oil producing microalgae are generally slower growing microalgae, the culture of choice should therefore be of an extremophilic nature to enable a competitive advantage in open pond culturing by the adaptation of culture conditions to favour the culture of choice over contaminant strains (Liu et al., 2011).

# Chapter 4: Optimisation of growth and lipid yields of selected strains of microalgae

## 4.1 Introduction

Optimisation of growth and lipid yield is essential to the economic viability of production of biodiesel from microalgae. Lipid accumulation occurs naturally as a mechanism for energy storage during unfavourable conditions (Li et al., 2011). The role of lipids in the growth of microalgae is as energy reserves and part of the structural components of the cell. Phospholipids and glycolipids are the primary components of cell wall structures and determine the fluidity of membranes under various conditions. This is achieved by being able to adapt quickly to changes in environment by recycling of lipids and *de novo* synthesis. A large proportion of phosphate is present in the cell wall (Williams & Laurens, 2010). Triacylglycerols are the primary storage components as energy reserves (Mairet et al., 2011; Singh et al., 2011b). The greater proportions of the lipids produced are TAGs which are produced as metabolic rate of microalgae slows (Williams & Laurens, 2010). Some species have high growth rates and the ability to produce high amounts of lipid under certain conditions. Lipid accumulation may be induced or effected by a variety of stress factors such as the removal or limitation of essential nutrients such as nitrogen as well as changes in inorganic carbon and light intensity (Johnson & Sprague, 1987). Change in cultural conditions may be used as a mechanism for the manipulation of metabolic pathways resulting in the redirection of cellular function to the production of desired products such as neutral lipid (Singh et al., 2011a). This method of metabolic manipulation is preferred over mutagenesis and the production of transgenic strains due to problems with stability of transformants and the potential impact on environmental security. Microalgal lipid is a valuable component of microalgal biomass as they are high in energy and similar to conventional fuels (Singh et al., 2011a). Lipid accumulation generally has an antagonistic relationship to growth rate. Therefore it is important to determine the trade off between neutral lipid production and algal growth as part of the optimisation for biodiesel production (Ahmad et al., 2011; Mairet et al., 2011).

## 4.2 Materials and Methods

Cultures A11.2 (*Scenedesmus* sp) and BM (*Chlorella* sp) were selected for further optimisation. Growth rate was determined spectrophotometrically using a Merck Pharo 300 Spectroquant with quartz cuvette (path length 10mm) at 680nm. Equations 1 and 2 were used to calculate growth rate and generation time.

### 4.2.1 Selection of Growth Media

Both *Scenedesmus* and *Chlorella* spp. were grown in 5 different media to establish the best media for growth of the cultures. Each culture was inoculated into AF6 medium (Appendix C), BG 11 medium (Appendix D), modified Bold's Basal medium (BBM; Appendix A) Bristol's NaCl medium (Appendix E) and CHU #10 medium (Appendix F). Media was selected on the basis of nutrient composition as well as concentration of specific nutrients essential for microalgal growth with special consideration given to nitrate and phosphate levels. AF6 medium is used as a general culture medium for microalgae requiring slightly acid media as well as for culture maintenance when used at half strength. BG11 medium is used as a general medium for freshwater cultures but is preferred for growth of microalgae with high nutrient requirements as the concentrations of nitrate and phosphate are exceptionally high. BBM is also a general culture medium but differs in that it contains high levels of trace elements. Bristol's NaCl is the base medium from which BBM is derived. It however contains no trace elements or vitamins and a significantly higher concentration of NaCl. Chu#10 media is a general medium which contains low amounts of nitrate and phosphate. Nitrate is supplied in the form of $Ca_2 (NO_3)_2$ as opposed to $NaNO_3$. In addition Chu #10 contains a silicate that is required for growth of certain types of microalgae. Cultures were grown in triplicate in 750ml glass jars with a working volume of 500ml under ambient conditions and exposure to 150µmol/$m^2$s with a light: dark cycle of 16h:8h for a period of 21 days. Culture density measurements were performed daily. Growth rates were calculated using equations 1 and 2 (Section 3.2.2).

### 4.2.2 Determination of optimum nitrate concentration for optimisation of growth

Cultures were grown in modified BG 11 (Appendix D). Concentrations of Sodium nitrate were amended to 0 g/L (control), 0.3g/L, 0.6g/L, 0.9g/L, 1.2g/L and 1.5g/L for growth trials on each of the algal species chosen. The experiment was done in triplicate. Cultures were grown in 750ml glass jars with a working volume of 500ml under ambient conditions and exposure to 150µmol/$m^2$s with a light: dark cycle of 16h: 8h for a period of 21 days. Culture density measurements were performed daily. Growth rates were calculated using equations 1 and 2 (Section 3.2.2).

### 4.2.3 Determination of optimum phosphate concentration for optimisation of growth

Similarly di-Potassium hydrogen ortho-phosphate concentrations were varied as 0g/L (control), 0.008g/L, 0.016g/L, 0.024g/L, 0.032g/L and 0.04g/L of the original concentration as per media (0.04g/L). The experiment was done in triplicate. Cultures were grown in 750ml glass jars with a working volume of 500ml under ambient conditions and exposure to 150µmol/$m^2$s with a light: dark cycle of 16h: 8h for a period of 21 days. Culture density measurements were performed daily. Growth rates were calculated using equations 1 and 2 (Section 3.2.2).

### 4.2.4 Determination of optimum nitrate and phosphate concentrations for optimisation of lipid yield

Cultures were grown in modified BG 11 (Appendix D). di-Potassium hydrogen ortho-phosphate concentrations were varied as Control, 0.008g/L, 0.016g/L, 0.024g/L, 0.032g/L and 0.04g/L of the original concentration as per media (0.04g/L). The experiment was done in triplicate. Cultures were grown in 750ml glass jars with a working volume of 500ml under ambient conditions and exposure to 150µmol/$m^2$s with a light: dark cycle of 16h: 8h for a period of 10 days. Lipid yields were determined *in situ* using whole cell relative fluorescent microscopy. Cultures were observed with 450-490nm excitation filter using a Zeiss Axiophot epiflourence microscope with Zeiss Axiolab 8.0 software for imaging with Nile Red as the vital stain for lipid

detection. Semi quantification was carried out using Olympus anaLYSIS ver.5.0 image analysis software. Lipid content is expressed as percentage lipid content per cell. Method modified from Qin, (2005)

### 4.2.5 Determination of the effect of pH on growth rate

Cultures were grown in modified BG 11 (Appendix D). pH values were adjusted using HCl and NaOH to give control (unaltered) and pH 7, 8, 9, 10, 11, 12. pH was read daily and adjusted to the pre-determined value. The experiment was done in triplicate. Cultures were grown in 750ml glass jars with a working volume of 500ml under ambient conditions and exposure to 150µmol/$m^2$s with a light: dark cycle of 16h: 8h for a period of 14 days. Culture densities were measure spectrophotometrically daily. Growth rates were calculated using equations 1 and 2 (Section 3.2.2).

## 4.3 Results

### 4.3.1 Selection of growth media

The medium chosen for growth of algal cultures plays an important role in the optimisation of growth of microalgae. Media components and concentrations vary thus giving the need for media selection as part of growth optimisation. The best performing media for growth of *Scenedesmus* sp. was observed to be BG11 (Figure 15 and Table 6). AF6 media showed similar growth trend to BG11 however with a consistent lower density, generation times for growth in BG11 and AF6 were 33.28 hours and 36.83 hours respectively.

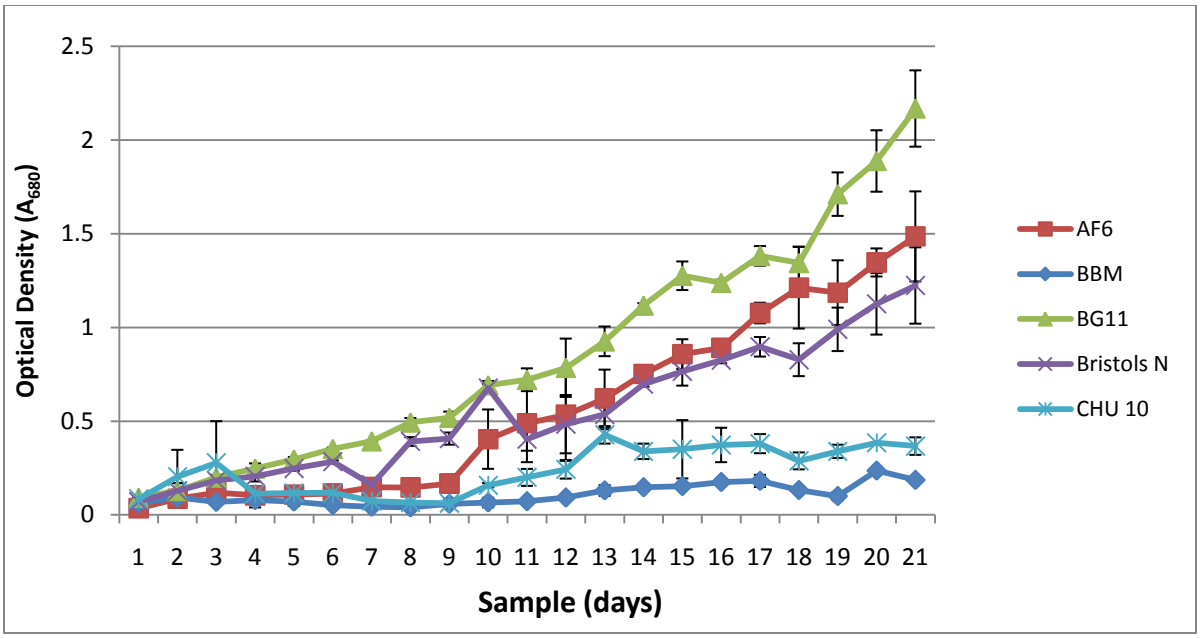


Figure 15: Growth rates of *Scenedesmus* sp in various media compositions to ascertain the most appropriate media for optimisation of growth

Table 6: Growth rates and generation time of *Scenedesmus* sp. grown in various media for a period of 21 days to ascertain best media composition for growth of the culture

| Media | Growth Rate | Generation Time (h) |
|---|---|---|
| **Bg11** | 0.2171 | 33.27 |
| **Af6** | 0.1962 | 36.82 |
| **Bristols** | 0.1956 | 36.93 |
| **Chu** | 0.1005 | 71.88 |
| **BBM** | 0.0755 | 95.68 |

BBM exhibited a consistently low culture density throughout the period of cultivation reaching a maximum OD of 0.236 (Figure 15). A steady increase in cell density was observed for Bristol's NaCl for the first 10 days of cultivation before the culture showed a slight decline in cell density. Generation time for the period of days 10 to 21 was calculated to be 42.28 hours, this differed from the overall (Table 6). Chu #10 exhibited unconventional growth characteristics in that the growth rate was exceptionally high from days 1 to 3 before declining and remaining at steady

state between days 4 to 10. The culture subsequently showed an increase growth rate till the end of the cultivation period with a generation time of 71.88 hours.

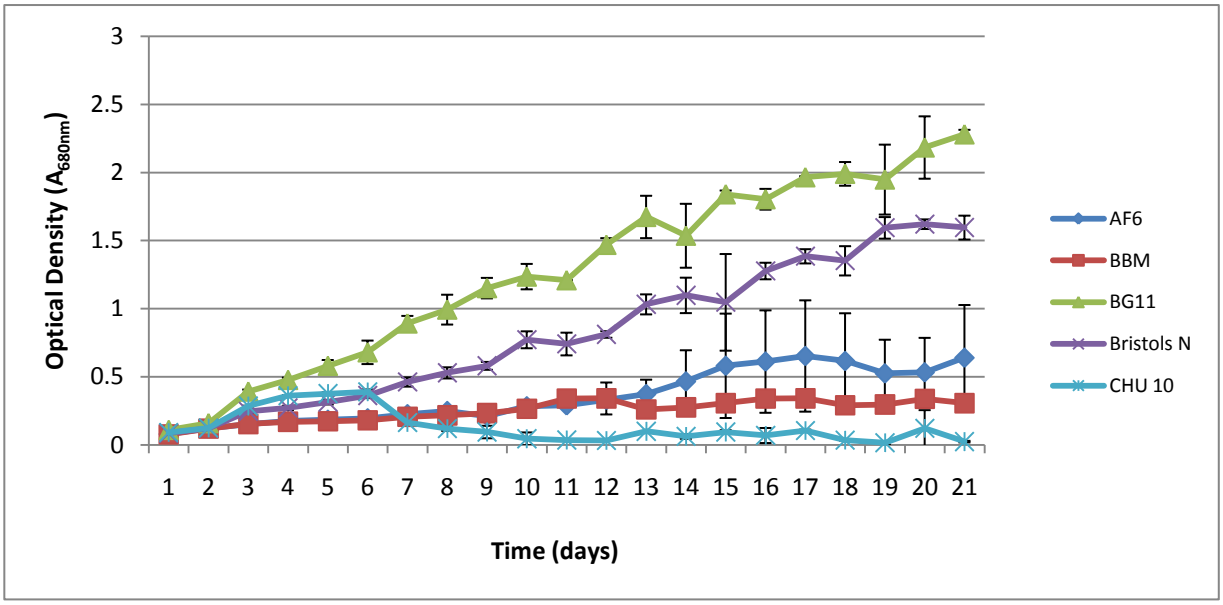


Figure 16: Growth rates of *Chlorella* sp. in various media compositions to ascertain the most appropriate media for optimisation of growth

Table 7: Growth rates and generation time of *Chlorella* sp. grown in various media for a period of 21 days to ascertain best media composition for growth of the culture

| Media | Growth Rate | Generation Time (h) |
|---|---|---|
| **BG11** | 0.2083 | 34.68 |
| **Bristols** | 0.1975 | 36.58 |
| **AF6** | 0.1433 | 50.41 |
| **BBM** | 0.0952 | 75.88 |
| **Chu** | 0.1105 | 65.38 |

*Chlorella* sp species showed the highest growth in BG11 with a generation time of 34.68 hours (Figure 16). The culture exhibited a short lag phase of 1 to 2 days before increasing at a steady rate for BG 11 and Bristol's NaCl media. Growth was slow in cultures AF6 and BBM with culture density remaining fairly constant for BBM and showing slight improvement in growth

for AF6 after 10days. Similar to growth of *Scenedesmus* culture, Chu #10 media performed better than AF6, Bristol's NaCl and BBM (Table 7), showing increased culture density for the first 6 days of cultivation with an initial generation time of 20.50 hours. However a decline was observed from days 6 to 10 after which culture death was observed. The final optical density reading being lower than the initial is due to loss of chlorophyll. The best performing growth media in both trials was observed to be BG 11. All subsequent trials were therefore carried out using BG11 media which is high in nitrate (1,5g/l).

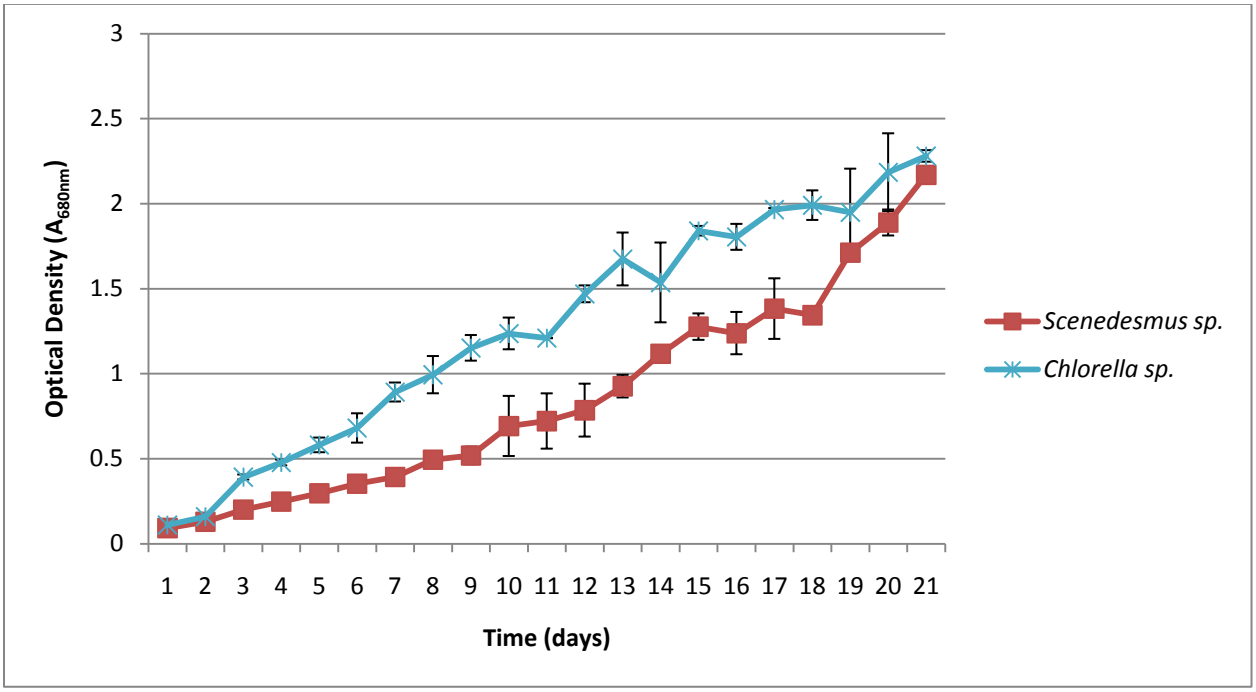


Figure 17: Growth of *Scenedesmus* sp and *Chlorella* sp. grown in BG11 media in broth culture for the determination of growth rate

Growth rate of both cultures were higher for the first 13 days of cultivation with growth rates of 0.236 and 0.302 for *Scenedesmus* sp. and *Chlorella* sp. respectively (Figure 17). For the overall cultivation period however, the *Scenedesmus* culture increased in growth rate from days 14 to 21 giving an overall growth rate of 0.204 and generation time of 35.41 hours. Conversely the

growth rate of *Chlorella* sp. dropped slightly from days 14 to 21, with an overall growth rate of 0.208 and generation time of 34.73 hours.

### 4.3.2 Optimisation of nitrogen concentrations for growth of *Scenedesmus* sp. and *Chlorella* sp.

*Scenedesmus* sp. exhibited the best growth in BG11 (Figure 18) with reduced $NaNO_3$ to 0.3g/L with a growth rate of 0.21 and generation time of 35.05 hours (Table 8). The culture performed almost identically for the first 4 days of cultivation. On day 5 deviation in the culture containing no nitrogen was observed.

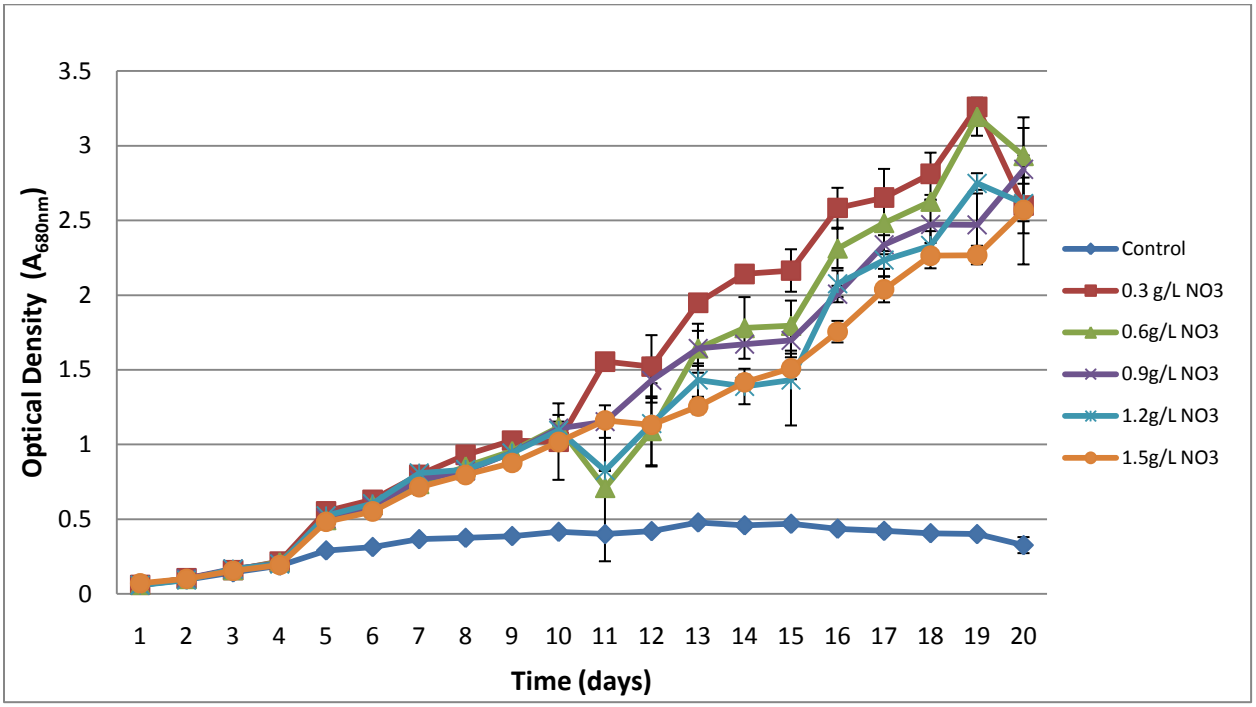


Figure 18: Effect of various nitrate concentrations on growth of *Scenedesmus* sp.

Table 8: Growth rates and generation time of *Scenedesmus* sp. grown in various nitrate concentrations to ascertain optimal nitrate concentration for growth of the culture

| Concentration | Growth Rate | Generation Time (h) |
|---|---|---|
| **Control** | 0.0573 | 126.00 |
| **0.3g/L NO3** | 0.2061 | 35.05 |
| **0.6g/L NO3** | 0.2058 | 35.10 |
| **0.9g/L NO3** | 0.1896 | 38.10 |
| **1.2g/L NO3** | 0.1992 | 36.27 |
| **1.5g/L NO3** | 0.1869 | 38.65 |

From days 10 to 20 the culture remained at steady state. This is likely due to depletion of any storage product that might have been accumulated in the first 4 days of cultivation. The highest growth rate was achieved by 0.3g/L $NO_3$ (Table 8). All cultures with the exception of $NO_3$ control showed similar performance from days 1 to 10. The culture grown in media 1.5g/L $NaNO_3$ for continued to increase in biomass after day 20. A slight decrease in culture density was observed on day 11, this may be explained by a power failure that occurred resulting in the cultures not receiving the 150µmol/$m^2$s light intensity for a period of 24 hours. The *Scenedesmus* culture exhibited the fastest growth rate between days 4 and 10 with generation times of 15.22, 24.90, 17.32, 21.16 and 16.76 hours for 0.3g/L to 1.5g/L $NO_3$ respectively. The first 4 days of growth being at a slower rate is due to adaptation of the culture to new conditions.

Both *Scenedesmus* sp. and *Chlorella* sp. showed a decline in optical density for some of the trials from days 12 to 15. A power failure occurred during this period. Chlorophyll density decline for *Scenedesmus* sp. was less pronounced with the control and culture containing 0.3g/L $NO_3$ appeared to be unaffected and only a small change for the rest of the cultures (Figure 18). *Chlorella* sp. however showed a significant change in optical density of 0.6g/L on day 13 and a further depletion on day 14. The culture containing 0.3g/L $NaNO_3$ was observed to be unaffected by the reduced light intensity (Figure 19).

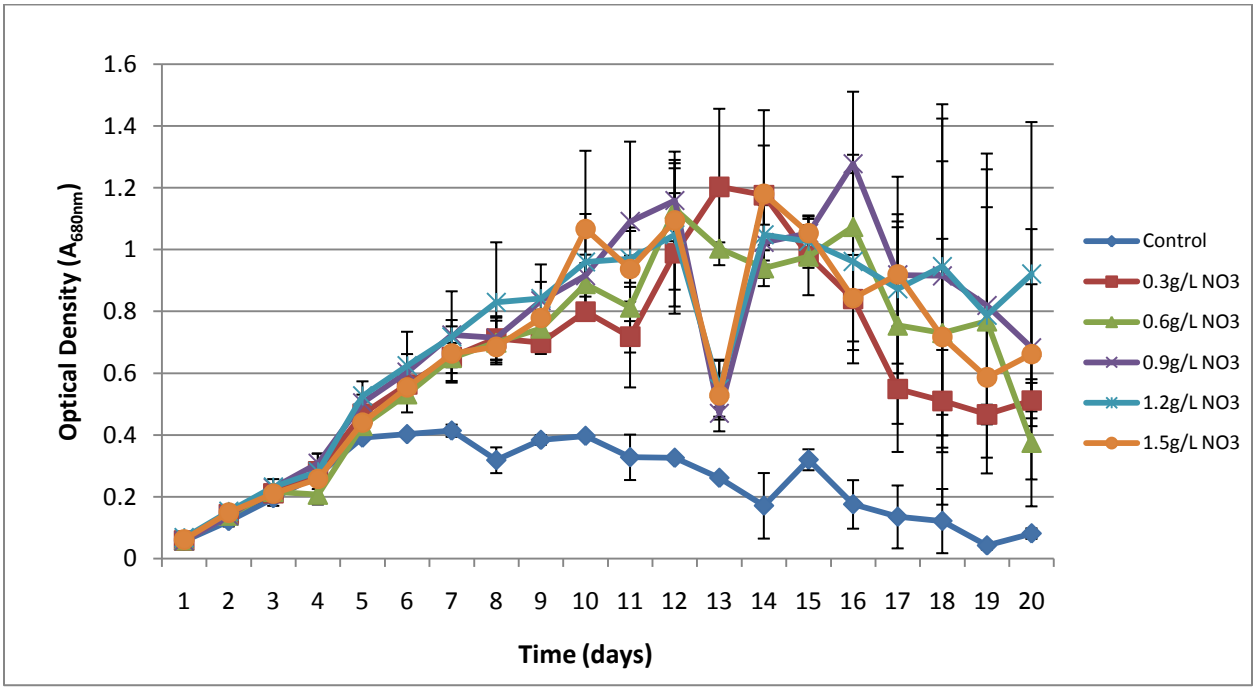


Figure 19: Effect of various nitrate concentrations on growth of *Chlorella* sp.

Table 9: Growth rates and generation time of *Chlorella* sp. grown in various nitrate concentrations to ascertain optimal nitrate concentration for growth of the culture

| Concentration | Growth Rate | Generation Time (h) |
|---|---|---|
| Control | 0.2326 | 31.06 |
| 0.3g/L NO3 | 0.3398 | 21.26 |
| 0.6g/L NO3 | 0.3552 | 20.34 |
| 0.9g/L NO3 | 0.3632 | 19.89 |
| 1.2g/L NO3 | 0.3297 | 21.91 |
| 1.5g/L NO3 | 0.3461 | 20.87 |

Growth of *Chlorella* sp. showed that, with the exception of $NO_3$ control, all cultures reached a maximum between days 12 and 16 of cultivation (Figure 19). It should be noted that growth rates and generation times were calculated for the exponential phase of growth only. Calculation for the overall period would result in growth rates that are far below the potential of the culture. As

expected the $NO_3$ control yielded the slowest growth rate and generation time (Table 9). Maximum growth rate was achieved on day 7 after a period exponential growth for 5 days followed by stationary growth for 2 days and subsequently decline (Figure 19). Growth rate for the exponential phase was calculated to be 0.558. A trend was observed for all cultures reaching highest growth was achieved between days 12 and 14 before the cultures showed a decline. Day 13 showed a significant reduction in chlorophyll for cultures growing in 1.5g/L, 1.2g/L and 0.9g/L $NO_3$. Growth of *Chlorella* species occurred in stages. Days 1 to 5 gave fast doubling times of 12.32+/-0.54 hours for all cultures. This is indicative of sufficient nitrogen availability and high light intensity for growth. Days 6 to 12 showed a considerably slow growth rate resulting in generation times of 30.66 +/- 4.32 hours for cultures containing 0.3g/L, 0.6g/L, 0.9g/L, 1.2g/L and 1.5g/L $NO_3$.

### 4.3.3 Optimisation of phosphate concentrations for growth of *Scenedesmus* sp. and *Chlorella* sp.

Phosphate is essential for the growth of microalgae and is required for the production of phospholipids and thus growth. Growth of *Scenedesmus* sp. was found to be the lowest in terms of cell density (Figure 20) and growth rate (Table 10) for the control. *Scenedesmus* sp. grown in 0.032g/L $PO_4$ showed the highest culture density (Figure 20) however not the highest growth rate when effecting conversion using the equation 1. It is also noteworthy that the standard deviation for the culture grown in 0.032g/L $PO_4$ is large in that the lower limit brings the culture remarkable close to the growth achieved by the media containing 0.024g/L $PO_4$.

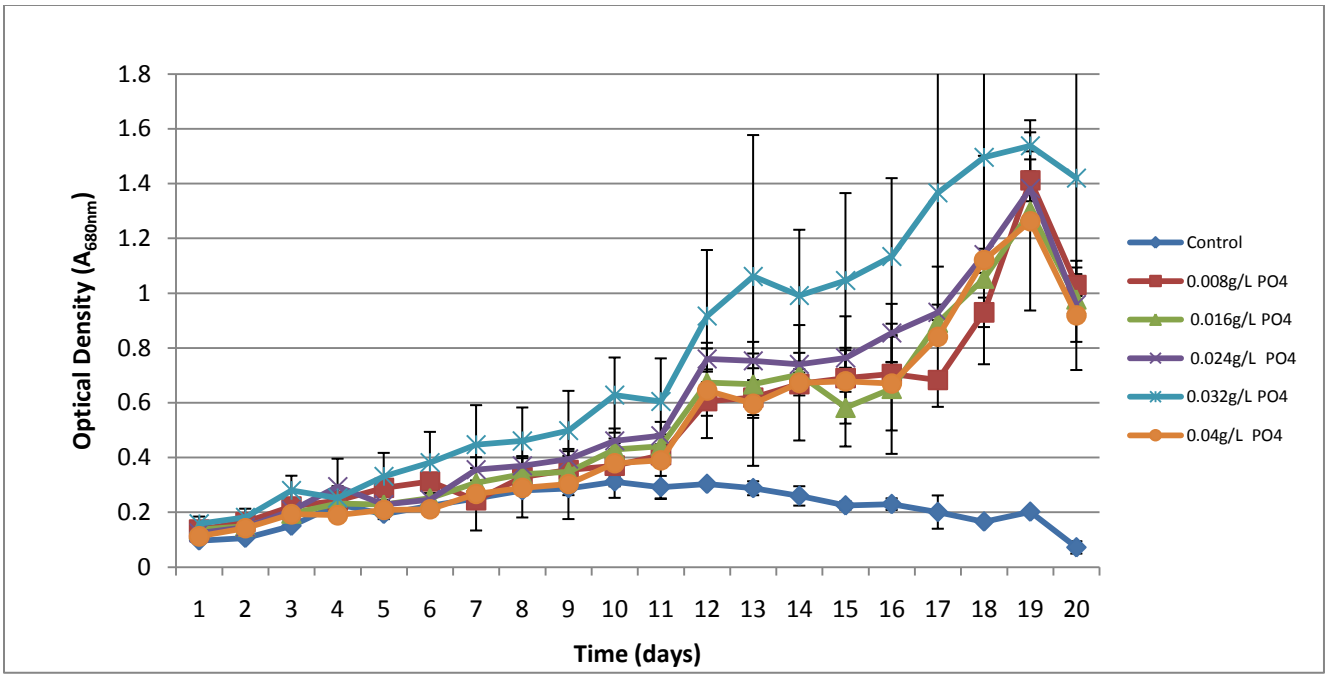


Figure 20: Effect of various phosphate concentrations on growth of *Scenedesmus* sp.

Table 10: Growth rates and generation time of *Scenedesmus* sp. grown in various phosphate concentrations to ascertain optimal phosphate concentration for growth of the culture

| Concentration | Growth Rate | Generation Time (h) |
|---|---|---|
| **Control** | 0.0557 | 129.69 |
| **0.008g/L PO4** | 0.1771 | 40.78 |
| **0.016g/L PO4** | 0.1708 | 42.29 |
| **0.024g/L PO4** | 0.1828 | 39.53 |
| **0.032g/L PO4** | 0.1726 | 41.86 |
| **0.04g/L PO4** | 0.1836 | 39.35 |

The highest growth rate was observed in culture containing 0.04g/L $PO_4$ with a growth rate of 0.183 and a generation time of 39.35 hours, followed closely by the culture containing 0.024g/L $PO_4$ at a growth rate of 0.182 and a generation time of 39.53 hours. The culture showed similar trends throughout the period of growth for all cultures. This indicated that there was no significant difference in growth of cultures with 0.008, 0.024 and 0.04g/L $PO_4$ (Table 10). Variations in the growth rate were not dependant on phosphate concentration and are likely as a

result of variation in temperature for the period. Reduction of phosphate in the media resulted in overall slower growth rates as compared to those achieved for nitrate limitation.

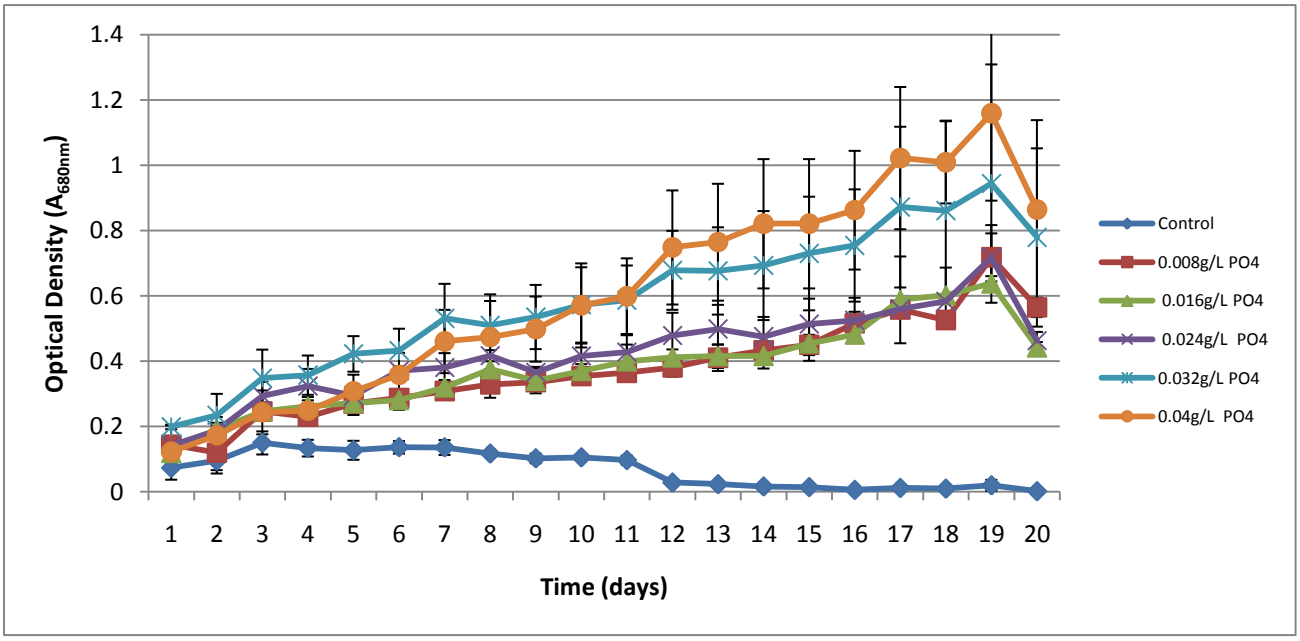


Figure 21: Effect of various Phosphate concentrations on growth of *Chlorella* sp.

Table 11: Growth rates and generation time of *Chlorella* sp. grown in various phosphate concentrations to ascertain optimal phosphate concentration for growth of the culture

| Concentration | Growth Rate | Generation Time (h) |
|---|---|---|
| **Control** | 0.1444 | 209.39 |
| **0.008g/L PO4** | 0.1217 | 59.34 |
| **0.016g/L PO4** | 0.1275 | 56.66 |
| **0.024g/L PO4** | 0.1231 | 58.70 |
| **0.032g/L PO4** | 0.1338 | 53.98 |
| **0.04g/L PO4** | 0.1706 | 42.34 |

Figure 21 shows the optimal $PO_4$ concentration to be 0.032g/L to 0.04g/L. The curves for all the cultures showed a decline after day 19 of cultivation (Figure 21). These results however do not give a clear indication of growth characteristics. Table 11 reflects growth rates for the exponential phase of growth. Generation times for the first 7 days of cultivation 46.21, 35.43,

35.56, 37.71 and 26.46 hours for cultures 0.008g/L to 0.04g/L $PO_4$ respectively. Generation times for growth from days 7 to 12 decreased considerably to 143.22, 119.46, 131.09, 122.56 and 61.84 hours for cultures 0.008g/L to 0.04g/L $PO_4$ respectively. This considerable increase in doubling time has a knock on effect of slowing the overall growth rate and generation times of the cultures. The full cultivation period must however be considered as our aim was to gauge the effect of phosphate depletion over time so as to select the most appropriate time and concentration. The control culture showed a steady decline in chlorophyll from day 3 onwards to a point below the initial inoculum chlorophyll content on day 11.

### 4.3.4 Optimisation of lipid yield for *Scenedesmus* sp. and *Chlorella* sp.

Nitrogen limitation yielded higher lipid concentrations as expected, with the highest level of lipid accumulated in the *Scenedesmus* sp control showing 15.79% lipid (Figure 22). Lipid decrease is observed with an increase in nitrogen concentration. Cultures grown in 0.3g/L, 0.6g/L, 0.9g/L and 1.2g/L nitrate yielded 14.98, 14.54, 5.96 and 10.28% respectively. The lowest accumulation had been observed in the culture containing 1.5g/L $NaNO_3$. This is indicative of no nutrient stress in the culture. The lipid yield of 2.4% may be attributed to phospholipids.

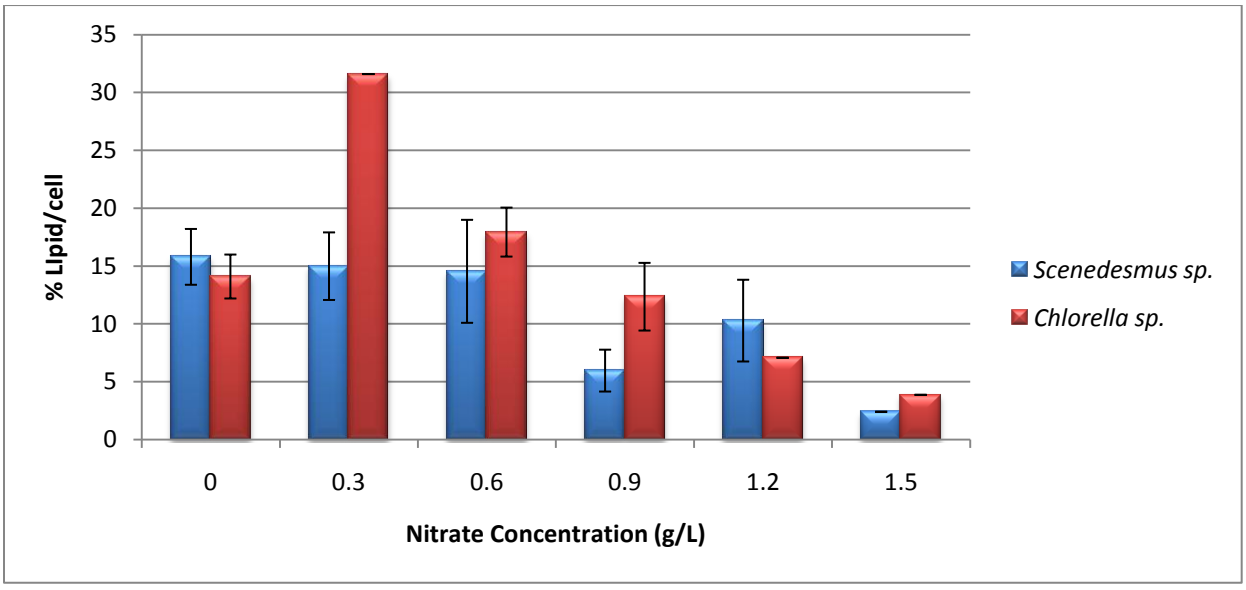


Figure 22: Effect of various concentrations of nitrate on percentage lipid accumulation per cell of *Scenedesmus* sp. and *Chlorella* sp.

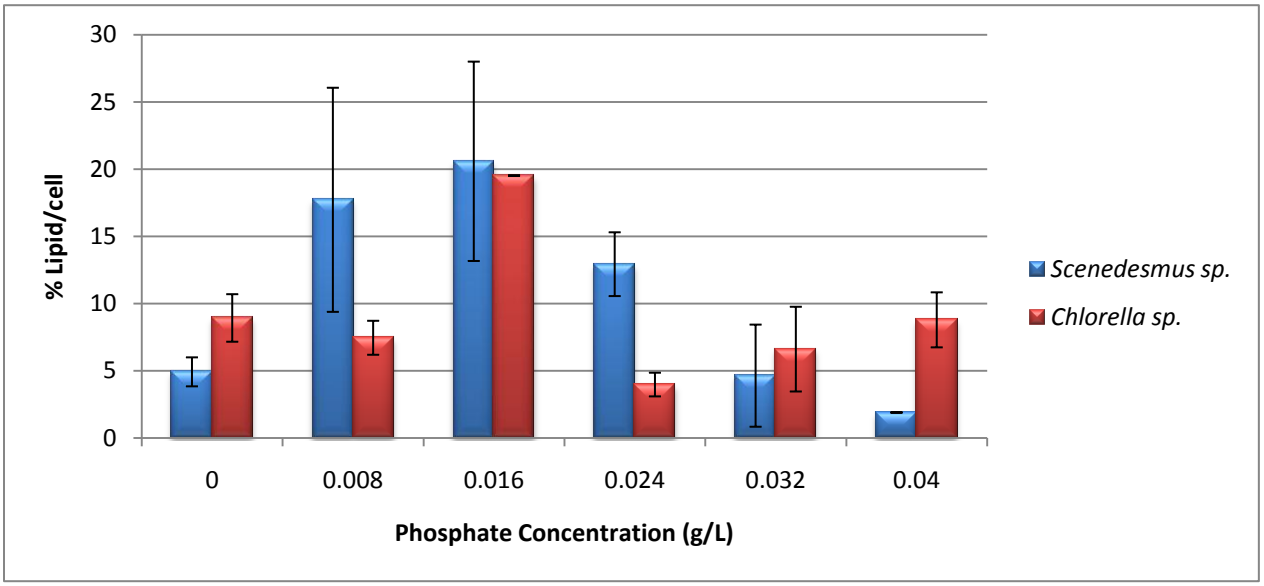


Figure 23: Effect of various concentrations of phosphate on percentage lipid accumulation per cell of *Scenedesmus* sp. and *Chlorella* sp.

The highest lipid yield for *Chlorella* sp. (Figure 22) was obtained from the culture containing 0.3g/L nitrate, yielding 31.59%. Culture grown in 0.6g/L nitrate yielded 17.93% followed by the 0% nitrate with 14.09%. The lowest lipid yield was achieved in 1.5g/L nitrate at 3.86%. Growth in varying phosphate concentrations exhibited a similar trend to the results obtained for thenitrate concentrations. Lipid levels for phosphate limitation peaked in the culture containing 0.016g/L phosphate at a yield of 20.58%. Cultures grown at 0.008g/L and 0.024g/L phosphate yielded 17.72% and 12.93%. Similar to the results achieved for nitrate, cultures grown in 0.04g/L phosphate yielded only 1.90% lipid for the *Scenedesmus* culture (Figure 23). Maximum lipid yield achieved was 19.52% at 0.016g/L phosphate. Yields obtained for 0.024, 0.032 and 0.04g/L were observed to be low at 4 to 9%. 0.3g/L- 0.6g/L nitrate and phosphate as per BG11 media yielded the highest amount of lipid for both *Scenedesmus* sp. and *Chlorella* sp. Lipid concentrations were determined after 7 days of cultivation as this period yielded the fastest growth rates for cultures as determined by nitrate and phosphate growth rate determination experiments (Figures18 to 21). Subsequent to this period growth rates decreased.

### 4.3.5 Optimisation of pH for growth of *Scenedesmus* sp. and *Chlorella* sp.

Various pH levels were tested to ascertain growth characteristics of the *Scenedesmus* sp. pH of cultures were adjusted daily using 0.1 M and 1 M HCl and 0.1 M and 1 M NaOH. The *Scenedesmus* culture exhibited the fastest overall generation time of 34.58 hours at pH 11, slightly better than the control exhibiting generation time of 35.52 hours (Table 12). pH 10, pH 11 and the control (Figure 24) achieved the highest densities of the trial.

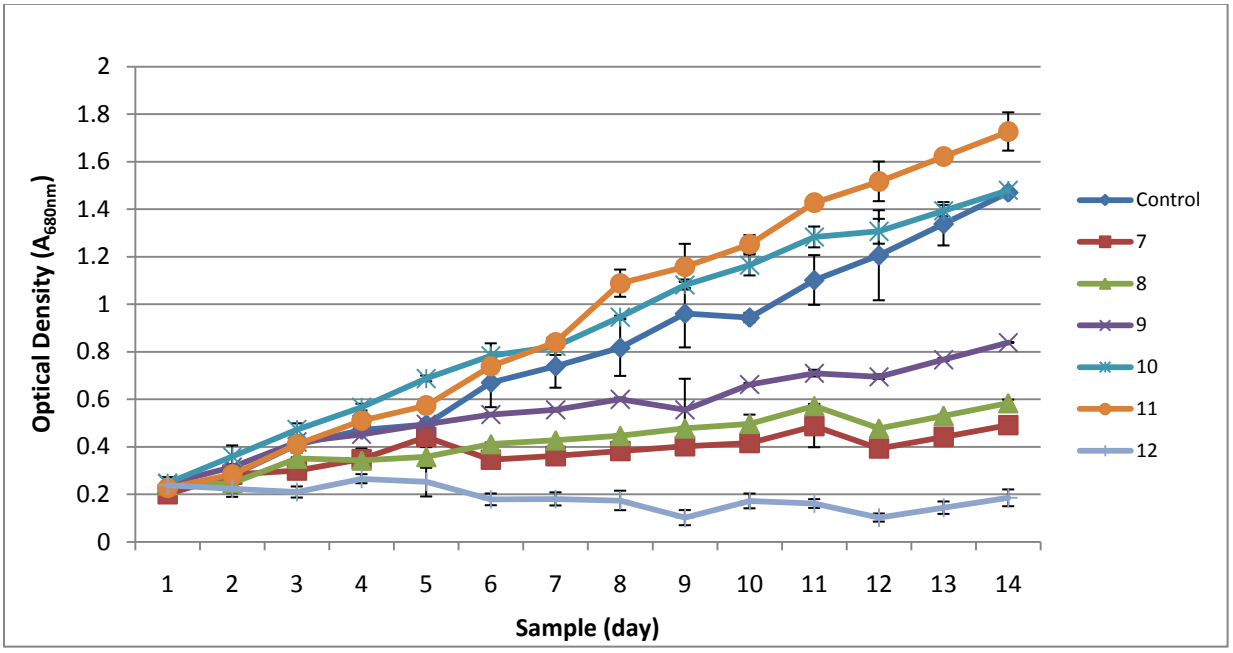


Figure 24: Effect of various pH concentrations on the growth of *Scenedesmus* sp.

Table 12: Growth rates and generation times of *Scenedesmus* sp. under various pH levels

| pH Levels | Growth Rate | Generation Time (h) |
|---|---|---|
| **Control** | 0.2033 | 35.53 |
| **pH 7** | 0.0920 | 78.49 |
| **pH 8** | 0.0921 | 78.46 |
| **pH 9** | 0.1269 | 56.95 |
| **pH 10** | 0.1843 | 39.20 |
| **pH 11** | 0.2089 | 34.58 |
| **pH12** | -0.0260 | -278.27 |

Growth was achieved in cultures with pH 7, pH 8 and pH 9, however at growth rates significantly lower than the control. Overall generation times achieved for the trial are reflected in Table 12. The pH of the control was unaltered and remained within a range of 9.44 to 10.63. Throughout the period of growth, culture pH for all cultures was observed to shift toward the pH of the unaltered culture. The culture grown at pH 7 initially shifted 3 pH points to pH 9.99 in the first 24hours of growth. The change in pH per 24 hours subsequently decreased on average throughout the period of growth and resulted on a change of 0.23 pH points on day 14. The same trend was observed in cultures adjusted to pH 8 and pH 9. The pH 11 trial showed a decrease in the pH towards the pH of the control and a similar trend in shifts to cultures pH 8 and pH 9. The culture grown at pH 12 did not exhibit sustained growth and exhibited loss of chlorophyll from day 5.

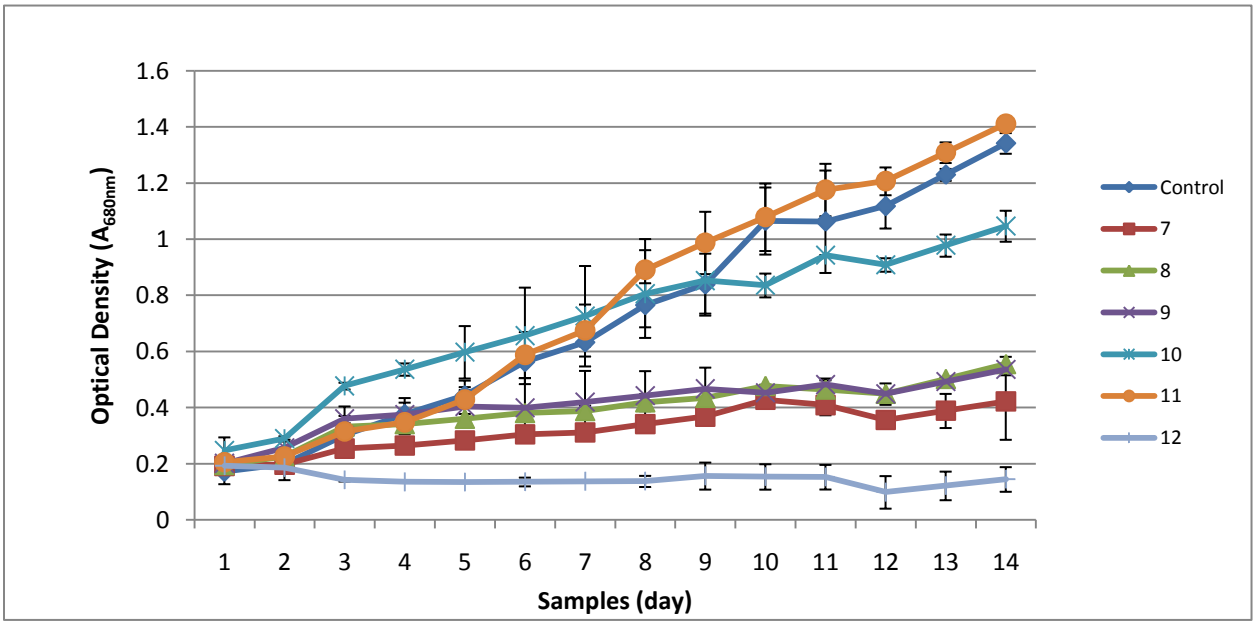


Figure 25: Effect of various pH concentrations on the growth of *Chlorella* sp.

Table 13: Growth rates and generation times of *Chlorella* sp. under various pH levels

| pH Levels | Growth Rate | Generation Time (h) |
|---|---|---|
| **Control** | 0.2117 | 34.12 |
| **pH 7** | 0.0809 | 89.31 |
| **pH 8** | 0.1078 | 67.02 |
| **pH 9** | 0.1008 | 71.65 |
| **pH 10** | 0.1486 | 48.60 |
| **pH 11** | 0.1985 | 36.39 |
| **pH12** | -0.0295 | -245.14 |

*Chlorella* sp. was found to have the highest growth rate of 0.2117 and generation time of 34.12 hours in the control. Growth at pH 11 exhibited similar growth with a growth rate of 0.1985 and generation time of 36.39 hours (Table 13). This result was expected as the pH of the control ranged from 10.15 to 10.69 throughout the period of cultivation. Growth in pH 10 was slightly slower with generation time of 48.60 hours. This deviation despite the similar pH to the control is due to the alteration of pH. Growth in cultures pH 7, pH 8 and pH 9 achieved considerably lower culture densities (Figure 25) as a result of slow generation times of 89.31, 67.02 and 71.65 hours respectively. The culture grown at pH 12 exhibited loss of chlorophyll and death by day 3.

## 4.4 Discussion

### 4.4.1 Selection of growth media

Production of microalgae requires important inorganic nutrients in the forms of nitrogen and phosphorus (Suh & Lee, 2003; Brennan & Owende, 2010). Microalgae are known to have different nutrient requirements, not only by composition but also by concentration of the nutrients supplied. Microalgal growth media are composed of macronutrients generally consisting of a nitrogen source, phosphate, metal chelator. Iron is generally supplied in the form of a micronutrient. BG media differs in nitrate concentration by factors of 6, 6, 10.7 and 37.5 times higher than BBM, Bristol's NaCl, AF6 and Chu #10 media respectively. This is not however the determining factor as to performance of the microalgal cultures as is seen in Tables 6 and 7. BG 11 media only slightly outperformed AF6 and Bristol's NaCl media (Table 6)

despite significantly different nitrate concentrations. The presence of vitamins present in AF6 medium and lack of trace metals in Bristol's NaCl is unlikely to be the contributing factors as BBM performed poorly despite the presence of both trace metals and vitamins. It is likely that *Scenedesmus* sp. has an inherent adaptability to cultural conditions due to the environment of isolation being in constant flux. *Chlorella* sp. is known to grow fairly well in nutrient rich media (Borowitzka, 1999). Illman et al. (2000) showed growth of various species of *Chlorella* in watanbe media containing 1.25g/l KNO3. Generation times of the strains ranged between 16.8 hours and 86.4 hours (Figure 17). Our results are in accordance with those achieved by Illman et al, (2000).

Some microalgae exhibit very high growth rates in that they are can have a generation time of 3 to 4 hours. Most microalgae have a generation time of 24 to 48 under favourable conditions (Williams & Laurens, 2010). Both *Scenedesmus* and *Chlorella* cultures exhibited similar growth trends (Figures 17). This may be as a result of diminishing nutrient values in the media. The growth rate achieved for *Chlorella* sp. (Table 7) was similar to those reported in literature for *Chlorella* sp. (Mandalam & Palsson, 1997). *Chlorella* sp. is a high nutrient requiring culture. As the nutrients in the media are consumed, the growth rate will decrease. Conversely, *Scenedesmus* sp. requires lower nutrient concentrations, thus there is sufficient nutrients to support growth of the culture after the period. Furthermore nutrients supplied in excess have been shown to hinder growth in certain species (Watanabe, 2005).

### 4.4.2 Optimisation of nitrogen concentrations for growth

*Scenedesmus* sp. showing no appreciable difference in growth rate from days 1 to 10 (Figure 18) is indicative of the culture having nitrates to meet growth requirements for that period. Subsequent to the initial lag phase the behaviour of the control was expected as lack of nitrate results non proliferation of cells. A similar trend is noted for *Chlorella* sp. (Figure 19). Nutrient toxicity resulting in slight inhibition of growth may have been responsible for better performance of *Scenedesmus* sp. higher growth rates of cultures containing 0.3 and 0.6g/L $NO_3$ (Table 8). Decline in optical density for *Scenedesmus* sp. (Figure 18) and *Chlorella* sp. (Figure 19) during

the period of the power failure occurred as the result of a drastic decrease in light intensity and subsequently chlorophyll content. A loss of up to 25% of biomass may be lost due to dark phase respiration. This is however dependant on light intensity at which biomass is grown (Chisti, 2007). The cultures containing 0.3g/L $NaNO_3$ was observed to be unaffected by the reduced light intensity (Figures 18 and 19). Under nutrient depleted conditions, cells do not proliferate. A lower amount of light is required for biomass maintenance and excess energy in the form of free electrons are directed to lipid production as an energy sink to prevent photooxidation (Packer et al., 2011). Growth of *Chlorella* sp. occurred in stages. The fast doubling times noted for days 1 to 5 is indicative of sufficient nitrogen availability and high light intensity for growth (Figure 19). Days 6 to 12 showed slower growth rate may be explained by the changing of metabolism pathways of the microalgae to produce lipid as a storage product due to the depletion of nitrogen.

The results of reduced growth rate under low nitrogen conditions are in accordance with results achieved by Illman et al. (2000). This trend occurred more rapidly in the culture with no added nitrogen (Figures 18 and 19) thus resulting in the low densities achieved and depletion of chlorophyll. *Chlorella* sp. is known to grow fairly well in nutrient rich media and is generally regarded as a high nutrient requiring algal species (Borowitzka, 1999). Illman et al., (2000) showed a decrease in growth rates and growth continued for extended periods under nitrogen limited conditions for a variety of *Chlorella* sp. Pruvost et al., (2009) observed microalgal growth in nitrate free medium. This was accompanied by a change in cellular composition whereby there was an increase in total carbohydrate and decrease of protein accompanying lipid accumulation. Nitrogen starvation induced the production of storage products accompanied by degradation of cellular nitrogen sources such as protein and chlorophyll (Pruvost et al., 2011). This phenomenon may be used to explain the growth of *Chlorella* sp. and *Scenedesmus* sp. observed in nitrogen free trials of our study (Figure 18 and 19). Figures 18-21 all exhibit a similar trend in that growth rates were found to be higher for the initial period of cultivation and slow as growth progressed. This trend was not only observed for cultures under nutrient limitation conditions whereby nutrient limitation may explain the decrease in growth rate. Under normal cultural growth conditions i.e. no nutrient stress, photosynthesis increases with an increase in light intensity until light saturation is attained at which point the maximum growth

rate will be attained (Richmond, 2004). Photoinhibition and consequently decrease in growth rate is as a result of irradiance of the culture above the level of light saturation (Park et al., 2011). Despite consumption of nutrients, it is unlikely that photoinhibition resulted in the decreasing growth rates as BG11 media is a nutrient rich medium and illumination was constant at 150µmol/$m^2$s. With an increase in culture density however penetration of light through the entire culture would decrease due to the shading effect, thus lowering the overall light intensity and subsequently growth rate.

### 4.4.3 Optimisation of phosphate concentrations for growth of *Scenedesmus* sp. and *Chlorella* sp.

Phosphorus is one of the most important growth limitation nutrients for algal cultivation (Ge et al., 2011). Phosphorus is required in small amounts as compared to nitrogen however, must be supplied in excess as not all phosphorus is bio-available (Chisti, 2007). The slowing of the growth rates is indicative of metabolic change in the microalgae as nutrients become depleted. Increase in culture density also restricts light from passing through the culture optimally thus also inhibiting the growth rate. Figures 20 and 21 show a decline in chlorophyll a. The decline of chlorophyll for the phosphate control is likely to be as a result of the cultures inability to produce phospholipids. Phospholipids form part of the major components of cellular membranes thus making phosphate essential for growth and active division of cultures (Williams & Laurens, 2010). Structural lipid determines the ability of the organism to adapt rapidly to changes in the growth environment. This is achieved by alteration of the level of fatty acid chains that determine cell membrane fluidity. Consumption of phosphorus leads to a decrease in algal biomass as observed in figures 21 and 22 negatively impacted growth. The growth rate for *Scenedesmus* sp. shows that phosphate is supplied in excess for the lowest concentration (0.008g/L). Growth trends for *Chlorella* sp. however indicate that phosphate may be rate limiting below 0.032g/L thus giving the variation seen in Figure 21. This is expected as *Chlorella* sp. are known to have high nutrient requirements. This is further denoted by the lower growth rates achieved for *Chlorella* sp. (Table 11) as compared to *Scenedesmus* sp. (Table 10).

### 4.4.4 Optimisation of lipid yield for *Scenedesmus* sp. and *Chlorella* sp

Chlorophyceae have been reported to contain high levels of neutral lipid thus the class may represent a large pool of lipid producers that may be useful for lipid production (Chen et al., 2011b). *Chlorella* sp. and *Scenedesmus* sp, fall within the Chlorophyceae family. Whilst certain microalgae have the ability to fix atmospheric nitrogen, most microalgae require the addition of nitrate in the growth media (Moreno et al., 2003). Lipid accumulation under standard growth conditions was observed to be very low and increased after starvation by (Pruvost et al., 2011). Under stressed conditions many microalgae alter their biosynthetic pathways towards the production of neutral lipid (Li et al., 2011). This phenomenon is clearly seen in figures 22 and 23. Nitrogen limitation or depletion is commonly used for the induction or increase of lipid content in microalgae and regarded as the most effective method to achieve this (Brennan & Owende, 2010; Mata et al., 2010). Increase in lipid production is gained at the expense of biomass yields. Lipid accumulation begins upon nitrogen becoming depleted to the point of becoming the growth limiting factor or being exhausted from the growth media. Therefore it is necessary to consider the production of increased amounts of lipid and growth rate simultaneously and they need to be optimised as a trade-off rather than optimisation for lipid alone (Chen et al., 2011a). Nitrogen deficiency inhibits the cell cycle and production of most cellular components (Singh et al., 2011b). Nitrogen starvation increases lipid and decreases growth rate, with a relationship of inverse proportionality. Nitrogen limitation also serves to convert free fatty acids to triacylglycerols (TAGs) over an extended period of time. Triacylglycerols are the best algal lipid feedstock for conversion to biodiesel (Chisti, 2007; Brennan & Owende, 2010; Pruvost et al., 2011). For continuous culture it is necessary to maintain nitrogen at a level that prevents cells from becoming starved and stop growing, yet be low enough to induce lipid production (Mairet et al., 2011).

Upon reaching nutrient limited status (Figure 22), carbon is assimilated into cells but cell proliferation does not occur (Meng et al., 2009). This carbon is converted to TAGs or carbohydrate within existing cells depending on species (Brennan & Owende, 2010; Feng et al., 2011). In the case of *Chlorella* sp. and *Scenedesmus* sp. isolated within the scope of this project, TAGs are produced in excess (Figures 22 and 23). Cell growth is a high energy consuming

process. Under nutrient limited conditions, electrons are not taken up further than the stage of carbon fixation resulting in a build-up of electron in the electron transport chain. This excess of electrons increases the risk of photooxidation. To mitigate this risk, electrons are diverted towards production of lipids, carbohydrates, pigments or excretion of photosynthate (Packer et al., 2011). Neutral lipid production is an effective energy sink, storing more than twice the energy than stored during carbohydrate production allowing many species of microalgae to maintain high photosynthetic rates (Hu et al., 2008). Under nutrient limited conditions newly fixed carbon is used for the synthesis of lipid thus acting as carbon storage. This trend is clearly shown in the results achieved (Figures 22 and 23).

Increasing irradiance has a similar effect to nutrient limitation in that it may provide an excess of free electrons that need to be taken up in order to prevent photooxidation. During neutral lipid synthesis it is likely that photosynthesis becomes decoupled from cellular growth resulting in high level of neutral lipid synthesis (Packer et al., 2011; Park et al., 2011). Packer et al., (2011) showed that the C:N ratio can be used as model to predict neutral lipid production. Under conditions with low C:N ratio or high irradiance, chlorophyceae had a tendency to produce lipid. TAGs are stored within the cytoplasm as densely packed lipid bodies after synthesis (Hu et al., 2008). The Nile Red semi-quantification method was employed as traditional methods of lipid determination require large microalgal samples and extraction procedures to be optimised for the extraction of the entire lipid fraction. Furthermore the extraction process may result in decomposition or oxidation of the lipid. Due to lipids having different cell wall components and consistencies, extraction procedures must be optimised for each isolate or at least genus. These processes are labour intensive, time consuming and make high throughput screening almost impossible (Chen et al., 2011b). Nitrogen limitation has variable effects on different types of microalgae in terms of growth and cellular content (Illman et al., 2000). Amounts of lipid accumulation may be variable depending on the amount of nitrogen available (Ge et al., 2011). In this study we used BG11 media which is a high nitrate containing media (1.5g/L $NaNO_3$) and might be the reason lipid levels achieved were lower than those found in literature. Irradiance was supplied at a constant level, therefore with an increase of biomass density, lower irradiance levels had penetrated the media resulting in slowing of growth and lipid production under nutrient stressed conditions.

### 4.4.5 Optimisation of pH for growth of *Scenedesmus* sp. and *Chlorella* sp.

It should be noted that growth rates of photosynthetic organisms in batch culture are dynamic. Unlike heterotrophic culture, where nutrients play the major role in determination of growth rate, phototrophic systems are governed by nutrient supply as well as light intensity. Despite light intensity is supplied at constant illumination at the surface of the reaction vessel and efforts are made to ensure even distribution by shaking at regular intervals, light intensities decrease over time as culture densities increase (Preisig & Anderson, 1995). The growth and maintenance of mono-cultures in open pond systems is exceedingly difficult (Brennan & Owende, 2010). The growth of both cultures at high pH can be explained by directed evolution over a period of time due to the nature of their ecosystems (Figures 24 and 25). The ability to cultivate species at high pH is highly beneficial for open pond systems as these systems require highly selective environments to prevent contamination by undesired microalgae, bacteria and other grazers (Brennan & Owende, 2010). By maintenance of high pH (10-11) it is possible to achieve growth of a single species of microalgae as a dominant culture, monoculture however, requires an extreme culture environment that supports only a small number of strains (Singh et al., 2011b). The proposed mechanism of physiological pH adjustment is via photosynthetic inorganic carbon fixation. Carbonic acid is taken up by the organism thus increasing pH of growth media. Carbon is then fixed and hydroxide ion efflux occurs that may further increase the pH of solution (Cardol et al., 2011). Elevated pH serves to chemically alter nutrient compositions by volatilisation of ammonia and precipitation of phosphorus with unchelated metals (Park et al., 2011). This needs to be accounted for during scale culturing as phosphate is an essential nutrient for production of cellular wall lipid (Williams & Laurens, 2010).

Bacterial contamination actively competes for nutrients and oxidise organic matter that could lead to putrification of the culture. Control of heterotrophic bacteria may be achieved by increase in pH. Aerobic bacteria generally found in microalgal pond systems have an optimum pH of 8.3. Increase in pH beyond this level gives effective inhibition thus preventing competition by influencing nitrogen efficiency (Craggs, 2005; Park et al., 2011). Open systems are susceptible to grazers in the form of protozoa and zooplankton. These organisms actively consume microalgae and can devastate algal concentration in relatively short periods of time (2 to 3 days). Reduction

of microalgal concentration by up to 90% of the original density in 48 hours by zooplankton (Oswald, 1980) and 99% reduction by Daphnia over a few days have been observed (Park et al., 2011). Several methods of control of these organisms are available including filtration, centrifugation, low DO, application of hormones and increase in free ammonia. These methods however have drawbacks in that filtration is difficult due to the size of algal species such as *Chlorella* sp. makes separation difficult. Centrifugation is prohibitively expensive at large scale requiring high capital and energy inputs. Photosynthetic microalgae produce oxygen thus actively increasing the DO as a function of growth. Increase in free ammonia as a control method may be achieved by pH elevation by volatilisation of ammonia. Oswald, (1988) reported that the toxicity brought about by high pH may be due to increased free ammonia levels due to volatilisation of ammonia at high pH. Thus the most appropriate method of control of zooplankton and bacteria is increase of pH to 11 (Park et al., 2011). The range of optimal pH for microalgae varies with species. Kong et al., (2010) stated that the optimal level of growth for many freshwater microalgae is close to 8 and deviation from this level gives reduction in biomass. Our experiments showed the optimal pH of the isolated strains to be 10-11 with decreases in growth rates below this level and inhibition at pH 12. Microalgae such as *Amphora* sp and *Ankistrodesmus* sp. have been shown to grow uninhibited at pH of 9 and 10 respectively (Park et al., 2011). pH exceeding 11 is reported to occur in high rate algal pond systems due to consumption of carbon dioxide and carbonic acid by the process of photosynthesis (Craggs, 2005).

# Chapter 5: Sustainability of *Chlorella* sp in open pond system

## 5.1 Introduction

Industrial cultivation of microalgae in the quantities required to make biodiesel from algal lipids viable, require certain criteria to be met (Chisti, 2007). Raceway ponds and photobioreactors are the main methods used for culturing large volumes of microalgae. Each of these methods has its merits and the deciding factor as to which best suits the production is dependent on the initial capital outlay as well as the strain to be cultured (Borowitzka, 1999; Chisti, 2007). This warrants trials in order to ascertain the viability of the species of choice in a raceway pond system. Raceway ponds are open circular ponds that are typically not deeper than 30cm and have a paddle wheel to constantly circulate the biomass and prevent sedimentation. The depth is limited by the fact that light can only penetrate approximately 30cm of water. Use of a pond with greater depth does not increase efficiency. Circulation of the biomass also aids in the reduction of the shading effect by constant mixing. Proper mixing may also be a requirement for high rate growth with certain species of microalgae (Borowitzka, 1999). The disadvantages to the open pond systems are that temperature cannot be easily controlled and fluctuates seasonally. Loss of water due to evaporation may be significant and raceway ponds are prone to contamination by other microalgae as well as microorganisms such as rotifers that consume microalgae (Chisti, 2007). Moreover only a small number of microalgae can be successfully culture in open pond systems. These disadvantages are outweighed by the initial capital cost of photobioreactors (Brennan & Owende, 2010). Closed systems are also extremely difficult to scale up (Borowitzka, 1999). *Chlorella* sp. requires a well mixed system such as a raceway pond. It has the ability to reach high biomass densities thus out-competing most contaminants. If a greater susceptibility to protozoa and microalgae exist, it becomes necessary to seed the system periodically (Borowitzka, 1999). Contamination problems with autotrophic growth has also been found to be less severe than heterotrophic systems and thus open pond systems are generally used for phototrophic cultivation (Chen et al., 2011a). It is however necessary to ascertain the sustainability of growth of the desired algal species in open pond system. The objective of this aspect of the research was to test the sustainability of *Chlorella* sp. in open pond under natural climatic conditions and to assess its ability to be maintained as the dominant culture in the system and not optimization of biomass or lipid.

## 5.2 Materials and Methods

### 5.2.1Design and construction of pond

A pre-pilot scale raceway pond was constructed for growth and assessment of culture at large scale. The pond was designed in a raceway configuration to ensure effective and efficient mixing of the culture. The pond was constructed using sheet metal with overlapping edges, fixed by pop rivets. The surface of the pond was subsequently waterproofed using conventional roofing sealant. To enhance stability of the structure the pond was encased within a wooden framework. The depth of the pond is 350mm with a capacity of 3000L when filled to 300mm (Figure 26). An electric motor was employed to drive the paddlewheel, thus enable adequate mixing of the culture in order to increase aeration and minimise the shading effect as culture density increases. Four paddles were constructed by fixing a semi-flexible plastic based material attached to a metal frame. Each of the paddles was situated at 90 degree angles to the previous to aid in continuous flow. Direction of flow was set towards the longest straight before the bend to avoid impeding of flow and formation of eddys. The paddlewheel speed was set to 4rpm to give a flow rate of 150 to 300mm per second depending on the volume used. The pond was covered with transparent plastic greenhouse material to prevent flooding due to excess rainwater accumulation, and served a dual role in preventing dust and debris from entering the pond. The induced greenhouse effect further prevents sharp drops in temperature and also serves to diminish contamination from airborne sources.

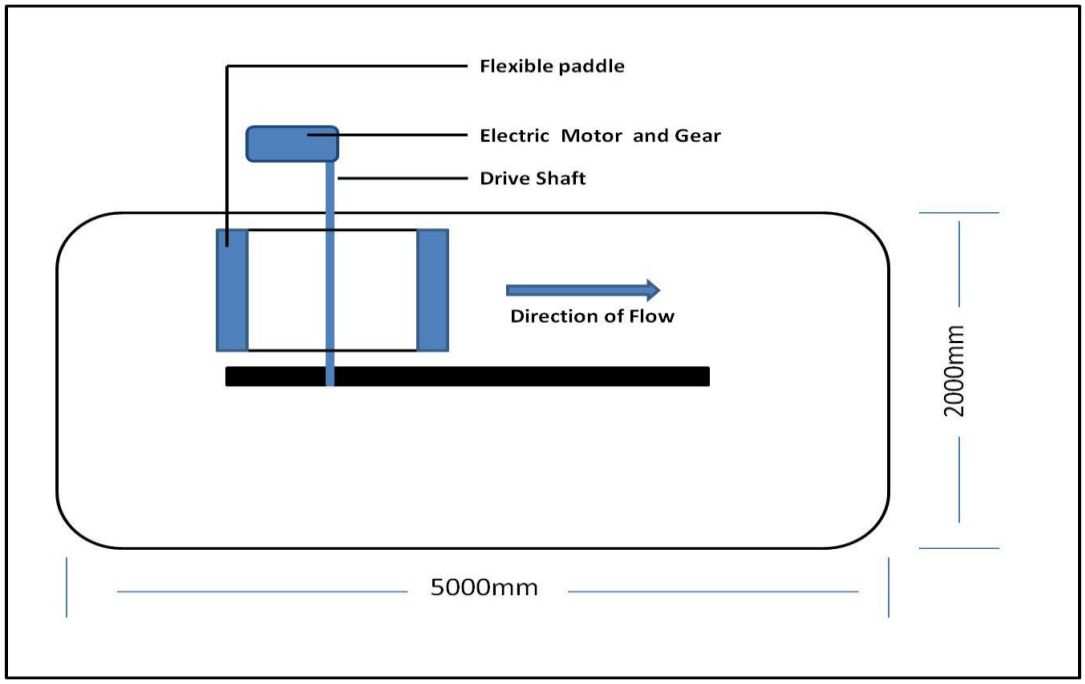


Figure 26: Schematic of pre-pilot scale raceway pond constructed for experimentation on selected algal strains

### 5.2.2 Pre-culturing preparation

A 20L seed culture of *Chlorella* sp. was grown up in two 10L Schott Bottles with natural illumination in modified BG11 media (Appendix D). Carbon dioxide was pumped in at 0.5l/min for 5mins every 24 hours. The culture was grown for a period of 21 days before inoculation. The raceway pond (Figure 27) was prepared by meticulous washing of the surface to remove dust, debris and dry biomass. The pond was air dried before being sanitised with 70% ethanol and further fumigated with formaldehyde for 10-12 hours.

### 5.2.3 Culturing and operation

The pond was inoculated with 500L BG11 and a pure starter culture of *Chlorella* sp. The *Chlorella* sp. was allowed to initially grow for 7days before water was added to bring the final volume of the pond to 2000 L. Media containing mineral salts was added to the pond on a

fortnightly basis. Water temperature, water levels and biomass concentrations were measured/calculated daily for the 6 month period. Harvesting of 1000L by a combination of settling and centrifugation was carried out weekly, the supernatant was returned to the pond. Biomass was determined gravimetrically by centrifugation for 15mins at 3000rpm and dried for 24h at 95°C. Cells were counted using a Nikon Eclipse 80i microscope with digital imaging using ACT2U imaging software and a Nikon DS1 camera. The pH was maintained between 9.5-10.5 to prevent contamination by predators

Figure 27: Pilot scale raceway pond in operation under sub-tropical conditions to ascertain sustainability of *Chlorella* sp.

## 5.3 Results

The sustainability trials were carried out during the period covering the cooler months of the year (autumn to early spring). Evaporation took place at approximately 50L/day at 22°C (water temperature) and was replenished every 3 days. The effect of evaporation could be negated since the focus of this aspect was to determine population dynamics with regards to dominant culture.

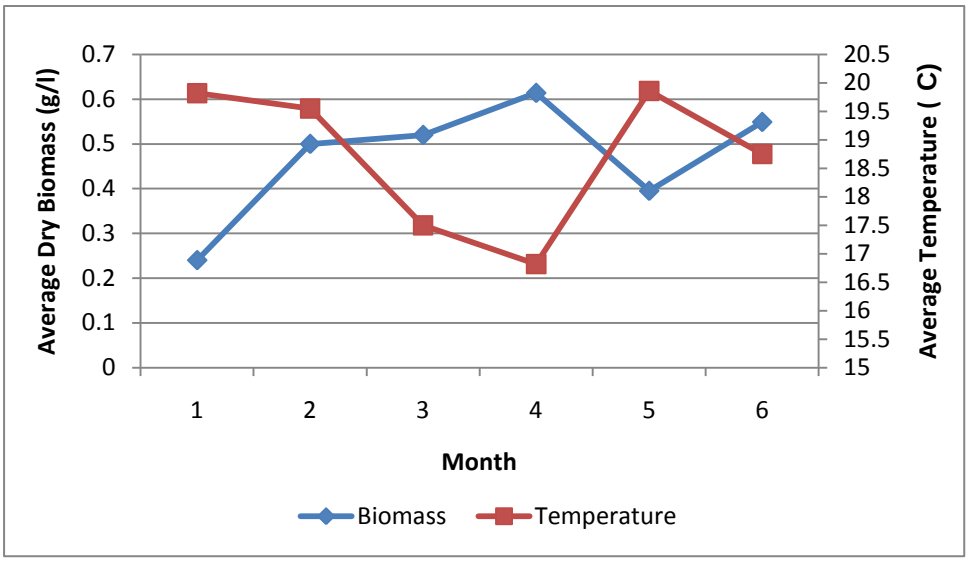


Figure 28: Average dry biomass and water temperature graphs for the 6 months of sustainability trial.

The raceway pond was started during month 1. This is evident from the lower dry biomass levels achieved in month 1 (Figure 28) averaging only 0.24g/L. Biomass for subsequent months attained a minimum of 0.39g/L in month 5 and a maximum of 0.61g/L in month 4. Dry biomass levels for months 2, 3 and 6 remained fairly constant and gave results of 0.5g/L, 0.52g/L and 0.54g/L respectively. The biomass levels achieved in the raceway were largely below the level of 1g/L, due to experimentation taking place during the cooler months of the year. Water temperatures were ranged between levels of 14°C to 24°C. Biomass showed an increase with a decrease in temperature during months 3 and 4 and a decrease in month 5 that correlated with an average increase in temperature for said period (Table 14).

Table 14: Average water temperatures for the 6 month period of the trial.

| Month | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Average Temperature(°C) | 19.8 | 19.5 | 17.5 | 16.8 | 19.8 | 18.7 |

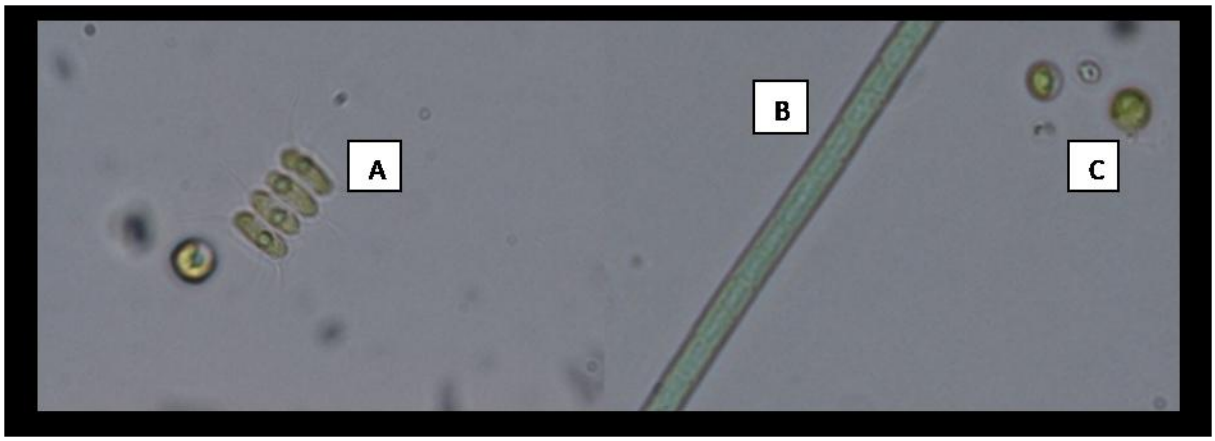


Figure 29: Light micrograph showing A) *Scenedesmus* sp. B) *Oscillatoria* sp. and C) *Chlorella* sp. at 1000X

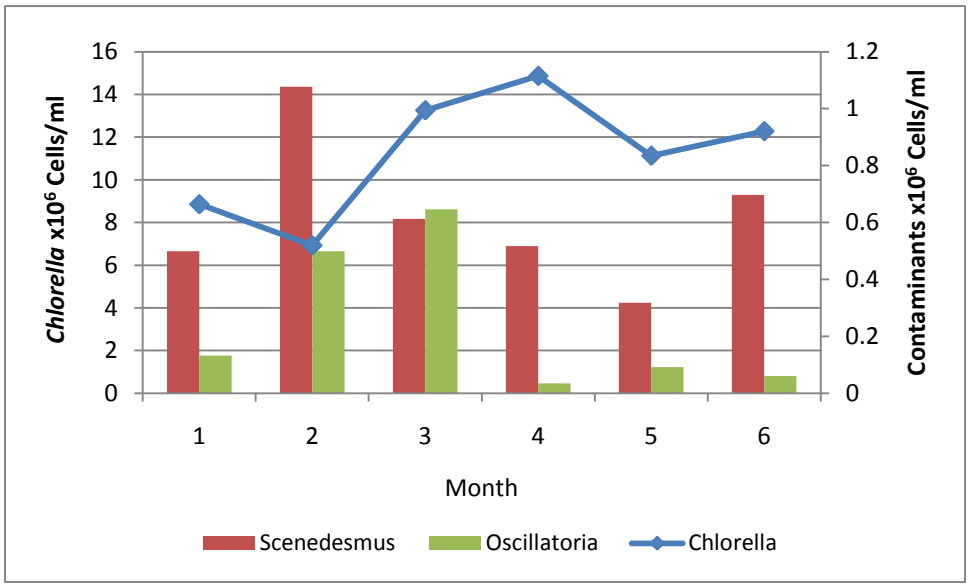


Figure 30: Population dynamics of pilot scale raceway pond growing microalgae for the production of biodiesel. (*Chorella* sp. is read of the primary Y axis as opposed to *Scenedesmus* sp. and *Oscillatoria* sp. which are read of the secondary Y axis.)

The raceway pond showed evidence of contamination 14 to 21 days subsequent to inoculation. Figure 25 shows the average number of algal cells per 15 fields of view per sample for each of the 6 months by direct counting. The data obtained per sample are averages that are reflected by the data for each month. It should be noted that Figure 30 reflects trends to show comparative population densities rather individual cell densities. The more prevalent of these contaminants were *Scenedesmus* sp. and *Oscillatoria* sp. species (Figure 30). Cyanobacteria were found to be present although in small amounts. Throughout the period of the trial, the *Chlorella* sp. remained dominant showing average numbers far greater than 10 fold the number of contaminants present at any given time.

Month 2 showed a decline in the average number of *Chlorella* cells, however, with regards to the contaminants, S*cenedesmus* sp. increased by greater than 100% to over $1.07x10^6$ cells/ml and there was a 378% increase in *Oscillatoria* sp. to approximately $4.99x10^5$cells/ml. Months 3 and 4 show an increase in concentration of *Chlorella* sp. with $13.25 \times 10^6$ cells/ml and $14.86 \times 10^6$ cells/ml respectively. A decrease in the total contaminants was noted for the period of months 4 to 5. *Oscillatoria* sp. showed a dramatic decrease in month 4 with $0.034 \times 10^6$ cells/ml and recovered only slightly in month 5. This correlated to the lowest average temperature for the experimental period. *Chlorella* sp. showed the highest numbers during the same period. *Chlorella* sp. and *Scenedesmus* sp. showed a decrease in number during month 5 which corresponded to higher temperature (Table 14) followed by an increase in cell concentration in month 6. Total biomass and numbers of *Chlorella* sp. over the experimental period follow the same trend (Figures 28 and 30). The minimum cell counts of *Chlorella* sp. were 6.4 times greater than the maximum of any contaminant for the experimental period.

## 5.4 Discussion

Open systems are prone to contamination. When using an open system it is necessary to select a culture that will have a competitive advantage in order to remain the dominant culture in the system. *Chlorella* sp. has such an advantage in that they are a high nutrient requiring genus of microalgae (Borowitzka, 1999). The best averaged annual biomass production for open ponds is

approximately 1g/l dry weight $m^{-1}$ $h^{-1}$ (Gordon & Polle, 2007). The biomass levels achieved (Figure 28) in the raceway were all well below this level. This may be to experimentation taking place during the cooler months of the year (April to September) (Table 14). Total biomass levels achieved were higher in the colder months of the experimental period (Table 14) and showed a decline in a warmer month before increasing. This was contrary to the expected results of an increase in biomass with an increase in temperature as is the norm in biological systems. This phenomenon may however be explained by an increase in cell volume with a decrease in temperature (Latala et al., 1991), however, it must also be considered that temperature is merely one of the factors that play a role in algal growth. Nutrient uptake by algal cells is enzymatically controlled and thus rates of nutrient uptake by algal communities is likely temperature dependent (Rugenski et al., 2008). Nutrient limitation may come into play when temperature becomes a limiting factor. Sterner & Grover, (1998) showed in 2 studies of algal growth in open pond systems that nutrients may become strongly and consistently limiting in warm temperatures i.e. above 20°C. Due to the open nature of the pond, it is extremely difficult to adequately quantify other external parameters and interactions.

The inverse relationship between *Chlorella* sp. and contaminants as seen in Figure 30 may be as a result of increased competition for nutrients due to proliferation of the desired *Chlorella* sp. culture amongst other factors. Furthermore *Chlorella* sp. showed the highest numbers during the period corresponding to the lowest temperatures may be as a result of *Chlorella* sp. having a greater tolerance to lower temperatures and may the benefit of increased nutrients due to less competition from contaminants thus resulting in better growth for this period. Photosynthesis in *Chlorella* sp. occurs in a temperature range of 5°C to 45°C reaching a maximum at 35°C (Nagashima et al., 1995). The ability of *Chlorella* sp. to adapt to lower temperatures is by the mechanism of conversion lipids resulting in a net decrease in the total amounts of saturated fatty acids and an increase in unsaturated fatty acids, thus correlating with the decrease in total biomass as seen in month 5 (Nagashima et al., 1995). The decrease of *Chlorella* sp. and *Scenedesmus* sp. seen during month 5 (Figure 30) is likely due to the microalgae undergoing metabolic change to allow the increased growth seen in month 6 (Suga et al., 2002). This will include the reversion of unsaturated fatty acids to saturated fatty acids thus diverting energy that

would otherwise been used for growth (Nagashima et al., 1995). Total biomass and numbers of *Chlorella* sp. over the experimental period follow the same trend (Figures 28 and 30). This coupled with the minimum cell counts of *Chlorella* sp. being 6.4 times greater than the maximum of any contaminant for the period leads us to conclude that *Chlorella* sp. can be maintained in open pond under natural climatic conditions as the dominant culture in the system.

# Chapter 6: Conclusion and Recommendations

## 6.1 Conclusions

One of the most important stages in the viable production of biodiesel from microalgae is the selection of appropriate strains for large scale culturing. This is accomplished preferentially by bioprospecting of regions in the vicinity of the proposed production area. Selection of wild type microalgae limits the degree of adaptation that may be required by the organism as well as providing the possibility for selection of novel strains and strains with inherent competitive advantages. From the research conducted, the following can be concluded:-

- Screening of aquatic environments yielded large number of microalgae strains of potential interest. Micromanipulation as a technique for isolation met with limited success. Future technological improvements may assist in making the technique a more powerful method for isolation of microalgae.
- Pre-screening and growth rate calculations enabled selection of two strains, *Chlorella* sp. and *Scenedesmus* sp. that showed potential to meet some of the important criteria for selection. Literature showed these microalgae to be good candidates for biofuels production.
- Optimisation of growth and lipid yields was initiated by media selection resulting in the selection of BG11 media for the cultures of choice. BG11 medium is seen as advantageous in that it is a high nutrient media thus giving a competitive advantage in that high nutrient requiring microalgae are easier to cultivate. Futhermore, conditions for induction of neutral lipid by nutrient limitation is more readily achieved. Moreover successful cultivation in open systems becomes more viable thus making the process more economical.
- Nutrient limitation induced stress was successful for increase in lipid yields. Nitrogen limitation rather than starvation yielded the best results for increase of neutral lipid. Phosphate limitation in moderate quantities resulted in lipid accumulation. This was however not suggested as the method for lipid induction as growth is severely retarded when compared to nitrogen limitation.

- Certain microalgal species not only have the ability to proliferate at high pH but can also perform better than when grown at pH 7 to pH 9 due to decreased competition from other microalgae.
- *Chlorella* sp. can be sustainably grown using open pond system and successfully outcompete microalgal contaminants. Population densities need to be monitored regularly and corrective action in the form of pure culture seeding be implemented if required.

## 6.2 Recommendations

The use of microalgae for the production of biodiesel is technically feasible. It is recommended that each strain be investigated individually for optimisation as conditions differ for the production of lipid. Further work is required with regard to ascertaining the role of other essential nutrients such an iron and how they may be manipulated in order to assure hyper-lipid production. Large scale cultivation has inherent challenges that are not experienced at bench scale. Some of these challenges cannot be foreseen or effectively simulated under laboratory conditions. Therefore further optimisation of conditions needs to be performed at large scale. Synergistic and/or antagonistic interactions between microalgae (desired and contaminative), bacteria and other organisms in open pond systems should be elucidated in order to understand the ecosystem and enable efficient optimisation of the production process.

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# Appendix A: Bolds Basal Medium (BBM) (Starr & Zeikus, 1987)

Modified bolds basal medium was made up by addition of:

| Component | g/L |
|---|---|
| **Macronutrients** | |
| $NaNO_3$ | 0.25 |
| $K_2HPO_4$ | 0.075 |
| $KH_2PO_4$ | 0.175 |
| $MgSO_4.7H_2O$ | 0.075 |
| $CaCl_2.2H_2O$ | 0.025 |
| NaCl | 0.025 |
| Thiamine | 1ml/L |
| Biotin | 1ml/L |
| Vitamin $B_{12}$ | 1ml/L |
| PIV Metal Solution | 6ml/L |
| | |
| **Vitamins Stocks** | |
| Thiamine | 1 |
| Biotin | $25 \times 10^{-5}$ |
| Vitamin $B_{12}$ | $15 \times 10^{-5}$ |
| | |
| **PIV Metal Solution** | |
| $Na_2EDTA$ | 0.750 |
| $FeCl_3 . 6H_2O$ | 0.097 |
| $MnCl_2 .4H_2O$ | 0.041 |
| $ZnCl_2$ | 0.005 |
| $CoCl_2 .6H_2O$ | 0.002 |
| $Na_2MoO_4.2H_2O$ | 0.004 |

Macronutrient components were made up as 100X stock solutions. 10ml of each macronutrient stock was added in 900ml distilled water. PIV metal solution and vitamin solutions were added after autoclaving for 15 mins at 121°C at 1 atmosphere. Media was made up to 1L with sterile distilled water. Final pH= 6.6

BBM agar was prepared by addition of 1.5% (w/v) agar bacteriological to BBM before autoclaving.

## Appendix B: Spirulina Medium (SP) (Aiba & Ogawa, 1977)

| Component | g/500ml |
|---|---|
| **Solution I** | |
| $NaHCO_3$ | 13.61 |
| $Na_2CO_3$ | 4.03 |
| $K_2HPO_4$ | 0.50 |
| $dH_2O$:500ml) | |
| **Solution II** | |
| $NaNO_3$ | 2.50 |
| $K_2SO_4$ | 1.00 |
| NaCl | 1.00 |
| $MgSO_4 . 7H_2O$ | 0.20 |
| $CaCl_2 . 2H_2O$ | 0.04 |
| **Chu Micronutrient solution** | mg/L |
| $Na_2EDTA$ | 50 |
| $H_3BO_3$ | 618 |
| $CuSO_4.5H_2O$ | 19.6 |
| $ZnSO_4$ | 44 |
| $CoCl_2.6H_2O$ | 20 |
| $MnCl_2.4H_2O$ | 12.6 |
| $Na_2MoO_4.2H_2O$ | 12.6 |

Solutions I and II were made up separately in 450ml distilled water for each solution. Solutions were autoclaved at 121°C for 15mins and were united after cooling and supplemented with 5 ml of Chu micronutrient solution and made up to 1L

SP agar was prepared by addition of 1.5% (w/v) agar bacteriological to solution II before autoclaving.

## Appendix C: Modified AF6 media (Watanabe et al., 2000)

| Component | g/L |
|---|---|
| **Macronutrient** | |
| Ferric Citrate | 0.002 |
| Citric acid | 0.002 |
| $NaNO_3$ | 0.140 |
| $NH_4NO_3$ | 0.022 |
| $MgSO_4.7H_2O$ | 30 |
| $KH_2PO_4$ | 0.010 |
| $K_2HPO_4$ | 0.005 |
| $CaCl_2.2H_2O$ | 0.010 |
| Trace metal solution | 1ml |
| Vitamin Solution | 1ml |
| | |
| **Trace Metal Solution** | |
| $Na_2EDTA$ | 5.00 |
| $FeCl_3$.6H2O | 0.98 |
| $MnCl_2.4H_2O$ | 0.18 |
| $ZnSO_4.7H_2O$ | 0.11 |
| $CoCl_2$. $6H_2O$ | 0.02 |
| Na2MoO4. $2H_2O$ | 0.0125 |
| | |
| **Vitamin Stock solution** | |
| Thiamine HCl (vitamin B1) | 0.01 |
| Biotin (vitamin H) | 0.002 |
| Pyridoxine HCl (vitamin B6) | 0.001 |
| Cyanocobalamin (vitamin B12) | 0.001 |

Macronutrient components prepared in the form of 1000X stock solutions. For each litre of media, 1ml of each macronutrient was dissolved in 900ml distilled water. Vitamin solution was filter sterilized. Trace metal solution and vitamin solutions were added after autoclaving for 15 mins at 121°C at 1 atmosphere. Media was made up to 1L with sterile distilled water.

AF6 agar was prepared by addition of 1.5% (w/v) agar bacteriological to AF6 medium before autoclaving.

## Appendix D: BG 11 medium (Allen & Stanier, 1968)

| Component | g/L |
|---|---|
| **Macronutrient** | |
| $NaNO_3$ | 1.5 |
| $K_2HPO_4$ | 0.04 |
| $MgSO_4.7H_2O$ | 0.075 |
| $CaCl_2.2H_2O$ | 0.036 |
| EDTA (disodium magnesium) | 0.001 |
| $Na_2CO_3$ | 0.02 |
| Ferric Citrate Solution | 1ml |
| Trace Metal Solution | 1ml |
| | |
| **Ferric citrate solution:** | |
| Citric Acid | 0.006 |
| Ammonium ferric citrate | 0.006 |
| | |
| **Trace Metal Solution** | |
| $H_3BO_3$ | 2.86 |
| $MnCl_2.4H_2O$ | 1.81 |
| $ZnSO_4.7H_2O$ | 0.22 |
| $Na2MoO_4.2H_2O$ | 0.390 |
| $CuSO_4.5H_2O$ | 0.079 |
| $Co(NO3)_2.6H_2O$ | 0.049 |

Macronutrient components prepared in the form of 1000X stock solutions (except $NaNO_3$). For each litre of media, 1ml of each macronutrient was dissolved in 900ml distilled water beginning with the ferric citrate solution. Media was made up to 1L with sterile distilled water before autoclaving for 15 mins at 121°C at 1 atmosphere. Final pH = 7.4

## Appendix E: Bristols NaCl medium (Starr & Zeikus, 1987)

| Component | g/L |
|---|---|
| **Macronutrient** | |
| $NaNO_3$ | 0.25 |
| $K_2HPO_4$ | 0.075 |
| $KH_2PO_4$ | 0.175 |
| $MgSO_4.7H_2O$ | 0.075 |
| $CaCl_2.2H_2O$ | 0.025 |
| NaCl | 3.6 |

Macronutrient components prepared in the form of 1000X stock solutions. NaCl was added directly. For each litre of media, 1ml of each macronutrient was dissolved in 900ml distilled water. Media was made up to 1L with sterile distilled water before autoclaving for 15 mins at 121℃ at 1 atmosphere.

## Appendix F: Chu #10 medium (Chu, 1942)

| Component | g/L |
|---|---|
| **Macronutrient** | |
| $Ca(NO_3)_2$ | 0.04 |
| $K_2HPO_4$ | 0.005 |
| $MgSO_4.7H_2O$ | 0.025 |
| $Na_2CO_3$ | 0.02 |
| $Na_2SiO_3$ | 0.025 |
| $FeCl_3$ | 0.0008 |

Macronutrient components prepared in the form of 1000X stock solutions. For each litre of media, 1ml of each macronutrient was dissolved in 900ml distilled water. Media was made up to 1L with sterile distilled water before autoclaving for 15 mins at 121℃ at 1 atmosphere.