

https://dspace.org/

The molecular characterisation, strain selection and induction of lipid synthesis in a suitable marine microalgal species for potential biodiesel production

Nambiar, Shari

2011-03-30
http://hdl.handle.net/10539/9287

Downloaded from a DSpace repository. https://dspace.org/

THE MOLECULAR CHARACTERISATION, STRAIN SELECTION AND INDUCTION OF LIPID SYNTHESIS IN A SUITABLE MARINE MICROALGAL SPECIES FOR POTENTIAL BIODIESEL PRODUCTION

Shari Nambiar

A research report submitted to the Faculty of Science, in partial fulfilment of the requirements for the degree of Master of Science at the University of the Witwatersrand, Johannesburg

Supervisor: Prof. V. Gray

Co-Supervisor: Prof. S. Sym

Advisor: Dr. C. A. Togo

Johannesburg 2010

DECLARATION

I declare that this research report is my own, unaided work. It is being submitted for the Degree of Master of Science (Biotechnology) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



_____

Signature of candidate

11th day of October 2010

ABSTRACT

The demand for alternative energy has increased exponentially and algal-based biofuels are viewed as a viable alternative. This study examined the lipid producing capability of *Isochrysis* and the role of phosphorus depletion in stimulating lipid production. The main objective of this study was to determine the relationship between varying levels of phosphorus depletion and total lipid production in a suitable marine microalga. The ability of the microalgal strain to grow and produce lipid were examined under varying concentrations of phosphorus in the f/2 medium. *Isochrysis* exhibited log growth after 8 days of cultivation in the various dilutions of the phosphorus concentration recommended for f/2 medium. A stationary phase was observed when the microalgal cells depleted the surrounding medium of phosphorus. The phosphorus concentration of the various media was measured using a phosphorus colorimetric assay which revealed that the phosphorus concentration of the media progressively decreased from day 2 to day 24. However, there was no further uptake of phosphorus from the surrounding medium when the cells entered a stationary phase. The medium containing no phosphorus supplementation stimulated a lipid production of 57.75 % after 24 days which was attributed to the microalgae accumulating lipid under phosphorus deprivation. This was a result of continuous lipid synthesis combined with a low rate of cell division and photosynthesis. Therefore, allowing *Isochrysis* to deplete the medium of soluble reactive phosphorus resulted in increased lipid production. Furthermore, a relationship between phosphorus deprivation and lipid content was attained for sustained lipid production over time.

ACKNOWLEDGEMENTS

The author would like to acknowledge the following people for their contribution:

- A sincere and heartfelt thank you to my supervisors Prof Sym and Prof Gray. I have grown immensely with your guidance,
- University of the Witwatersrand and the Gauteng Department of Conservation Agriculture and the Environment for funding and
- My parents and friends from International house for their continuous support.

CONTENTS PAGE

CONTENTS **Page**

DECLARATION ….....2

ABSTRACT ……..……..……..3

ACKNOWLEDGEMENTS ….....4

LIST OF FIGURES ….....7

LIST OF TABLES ……..…….....10

LIST OF ABBREVIATIONS ……..……...11

CHAPTER 1 - LITERATURE REVIEW

1.1 Introduction.....13

1.2 Utilisation of microalgae as a biofuel feedstock.....14

1.3 Mechanisms by which microalgae produce lipids for potential biodiesel production.....15

1.4 Lipid production from the marine microalgae.....17

1.5 Hypothesis.....18

1.6 Objective.....18

1.7 Aims.....19

CHAPTER 2 - SELECTION OF MARINE MICROALGAL SPECIE

2.1 Introduction.....20

2.2 Materials and Methods.....20

2.2.1 Culture maintenance.....20

2.2.2 Set-up and inoculation of culture vessels.....20

2.2.3 Lipid extraction.....21

2.2.4 Measurement of lipid production.....22

2.3 Results and Discussion.....23

2.3.1 Lipid standard curve for Isochrysis sp. *and* Tetraselmis JJII.....23

CHAPTER 3 - MOLECULAR CHARACTERISATION OF SELECTED SPECIE

3.1 Introduction.....27

3.2 Methods and Materials.....27

3.2.1 Inoculation of culture vessels for DNA extraction.....27

3.2.2 Extraction of the Isochrysis sp. culture.....27

3.2.3 PCR amplification of 18S rDNA.....29

3.2.4 Detection of purity and fragment length of PCR products.....30

3.2.5 Sequencing and bioinformatic analysis of the amplified 18S rDNA.....30

3.3 Results and Discussion.....31

3.3.1 Detection of the amplified DNA fragments.....31

3.3.2 Sequencing of the amplified 18S rDNA fragments.....32

3.3.3 Bioinformatic analysis of the microalgal DNA.....33

CHAPTER 4 - LIPID PRODUCTION ANALYSIS

4.1 Introduction.....34

4.2 Materials and Methods.....34

4*.2.1 Phosphorus standard curve*.....34

4*.2.2 Preparation of f/2 medium with various phosphorus concentrations*.....35

4.2.3 Inoculation and set-up of culture vessels.....36

4.2.4 Sampling.....37

4.2.5 Light microscopy.....38

4*.2.6 Measurement of algal growth using a haemocytometer*.....38

4.2.7 Measurement of algal growth using Spectrophotometer.....39

4.2.8 Detection of lipid production using flow cytometry.....39

4.2.9 Measurement of phosphorus during algal cultivation.....39

4.3 Results and Discussion.....40

4.3.1 Soluble reactive phosphorus standard curve.....40

4.3.2 Algal growth curves obtained using absorbance and cell counts.....41

4.3.3 Lipid production at various phosphorus concentrations.....44

4.3.4 Light microscopic images of the microalgal culture.....47

CHAPTER FIVE - OVERALL DISCUSSION AND CONCLUSION

5.1 Discussion 51

5.2 Conclusion.....52

6 REFERENCES.....53

7 APPENDICES.....58

LIST OF FIGURES

Figure 1.1: Schematic diagram depicting the process of photosynthesis in algae that feeds a separate anabolic reaction series, fatty acid synthesis.....16

Figure 2.1:.Linear correlations between the standard curve of percentage of lipid content versus the total Nile Red fluorescence of *Isochrysis* sp. microalgal cells.....24

Figure 2.2: Linear correlations between the standard curve of percentage of lipid content versus the total Nile Red fluorescence of *Tetraselmis* microalgal cells.....25

Figure 3.1: Agarose gel showing the U.V. fluorescence of the amplified 18S rDNA fragments on a U.V. trans-illuminator.....32

Figure 3.2: Phylogenetic tree comparing the homology between the unknown microalgal sequence and known microalgal 18S rDNA sequences.....33

Figure 4.1: Set-up of the microalgal culture vessels.....37

Figure 4.2:.Soluble reactive phosphorus standard curve obtained from the colorimetric molybdate antimony method.....41

Figure 4.3: Growth of *Isochrysis* measured by haemocytometer cell counts showing the ability of the microalgal cells to undergo cell division in f/2 media with various phosphorus concentrations.....42

Figure 4.4: Growth of *Isochrysis* measured by absorbance measurements showing the ability of the microalgal cells to undergo cell division in f/2 media with various phosphorus concentrations.....43

Figure 4.5: The change in the concentration of soluble reactive phosphorus in various f/2 media during the 24 day growth period of *Isochrysis*.....45

Figure 4.6: The percentage of lipid produced in the *Isochrysis* cells as a response to various phosphorus concentrations in f/2 medium.....46

LIST OF FIGURES

Figure 4.7 Visible lipid produced in *Isochrysis* after a cultivation time of 24 days in 0 ppm of P in f/2 medium.....47

Figure 4.8: Visible lipid produced in *Isochrysis* after a cultivation time of 24 days in 0.01 ppm of P in f/2 medium.....48

Figure 4.9: Visible lipid produced in *Isochrysis* after a cultivation time of 24 days in 0.10 ppm of P in f/2 medium.....48

Figure 4.10: Visible lipid produced in *Isochrysis* after a cultivation time of 24 days in 0.25 ppm of P in f/2 medium.....49

Figure 4.11: Visible lipid produced in *Isochrysis* after a cultivation time of 24 days in 0.50 ppm of P in f/2 medium.....49

LIST OF TABLES

Table 1.1: Comparison of the ability of first generation feedstocks and algae to produce biodiesel to meet the current demand.....14

LIST OF ABBREVIATIONS

In alphabetical order:

BLAST: basic local alignment search tool

CO_2: carbon dioxide

°C: degree Celsius

CTAB: cetyl trimethylammonium bromide

EDTA: ethylene diamine tetra acetic acid

H_2O: water

H_2SO_4: sulphuric acid

L: litre

18S rDNA: small ribosomal subunit deoxyribonucleic acid

ml: millilitre

mins: minutes

µl: microlitre

µg: microgram

mm^2: millimetre squared

mm: millimetre

g: gram

$NaH_2PO_4.2H_2O$: sodium dihydrogen orthophosphate dihydrate

N: normality of a solution

nm: nanometre

NCBI: national centre for biotechnology information

P: phosphorus

ppm: part per million

PCR: polymerase chain reaction

rpm: revolutions per minute

SSU: small subunit

secs: seconds

TAE: tris base, acetic acid and ethylene diamine tetra acetic acid

TAG: triacylglycerol

UV: ultra violet

1 LITERATURE REVIEW

1.1 Introduction

The demand for biofuels has increased significantly over the past decade in order to reduce gaseous emissions and to promote environmental sustainability (Schenk *et al*., 2008). The atmospheric carbon dioxide levels have been reported to be above 450 ppm CO_2-e, where *e* is the equivalent contribution of all greenhouse gases (Schenk *et al*., 2008). The CO_2 level in the atmosphere of 450 ppm was reached ten years earlier than expected which has resulted in a concerted effort to develop biofuels (Schenk *et al*., 2008; Gouveia and Oliveira, 2009).

Biomasses from forestry, agricultural and aquatic sources have been studied for their potential use as a feedstock for biofuels (Vasudevan and Briggs, 2008). At present, only biodiesel and bioethanol have been produced on an industrial scale (Vasudevan and Briggs, 2008). They are viewed as a fuel replacement for internal combustion engines and have been derived from sugarcane, sugar beet, maize, sorghum and wheat (Schenk *et al*., 2008). First generation feedstocks are primarily crops such as cotton, soybean, palm and soybean which produce carbohydrates that are extracted for biofuel production. These first generation feedstocks have numerous problems, such as the supplementation for fossil fuel input as the biofuel cannot be utilised alone, limited harvesting frequency as the crops have to reach a particular size prior to harvesting, requirements for arable land which is only 13 %, competition with food as these feedstocks form a staple diet in certain countries and a poor carbon balance due to the use of machinery for harvesting and transport of the crops which emit fossil fuel derived CO_2 (Schenk *et al*., 2008; Gouveia and Oliveira, 2009). However, total reliance on first generation feedstocks would require a significant amount of land to meet our current fuel consumption as shown in table 1.1 (Schenk *et al*., 2008). There is insufficient land for the production of first generation feedstocks to replace 50 % of current fossil fuel (Table 1.1). In contrast to first generation feedstocks, algae are more efficient at utilising sunlight than terrestrial plants, consuming harmful pollutants, minimal usage of arable land area and they do not compete with food or agriculture for precious resources such as maize (Chisti, 2007).

Table 1.1: Comparison of the ability of first generation feedstocks and algae to produce biodiesel to meet the current demand (Adapted from Schenk *et al*., 2008)

Plant Source	**Biodiesel (L/ha/year)**	**Area to produce global oil demand (hectares x 10^6)**	**Area as percent global arable land**
Cotton	325	15002	756.9
Soybean	446	10932	551.6
Sunflower	952	5121	258.4
Palm oil	5950	819	41.3
Algae*	98500	49	2.5

*The biodiesel produced is dependent on a 50 % TAG content and if algal ponds and bioreactors are situated on non-arable land.

1.2 Utilisation of microalgae as a biofuel feedstock

The microalgae are a diverse group of photosynthetic prokaryotic and eukaryotic organisms that convert carbon dioxide to potential biofuels (Li *et al*., 2008). Numerous biofuels can be generated by microalgae, as stated by Chisti (2007). These include methane produced by the anaerobic digestion of the algal biomass; biodiesel which can be derived from the microalgal oil and biohydrogen produced photobiologically (Li *et al*., 2008). Algae are an appealing feedstock because they have many biological and technical attributes that help us overcome problems that are presented by many first generation biofuel feedstocks (Li *et al*., 2008; Patil *et al*., 2008; Schenk *et al*., 2008). The advantages that algae can offer as a feedstock can be summarised as follows:

(1) Resource requirements of algae are less intensive compared to other crops and plants (Li *et al*., 2008; Schenk *et al*., 2008). Algae only require basic resources to grow which are: carbon dioxide, water, sunlight and nutrients,

(2) The higher photosynthetic efficiency of algae allows for larger amounts of energy to be utilised and subsequently stored within the algal cell, which can be extracted later in the form of oil or biomass (Schenk *et al*., 2008),

(3) Algae have the ability to sequester carbon dioxide or nitrogen oxides from the atmosphere (Li *et al*., 2008; Schenk *et al*., 2008), and

(4) Algae are often oil-rich organisms and the percentage of oil per weight of dry biomass typically ranges from 20 % to 50 %, depending on the species (Chisti, 2007).

The algae have a faster growth rate than terrestrial crops and the per unit area yield of oil from algae is estimated to be between 18 927 to 75 708 litres per acre per year; this is 7 to 31 times greater than that of palm oil, which is the largest oil-producing crop (Schenk *et al*., 2008). Algae have the ability to grow in either freshwater or marinewater thus avoiding the use of excessive land (Li *et al*., 2008). Furthermore, two thirds of the earth's surface is covered with water, therefore algae can truly be a renewable option of immense potential for global energy needs (Patil *et al*., 2008).

1.3 Mechanism by which microalgae produce lipids for potential biodiesel production

Photosynthesis is the main process by which light energy is converted to chemical energy thus driving the production of oils for biodiesel production (Schenk *et al*., 2008). The Calvin cycle is vital for lipid synthesis and will therefore be explained. The Calvin cycle is driven by the NADPH and the ATP that were produced in the light reactions (Schenk *et al*., 2008).

It is the Calvin cycle that plays an integral role in the ability of microalgae to fix carbon dioxide. There are three reactions that occur in the Calvin cycle, namely: carboxylation, reduction and substrate regeneration (Schenk *et al*., 2008). Rubisco (ribulose-1, 5-bisphosphate carboxylase/oxygenase) catalyses the formation of 3-phosphoglycerate (3PG) molecules from the substrate, ribulose-1.5-bisphosphate (RuBP), CO_2 and H_2O (Schenk *et al*., 2008). The carboxylic acids are reduced to form glyceraldehyde-3-phosphate (G3P). In the final stage of the Calvin cycle, a portion of the produced glyceraldehyde-3-phosphate is converted to the initial substrate RuBP (Figure 1.1) (Schenk *et al*., 2008). The biosynthesis of triacylglycerol (TAG) in algae was suggested to take place via the direct glycerol pathway (Hu *et al*., 2008). The G3P serves as the substrate for TAG synthesis which is required for biodiesel production (Hu *et al*., 2008).

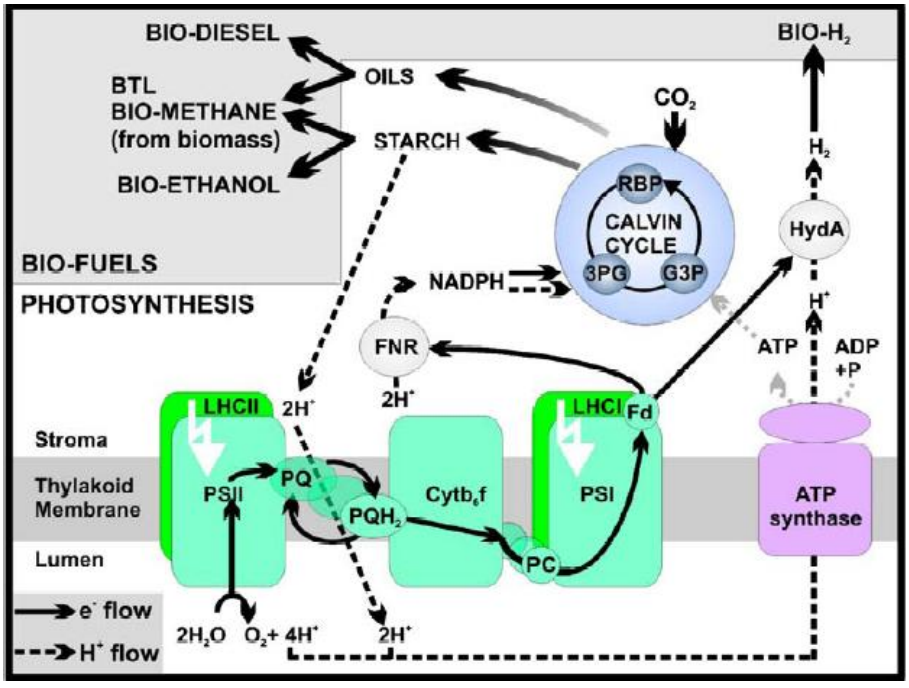

Figure 1.1: Schematic diagram depicting the process of photosynthesis in algae that feeds a separate anabolic reactions series, fatty acid synthesis. (Schenk *et al*., 2008).

1.4 Lipid production from the marine microalgae

Marine microalgae such as *Isochrysis*, *Nannochloropsis*, *Chlorella* and *Tetraselmis* have often been characterised by their ability to produce high concentrations of long-chain polyunsaturated fatty acids unlike other microalgae and terrestrial plants (Hu *et al*., 2008). The marine microalgal strains can grow in brackish water thus eliminating competition with other land-based technologies for generating energy. The quantity and type of lipid produced by the various classes of microalgae is largely influenced by the growth factors which are light, temperature, nitrogen concentration and phosphorus concentration. Reitan *et el*., (1994) showed that *Isochrysis galbana* and *Pavlova lutheri* increased their lipid content when the extent of nutrient limitation was greater. The microalgae tend to accumulate neutral TAG under nutrient deprivation as a means of recovering from the stress when normal growth conditions have been restored (Reitan *et al*., 1994). All microalgal species accumulate lipids and starch in most instances during nutrient depleted conditions. The accumulation of a storage product (starch and lipid) occurs for the microalgal cell to derive energy and continue with cell growth and development.

The microalgae *Tetraselmis* sp. was shown to have decreased lipid content under phosphorus-limited growth conditions. These microalgae belong to the Prasinophyceae class which tend to accumulate photosynthetic products other than lipids (e.g. carbohydrates) when nutrients are limited (Reitan *et al*., 1994). The oleaginous microalgae tend to have the unique ability for *de novo* lipid synthesis when cultivated under nutrient deprived conditions (Rodolfi *et al*., 2008). The excess carbon and energy is channelled into storage lipid which mainly consists of triacylglycerides. Triacylglycerides are glycerides in which the glyceride is esterified with three fatty acids. When the microalgal cell requires energy, the triacylglyceride is broken down to release the energy-rich fatty acids. These triacylglycerides are predominantly composed of saturated and mono-unsaturated fatty acids which can be packed into the cell (Rodolfi *et al*., 2008).

Subsequently, they generate larger quantities of energy compared to carbohydrates upon oxidation, thus serving as an energy reserve to build the cell after nutrient depletion (Rodolfi *et al*., 2008). Thus, the growth conditions of the microalgae can be manipulated such that large quantities of lipids are accumulated but sufficient algal biomass is required to extract the produced lipid.

The near depletion of fossil fuel reserves and the drastic consequences on the environment has driven research into alternative fuels. The generation of second-generation biofuels from algae can be viewed as a renewable alternative that promotes environmental sustainability (Chisti. 2007). Current research has focused on the effect of nitrogen and light intensity on the ability of the marine microalgae, *Isochrysis* to produce lipids. However, there has been one research paper which has been published regarding the effect of phosphorus limitation on neutral lipid production in *Isochrysis*. The findings in Reitan *et al*., 1998, indicated that prymnensiophytes accumulate lipids as result of phosphorus limited growth conditions. In the present study, the lipid content of *Isochrysis* sp. was examined. The goal was to study the effect of phosphorus-limited growth on the quantity of neutral lipid produced. The lipid production and growth were analysed at different phosphorus concentrations.

1.5 Hypothesis

Phosphorus supply controls the fate of photosynthate partitioning into either growth of new cells or accumulation of storage products.

1.6 Objective

To investigate the affect of phosphorus supply on cell growth and lipid accumulation in a selected lipid-accumulating marine microalgal species.

1.7 Aims

a. Select and characterise one suitable marine microalgae for lipid production.

b. Molecular characterisation of the selected microalgal species using 18S rDNA.

c. To evaluate growth and lipid accumulation under conditions of phosphorus depletion.

2 SELECTION OF MARINE MICROALGAL SPECIES

2.1 Introduction

It is vital for researchers to measure the amount of neutral lipid produced by the microalgae in order to utilise the lipids for biodiesel production. Flow cytometry allows for the monitoring of the total lipid content, *in situ* and in real time during the growth of the microalgae. *Isochrysis* and *Tetraselmis* were selected from an algal culture collection to conduct a standard curve of total Nile Red fluorescence against the percentage of lipid. These microalgae were selected as they are known to produce large amounts of lipid and the lipid production can be manipulated via changing the growth conditions.

2.2 Materials and Methods

2.2.1 Culture maintenance

Unialgal cultures of *Tetraselmis* and *Isochrysis* were maintained in f/2 medium (Appendix a). A drop of the respective culture was inoculated aseptically using a sterile micropipette into 50 ml of f/2 medium in sterile 50 ml conical flasks. The cultures were incubated in an algal growth room with constant illumination at 110 μmol.$m^{-2/}$s at 25 °C. This procedure was performed on a weekly basis.

2.2.2 Set-up and inoculation of culture vessels

The culture vessels utilised for growth and lipid production studies was a modified 1 L Schott bottle with a quick-fit aperture into which a quick-fit gas sparger consisting of a gas exhaust and aeration tubes. A rectangular (2.5 cm x 1.5 cm) sand stone fish tank sparger was fitted via plastic tubing to the end of the glass aeration tube .All 1 L culture vessels and glass sparger stoppers were autoclaved prior to use, to eliminate contamination.

The sand stone fish tank spargers were cleaned by pumping air through them in Milli-Q water, to remove any debris or dust from inside the spargers. The glass sparging apparatus were incubated at 70 °C for 15 mins to sterilise the glass prior to inoculation. The f/2 medium was autoclaved and then filtered into the culture vessels under the laminar flow hood, to a volume of 900 ml. The medium was filtered to remove salt crystals that could cause clumping of the microalgal cells during growth. The culture vessels were then inoculated with 1 ml of the respective cultures which had a cell count of approximately 15 cells per ml, using a sterilised Pasteur pipette. The vessels were then connected to an air pump (Labotec with a P_{max} of 2.4 bar) which aerated the cultures with air at a gas flow rate of 15 ml/ min. The air was filtered through a 0.2 µm filter (Whatman Uniflo) and cultures were incubated with continuous illumination at 110 µmol.$m^{-2/}$s at 25 °C. The cultures were monitored on alternate days for growth and lipid production. This was performed in triplicate.

2.2.3 Lipid extraction

Samplings and measurements were performed in triplicate. After one week of cultivation, a 100 ml sample was taken aseptically from each culture vessel on alternate days. The 100 ml culture samples were centrifuged at 5000 rpm for 15 mins at 4 °C. The supernatant from each tube was discarded and the pellet was retained. The pellet was dried for 36 h in a dessicator at room temperature. A 2 ml eppendorf tube was weighed and the dry pellet which was dried was added to the eppendorf tube and weighed. The algal biomass dry weight was then calculated. The lipid extraction method was adapted from Bligh and Dyer. (1959).

The extraction procedure was initiated with the addition of 500 µl methanol, 250 µl chloroform and 200 µl of Milli-Q water to the biomass in the tube. Ten autoclaved glass beads with a 0.1 mm diameter (Inqaba Biotech) were added to cover the biomass and the tube was vortexed for 10 mins, to break down the algal biomass. Subsequently, 250 µl chloroform and 250 µl Milli-Q water was added to the tube.

The contents of the eppendorf tubes were transferred to two Zymo spin column tubes with micro-filters (Zymo research DNA clean and concentrator^{TM}-5 kit). The tubes were centrifuged for 5 mins at 1500 rpm. After phase separation following centrifugation, the aqueous phase was denser than the non-aqueous phase. The aqueous phase was removed using a micropipette and the non-aqueous phase was decanted into a clean 2 ml eppendorf tube. The eppendorf tubes were then placed in a 60 °C water bath to allow the chloroform to evaporate. Tubes were placed in a dessicator to dry for 24 h at room temperature. Thereafter, the tubes were weighed and the lipid dry weight was calculated as follows:

Tube weight (g)= t

Tube weight (g) + Lipid dry weight (g) = tl

Therefore, lipid dry weight (g) = tl – t

The algal dry weight and lipid dry weight were subsequently utilised to calculate the percentage of lipid, as follows: Percentage of lipid (%) = $\frac{\text{Lipid dry weight (g) X 100}}{\text{Algal dry weight (g)}}$

2.2.4 Measurement of lipid production

Samplings and measurements were performed in triplicate. The quantity of neutral lipid produced by the microalgal cultures was measured using flow cytometry. For the measurements, a 4 ml culture sample was aseptically collected from each respective culture vessel. The autofluorescene control tube contained 2 ml of the culture only while the sample tube contained 2 ml of the culture and 200 µl of a 0.1 mg/ml Nile Red solution (Appendix b) (Silva *et al*., 2008). The sample tubes were gently vortexed for 1 min and incubated for 10 mins at 37 °C in darkness (Silva *et al*., 2008). The flow cytometer was calibrated with calibrate beads as per the operators manual.

Nile Red fluorescence was determined using a BD FACSCalibur^{TM} flow cytometer (Becton-Dickinson Instruments) equipped with a 488 nm argon laser. The Nile Red fluoresces yellow and red when dissolved in neutral and polar lipids respectively, which are detected in the FL2 and FL3 channels. The total fluorescence of the microalgal culture was calculated as follows, where FL2 is the red fluorescence detector, FL3 is the yellow fluorescence detector and AF is the autofluorescence in the respective channels (Silva *et al*., 2008):

$$\text{Total fluorescence} = \frac{FL2}{AF} + \frac{FL3}{AF}$$

A standard curve of total fluorescence against dry weight of lipid was constructed from which future readings of dry weight of lipid could be determined from fluorescence readings.

2.3 Results and Discussion

2.3.1 Lipid standard curve for Isochrysis sp. and Tetraselmis JJII

The marine microalgae, *Isochrysis* and *Tetraselmis* were selected as they are known for their ability to produce high amounts of lipid under normal and stressful growth conditions. Therefore, a standard curve was constructed to determine the reproducibility of the amount of lipid produced against the total Nile Red fluorescence. Cellular lipid content increased with time (Figure 2.1). Lipid content was measured at different time intervals and the percentage of lipid content increased from 12.1 % to 54.9 % over 14 days (Figure 2.1). Each time interval had different percentage lipid content with respect to dry mass. Thus, a range of percentage of lipid contents was generated and these could be used to calibrate the intensity of Nile Red fluorescence with respect to percentage lipid content.

The Nile Fluorescence allowed for the specific detection of neutral lipids within the microalgal cells thus allowing for quantitative accuracy. A maximum Nile Red fluorescence of 38.46 was achieved while the minimum Nile Red fluorescence of 7.55 was observed (Figure 2.1). Therefore, as the growth period increased, the lipid as measured by the total Nile Red fluorescence increased during the growth of the microalgal cell (Figure 2.1).

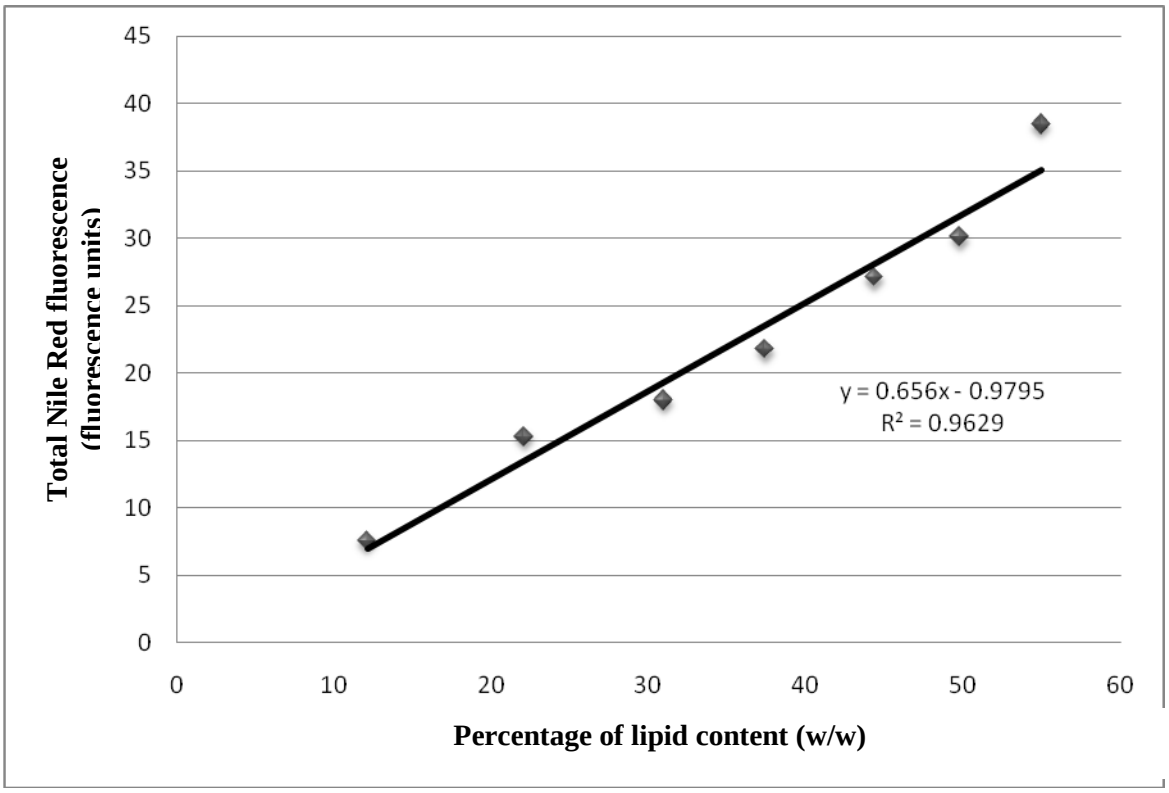

Figure 2.1 Linear correlations between the standard curve of percentage of lipid content versus the total Nile Red fluorescence of *Isochrysis* sp. microalgal cells. The lipid produced was a percentage of the dry mass weight. Each point is data that was expressed as the average of four replicates.

In *Tetraselmis*, the percentage of lipid content also increased with time. However, compared to *Isochrysis* the percentage of lipid content achieved over the 14 days culture period was lower (Figure 2.1 and Figure 2.2). A lipid content of 43.1 % was achieved after the growth period while the minimum lipid content in the cells was 11.1 %, as seen in figure 2.2. A maximum Nile Red fluorescence of 27.78 was achieved while the minimum Nile Red fluorescence of 7.81 was observed (Figure 2.2). In comparison to the *Isochrysis* standard curve, the *Tetraselmis* standard curve did not show a directly proportional relationship between total Nile Red fluorescence and lipid content. This indicated that the *Tetraselmis* did not have stable lipid production and the entire lipid content of the cells was not extracted.

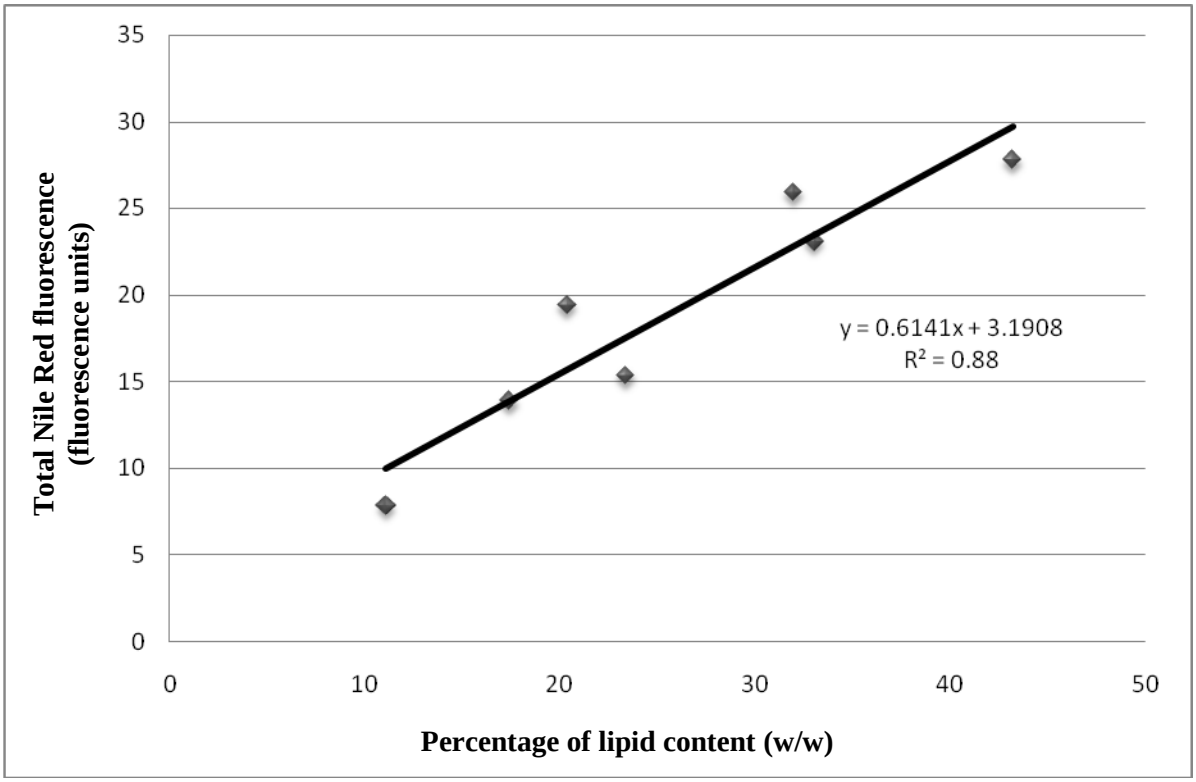

Figure 2.2 Linear correlations between the standard curve of percentage of lipid content versus the total Nile Red fluorescence of *Tetraselmis* microalgal cells. The lipid produced was a percentage of the dry mass weight. Each point is data that was expressed as the average of four replicates.

The lipid extraction procedure did not show qualitative accuracy as the percentage of lipid extracted was highly variable with *Tetraselmis* as shown in figure 2.2. The *Isochrysis* standard curve was highly reproducible indicating that the extraction procedure was more effective. The standard curves of the microalgae depicted the lipid content of the microalgal cells under normal growth conditions in f/2 medium. The *Isochrysis* cells had 54.9 % lipid content while the *Tetraselmis* cells had 43.1 % lipid content after the growth period. Therefore, the *Isochrysis* had a maximum lipid content that was 11.7 % greater than the *Tetraselmis* maximum lipid content which was shown in figure 2.1 and figure 2.2. It must be taken into consideration that *Tetraselmis* is known to produce carbohydrates as well as lipids, thus for the purpose of this study, *Isochrysis* was selected as it tends to produce lipids only.

The Nile Red fluorescence flow cytometric method of measuring total microalgal lipid was highly reproducible and allowed for specific detection of neutral lipids. Thus, *the Isochrysis* species was selected as the most suitable microalgal specie for further lipid induction studies.

3 MOLECULAR CHARACTERISATION OF SELECTED SPECIE

3.1 Introduction

Molecular biology techniques have allowed for the identification of numerous marine microalgal strains. Bioinformatics allows for sequence comparison of the unknown sequence with highly similar known microalgal strains that are available in databases such as the NCBI.

3.2 Materials and Methods

3.2.1 Inoculation of culture vessels for DNA extraction

All glassware were thoroughly cleaned and autoclaved prior to use. The filtering of the f/2 medium and inoculation was performed under the laminar flow hood, to ensure sterility of the medium. Into 250 ml of f/2 medium (Appendix A), 1 ml of *Isochrysis sp*. culture that had a cell count of approximately 15 cells per ml was inoculated into the medium using a sterilised Pasteur pipette. The inoculated flasks were incubated for one week in the growth room at 25 °C under continuous illumination of 110 μmol.$m^{-2/}$s. The microalgal culture was grown for one week to allow for sufficient production of biomass for subsequent DNA extraction and to minimise interference by by-products of algal growth.

3.2.2 Extraction of DNA from the Isochrysis sp. culture

Sterile conditions were maintained at all times under the laminar flow hood. Approximately 200 ml of dense *Isochrysis sp*. culture was transferred to two 100 ml plastic centrifuge tubes and centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was discarded and the algal biomass pellet was transferred to a 2 ml eppendorf tube. Freshly prepared f/2 medium was added to the tube to bring the volume to 2 ml. This lead to the being pellet re-suspended which washed off any excess polysaccharides that was present. The tube was then centrifuged at 1500 rpm for 15 min and the supernatant was decanted using a Pasteur pipette.

Ten autoclaved glass beads with a diameter of 0.1mm (Inqaba Biotech) were added to the tube to cover the cells and f/2 medium was subsequently added to a volume of 0.5 ml. The tube was immersed in liquid nitrogen to freeze all the liquid. A 60 °C water bath was prepared, to preheat 2.5 % CTAB buffer (Appendix C). Subsequently, 1 ml of 2.5 % CTAB buffer and 2 µl of mercaptoethanol were added to the frozen sample. The tube was vortexed to allow the sample to melt. The tube was then maintained at 60 °C in a water bath for 30 min with vortexing every 5 min.

Into the tube, 1 ml of chloroform:isoamyl alcohol (24:1) was added which extracted hydrophobic components. The tube was gently agitated for 20 min and then centrifuged at 1500 rpm for 15 min. The water phase which was the top phase was removed and transferred to a clean 2 ml eppendorf tube. The extraction was repeated into a third eppendorf tube. The tube was centrifuged for 15 min at 1500 rpm at 4 °C and samples were cooled by placing them on ice. The volume of the tube was made up to 0.6 ml with autoclaved Milli-Q water and 0.4 ml of cold isopropanol was added to the tube. The samples were placed in the freezer for 20 min and subsequently centrifuged at 4 °C at 1500 rpm for 15 min. The isopropanol was removed using a Pasteur pipette and samples were re-placed on the ice. Subsequently, 1 ml of cold 80 % ethanol was added to the tubes which functioned as a washing step.

The sample was centrifuged again at 1500 rpm for 15 min and the supernatant was removed. The remaining pellet was allowed to dry overnight in a dessicator. The DNA pellet was re-suspended in 50 µl of TE buffer (Appendix E) and placed on ice. The volume was then increased to 200 µl with autoclaved Milli-Q water. The samples were stored in the freezer.

3.2.3 PCR amplification of the 18S rDNA

PCR tubes with a 0.2 ml thin wall and cap (Whitehead Scientific) were autoclaved prior to use. The following components were added to four tubes: 25 µl of 2x PCR mix (Fermentas), 1 µl of forward primer 1F (Inqaba Biotec), 1 µl of reverse primer 1528 R (Inqaba Biotec), 4 µl of the template DNA and 17 µl of autoclaved Milli-Q water. The SSU nuclear ribosomal encoding region was amplified using the 1528R (5□-TGATCCTTCTGCAGGTTCACCTAC-3□) and 1F (5□-AACCTGGTTGATCCTGCCAGT-3□) primers (Metfies *et al.,* 2007). A negative control tube that contained all the above components with the exception of the template DNA was also set-up. In the control tube, 23 µl of autoclaved Milli-Q water was added to the tube, in order to make up a PCR reaction volume of 50 µl.

A PCR cycling profile was utilised that consisted of initial denaturation, amplification and final extension. A 2720 thermal cycler (Applied Biosystems) was used to perform the cycling profile. The cycle used was as follows (Seoane *et al*., 2009):

1) Initial denaturation before cycling at 94 °C for 3 min.
2) Amplification series for 35 cycles which consisted of:
    a. denaturation at 94 °C for 60 secs.
    b. annealing at 50 °C for 60 secs.
    c. extension at 72 °C for 2.5 min.
3) Final extension after cycling at 72 °C for 10 min.

3.2.4 Detection of purity and fragment length of PCR products

A 0.4 % agarose gel was prepared by the addition of 0.4 g agarose powder (Bio-Rad Laboratories) in 10 ml 5x TAE (Bio-Rad Laboratories GmbH) and 90 ml Milli-Q water (Seoane *et al*., 2009). The mixture was heated until all the agarose was dissolved. Subsequently, 3 µl of ethidium bromide was added to the agarose gel mixture. The ethidium bromide was incorporated into the gel as it allowed for staining during electrophoresis thus allowing visualisation of the DNA. Electrophoresis buffer was prepared by adding 100 ml of 5x TAE to 900 ml of autoclaved Milli-Q water. The electrophoresis buffer was then filled into the chamber. Samples were prepared by mixing 5 µl of the DNA sample, 1.5 µl of the tracking dye and 5 µl of 6x Orange loading dye (Fermentas).

A 100 bp DNA ladder (Fermentas) was used as the molecular ladder. Samples were loaded in aliquots of 6 µl to the respective lanes using a sterile pipette. The gel was run in a Power Pac Basic™ (Bio-Rad) at 80 V for 35 min. After the gel was allowed to run, the bands were visualised using a GelDoc XR Imager (Bio-Rad).

3.2.5 Sequencing and bioinformatic analysis of the amplified 18S rDNA

The amplified 18S rDNA was sent to the University of Stellenbosch DNA Sequencing Facility for post-PCR cleaning and sequencing. A nucleotide sequence was obtained as in Appendix E. The nucleotide sequence was utilised as an input sequence into BLAST at the NCBI which allowed for a homologue search whereby the unknown sequence was compared to known identified nucleotide sequences in the database that were highly similar (Krawetz and Womble. 2003; Lesk. 2001). Similarities between the sequences were examined and six sequences with biological relevance were downloaded and aligned with the input (unknown) sequence.

Thereafter, a phylogenetic tree was constructed using fdam1 in wEMBOSS via maximum likelihood phylogenetics. The most distantly related organism was used as the out-group to root the tree (Krawetz and Womble. 2003; Lesk. 2001). This step allowed for the identification of the unknown microalgal sequence as the *Isochrysis* genome has been sequenced previously.

3.3 Results and Discussion

3.3.1 Detection of the amplified DNA fragments

The marine microalgal 18S rDNA had a negative charge at a neutral pH due to the presence of the phosphate backbone. At an electrical potential of 80 V, the DNA migrated from the cathode to the anode (Figure 3.1). The DNA molecular weight marker consisted of DNA fragments with a known molecular size which was utilised to determine the molecular size of the unknown DNA fragment. The 0.4 % agarose gel has a lattice primary structure which allowed for small DNA fragments to migrate through the structure. However, large DNA fragments were hindered from migrating through the lattice structure. Therefore, DNA fragments were separated on the basis of their molecular size and if the DNA was complementary to the primers.

The separation of the various DNA fragments in the molecular weight marker showed the various molecular sizes in lane 1 (Figure 3.1). There was no amplified DNA fragment in lane 3 as this was the control. However, there was a faint band at the bottom of the gel which was a primer dimer. Primer dimmers were by-products of the PCR amplification process which consisted of primer molecules that hybridised to each other due to complementary base pairing. The primer dimmers were found at the bottom of the gel as they are 28 bases long which allowed for their migration to the bottom of the gel. Lane 4 and lane 6 consisted of 2 µl of the microalgal DNA and bands were visualised on the gel.

Primer dimers were also observed on the bottom of the gel (Figure 3.1). Lane 7 and 8 consisted of 4 µl of the microalgal DNA and bands were also visualised on the gel (Figure 3.1). The sizes of the bands were in correspondence with that of microalgal DNA.

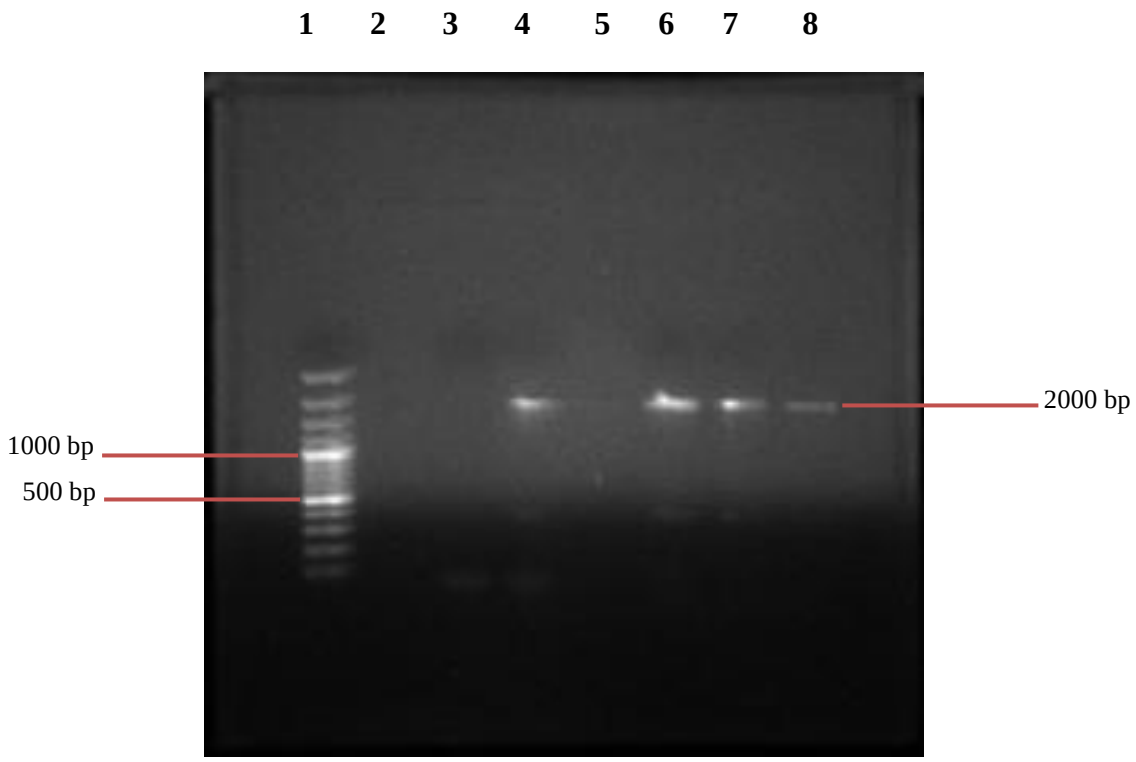

Figure 3.1: Agarose gel showing the U.V. fluorescence of the amplified 18S rDNA fragments on a U.V. trans-illuminator

3.3.2 Sequencing of the amplified 18S rDNA fragments

The sequencing of the microalgal DNA yielded a nucleotide sequence as in Appendix E. The sequence was utilised for all subsequent bioinformatic analysis.

3.3.3 Bioinformatic analysis of the microalgal DNA

The phylogenetic tree obtained using the ClustalW program indicated that the unknown microalgal sequence was closely related to *Isochrysis* sp. 0318 (Figure 3.2). The distance between the unknown sequence and the *Isochrysis* sp. 0318 was the smallest compared to the distances between the unknown and other *Isochrysis* sp (Figure 3.2). However, all the sequences that were highly similar to the unknown microalgal sequence were *Isochrysis*, as in figure 3.2.

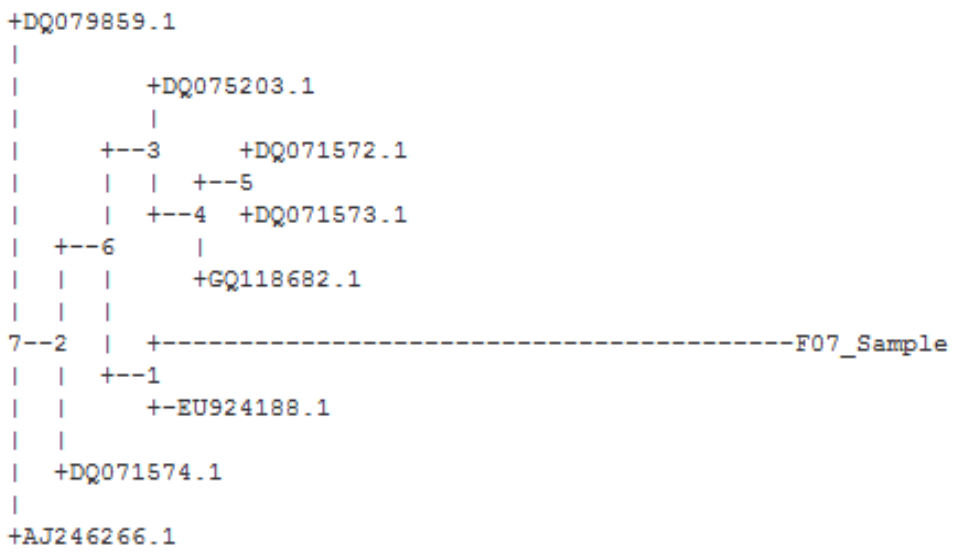

Figure 3.2: Phylogenetic tree comparing the homology between the unknown microalgal sequence (F07_Sample) and known microalgal 18S ribosomal RNA sequences (DQ07859: *Isochrysis* sp. CCAP, DQ75203: *Isochrysis* sp. *zhangjiangensis*, DQ071572: *Isochrysis* sp. 3011, DQ071573: *Isochrysis* sp. 8701, GQ118682: *Isochrysis galbana* strain DB, EU924188: *Isochrysis* sp. 0318, DQ071574: *Isochrysis* sp. *santou* 2, AJ246266: *Isochrysis galbana* strain UIO 102).

4 LIPID PRODUCTION ANALYSIS

4.1 Introduction

Research on developing microalgal biodiesel has focused on growth factors that can stimulate lipid production. Growth factors such as phosphorus, nitrogen, temperature and light intensity have been examined extensively for their effect on microalgal lipid production. Research that has examined phosphorus depletion as a factor to promote lipid production is limited. Therefore, this chapter examines the effect of phosphorus depletion on lipid accumulation in *Isochrysis*. Phosphorus is necessary in microalgal cells for mRNA production, glycolytic phosphorylation reactions, ATP production and Calvin cycle reactions. The effect of various phosphorus concentrations on growth and lipid production was examined to determine the relationship between phosphorus, growth and lipid production in *Isochrysis*.

4.2 Materials and Methods

4.2.1 Phosphorus standard curve

In order to prepare a phosphorus standard curve, the following reagents were made up which were required for the colorimetric detection of total phosphorus. The molybdate antimony solution was prepared by dissolving 2.4 g of ammonium molybdate and 0.05 g of potassium antimony tartrate in 500 ml of 4 N H_2SO_4 (Golterman, 1970). An ascorbic acid solution (0.1 M) was prepared by dissolving 2 g of ascorbic acid in 100 ml of Milli-Q water (Golterman, 1970). The solution was stored in the refrigerator. Phosphorus standard solutions with concentrations of 0.5 mg /L, 1 mg/L, 2 mg/L , 4 mg/L , 6 mg/L , 8 mg/L , 10 mg/L were made up in autoclaved filtered natural seawater. The solutions were preserved by adding 2.5 ml of 20 N H_2SO_4 and a few drops of chloroform (Golterman, 1970).

A 100 ml sample of each phosphorus standard solution was taken and into each conical flask ,10 ml molybdate and 4 ml ascorbic acid was added (Golterman, 1970). The volume was increased to 150 ml with the respective standard solution. The contents of the flask were transferred to plastic centrifuge tubes which were vortexed briefly and allowed to settle for 10 mins. The coloured sample was transferred to a separatory funnel and 10 ml n-hexanol was added to the sample (Golterman, 1970). The lower layer was discarded and the n-hexanol was transferred to another measuring cylinder. The volume was subsequently increased to 10 ml with isopropanol. The absorbance was measured at 690 nm with zeroing of the spectrophotometer with a distilled water blank cuvette prior to each measurement. The standard curve was constructed in triplicate per phosphorus standard solution. A blank tube consisted of the autoclaved filtered natural seawater only was also set-up. Each sample was gently agitated and allowed to settle for 10 mins prior to the absorbance reading. Samples were added to cuvettes and the absorbance was measured at 690 nm, with the zeroing of the spectrophotometer prior to each measurement (Golterman, 1970).

4.2.2 Preparation of f/2 medium with various phosphorus concentrations

The f/2 medium was prepared in 2 L of autoclaved filtered natural seawater (Appendix A). All the components were added as in the recipe stated in appendix A. However, the $NaH_2PO_4.2H_2O$ solution was added in various volumes to yield f/2 with different phosphorus concentrations. The molecular mass of $NaH_2PO_4.2H_2O$)is 156.01. The relative atomic mass of phosphorus (P) is 30.97376 which was rounded off to 31. One mole of $NaH_2PO_4.2H_2O$ equaling 156.01 g contains one mole of phosphorus (P) which equals 31 g. 5 g of $NaH_2PO_4.2H_2O$ will contain (5/156.01) x 31 = 0.993526056 g of P which was rounded off to 1.0 g. Hence the $NaH_2PO_4.2H_2O$ stock for making up the f/2 medium contains 1 g of P per 1000 ml.

If we then take 1 ml of the concentrated P stock and add it to 999 ml of natural seawater to make up the f/2 medium (as per appendix A), the P was diluted as follows: (1/1000) x 1 = 1.0 mg of P in 1000 ml of the final f/2 medium. If mg/ml equals ppm then, 1 mg of P in 1000 ml equals 1 ppm. Thus the normal P concentration strength f/2 as per appendix A would be 1 mg/1000 ml or 1 ppm P. Therefore the f/2 medium contains 1000 µg of P per L. The molar concentration of P in the full strength f/2 medium is (0.001/31) = 32.258 µM.

Therefore, the dilutions of the normal P concentration in f/2 medium were made and yielded the following solutions: 0.05 ppm of P (50 % reduction in P), 0.25 ppm of P (75 % reduction in P), 0.1 ppm of P (90 % reduction in P) and 0.01 ppm of P (99 % reduction in P). All the media were prepared in triplicate under the laminar flow hood to ensure sterility.

4.2.3 Inoculation and set-up of culture vessels

The culture vessels and glass sparger stoppers were autoclaved prior to set-up. 900 ml of the f/2 medium was added to the 1 L culture vessels after filtration under the laminar flow hood. For each different phosphorus concentration, four culture vessels were set-up. Four vessels were set-up to allow for statistical significance of the data attained. Natural seawater that was autoclaved and filtered was also used as a growth medium by itself. Each culture vessel contained 900 ml of the respective medium. All the culture vessels were inoculated with *Isochrysis* sp. culture that had a cell count of 15 cells per ml. These vessels were connected to air pumps which aerated the cultures with filtered air and subsequently incubated in the growth room with continuous illumination of 110 µmol.$m^{-2/}$s at 25 °C (Figure 4.1).

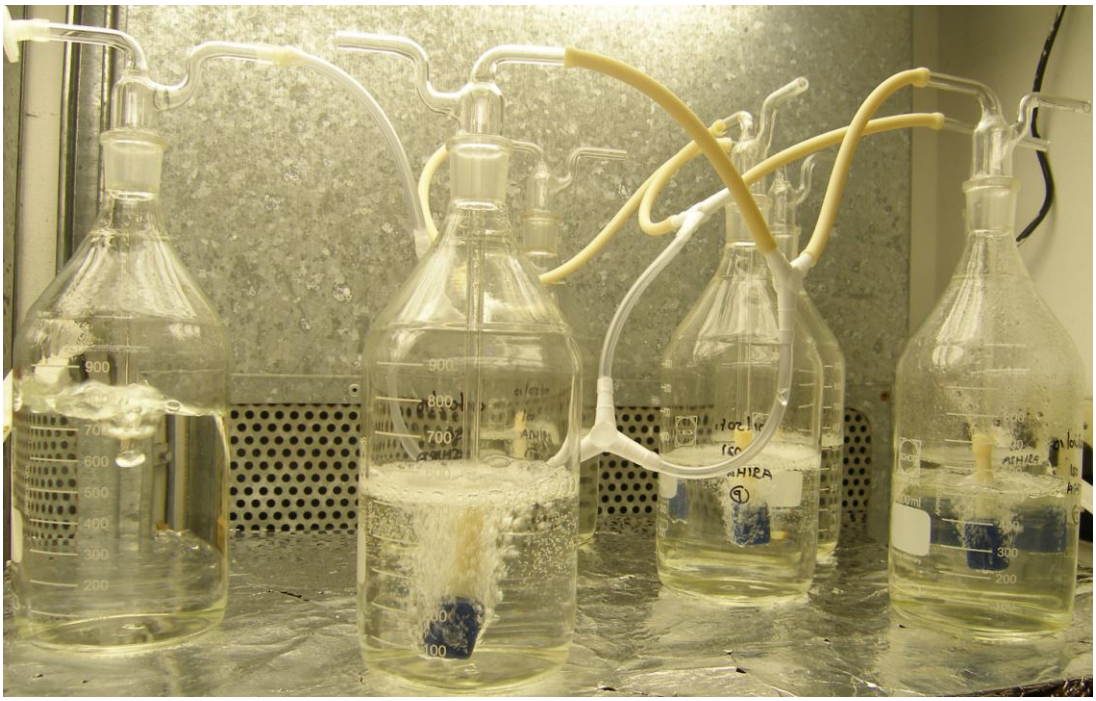

Figure 4.1: Set-up of the microalgal culture vessels

4.2.4 Sampling

The growth, lipid production and phosphorus concentration was monitored for 24 days every alternate day. To maintain sterile conditions, samples were taken in the laminar flow hood. Samples (10 ml) were withdrawn from culture vessels on alternate days in order to monitor growth, phosphorus measurements and lipid levels measurements. Thus, a 10 ml sample was taken from each vessel using a sterile pipette for each vessel, to avoid contamination. After sampling, culture vessels were re-connected to air pumps and incubated in the growth room.

4.2.5 Light microscopy

In order to visualise the microalgal cells after a certain period of growth, light microscopy was performed. After 1 wk, 2 wks and 3 wks, single 1 ml samples were taken from culture vessels containing 0.01 ppm of P, 0.10 ppm of P, 0.25 ppm of P, 0.50 ppm of P and 0 ppm of P. The microalgal cultures were prepared by adding a few drops of the culture onto a glass slide. Subsequently, the slides were viewed under the microscope (AxioPhot microscope from Zeiss) and pictures were taken at a magnification of 40x for comparative analysis. The program Axio Vision 4 and an Axio Vision camera were utilised to visualise and capture the pictures.

4.2.6 Measurement of algal growth using a hemocytometer

A 1 ml sample from each culture vessel was utilised for cell counts every alternate day. One drop of Lugol's solution was added to the culture sample and vortexed briefly. Lugol's solution was utilised to immobilise the microalgal cells as the cells were moving in the medium. To allow for accurate counting of the cells, the Lugol's solution fixed the cells onto the haemocytometer chamber. The haemocytometer chamber was filled with the culture using a Pasteur pipette.

The number of cells in four outer 1 mm squares was counted under the light microscope (Zeiss) at 40x magnification. The number of cells in 900 ml of medium was calculated as follows:

Cell concentration/ml = total cell count in 4 squares x 2500 x dilution factor (10^3)

Therefore, the cell concentration per 900ml = cell concentration/ml x 9

4.2.7 Measurement of algal growth using Spectrophotometer

The growth of the microalgal culture was monitored using cell counts and absorbance measurements to ensure that the growth curve obtained was of statistical significance and clearly represented the growth pattern of the algae. The absorbance measurements were also monitored with haemocytometer counts to construct a standard curve of cells counts versus absorbance which could be utilised for future studies where only absorbance readings will be needed. The increase in biomass of the *Isochrysis* culture was measured by reading absorption at 750 nm. Four samples were taken every alternate day for the 24 day growth period from each culture vessel. A sample of 3 ml was added to a cuvette and the spectrophotometer was zeroed with a natural seawater blank cuvette at 750 nm. The spectrophotometer was zeroed with the natural seawater blank cuvette in between measurements.

4.2.8 Detection of lipid production using flow cytometry

Samples were taken every alternate day for 24 days in triplicate from each of the respective culture vessels. A control tube and sample tubes were set-up as described previously in section *2.2.5*. The total fluorescence of the microalgal culture was calculated as follows, where FL2 is the red fluorescence detector, FL3 is the yellow fluorescence detector and AF is the autofluorescence in the respective channels:

$$\text{Total fluorescence} = \frac{FL2}{AF} + \frac{FL3}{AF}$$

4.2.9 Measurement of phosphorus during algal cultivation

Samples were taken every alternate day to measure the concentration of phosphorus with respective to time. Phosphorus measurements were carried out by taking a sample (100 ml) from each culture vessel. The samples were then centrifuged at 1500 rpm at 4 °C for 15 mins to remove the microalgal cells. The supernatant was decanted into conical flasks. The reagents were added and the phosphorus measured as described in section *4.2.1*.

4.3 Results and Discussion

4.3.1 Soluble reactive phosphorus (PO_4-P) standard curve

The PO_4-P standard curve (Figure 4.2) was obtained via the colorimetric molybdate-antimony method (Golterman, 1970). The increase in the concentration of PO_4-P in the medium corresponded to an increase in the absorbance as shown in figure 4.1. The molybdate ions were reduced to a blue coloured complex. However, because ascorbic acid was utilised, it functioned as a reducing agent. Therefore, the formation of the blue coloured complex was encouraged by the antimony. The curve also showed that there are no interferences by other substances that were present in the f/2 medium. Therefore, a directly proportional relationship exists between the PO_4-P concentration and absorbance measured. This standard curve was utilised to monitor the PO_4-P concentration of the various media every alternate day for the 24 day growth period.

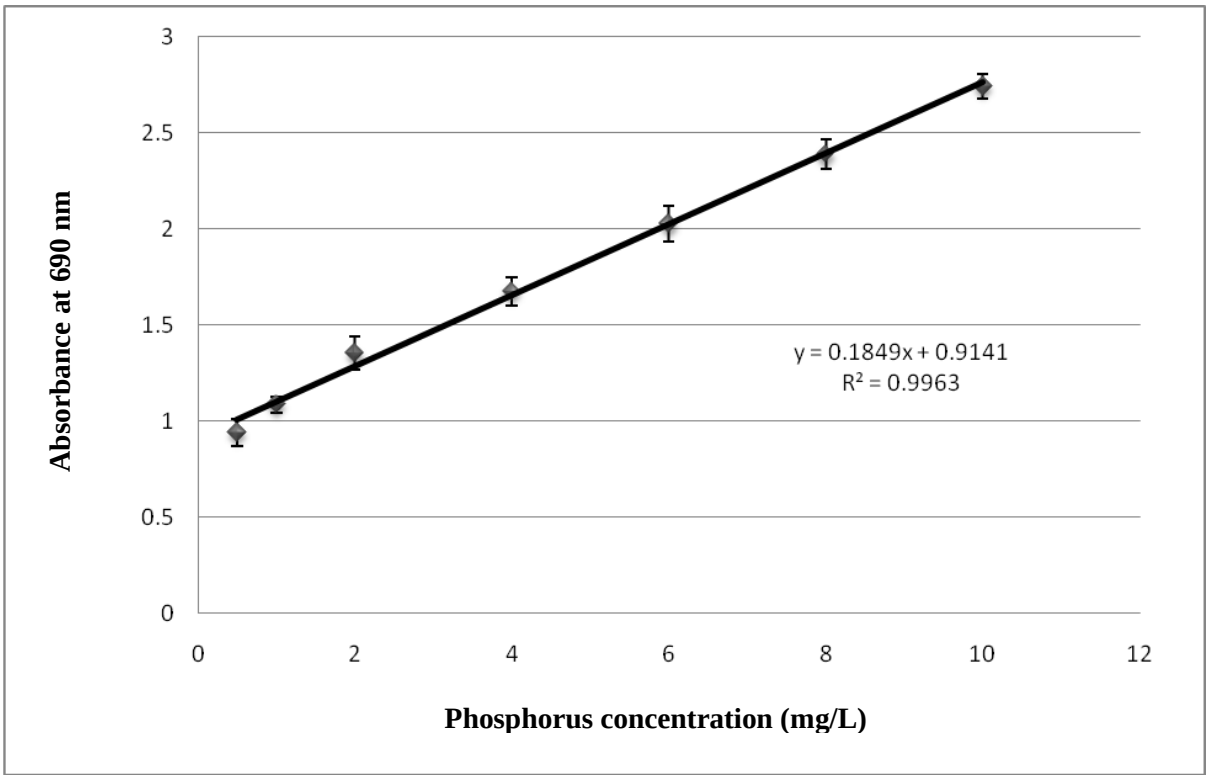

Figure 4.2: Soluble reactive phosphorus standard curve obtained from the colorimetric molybdate antimony method (Golterman. 1970). Error bars represent standard deviation (n=4).

4.3.2 Algal growth curves obtained using absorbance and cell counts

The microalgal cultures exhibited three growth phases as seen in figure 4.3. A lag phase was observed from day 3 to day 7 due to the microalgal cells adapting to the growth conditions. The duration of log phase was dependent on the capacity of the medium to support microalgal growth. The microalgal cells started to undergo an increased rate of cell division on the 9th day of the 24 day total growth period as seen by the large increase in the cell count (Figure 4.3). However, the cells that were grown in 0 ppm P only exhibited minimal change in growth and there were no distinct growth phases observed (Figure 4.3). The *Isochrysis* cells in the 0.25 ppm P and 0.50 ppm P medium exhibited similar growth patterns (Figure 4.3).

It was expected that the microalgal cells would grow better in the 0.5 ppm P compared to the other media this medium contained phosphorus at a level that exceeded their metabolic requirements. Therefore, the microalgae did not consider this media to be growth limiting immediately but rather have an excess of phosphorus which was utilised to build an internal pool of PO_4. On the other hand, the cells in the 0.01 ppm P medium and 0 ppm P exhibited the least growth over the 24 day growth period. The growth patterns of *Isochrysis* were observed to be more different when absorbance measurements were monitored as opposed to the cell counts. The methods were highly reproducible as indicated by the low standard deviation.

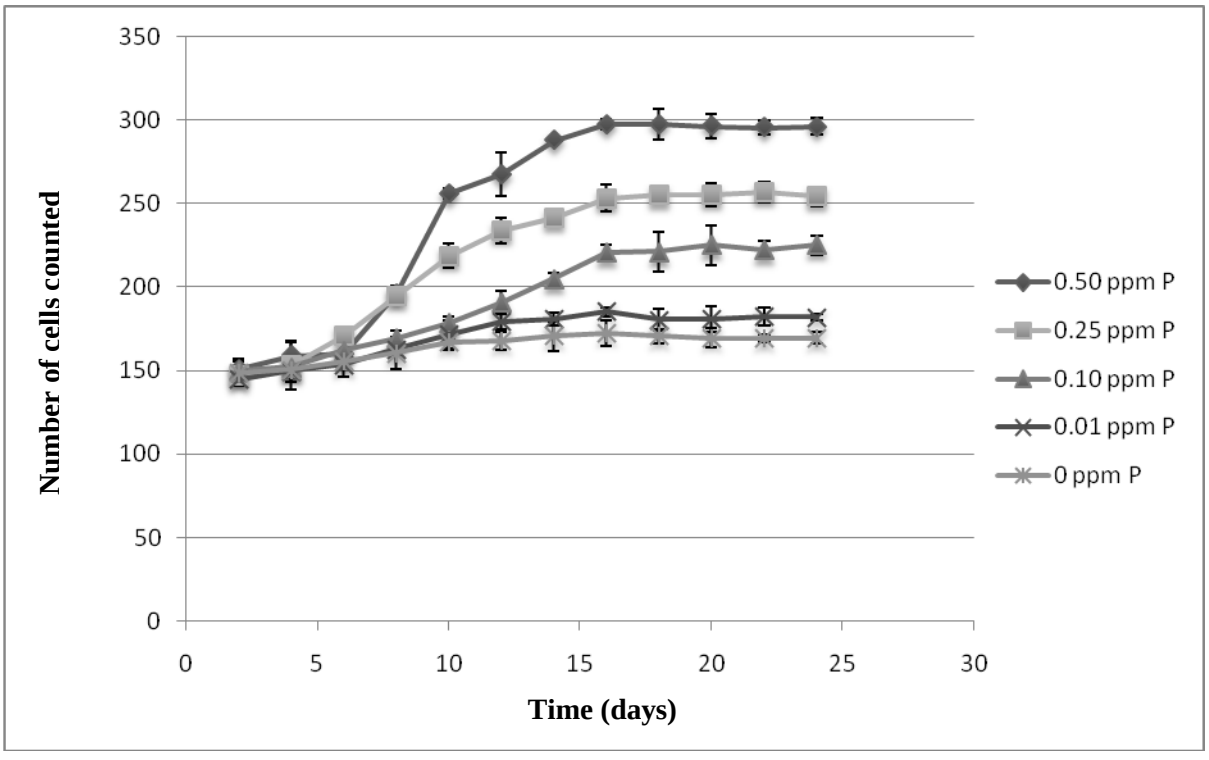

Figure 4.3: Growth of *Isochrysis* measured by haemocytometer cell counts showing the ability of the microalgal cells to undergo cell division in the f/2 media with various phosphorus concentrations. Error bars represent standard deviation (n=4).

The Isochrysis growth curve measured by absorbance (Figure 4.4) showed that the microalgal cells had an increased rate of growth from the 7th day of the 24 days growth period. The cells in the 0.1 ppm P and 0 ppm P maintained a low level of growth compared to the other media. The cells showed a greater ability to undergo cell division in the 0.50 ppm P compared to the other media as was observed in figure 4.3. However, the difference in the growth rate between the 0.50 ppm P and 0.25 ppm P was better observed in the absorbance measurements than the cell counts. The microalgal cells entered a stationary phase on the 17th day of the 24 day growth period as shown by no subsequent increase in the biomass (Figure 4.3 and Figure 4.4).

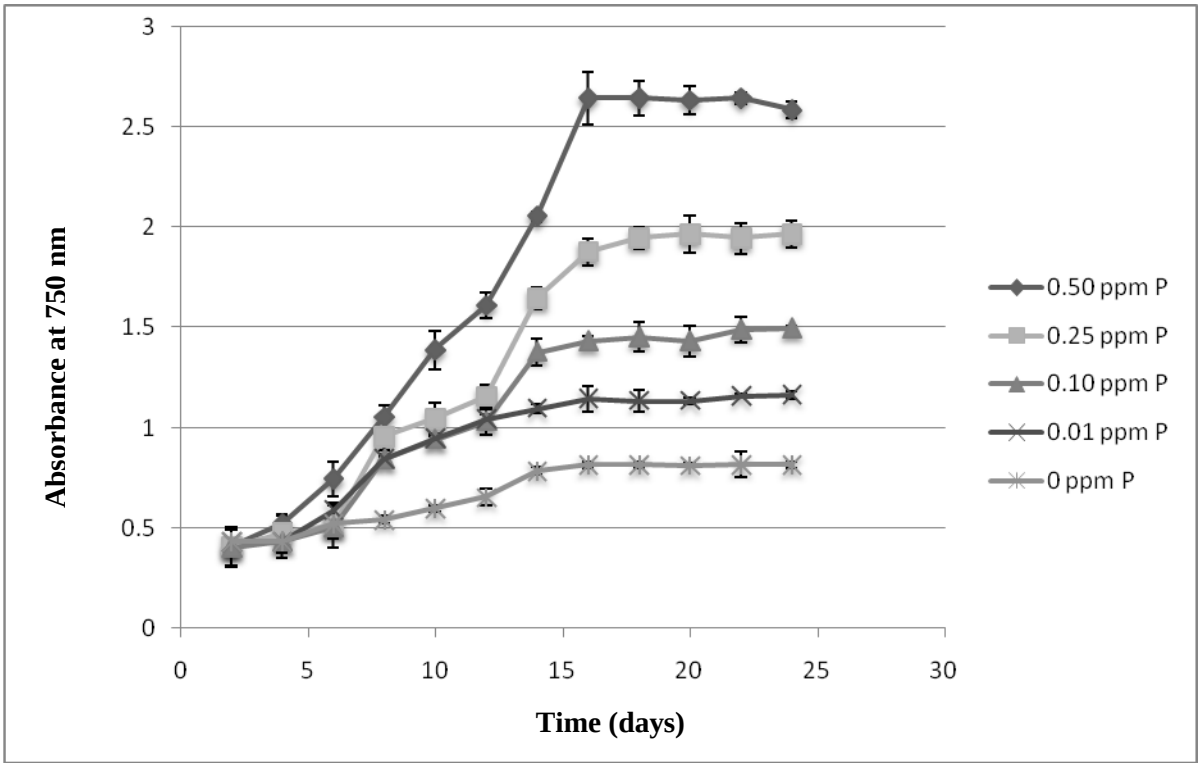

Figure 4.4: Growth of *Isochrysis* measured by absorbance measurements showing the ability of the microalgal cells to undergo cell division in the f/2 media with various phosphorus concentrations. Error bars represent standard deviation (n=4).

4.3.3 Lipid production at various phosphorus concentrations

The phosphorus concentration of all the f/2 media decreased over the 24 day growth period due to utilisation of the phosphorus for microalgal cell growth. There was a decrease in the phosphorus concentration from day 2 to day 24 of the growth period in the f/2 medium containing 0.50 ppm of P. It can be attributed to the microalgal cells luxury uptake of phosphorus from the f/2 medium. The cells were passively taking up the PO_4 from the surrounding medium which the cells did not necessarily need, thus forming an internal pool of PO_4. The uptake of the PO_4 from the f/2 media increased during the growth period of the algal uptake of PO_4 increased to prevent the cells from entering a limited state until a later stage. The growth curves obtained for the 0.50 ppm P also indicated that the medium was not limiting for the algal growth as the growth rate increased rapidly (Figure 4.4) and a high cell count was observed (Figure 4.3).

The ambient concentration of PO_4 in the cultures initially grown in 0.25 ppm of P and 0.10 ppm of P media also showed a decrease in the concentration of PO_4 in the surrounding medium as the cells were utilising the PO_4 for growth (Figure 4.5). However, from the 20th day of growth, the PO_4 concentration in the medium did not change (Figure 4.5). This was expected as the microalgal cells entered a stationary phase on day 18 of the growth period. The 0.01 ppm P and 0 ppm P media had PO_4 concentrations that were limiting to the microalgal cells as indicated by the low cell counts (Figure 4.3). The cells entered a stationary phase on day 16 of the 24 day growth period whereby no further PO_4 was extracted from the medium (Figure 4.5). The algal response to the limited PO_4 concentrations in the surrounding media for the 0.01 ppm of P and 0 ppm of P overlapped as shown in figure 4.5. Therefore, the growth curves of were highly similar (Figure 4.3 and Figure 4.4). The PO_4 concentration of the media was limiting algal growth in the 0.25 ppm P, 0.10 ppm P, 0.01 ppm P and 0 ppm P media. This nutrient limitation pushed the cells into stationary growth phase as shown in figure 4.3 and figure 4.4.

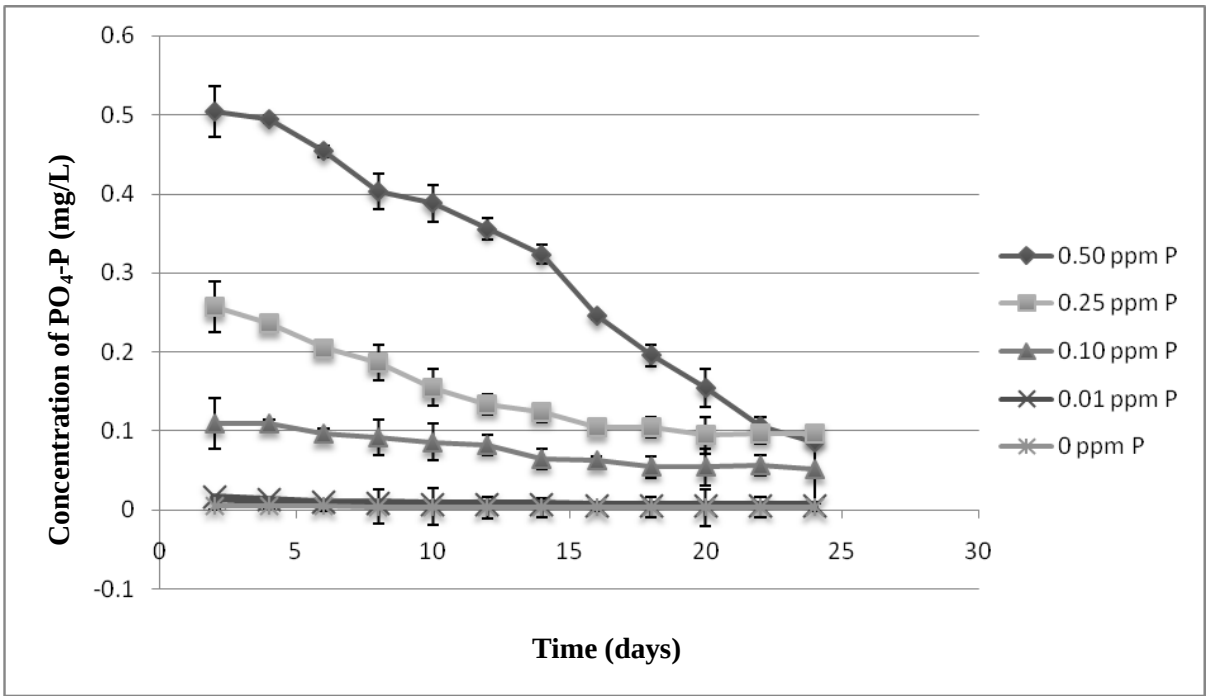

Figure 4.5: The change in the concentration of soluble reactive phosphorus in the various f/2 media during the 24 day growth period of *Isochrysis*. Error bars represent standard deviation (n=4).

The percentage of lipid produced by the microalgal cells was directly related to the change of the phosphorus concentration in the surrounding medium. The cells produced the same amount of lipid on day 2 and day 4 of the growth period as the same inoculum was utilised for all the media. The difference in the percentage of lipid produced in each media was observed from day 12. The lipid produced by the cells increased from day 2 to day 24 of the growth period (Figure 4.6). The 0 ppm P medium showed the highest lipid production which was expected as Reitan *et al*., 1994 showed a similar relationship between low phosphorus concentrations and high lipid production. The 0.01 ppm P and 0 ppm P media showed similar lipid production rates as the phosphorus concentrations of the media did overlap, as was shown in figure 4.5. The 0.50 ppm P medium showed the least ability to produce lipids which was a function of the phosphorus concentration of the medium. There was luxury uptake of phosphorus as mentioned previously and the cells stored the phosphorus.

Therefore, the lipid produced by the algae was greatly affected by the phosphorus concentration of the media. The higher the ambient phosphorus, the less the lipid produced by the cells. This trend is clearly shown in figure 4.6. The insufficient phosphorus in 0.25 ppm P, 0.10 ppm P, 0.01 ppm P and 0 ppm P supported carbon allocation into the production of progeny cells. The algal cells needed to utilise the carbon, thus lipids were produced.

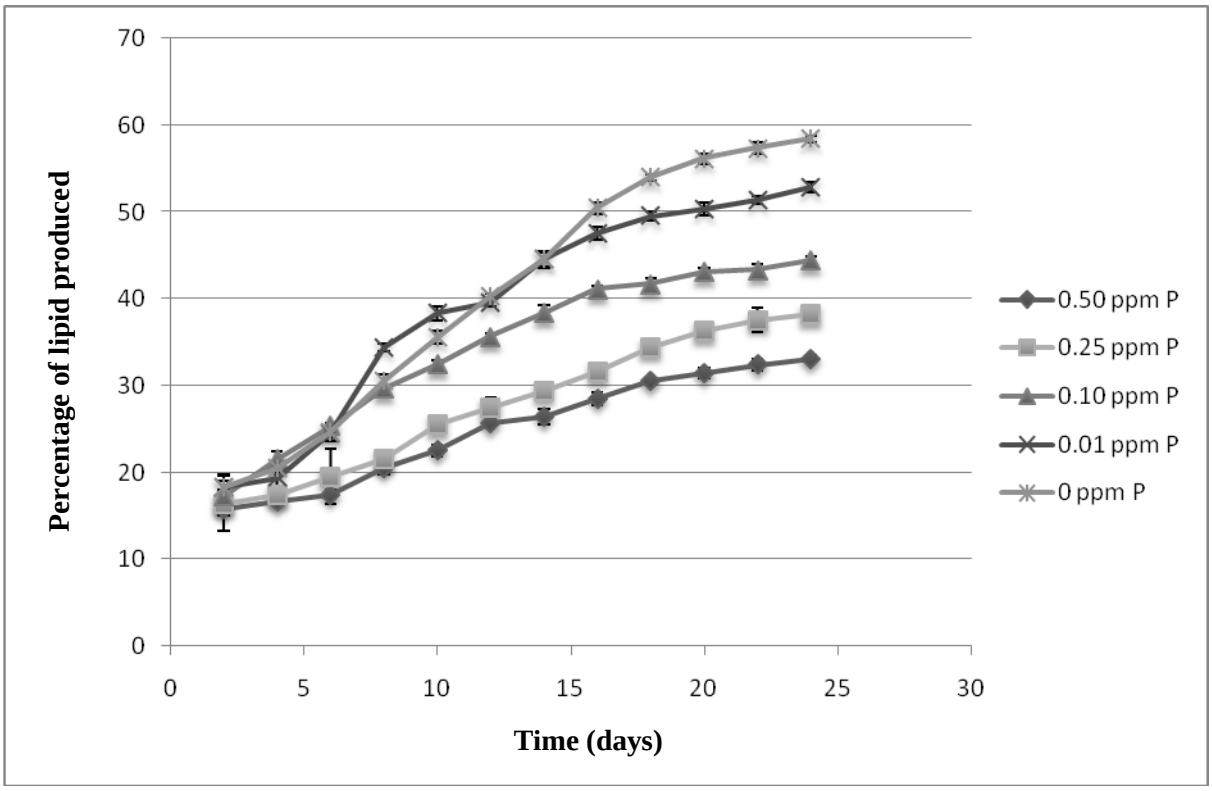

Figure 4.6: The percentage of lipid produced in the *Isochrysis* cells as a response to various phosphorus concentrations. The lipid produced was a percentage of the dry mass weight. Error bars represent standard deviation (n=4).

4.3.4 Light microscopic images of the microalgal cultures

The longer the *Isochrysis* cells were subjected to phosphorus limited conditions; there was an increase in the lipid produced within the cells. The production of lipids within the cells directly related to the Nile red fluorescence of the cell's lipids. The images showed an increase in phosphorus concentration of the medium resulted in less lipid production within the cells (Figure 4.11). The phosphorus depletion resulted in a visible enhancement of lipid presence in the cells as shown in figure 4.7. Thus, the greater the extent of the phosphorus depletion, the higher the total lipid percentage of the microalgal cell. Figure 4.7 showed that the 0 ppm P medium yielded the largest lipid accumulation, whereas the 0.50 ppm P medium (Figure 4.11) yielded a smaller lipid production.

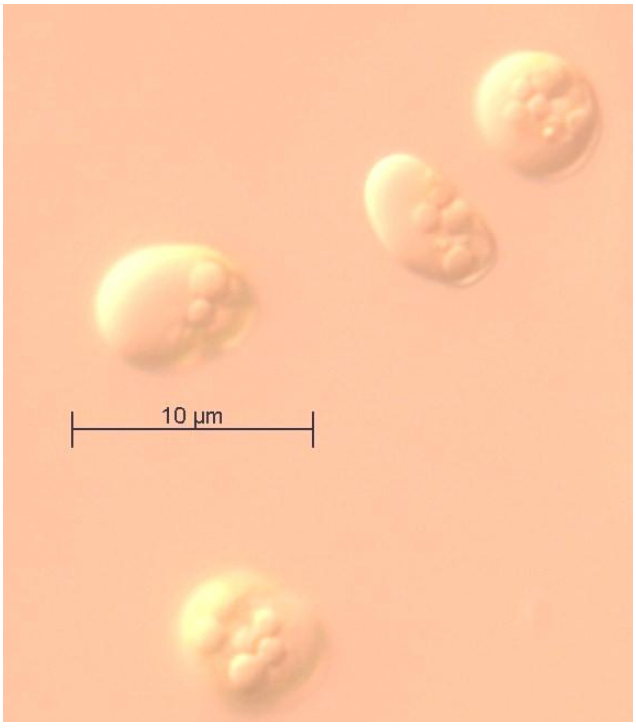

Figure 4.7: Visible lipid produced in *Isochrysis* after a cultivation time of 24 days in 0 ppm P medium.

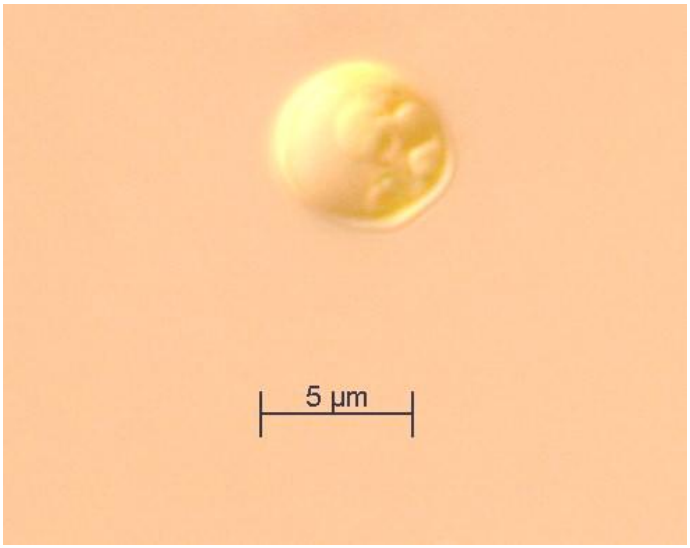

Figure 4.8: Visible lipid produced in *Isochrysis* after a cultivation time of 24 days in 0.01 ppm P medium.



Figure 4.9: Visible lipid produced in *Isochrysis* after a cultivation time of 24 days in 0.10 ppm P medium.

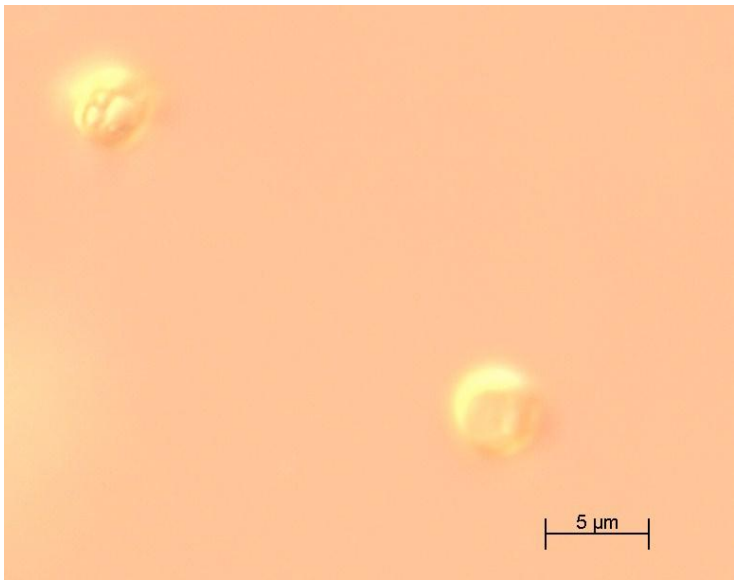

Figure 4.10: Visible lipid produced in *Isochrysis* after a cultivation time of 24 days in 0.25 ppm P medium.

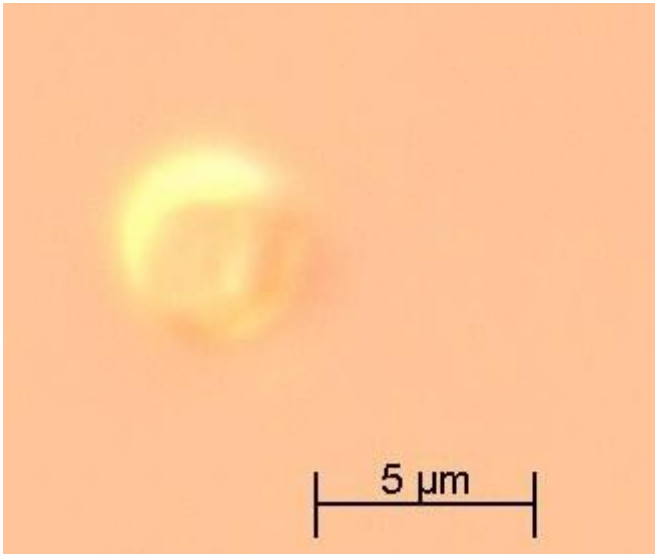

Figure 4.11: Visible lipid produced in *Isochrysis* after a cultivation time of 24 days in 0.50 ppm P medium.

The microalgae *Isochrysis* have the ability to accumulate more lipid under phosphorus limited growth conditions as opposed to normal growth conditions. The greater the extent of phosphorus limitation, the larger the amount of lipid accumulated. The 0 ppm P medium was best for lipid production however; the growth of the algae in this medium was limited which impacts negatively on the amount of biomass that will be available from which to extract all the lipid. Therefore, a balance between lipid production and growth has to be attained for successful lipid extraction from the cells. The 0.01 ppm P medium would be optimal for biomass production and subsequent lipid production.

5 OVERALL DISUSSION AND CONCLUSION

5.1 Discussion

In order to adapt to the stressful nutrient depleted environment, the microalgae produce and store lipids within the cells. This phenomenon has been observed previously by Reitan *et al*., 1994 which showed that phosphorus limitation resulted in increased lipid content in *Isochrysis*. Phosphorus is required in microalgal cells for the production of nucleic acids, phospholipids, phosphorylated sugars, ATP and NADP (South and Whittick, 1987). The role of phosphorus in regulating the photosynthetic rate and the partitioning of photosynthate has been studied extensively (Theodorou *et al*., 1991). The growth of microalgae and for the purpose of this study *Isochrysis* is greatly affected by the phosphorus concentration of the medium as limited phosphorus leads to decreases in the levels of ADP, ATP and inorganic phosphate. Ji and Sherrell, 2008, showed that phosphorus limitation leads to lower cell activities of superoxide dismutase and peroxidase *in vivo*, compared to normal growth conditions. There was also a lower photosynthetic efficiency due to decreased chlorophyll levels in the cells. Therefore, the cells enter a stressed-state which shifted the allocation of carbon away from protein synthesis to lipid synthesis (Smith *et al*., 1997). The growth of the microalgal cells was limited in the various phosphorus depleted media as the rate of synthesis and regeneration of substrates for the Calvin cycle (Figure 1.1) was lowered due to low rate of light utilisation for carbon fixation (Barsanti and Gualtieri, 2006). The microalgal cells that accumulate lipids have the ability to adjust the photosynthetic pathway from carbon dioxide fixation to TAG synthesis. The synthesis of the lipids are stimulated under the phosphorus limitation whereas the photosynthetic activity decreases (Becker, 1994).

The uptake and storage of phosphate in polyphosphate granules beyond the immediate metabolic needs of the cells is called luxury consumption. The stored phosphate provides the algal cells with a supply of phosphorus when the external levels of the nutrient may be limiting. Luxury consumption of phosphate was observed in the 0.50 ppm P medium as the phosphate concentration was greater than that of the cells immediate metabolic need and to prevent the cells from entering nutrient stress at an early stage. Therefore, the phosphate concentrations in the 0.50 ppm P medium continued to fall throughout the 24 day growth period. Since the accumulation of lipids occurs under phosphorus depleted conditions when growth is slow, a balance is required between biomass production and lipid production (Widjaja *et al*., 2009). Environmental stress which in this case was phosphorus depletion led to an inhibition of cell division and promotion of lipid accumulation within *Isochrysis*. The limited phosphorus concentration of 0.01 ppm stimulated 52.7 % of lipid to be produced but the cells entered a stationary phase as the nutrients present in the medium were not utilised for further cell division.

5.2 Conclusion

The prymnensiophyte, *Isochrysis galbana* exhibited the ability to produce lipids under phosphorus limited conditions. The rate of lipid production was accelerated by a decrease in the phosphorus concentration of the medium, as the microalgae were under nutrient stress. Carbon fixation was shifted to triacylglycerol synthesis and not photosynthesis. Luxury consumption of phosphorus into phosphorus pools was also observed which indicated that cells prevented itself from entering nutrient stress until a later stage.

6 REFERENCES

Altschul. S. F, Gish. W, Miller. W, Myers. E. W. and Lipman. D. J., (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology*. 215: 403-410.

Barsanti. L. And Gualtieri. P., (2006). *Algae: Anatomy, biochemistry and biotechnology*. United States of America. CRC Press. Accessed online on the 20th of February 2010 at google books.

Becker. E. W., (1994). *Microalgae: Biotechnology and Microbiology*. Cambridge. Cambrige University Press.

Bligh. E. G. and Dyer. W. J., (1959).A rapid method for total lipid extraction and purification. *Journal of Biochemistry and Physiology*. 37: 911-917.

Chisti. Y., (2007). Biodiesel from microalgae. *Biotechnology Advances*. 25: 294-306.

Fawley. M. W. and Fawley. K. P., (2004). A simple and rapid technique for the isolation of DNA from microalgae. *Journal of Phycology*. 40: 223-225.

Godhe. A, Anderson. D. M. and Rehnstam-Holm. A. S., (2002). PCR amplification of microalgal DNA for sequencing and species identification: studies on fixatives and algal growth stages. *Harmful Algae*. 1: 375-382.

Golterman. H. L., (1970). *Methods of Chemical Analysis of Fresh Waters*. Oxford and Edinburgh. Blackwell Scientific Publications. 71-75.

Gouveia. L. and Oliveira. A. C., (2009). Microalgae as a raw material for biofuels production. *Journal of Industrial Microbiology and Biotechnology*. 36: 269-274.

Gouveia. L, Marques. A. N, Da Silva. T. and Reis. A., (2009). *Neochloris oleabundans* UTEX #1185: a suitable renewable lipid source for biofuel production. *Journal of Industrial Biotechnology*. 36: 821-826.

Hu. G, Sommerfeld. M, Jarvis. Ghiradi. M, Posewitz. M, Seibert. M. Darzins. A., (2008). Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and Advances. *The Plant Journal*. 54: 621-639.

Ji. .Y. and Sherrell. R. M., (2008). Differential effects of phosphorus limitation on cellular metals in Chlorella and Microcystis. American Society of Limnology and Oceanography. 5: 1790:1804.

Krawetz. S. A. and Womble. D. D., (2003). *Introduction to Bioinformatics: a theoretical and practical approach*. Totowa, New Jersey, United States. Humana Press.

Lesk. A., (2001). *An Introduction to Bioinformatics*. Oxford. Oxford University Press.

Li. Y, Horsman. M, Wu. N, Lan. C. Q. and Dubois-Calero. N., (2008). Biofuels from microalgae. *Biotechnology progress*. 24: 815-820.

Li. Q, Du. W. and Liu. D., (2008). Perspectives for microbial oils for biodiesel production. *Applied Microbiology and Biotechnology*. 80: 749-756.

Metfies. K, Berzano. M, Mayer. C, Rooksen. P, Gualerzi. C, Medlin. L. and Muyzer. G., (2007). Am optimized protocol for the identification of diatoms, flagellated algae and pathogenic protozoa with phylochips. *Molecular Ecology Notes*. 7: 925-936.

Patil. V, Tran. K. Q. and Giselrod. H. R., (2008). Towards sustainable production of biofuels from microalgae. *International Journal of Molecular Sciences*. 9: 1188-1195.

Reitan. K.I, Rainuzzo. J. R. Olsen. Y., (1994). Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. *Journal of Phycology*. 30: 972-979.

Rodolfi. L, Zittelli. G.C, Bassi. N, Padovani. G, Biondi. N, Bonini. G. and Tredici. M .R., (2008). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering*. 102:100-112.

Schenk. P. M, Thomas-Hall. S. R, Stephens. E, Marx. U. C, Mussgnug. J. H, Posten. C, Kruse. O. and Hankamer. B., (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. *Bioenergy Research*. 1: 20-43.

Seoane. S, Eikrem. W, Pienaar. R. and Edvarsen. B., (2009). *Chrysochromulina palpebralis* sp. nov. (Prymnesiophyceae): a haptophyte, possessing two alternative morphologies. *Phycologia*. 48: 165-176.

Silva. T. L, Reis. A, Medeiros. R, Oliveira. A. C. Gouveia. L., (2008). Oil production towards biofuel from autotrophic microalgae semicontinous cultivations monitorized by flow cytometry. Applied Biochem Biotechnology. DOI 10.1007/s12010-008-8443-5.

Smith. R. E. H, Gosselin. M. And Taguchi. S ., (1997). The influence of major inorganic nutrients on the growth and physiology of high artic ice algae. *Journal of Marine Systems*. 11: 63-70.

South. G. R. And Whittick. A., (1987). *Introduction to phycology*. Oxford, London and Edinburg. *Blackwell* Scientific Publications.

Theodorou. M. E, Elrifi. I. R, Turpin. D. H. and Plaxton. W. C., (1991). Effects of phosphorus limitation on respiratory metabolism in the green alga *Selenastrum minutum*. *Plant Physiology*. 95: 1089-1095.

Vasudevan. P. T. and Briggs. M., (2008). Biodiesel production-current state of the art and challenges. *Journal of Industrial Microbiology and Biotechnology*. 35: 421-430.

Widjaja. A, Chien. C. C, Ju. Y.H., (2009). Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. *Journal of Taiwan Institute of Chemical Engineering*. 40: 13-20.

7 APPENDICES

Appendix A: Preparation of f/2 medium

Into 950 ml of filtered, autoclaved natural seawater, the following components were added and the final volume was increased to 1000 ml with filtered, autoclaved natural seawater:

Component	**Stock Solution (g/L dH_20)**	**Quantity Used**	**Concentration of Component in Final Medium (M)**
$NaNO_3$	75	1 ml	8.82×10^{-4}
$NaH_2PO_4. 2H_2O$	5	1ml	3.205×10^{-5}
Trace metals solution	-	1ml	-
Vitamins solution	-	0.5 ml	-

Appendix B: Preparation of Nile Red solution

Into 10 ml of acetone, 1 mg of Nile Red was dissolved. The solution was stored at room temperature in the dark.

Appendix C: Preparation of CTAB buffer

The CTAB buffer was prepared by the addition of 1 g CTAB, 4.091 g NaCl, 0.7305 g EDTA and 0.788 g Tris. The volume was increased to 50 ml with Milli-Q water. The buffer was stored in the fridge when not in use.

Appendix D: Preparation of 5x TBE

Into 1 L of Milli-Q water, 54 g of Tris base, 27.5 g boric acid and 20 ml of 0.5 M EDTA was added. The solution was subsequently autoclaved.

Appendix E: Microalgal DNA sequence obtained after sequencing

TTGCTCCTKCYTCGACTTCTGCTTCCTCTYSTGATAGGTTCGGACAGCTTCCMGCG
ACGCCAGSACTGGAGAACCAGCGGSGGCGCCGCASTCCGGGGGCCTCACCGGAT
CATTCAATCKGTAGGAGCGACGGGCGGTGTGTACAAAGGGSAGGGACGTAATCA
ACGTGCGCTGATGACACACGCTTACTAGGAATTCCTCGTTGAARATTAATASTTG
CAATAATCTATCCCCATCACGATGCRMGTTCACAAGATTACCCGGACCTCTCGGY
CAAGGGAGACGACTCGCTGAATGCATCASTGTAGCGCGCGTGCGGCCCASAACA
TYTAAGGGCATCACAGACCTGTTATTGCCGCGAACTTCCACTTGTTGAAGACAAG
TTGTCCCTCTAAGAAGCGAGCCCCAACAAGGGGTTGGGGACACTATTTAGCAGG
CTGCGGTCTCGTTCGTTAACGGAATTAACCAGACAAATCACTCCACCAACTAAGA
ACGGCCATGCACCACCACCCATCGAATCAAGAAAGAGCTCTCAATCTGTCAATC
CTCACAATGTCTGGACCTGGTAAGTTTCCCCGTGTTGAGTCAAATTAAGCCGCAG
GCTCCACTCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACC
ATACTCCCCCCGGAACCCAAAGACTTTAGTTTCCCGAAAGGTGCTGAAGGAGTC
ACAAACGGAACATCCTCCAATCCCTAGTCGGCATGGTTTATGGTTAAGACTACGA
CGGTATCTGATCGTCTTCGATCCCCTAACTTTCGTTCTTGATCAGTGAAAACATCC
CTGGCAAATGCTTTCGCAGTCGTTCGTCTTCCGCTGGTCTGAGAATTTCACCTCTC
TCGGCGGAATACGAGTGCCCCTGACTGTCCCTGTTCATCATTACTCCGGTGCTCG
AAACCAACAAATAGCACCAGAGTCCTATTTCATTATCCCATGCTAATCCATGCAG
ARCGACTGCCTGCTTGAAACMCTCTGATTTTTTCAAGTAAACATCCCGTCTCCGA
CCACCGCTCAGTTAAGAGTASCGKCCGTCTCCGGGAGGAGGACSCGCCCGCAKK
KSCGTACCCATCGGCAGAACCGGCGGTCCCGCCCGAAATCCGACTACGAGCGTTT
ACTGCCAMCACCTTTTAATTACGCTATTGGAGCKGAATACCGCGGCTGYCTGACC
GACTTGCCYCAGTGATCCTGKAARRRTKTAATTGTAMYCATYTCCATATCTCC

Appendix F: Preparation of TE buffer

A 10 mM Tris-Cl (pH 7.5) and 1 mM EDTA was made by adding 10ml 1 M Tris-Cl (pH 7.5) per L and 2 ml 500 mM EDTA (pH 8.0). The stock solutions were made as follows: 1 M Tris (crystallized free base) was made by adding 60.57 g in 0.5 L Milli-Q water. The pH was decreased to 7.5 using HCl. 0.5 M EDTA was made by adding 18.6 g in 100 ml Milli-Q water. The pH was increased to 8.0 using NaOH and the solution was heated to dissolve all the EDTA.