Effect of Heat and Nitrogen Stress on Growth and Lipid contents of *Chlamydomonas reinhardtii*

By

Sidra Saeed

Department of Biochemistry
Faculty of Biological Sciences
Quaid-e-Azam University
Islamabad

2016

CERTIFICATE

This thesis, submitted by **Ms. Sidra Saeed** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

Supervisor:

Dr. Samina Shakeel
Assistant Professor

External Examinar:

Dr. Muhammad Sheeraz Ahmad
Assistant Professor
PMAS Arid Agriculture, University
Rawalpindi

Chairperson:

Dr. Muhammad Ansar
Associate Professor

Dated: March 24, 2016

"In (or with) the name of Allah, the Beneficent, the Merciful"

Dedicated To

My Mother Rahat Saeed,

My Father Dr Muhammad Saeed,

My Teacher Dr Samina Shakeel

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and that this thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Sidra Saeed

ACKNOWLEDGEMENTS

All the praises are for my Lord, **Allah** (SWT), the most beneficent and merciful. He blessed me with all the golden opportunities, made all the required circumstances and assigned me the best people to help me accomplish my goals. Peace and blessings of Almighty Allah (SWT) be upon His Prophet **Muhammad** (P.B.U.H) who exhorted his followers to seek knowledge from cradle to grave.

I feel pride in extending my sense of gratitude and sincere feelings of reverence and regards to **Dr. Samina Shakeel,** Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad for assigning me this project and guiding me throughout the duration of M.phil. I am thankful to her for her scholastic guidance and valuable suggestions throughout the study and presentation of this manuscript. I am thankful to her for her inspiration, reassurance, counseling from time to time and specially a friendly environment. I am also thankful to **Dr. Ansar**, Chairperson, Department of Biochemistry, Quaid-i-Azam University, Islamabad, for his co-operation in providing me all possible facilities in this course of study.

I am also obliging to all the people who contributed a lot in my studies, especially **Mr. Safdar Abbas** for his support and guidance during my research work. I am thankful to my lab members, **Alveena Aleena, Imtiaz Baloch, Sania, Adnan, Saadia, Asiya, Binish Neelam, Huma, Samina, Siddra and Samia** for their support and their beautiful company. I am grateful to all my fellows and friends **Rubina, Fatima, Zainab**, especially **Sarah Rasheed** for their awesome company which helped me get rid of all stress related to research. I would like to acknowledge clerical staff of Department of Biochemistry especially to Mr. Tariq, Mr. Fayyaz, Mr. Saeed, Mr. Ashraf and other staff members for their help.

I am blessed with cooperative and loving family members who are always on my back through thick and thin. I pay my regards to my father **Dr Muhammad Saeed**, My mother **Rahat Saeed,** My aunty **Rukhsana Jahangir**, My Sister **Romana Saeed,** my brothers **Ilyas, Shoaib, Owais, Saad, Fahad and Haseeb Saeed** for their support and prayers.

May Almighty Allah shower his blessings and prosperity on all those who assisted me in any way during this research work.

Sidra Saeed

Table of Contents

List of Abbreviations

µg	Micro Gram
µl	Micro Liter
ABA	Abscisic Acid
ALA	α-linolenic Acid
ASTM	American Society for Testing Materials
ATP	Adenosine Tri Phosphate
BSA	Bovine Serum Albumin
BTL	Biomass-to-Liquid
C	Carbon
CCLM	CSIRO Collection of Living Microalgae
CCM	Carbon Concentrating Mechanism
CHO	Carbohydrates
CO	Carbon Monoxide
CO_2	Carbon Dioxide
$CuSO_4$	Copper Sulfate
DHA	Docosahexaenoic Acid
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EPA	Eicosapentaenoic Acid
FAs	Fatty Acids

FFA	Free Fatty Acids
FTIR	Fourier Transform Infrared Spectroscopy
g/L	Gram Per Liter
g/L d^{-1}	Gram Per Liter Per Day
GHG	Green House Gas
HC	Hydrocarbons
HCO3	Bicarbonate
Ml	Milliliter
N	Nitrogen
$Na_2C_4H_4O_6$	Sodium Tartrate
Na_2CO_3	Sodium Carbonate
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NH_4NO_3	Ammonium Nitrate
NIES	National Institute for Environmental Studies Collection
Nm	Nanometer
NO	Nitrous Oxide
NO_2	Nitrogen Dioxide
PG	Phosphatidyl Glycerol
PO_4^{-3}	Phosphate
PUFA	Polyunsaturated Fatty Acids

RNA	**Ribonucleic Acid**
Rpm	**Revolution Per Minute**
SO_2	**Sulphur Dioxide**
SQDG	**Sulfoquinovosyl Diacylglycerol**
TAGs	**Triacylglycerols**

List of Figures

List of Tables

Abstract

Microalgae are present in all existing earth ecosystems, not just aquatic but also terrestrial lands, represent a huge diversity of species living in a wide range of environmental conditions. Microalgal species are widely identified as a potential and talented resource of organic material to produce biofuel as a renewable source of energy in today's scenario because microalgae are exceedingly rich in oil, and can be used as a source for the production of biofuel if we can have a suitable algal strains which possess high amounts of total lipids or are capable of rapid accumulations of large quantities of neutral lipids under different culture conditions. To meet the current needs, we need to make the algae resistant to different adverse environments and increase the lipid contents of selected algae. The aim of the present work was to study the effects of high temperature and nitrogen concentration on the growth and lipid content of different mutant strains of *Chlamydomonas reinhardtii* for their possible utilization as novel crude materials for production of biodiesel. In addition, the contents of the carbohydrates, proteins, chlorophyll a, chlorophyll b, carotenoids and biomass were also identified. Initially different lipid extraction techniques were explored. The extracted lipids were quantitatively analyzed by gravimetric method, to check their suitability as per the European Standard for Biodiesels. Fourier transform infrared spectroscopy (FTIR) was used to determine lipid and carbohydrate content over time in the freshwater microalgae *C. reinhardtii* in batch culture at 25 and 33 °C.

We used Yellow in Dark Mutants *C. reinhardtii* and were grown in different nitrogen concentrations (0-0.50 mg/L NH_4NO_3) to check its effects on lipid content and growth. The contents of lipid in microalgae were strongly induced by an increase in temperature from 25 to 33 °C. Similarly, we observed 1 to 1.6 fold increase in the lipid contents when treated with NH_4NO_3 with respect to the optimal growth conditions. Taking these growth rates and the lipid accumulations into consideration, these strains were considered having significant potential for biofuel production and applications. In addition, different lipid productivities of these strains were also further investigated.

Introduction

Global warming and the exhaustion of fossil fuels are major world-wide problems. Energy demand is increasing day by day and human are aggressively using fossil fuels to fulfill their needs due to which it is unsustainable, because of the increasing level of consumption and inadequacy of novel sources for these non renewable. The increasing demand of energy is making scientist, to find an alternative reliable energy source like solar, water, wind and biomass to replace fossil fuels.

The transesterification of fats and oils derived a fuel called biodiesel and it is biologically fully degradable. Different kind of materials such as plants, microalgae, and animal fat has been undertaken as an alternative energy source for the production of biodiesel (Vasudevan and Briggs, 2008). The derivates of biofuel are sugar, starch and lipid rich substance, substitutes of liquid fossil fuels. These substances can be obtained from feedstock like cereal crops, together with corn and wheat (Nichols and Bothast, 2008), sugar crops, sorghum, sugarcane; energy crops, such as switch grass, horticultural waste products, such as straws and dried leaves of corn, domestic wastes and many marine species. Presently a great amount of ethanol can be obtained from corn and sugarcane as a derivative fuel, and is substitute for gasoline. In particular, biodiesel has two important advantages, the reduction of carbon dioxide and as alternative for petroleum (Chisti, 2008).

Studies conducted by Awang and May (2007) have demonstrated that palm oil can be utilized as natural friendly substituent source of fuel. Biodiesel produced from waste cooking oil has less amount of sulfur content than existing diesel fuel has also been proved and the aggregation of carbon mono oxide and hydrocarbon emissions have also decreased significantly (Najafi *et al.*, 2007). Due to their competition for land and water used for agricultural purposes, these sources of fuels are not advantageous for expansive scale biodiesel production (Takeshita, 2011). So as to decrease the expense of biodiesel production, ease waste materials are required as feedstock which will make environment friendly fuel and in addition reduces the contamination capability of the waste products (Ghaly *et al.*, 2010). Less amount of solid particles are present in the exhaust fume in

examination with the standard fossil diesel fuel (naphtha) and the substance of polycyclic aromatic hydrocarbons in fumes gasses with endorsed mutagenic and carcinogenic impacts is essentially lower. The carbon dioxide (CO_2) produced in the burning of biofuel enters a close cycle with a comparable sum bound by photosynthesis; in spite of the fact that it has no contribution to the effect of greenhouse (Cvengros and Cvengrosova, 2004). Numerous physiological and chemical conditions have been tried to recover or improve atmospheric CO_2. In organic methodologies, microalgae have all the remarks of being photosynthetically more effective than physical plants and are productive CO_2 fixers (Brown and Zeiler, 1993). Microalgae are the most efficient and fastest growing photosynthesizing organisms. Diatom algae can produce 46 tons of oil/ha/year approximately. Different quantity of oil is produced from different algae species. Up to 50% oil by weight is produced by some algae (Demirbas, 2009). In algal oil the most important difference is in productivity, and consequently the productivity of biodiesel. The yield (per acre) of oil acquired from microalgae is over 200× that from the best-performing plant/vegetable oils according to some evaluations (Sheehan *et al.*, 1998). The great biodegradability of biodiesel fuels in aqueous climate and soil was confirmed by many researchers. The biodegradability was also seen in variety of biodiesel and diesel fuel (Pasqualino *et al.*, 2006). The trials intended for vegetable oils, in which oiled shores were cleaned, have demonstrated the capacity of biodiesel, to upgrade the biodegradability of mineral oils. The production of biodiesel from vegetable starting point appears to improve the biodegradability as rather than edible oils (Pereira and Mudge, 2004). Transesterification is a chemical reaction through which biodiesel is produced (Ramos *et al.*, 2009). Acid or base are used to catalyze transesterification generally, However basic catalysis (sodium or potassium hydroxide or the comparing alkoxides) is a much speedier process than acidic catalysis in homogeneous catalysis (Freedman *et al.*, 1984).

1.1 Microalgae

Microalgae are defined as unicellular or simple multicellular photosynthetic microorganisms that are able to cultivate quickly as well as survive during bitter conditions because of their unicellular or simple multicellular organization.

Cyanobacteria (Cyanophyceae) are examples of prokaryotic microorganisms and green algae (Chlorophyta) and diatoms (Bacillariophyta), are examples of eukaryotic microalgae. Microalgae are explained in more detail by Richmond (2008).

Microalgae can be found in all existing earth ecosystems, both in aquatic and terrestrial, it represents different types of species living in a large range of environmental conditions. According to rough estimation more than 50,000 species of microalgae exist, but round about only 30,000, have been studied and analyzed (Richmond, 2008).

For instance, almost 30 to 50 percent of the environmental net photosynthetic yield (the variation among respiration and autotrophic gross photosynthesis) is derived from marine ecosystem and depends on phytoplankton biomass (Boyce *et al.*, 2010; Field *et al.*, 1998). Acknowledgment has been gained by the immense capability of microalgae as feedstocks for the production of inexhaustible biodiesel currently (Hu *et al.*, 2008; Scott *et al.*, 2010; Wijffels and Barbosa, 2010). Single celled microalgae are capable of utilizing sunlight and CO_2 for the production of energy rich biochemical compounds, like lipids and carbohydrates (CHO), that could be transformed into a source of energy (Hu *et al.*, 2008; Rodolfi *et al.*, 2009; Wijffels and Barbosa, 2010). While, due to restrictions in the organic profitability of strains, culturing system and extraction processes manufacturing of industrial algal biodiesel could be presently caught up (Hu *et al.*, 2008; Scott *et al.*, 2010; Sheehan *et al.*, 1998; Wijffels and Barbosa, 2010). Since currently anticipated, a multidisciplinary approach, as well as advancement in fundamental biology and metabolic engineering of algal strains including culturing system, bioprocessing and coordinated biorefinery outline, might be mandatory to understand microalgae maximum capacity as economical biofuel sources.

The most desirable characteristics of algal strains includes fast development rate, high productivity, tolerance to changes in natural conditions, resistance to predators and infections, as well as easiness to harvesting and extractions (Griffiths and Harrison, 2009; Radakovits *et al.*, 2010; Rodolfi *et al.*, 2009). Another important characteristic for the production of biodiesel from algae is the appropriateness of the lipid synthesis, with triacylglycerols (TAGs) that is the best substrate (Radakovits *et al.*, 2010; Schenk *et al.*, 2008). In ideal development circumstances algae can synthesized, unsaturated fats

essential for esterification into glycerol based layer lipids (Guschina and Harwood, 2006; Hu *et al.*, 2008; Khozin-Goldberg and Cohen, 2011). On the other hand in numerous algal species factors like temperature, irradiance and nutrients accessibility affects both lipid composition and lipid contents (Guschina and Harwood, 2006; Hu *et al.*, 2008; Khozin-Goldberg and Cohen, 2011; Siaut *et al.*, 2011). With some natural hassles (for the most part supplement confinement), a variety of algae collect vitality rich complex like starch and TAGs (Fan *et al.*, 2011; Guschina and Harwood, 2006; Hu *et al.*, 2008; Li *et al.*, 2010; Moellering and Benning, 2010; Rodolfi *et al.*, 2009; Siaut *et al.*, 2011; Wang *et al.*, 2009; Work *et al.*, 2010). Fatty acids seems as a common precursors in support of development of both membrane lipids (required for development) and TAGs (concerned in energy storage and fatty 49 corrosive homeostasis), however it is still needed to cleared that in what manner microalgal cells sort out division of the above precursors to various destinations because of natural boost (Guschina and Harwood, 2006; Hu *et al.*, 2008; Radakovits *et al.*, 2010; Sheehan *et al.*, 1998). In various algae, lipid content and biomass productivity appeared to be conversely interrelated in an interesting manner (Hu *et al.*, 2008; Rodolfi *et al.*, 2009; Sheehan *et al.*, 1998) and deficiency of supplement encourages TAG gathering yet at the consumption of development (Li *et al.*, 2011; Rodolfi *et al.*, 2009).

To improve algal strains performances, a more noteworthy comprehension of carbon distribution among biosynthetic pathways and administrative systems controlling this dispersion is required, primarily in response to ecological burdens. Through direct building of single lipid biosynthesis segments the present requirement is again highlighted with inadequate achievement in rising entire oil content in higher plants and microalgae (Durrett *et al.*, 2008; Li *et al.*, 2010; Radakovits *et al.*, 2010; Work *et al.*, 2010). The single celled microalga *C. reinhardtii* emerges as a standard organism for the study of various parameters like; microalgae physiology, photosynthesis, metabolism, structure and function of flagella (Harris, 2001; Merchant *et al.*, 2007). Its atomic, plastid and mitochondrial genomes had been sequenced and an arrangement of genomic, sub-atomic and hereditary instruments are likewise existing for this specie (Grossman *et al.*, 2007; Harris, 2001; Merchant *et al.*, 2007). Under nitrogen limitation or salt stress

Chlamydomonas is shown to accumulate important amount of TAGs (Dean *et al.*, 2010; Fan *et al.*, 2011; James *et al.*, 2011; Li *et al.*, 2010; Moellering and Benning, 2010; Siaut *et al.*, 2011; Wang *et al.*, 2009; Work *et al.*, 2010). While, majority of experiments characterizing storage lipid production in *C. reinhardtii* had been approved under 50 photo heterotrophic conditions, in media having acetate (Fan *et al.*, 2011; James *et al.*, 2011; Li *et al.*, 2010; Moellering and Benning, 2010; Siaut *et al.*, 2011; Wang *et al.*, 2009; Work *et al.*, 2010).

Somewhat everywhere throughout the world, other algal accumulations demonstrate for the significance that algae have ascended, for a wide range of creation purposes. The collection of the Goettingen University, Germany (SAG) is an example, which began during mid of 1920s and have around about 2213 strains and 1273 species. Around 77% of the considerable numbers of strains in the SAG accumulation are microalgae and around 8% cyanobacteria (61 genera and 230 strains). It consists of both saline and freshwater red algae.

The University of Texas Algal Culture Collection is another extremely surely understood collection of algal cultures which has been established in 1953. This incorporates 2300 unique freshwater algal strains (edaphic green growth and cyanobacteria), however incorporates agents of the majority of algal taxa and includes numerous aquatic macrophytic green and red algal species.

In the Asian landmass, the National Institute for Environmental Studies (NIES), in Ibaraki, Japan, had round about 2150 strains, having almost 700 types of diverse algae. The CSIRO Collection of Living Microalgae (CCLM), at Australia had round about 800 strains of distinctive algae, as well as agents from larger part of classes of some of marine and some freshwater microalgae, most of which were obtained from Australian waters.

1.2 Advantages of Using Microalgae for Biodiesel Production

According to most of the researchers reports as well as publications microalgae is advantageous for the production of biodiesel in comparison with other available feedstocks (Chisti, 2007; Hossain *et al.*, 2008; Hu *et al.*, 2008; Li *et al.*, 2008; Rodolfi *et al.*, 2009; Rosenberg *et al.*, 2008; Schenk *et al.*, 2008; Tsukahara and Sawayama, 2005).

From a practical point of view microalgae can grow easily without giving much attention in water that is incompatible for human consumption, they can also be easily cultivated and easy to obtain nutrients.

Photosynthesis is a factor that reproduce microalgae by converting sunlight energy into chemical energy, and repeat its growth cycle in a few days (Sheehan *et al.*, 1998). Moreover they can grow almost anywhere, requiring sunlight and some simple nutrients, although the growth rates can be accelerated by the addition of specific nutrients and sufficient aeration (Aslan and Kapdan, 2006; Pratoomyot *et al.*, 2005; Renaud *et al.*, 1999).

Various algal species can be modified to survive in different ecological circumstances. Therefore, it can be feasible to discover species that are suitable to restricted environment or particular characteristics of growth, which cannot be feasible doing among recent biodiesel feedstocks (e.g. soybean, rapeseed, sunflower and palm oil).

The growth rates and productivity are much higher in comparison to conservative forestry, horticultural crops, and other aquatic plants, and also require small region as compared to additional biofuel feedstocks of agricultural origin, upto 49 or 132 folds fewer in comparison to soybean crops, for a 30% (w/w) of oil contents in algal biomass (Chisti, 2007). Thus, the struggle intended for arable soil among different crops is greatly reduced for human consumption.

For a variety of renewable fuels microalgae can provide feedstocks, for example, biodiesel, methane, hydrogen, ethanol, in comparison to others. Algal biofuel do not have sulfur and execute like petroleum diesel, whereas emission of particulate matter is reduced, CO, HCs, and SO_2. Yet emission of NO_2 might be elevated among several types of engine (Delucchi and Model, 2003). The use of microalgae for biofuels generation can likewise fill different needs. A few conceivable outcomes presently being considered are listed below.

Expulsion of CO_2 from industrial flue gasses by microalgae bio-fixation (Wang *et al.*, 2008) decreasing the GHG outflows of an organization or process while delivering biodiesel (Directive 2003/30/EC). Waste water administration by removal of ammonium

nitrate, nitrous oxide, phosphate (NH_4^+, NO, PO_4^{-3}), making algae to develop utilizing these water contaminations as supplements (Wang *et al.*, 2008). After oil extraction the subsequent algae biomass can be processed into ethanol, methane, utilized as organic fertilizer because of its high N:P proportion, or basically burned for cogeneration of energy (Wang *et al.*, 2008). The capacity of developing under vigorous situations, and their decreased requirements intended for supplements, it might be developed within range that is inappropriate for farming purposes independent of regular climate variations; therefore they do not compete for use of arable land, and also able to utilize wastewaters as the culture medium without demanding utilization of freshwater. Different compounds might be removed depending upon the microalgae species, by means of profitable applications of various industrial sectors, along with a wide range of fine chemicals and bulk products, for example, FAs, polyunsaturated unsaturated fats, oil, characteristic colors, sugars, pigments, cancer prevention agents, high-value bioactive compounds, and other fine chemicals and biomass (Li *et al.*, 2008; Raja *et al.*, 2008).

Due to increased development rate, increased lipid content and excellent CO_2 obsession capacity in comparisons to other plant sources, microalgae are treated as inexhaustible alternative biodiesel feedstock (Chen *et al.*, 2011; Ho *et al.*, 2010). With suitable environmental stress several microalgal species like *Chlorella emersonii*, *Chlorella minutissima*, *Chlorella protothecoides*, *Chlorella vulgaris*, *Neochloris oleabundans*, and *Dunaliella tertiolecta* can acquired round about 50 to 70% of lipid content per dry weight of biomass, and the lipid obtained is appropriate for biodiesel production.

The world total biodiesel production was predicated to be almost 1.8 billion liters in 2003 (Fulton, 2004). Whereas no increment was observed in the creation of biodiesel from 1996 to 1998, quick increase in manufacturing of biodiesel has been reported few years back. A hypothesis has been created for production of biodiesel that it could be moreover enormously increased due to greater requirement of fuels globally cleaned energy.

To compete with the worldwide energy limitation crisis, lipid-rich biological resources are very necessary for effective production of biodiesel and it has attracted a great interest. Due to the small developmental rate, increased content of lipid and easily being adapted by biotechnological means oleaginous microorganisms are satisfactorily

considered. A few microalgae appeared to be an appropriate collection of oleaginous microorganism to produce lipids (Chisti, 2007). Microalgae seems to be a great applicant for the manufacturing of fuel due to some advantages like, higher photosynthetic efficiency, higher production of biomass and high developmental rate in comparison to other plants (Dote *et al.*, 1994; Milne *et al.*, 1990; Minowa *et al.*, 1995). Additionally, in accordance with biodiesel standard published by, the American Society for Testing Materials (ASTM) the production of biodiesel from microalgal oil is related in assets to the standard biodiesel.

Earlier studies on liquid fuel from microalgae had begun in mid 1980s. During the world war II, although some German scientists attempted to extract lipids from diatom in order to resolve energy crisis (Cohen *et al.*, 1995), and almost immediately in the USA, research has been conducted by a group of researchers at the Carnegie Institution of Washington, and the experience gained by them had been summarized in a book (Burlew, 1953) named "Algal Culture from Laboratory to Pilot Plant", however the technologies of manufacturing microalgae as fuels had not been completely exploited. The argumentation might be as follows. First, microalgae are less known as a source of lipids than plants and animals. Secondly, plant oils costs relatively low and animal fats are yet more cheaper; therefore, techniques for the microbial oils production had largely paying attention on highly appreciated products that cannot be manufactured by plants, like omega-3 polyunsaturated fatty acids, particularly Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) (Wen and Chen, 2001).

1.3 Algae Species

One distinctive aspect of algae is the variety of species available for acceptance in comparison to other advanced sources for producing biofuel. For optimizing the manufacture of different biofuel, variety of species may be selected. A verity of valuable products and pollution sources was offered by algae, such as nutritional compounds, food, omega-3 fatty acids, animal feed, energy sources (including jet fuel, aviation gasoline, gas, biodiesel, and bioethanol), biodegradable plastics, pigments, medicines, organic fertilizers, recombinant proteins, pharmaceuticals, and vaccines (Pienkos and Darzins, 2009).

1.3.1 Chlamydomonas sp

The green algal species *Chlamydomonas reinhardtii* is a unicellular, photosynthetic microbe that inhabits aquatic environments (Harris, 2012; Nichols and Bothast, 2008). In 1945 (Massachusetts, USA) the very first uncultivated strain of this species was collected for research purposes by Gilbert M. Smith (Nichols and Bothast, 2008). The cells of *C. reinhardtii* are encapsulated by means of cell wall along with two large flagella to facilitate the optimization of exposed light and nutrients (Govorunova and Sineshchekov, 2005; Rochaix, 1995). In round about 6 and 12 hours these species were easily cultured in laboratory conditions. The nuclear genome of *C. reinhardtii* exists in the haploid state during normal vegetative growth (Harris *et al.*, 1989), representing the genetic analysis and construction of interesting mutant phenotypes in relation to additional organisms like higher plants where diploidy and polyploidy cause difficulties (te Beest *et al.*, 2011; Weiss-Schneeweiss *et al.*, 2013). Genetic transformation of *C. reinhardtii* knowing the complete genomic DNA sequence of any organism provides numerous advantages to those who study it. Thus it is important that the nuclear (Kumar and Murthy, 2011), mitochondrial and chloroplast genomes of *C. reinhardtii* have all been sequenced and annotated.

1.3.1.2 Photosynthetic properties of *C. reinhardtii*

An important property of *C. reinhardtii* is that it can grow phototrophically, as well as heterotrophically by using acetate as a sole carbon source (Levine, 1968; Spreitzer and Mets, 1981). Due to this property of *C. reinhardtii* the mutants that are not able to maintain photosynthesis can created and kept up in medium containg acetate (Khrebtukova and Spreitzer, 1996). As for the most part it is difficult to proliferate practical photosynthetic mutants of area plants (Grossman, 2000), this aspect has enabled researchers to conduct a significant photosynthetic research which would be otherwise infeasible (Dent *et al.*, 2001; Gutman and Niyogi, 2004; Rochaix, 2002). An illustration can be found in investigations of a particular procedure special to algal cells that had the control to produce and manage photosynthetic mutants of *C. reinhardtii* recognized as the Carbon Concentrating Mechanism (CCM) (Spalding, 2008; Wang and Spalding, 2006).

The cell of every *C. reinhardtii* contains a single large chloroplast that can almost occupy 40% of cellular volume and a structure called pyrenoid develop inside every chloroplast under atmospheric CO_2 levels (Fukuzawa *et al.*, 2001; Rochaix, 1995). This globular structure is for the most part encased by a starch sheath that possesses just little segment of the chloroplast and contains its own particular subset of proteins too. Above 90% of the number of inhabitants in the Rubisco holoenzyme is situated in this structure under climatic CO_2 levels in *C. reinhardtii* (Borkhsenious *et al.*, 1998). *C. reinhardtii* cells make utilization of different transporters and different proteins to viably "pump" CO_2/bicarbonate into the pyrenoid structure, effectively expanding the CO_2 focus at the Rubisco dynamic site in order to boost up the productivity of the CO_2 fixation response (Brueggeman *et al.*, 2012; Fang *et al.*, 2012). Presence of such sort of methodology permits photosynthesis to happened in most efficient way in aquatic environments where the availability of CO_2 is limited in contrast with terrestrial environments (Wang *et al.*, 2011). The manufacturing and classification of different mutants having interruptions in a variety of procedures connected with the CCM has uncovered much about this complicated organization (Wang *et al.*, 2011). To increase the photosynthetic productivity of essential land plants more reveled clarification of the workings of the *C. reinhardtii* CCM might ultimately proved valuable. Utilization of the *C. reinhardtii* chloroplast as a protein production more recently, as greater focus has been placed on the development of renewable fuel sources, *C. reinhardtii* and other algal species have also been used to study the production of renewable biofuels in the form of hydrogen gas or oils (Grossman *et al.*, 2011; Merchant *et al.*, 2012; Radakovits *et al.*, 2010).

1.3.2 Chlorella

The capability of capturing carbon dioxide is higher in *Chlorella vulgaris*. Its growth rate is fast (0.6 g/L day) and has the ability to absorbed 10-15% CO_2 (Lee *et al.*, 2000). *C. vulgaris* has the ability to grow in extreme conditions for example, elevated temperature of 30-35 °C (Converti *et al.*, 2009) and acidic environment, like pH of 3 (Mayo, 1997). On the other side in case of flue gas, it has the ability to absorb up to 50 ppm of So_2 and 200 ppm of NO_2 (Lee *et al.*, 2000). It can also be use in secondary process after it has been used for consumption of carbon dioxide for example animal feed. *C. vulgaris* have a

high percent of proteins, minerals, and vitamins for secondary processes, (Lee *et al.*, 2000).

1.3.3 Scenedesmus

Scenedesmus has the ability to take up CO_2 and provide oxygen to bacteria in sewage treatment plants as it uses organic matter. As Scenedesmus can tolerate growth in wastewater therefore it is an important agent for CO_2 remission with chimney gas. The daily consumption time of CO_2 is 28.08% at a 6% CO_2 level (De Morais and Costa, 2007). Scenedesmus can grow at a range of 10 to 40 °C temperature (Christov *et al.*, 2001).

1.3.4 Spirulina

At present, Spirulina is generally utilized as a part of nourishment applications and can possibly expend CO_2. Its Carbon (C) obsession time is 318.6 mg CO_2/L day at 5% CO_2 (Sydney *et al.*, 2010). It have the capacity to develop at temperature of 20 to 40 °C, yet the temperature will influence the protein and starch level (De Oliveira *et al.*, 1999). The composition of Spirulina is mostly protein (Sydney *et al.*, 2010). It had additionally a possibility of developing on dung, catch CO_2 and produce biogas (Shelef *et al.*, 1980).

1.4 Autotrophic Versus Heterotrophic

Algae can grow requiring organic compounds of carbon and nitrogen for nourishment (heterotrophically) as well as autotrophically (in presence of light). Algae that grow autotrophically can make complex organic nutritive compounds from simple inorganic sources by photosynthesis including biomass. An increment in light source with autotrophic development can impact the general rate of growth. With one examination, the luminosity was improved, (163 $\mu mol/m^2s^{-1}$ to 310 $\mu mol/m^2s^{-1}$) and developmental rate was expanded (2 g/L d^{-1} to 4 g/L d^{-1}) (Ogbonna *et al.*, 1997). Autotrophic growth most significantly leads to the reduction of CO_2 from the environment.

In fermentation processes heterotrophic growth is used for the manufacture of notorious or healthy food (Apt and Behrens, 1999). Organic carbon (i.e. carbohydrates) is used for this kind of growth to deliver carbon dioxide, a straightforward inorganic compound, for

which sunlight energy is not required. To increase the production of biomass autrophic as well as heterotrophic growth can be pooled within a single framework. Within one report Chlorella was utilized to grow both heterotrophically as well as autrophically, for increment in the biomass, and CO_2 production from the heterotrophic phase was utilized as part in the autotrophic time period (Ogbonna *et al.*, 1997). Inconveniences linked among heterotrophic growth are the production of carbon dioxide, due to the accumulation of a carbon source nutrient media cost more, and the risk of bacterial contamination becomes higher, as bacteria could likewise grow in the existence of a CHO carbon source (Feofilova *et al.*, 2010).

1.5 Importance of Algal Species for Biodiesel

Microalgae might be very soon one of the world's great vital inexhaustible oil crops (Campbell, 2005). Fundamental points of interest of microalgae are: (Chisti, 2007; Huntley and Redalje, 2007; Li *et al.*, 2008; Rodolfi *et al.*, 2009; Schenk *et al.*, 2008). A higher efficiency of photon conversion (round about three to eight percent against 0.5 percent for land plants), that corresponds to increased biomass productivity per hectare) as well as developed at high rates (e.g. one to three doublings per 24 hours), the capability of sequestering higher CO_2. With better handling it has also the ability to develop in a liquid medium, and can grow in salt and waste water streams (saline/brackish water/coastal seawater), by this means the use of freshwater is reduced. It utilizes N as well as P from different sources of waste water which provides further advantage of wastewater bioremediation. Also it uses the areas that are inappropriate agricultural purposes (e.g. deserts and seashore lands) therefore there is no competition with arable land uses for the manufacturing of food. Most importantly there is no specific time for it production and it can be harvested any time in year. A high concentration of lipids, carbohydrates and biomass can be produced by inducing these cultures. The production systems of algal biomass can be effectively modified to different types of technologies and skills. Microalgae can also be cultivated without any use of manures and pesticides, playing role in reduction of pollution. Production of biofuel from microalgae can also reduced nitrous oxide (Li *et al.*, 2008). The effect of environment is also insignificant for example, deforestation. The change of light energy into chemical

energy may also be in charge of an extensive range of fuel synthesis for example protons and electrons (for biohydrogen), sugars and starch (for bioethanol), oils (for biodiesel) and biomass (for BTL and biomethane), by means of biochemical, thermo chemical, chemical and direct combustion processes, which produces valuable co-products or by-products (e.g. proteins, polysaccharides, pigments, biopolymers, animal feed and fertilizers.

1.6 Environmental Factors Affecting Algal Growth

Environmental factors may affect diverse frameworks elements connected with algal development or cell structure. For example, increase in temperature can prompt diminishment in supplements accessibility (Sterner and Grover, 1998). For instance in the examination of phytoplankton in Antarctic Ocean, Smith and Morris, (1980) has been observed that phytoplankton intertwined more carbon into the protein segment with decline within the lipid content at higher temperatures while according to Morris *et al.* (1974) *P. tricornutum* accumulate more protein at lower temperatures. This inconsistency may possible because of the species-specific impacts, changes in intensity of light as well as variability in the principle developmental conditions.

Converti *et al.* (2009) studied the effects of temperature and nitrogen concentration on cell growth and lipid content in two strains of algae—*Chlorella vulgaris* and *Nannochloropsis oculata.* Reducing the nitrate concentrations in the growth media by 75%, lipid accumulation increases, with only a small reduction in growth rate at optimal growth temperature. This result indicates that it may be possible to achieve higher lipid productivity for biofuels production by employing nitrogen limitation with fine temperature control. It has been observed that salinity stress result in increase in protein content. The conversion of nitrate to protein content increases with increase in salinity while decreases with increase in nitrate concentration.

Environmental stresses often trigger the production of microalgal lipids for example depletion of nitrogen. Yet, the operation under conditions of stress is constantly connected with moderately low growth rate, thus resulting in low productivity of lipid. Most of the investigations had been reported that in microalgae the developmental rate

and accumulation of lipid content are extensively affected by culture conditions (for example irradiance, concentration of salts, and selection of medium) and operational procedures (for example two-stage cultivation, semi-batch cultivation, or continuous cultivation). In spite of the fact the data provided in the literature shows that the production of lipid from microalgae might be enhanced by promotion of appropriate operational strategies and conditions, an efficient examination for optimization of lipid productivity and metabolic mechanism, but behind this the advances in lipid accumulation is still lacking. Although the uses of marine microalgae has been little studied for the production of lipid.

1.6.1 Temperature Stress

It has been observed by Chinnasamy *et al.* (2009) that most favorable temperature for *Chlorella vulgaris* ranges from 25 to 30 °C and (Converti *et al.*, 2009) reported that decrease in temperature from 30 °C to 25 °C can trigger increase in lipid contents from 5.9 to 14.7%. Furthermore at temperatures above 38 °C oleic acid production increased.

Under heat stress or heat shock it was observed that reduction in the microalgal protein occurs and produces a stress hormone know as abscisic acid (ABA) (Bajguz, 2009). This stress hormone is considered as an important aspect for the control of downstream responses such as growth rate and gene expressions. It has been reported that at the temperature above 40 °C, *C. vulgaris* was found to be less resistant to acidic pH than when it was grown at 35 °C or lower temperatures (Mayo, 1997).

Scenedesmus sp. can grow in a temperature between 20-40 °C (Schenk *et al.*, 2008). It has been studied that at lower temperatures of 15 to 36 °C, the chlorophyll and protein levels got to be decreased in *Scenedesmus sp.*, whereas the levels of carotenoids, saccharides, and lipid content got to be increased (Christov *et al.*, 2001). There was also an increase of 30% observed in sugars and lipid content at high temperature of 36 °C. Using algae the effect of temperature on phosphorus content of wastewater was studied and it has been reported that at higher temperature of 25 °C phosphorus content is present in higher concentration in biomass than at lower temperatures (Powell *et al.*, 2008). On the uptake of arsenic, cadmium, copper, lanthanum, tungsten, and zinc the effect of

temperature (0-22 °C) has been observed, and a rise in arsenic, tungsten, zinc and cadmium uptake has been noticed with increase in temperature (Demon *et al.*, 1989).

The mobility in the cell membrane was affected by lower temperature and with increase in level of unsaturated FA mobility of cells can be increased. This can be demanding for cell membrane as it will favor the free radicals (Nishida and Murata, 1996; Raven and Geider, 1988). High level of unsaturated fatty acids in combination with its greater fluidity have tendency to upgrade the constancy and steadiness of cell membranes (mainly the thylakoid membrane). Which in turns results in protection of photosynthetic machinery from photoinhibition at low temperatures (Nishida and Murata, 1996). *Botryococcusbraunii* was observed to change its lipid content as well as composition when grown to different temperatures and lipid synthesis was suppressed at 32 °C. The content of lipid was reduced to 5 % at 32 °C as compared to 22 % at 25 °C (Kalacheva *et al.*, 2002).

Same effects were seen in *Nannochloropsis oculata* and *C. vulgaris*, each of which grows at most favorable temperature of 25 °C. The lipid content seems to be doubled (from 7.90% to 14.92%) with increase in temperature from 20 to 25 °C in *N. oculata.* In *C. vulgaris* with increment in temperature from 25 to 30 °C a decrease in the content of lipid from 14.71% to 5.90% has been observed (Converti *et al.*, 2009). As the temperature increases above the optimum synthesis of protein is reduced and therefore results in decreased developmental rates (Konopka and Brock, 1978). Lower temperature induced high protein synthesis in *Phaeodactylumtricornutum, Scenedesmus* sp. (Cuhel *et al.*, 1984; Morris *et al.*, 1974). The impact of temperature on content of lipid and composition has been summarized in table 1. It is for the most part recognized that the vast majority of changes in the profile of lipid adjust the physical properties of membranes with the goal that ordinary capacities, for example, ion penetrability, photosynthetic and respiratory procedures can proceeded as a whole (Somerville, 1995).

Patterson (1970) observed that change in temperature has no effect on the lipid content of *Chlorella sorokiniana.* In *C. reinhardtii* the impact of temperature was not observed on the contents of the acidic thylakoid lipids, Sulfoquinovosyl diacylglycerol and phosphatidyl glycerol (SQDG and PG), (Sato *et al.*, 2000). An inadequate data on this

subject is available and it must be noticed. Furthermore investigations were done at laboratory scale where retaining the desired temperature is very easy. Therefore only in closed system photobioreactors the maintaining of increasing and decreasing temperature is feasible which is very expensive in comparison to open setup. Presently there is no awareness of investigations which had advertised impact of temperature to energize lipids generation on vast platform; however as at various temperatures lipid profiles plainly changes, properties of biodiesel obtained from algae would likewise change for distinctive atmospheres and seasons (e.g., summer or winter strains) and efforts are in progress for utilization of flue gases as well as sources of temperature to increase algal growth during cold atmosphere

Table 1: Microalgal Lipid Production with Change in Temperatures

Microalgae Species or	Strain Stressing Agent	Lipid Profile Change After Induction	Reference
Chaetoceros Rhodomonas sp.,	Grown at 25 °C	Total lipid increased by 16.8%	(Renaud *et al.*, 2002)
Cryptomonas sp., Isochrysis sp.	Range of 27 °C to 30 °C	Lipid production increased by 15.5, 12.7, and 21.7% respectively	(Renaud *et al.*, 2002)
Nannochloropsis oculata	Increase from 20 °C to 25 °C	Lipid production increased by 14.92%	(Chen *et al.*, 2011)
Isochrysis galbana	Increase from 15 °C to 30 °C	Increase in neutral lipids	(Zhu *et al.*, 1997)
Chlorella ellipsoidea	Lowering temperature	Unsaturated FA was increased by 2-fold	(Joh *et al.*, 1993)
Nannochloropsis salina	Increase in temperature	Increase in total lipids	(Boussiba *et al.*, 1987)
Dunaliella salina	Shift from 30 °C to 12 °C	Increase in unsaturated lipids	(Thompson, 1996)
Ochromonas danica	Increase from 15 °C to 30 °C	Increase in total lipids	(Aaronson, 1973)
Selenastrum capricornutum	Temperature from 25 °C to 10 °C	Increase in oleate fatty acid	(McLarnon-Riches *et al.*, 1998)
Phaeodactylum tricornutum	Shifted from 25 °C to 10 °C for 12 h	Highest yields of PUFA and EPA	(Jiang and Gao, 2004)
Pavlova Spirulina platensis, Chlorella	lutheri Grown at 15 °C	Increased relative amount of EPA	(Tatsuzawa and Takizawa, 1995)
vulgaris, Botryococcus braunii	Increase in temperature	Saturated FAs increased	(Sushchik *et al.*, 2003)

1.6.2 Light

Light has been crucial factor for algal growth as they contain chlorophyll a and b and also important for photosynthesis. Light harvesting pigments (Chl a and b) has been reported to be more sensitive to red and blue light. (Yun and Park, 2001). Different light intensities have an influence on lipid content (Siegenthaler and Murata, 2006) (Table 2). High intensity of light causes oxidative damage to the poly unsaturated fatty acids and also increase the level of these fatty acids in microalgae. (Siegenthaler and Murata, 2006). Quantity of Polar lipids get rise by low light intensity e.g lipids present on the membrane connected with chloroplast. Polar lipid content get damaged by high light intensity but instantly increase the amount of neutral storage lipids e.g TAGs (Brown *et al.*, 1996; Khotimchenko and Yakovleva, 2005; Napolitano, 1994; Orcutt and Patterson, 1974).

The decreased in total phopholipid content and increase in TAG has been linked with exposure to high light in filamentous green algae *Cladophora* by Napolitano (1994). When red algae *T. Crinitus* was exposed to lower light intensity then increased level of plasma membrane lipid was observed (Khotimchenko and Yakovleva, 2005). High light intensity act as biological catalyst as in the haptophyte *Pavlova lutheri*. It raises the lipid content, low the dilution rate and increase the cell weight per cell.

In strong constant light or under 12:12 h continuous light/dark conditions, the culture grown to stationary phase have increased amount of triglycerides with saturated and monounsaturated fats in correlation to batch cultures developed in less light (Brown *et al.*, 1996). For the production TAG light is essential, yet if high light illumination is utilized as a forerunner precursor for increasing the assembly of TAG, as appeared in the illustrations above and in Table below it can be anticipated that for various species it will be distinctive. Omega-3 unsaturated fats (twenty eight percent to eight percent of aggregate fatty acids), brought about primarily by a diminishing of EPA (20:5n-3).

Table 2: Microalgal Lipid Production Due to Various Light Irradiation Stresses

Microalgal Species	Illumination Types	Lipid Profile Change After Induction	Reference
Tichocarpus Crinitus	Low illumination	TAG Increases	(Khotimchenko and Yakovleva, 2005)
Pavlova lutheri	High illumination	Total lipid content Increases	(Carvalho and Malcata, 2005)
Thalassiosira pseudonana	Light/dark or continuous cycled, high light intesity at exponential growth	PUFA Increases	(Brown *et al.*, 1996)
Thalassiosira pseudonana	Light/dark or continuous cycled, high light intensity at stationary phase	TAG increases	(Brown *et al.*, 1996)
Selenastrum capricornutum	Grown in dark	Linoleate fatty acids increases	(McLarnon-Riches *et al.*, 1998)
Prorocentrum minimum	Grown in dark	Insignificant increase in phospholipids	(McLarnon-Riches *et al.*, 1998)
Isochrysis galbana	Lower light phase	Increase in PUFA	(BANDARRA *et al.*, 2003)
Nannochloropsis oculata	Ultra violet (UV) –A	PUFA got increases, structural lipids	(Srinivas and Ochs, 2012)
P. Antarctica	Low UV-B	PUFA got increases, structural lipids	(Jiang and Chen, 1999)
C. simplex	elevated UV-B	Total lipid contents got increased	(Jiang and Chen, 1999)
Tetraselmis sp.	UV-B radiation	Saturated and monounsaturated fatty acids got increases	(Goes *et al.*, 1995)
Phaeodactylum tricornutum	UV radiation	Increased EPA and PUFA got increased	(Liang *et al.*, 2006)
Chaetoceros muellerii	UV-A	Monounsaturated FA got increased	(Liang *et al.*, 2006)

1.6.3 pH

pH is the most important factor for algal cultivation as it determines the solubility and availability of CO_2 and crucial nutrients. It also have a major impact on algal metabolism(Chenl and Celia, 1994; Shapiro, 1973). pH of algal culture increased due to inorganic carbon and it has been reported that microalgae grow maximum at neutral pH (Hansen, 2002). Different species of microalgae requires different pH of algal culture which ranges from low pH 6.5 to high pH 8.8 (Pruder and Bolton, 1979). Chenl and Celia (1994) found the rate of photosynthesis and algal growth at pH 9.0.

pH effect the availability of CO_2 in carbonaceous species in water. CO_2 is found to be restricted at high pH which, in turn suppress algal growth (Azov, 1982; Chenl and Celia, 1994). Normally at high pH carbon dioxide is available in the form of carbonates and in photoautotrophic culture substitution of CO_2 used for photosynthesis is reluctant, as a result reduction in partial pressure of CO_2 occurs which leads to increase in pH. Basic pH leads to increase the elasticity of the cell wall of parent cells, that inhibits its break down and block the libration of auto spores, rising the time for completion of cell cycle (Guckert and Cooksey, 1990).

1.6.4 Salinity

A critical variable that affect the synthetic synthesis of microalgal cells is salinity. Salinity effect the growth rate of microalgae when exposed to higher level of salinity. For instance, rise in salinity boost up the lipid content of algae (Fabregas *et al.*, 1984; Renaud and Parry, 1994; Zhila *et al.*, 2011). Salt concentration is an important factor that affects the biochemical processes of algae. High and low salt concentration have an impact on algal growth and lipid content Studies showed that when concentration of Sodium chloride raises from 0.4 to 4M the quantity of monounsaturated and saturated fatty acids increased in marine alga like *Dunaliella*. Another study revealed that when sodium chloride concentration increase from 0.5 to 1M it elevated the level of triglycerides and intracellular lipids (Takagi and Yoshida, 2006). Also higher level of sodium chloride, alter the growth rate, carbohydrate and lipid content in fresh water alga like *Botryococcusbraunii*. At the lowest salt concentration highest biomass quantity was

observed. (Rao *et al.*, 2007). These outcomes were upheld by another study in which lipid substance of *Botryococcusbraunii* developed in 0.50 M NaCl was higher in examination to media having no addition of NaCl, yet the contents of protein, starch, and pigments were lower (Ben-Amotz and Avron, 1990). A reduction in protein content with unaltered starch and lipid content with an increment in salinity has been reported in another study with the same alga (Vazquez-Duhalt and Arredondo-Vega, 1991). Reduction in growth at higher salinities has also been reported in this study, which may be because of a failure of the alga to change in accordance with high salinity. Protein content decrease as salt level elevated up to 20% was observed in *Tetraselmis suecica* (Fabregas *et al.*, 1984).

1.7 Affect of Nutrient Availability on Growth and Lipid Content of Microalgae

Abundant possibilities in the biochemical arrangement under states of supplement inadequacy can be found in algae relying on which supplement is restricted and to what degree. Under proper states of temperature and pH the proportion of development of microalgae is identified with the uptake rate of the most restricting supplement in general, and is for the most part reveled by Michaelis-Menten equation. N and P are main elements used in improvement plus breakdown of microalgal cells. Nitrogen is the significant component for growth of proteins and nucleic acids. Another fundamental nutrient is phosphate. It is one of the essential molecules, e.g adenine tri phosphate, the energy transporter in cells. Significant macromolecule for every single living cell is phosphate which is likewise fundamental component of DNA and RNA. Phosphorus is likewise critical constituent of phospholipids. It is not remarkable for algae to become nutrient-restricted (i.e., nitrogen-and phosphorus-restricted) in the ordinary habitat. The metabolic pathway of the organism swings with the limitations of these nutrients.

1.7.1 Nitrogen Limitation

In algal cells, nitrogen is a significant element of structural and functional proteins and constitutes about 7%–20% of cell dry weight. Nitrogen is rapidly used by algae in many biological processes to fulfill the needs of functional requirements. (Fujita *et al.*, 1988; Vergara *et al.*, 1995). Studies revealed that low nitrogen level boosted the synthesis of lipids and triglycerides in algae (Stephenson *et al.*, 2010; Takagi and Yoshida, 2006)

with the decrease in protein level. (Kilham *et al.*, 1997; Morris *et al.*, 1974). Through experimental analysis it was cleared that output of lipids get elevated as the nitrogen level get low (Metting Jr, 1996). It was observed that if nitrogen level get decreased, algae halts to fix carbon into carbohydrate production during photosynthesis process (Hu, 2004). Also low level of nitrogen decreases the production of oxygen, chlorophyll content, fixation of carbon dioxide and synthesis of tissues. (Kolber *et al.*, 1988; Richardson *et al.*, 1969). A rise in amino acid content of *Chlorella pyrenoidosa* has been reported on the expense of sugar phosphate (like glucose-6-phosphate, fructose-6-phosphate) with addition of ammonium (nitrogen source) to the emergent culture (Holm-Hansen *et al.*, 1959).

Nitrogen is one of the most perilous constituent affecting lipid metabolism in algae. In response to nitrogen deficiency a general trend towards accumulation of lipids particularly TAG, has been observed in numerous species or strains of different microalgae. (Hsieh and Wu, 2009; Praveenkumar *et al.*, 2012; Yeh and Chang, 2011). Also nitrogen stress show increase lipid content in several microalgae, cyanobacteria and diatoms (Hu, 2006). A study carried out by Rodolfi *et al.* (2009) show lipid production by the depletion of nitrogen and phosphorous in several green and red algae, diatoms, eustimatophytes and prymnesiophytes. Shortage of silicon rises the level of neutral lipids and mono-unsaturated and saturated fatty acids in the diatoms (Miao and Wu, 2006) and also little rise in triglycerides levels (from 69 to 75% from whole lipids) in addition with phospholipids (from 6 to 8%) was discovered in the microalga *Phaeodactylum tricornutum* in low nitrogen level (Alonso *et al.*, 2000). Low nitrogen level increase lipid level 40% in *Chlorella vulgaris* (Illman *et al.*, 2000). Also lipid synthesis increased in Chlorella with decreased in silicon level (Griffiths and Harrison, 2009).

Additionally it was observed that if media was changed from normal to nitrogen depleted media a changed occur in lipid composition i-e from free fatty acids to triglycerides. (Widjaja *et al.*, 2009). Nitrogen limitation also affect the pigment composition e.g an ıcrease ratio of carotenoids and chlorophyll was detected in *Parietochloris incise* ʏvchenko *et al.*, 2008).

Table:3 Microalgae Species Under Nitrogen Deficiency for Oil Production

Species	Lipid Content (%)	References
Cyclotella cryptica	18	(Feinberg, 1984)
Dunaliella salina	18.5	(Borowitzka, 1999; Feinberg, 1984)
Nitzchia sp.	14.4	(Feinberg, 1984)
Botryococcus braunii	54.2	(Feinberg, 1984; Sawayama *et al.*, 1995)
Chlamydomonas sp.	23	(Feinberg, 1984)
Chlorella vulgaris	18	(Huntley and Redalje, 2007; Illman *et al.*, 2000)
Nannochloropsis salina	54	(Huntley and Redalje, 2007)
Tetraselmis sueica	20-30	(Huntley and Redalje, 2007)
Isocttrysis sp.	26-45	(Feinberg, 1984)

Objectives

This study demonstrated an effect of high temperature and nitrogen limitation on the growth and lipid accumulations on the selected microalgae (Yellow in Dark mutants of *Chlamydomonas reinhardtii*) to meet the demand for biodiesel feedstock.

The main objective of the studies were

- To examine the effect of temperature on biomass and lipid accumulation of *C. reinhardtii*.
- To investigate growth and lipid contents of *C. reinhardtii*, by varying concentrations of nitrogen in growth media.

Materials and Methods

This research was done at Stress Signaling Lab, Department of Biochemistry, Quaid-i-Azam University, Islamabad Pakistan. The study was approved by Institutional Review Board of Quaid-i-Azam University Islamabad in collaboration with Professor Dr. Yi-Feng Chen, Jiangsu Academy of Agricultural Sciences, Nanjing, China.

2.1 Algal Material

Chlamydomonas reinhardtii is a haploid single-cell green alga and widely distributed worldwide in soil and fresh water. It is about 10 micrometers in diameter that swims with two flagella. It has a cell wall, a large cup-shaped chloroplast, a large pyrenoid, and an "eyespot" that senses light. *C. reinhardtii* is an especially well studied biological model organism, partly due to its ease of culturing and the ability to manipulate its genetics.

Culture were grown in glass Erlenmeyer flasks ranging in size from 125 to 250 mL. Flasks were stopped with non-absorbent cotton wool covered with aluminum foil to prevent contamination with dust. Level of MS media added never exceeded the widest diameter of vessel, which ensured adequate gas exchange between media and air. Cultures were incubated in temperature controlled incubators se at 25 ± 2 °C. Lighting was set to 12:12 light/dark cycle.

The Yellow in Dark mutants of *C. reinhardtii* were screened for phenotypic analysis as well as for growth and lipid analysis. The pre-cultures were grown on agar MS media at room temperature and with a 16 hour light/8 hour dark cycle. The media and the petri plates were sterilized by autoclaving at 121 °C for twenty minutes to avoid all contamination in the early developmental stages.

Table: 2.1 List of selected mutant strains of Chlamydomonas reinhardtii which were used for growth and lipid accumulation

Classes	Strains	Genotype	Phenotype	References or source
Wild type	CC-735	y1 mt-	yellow in the dark	Chlamydomonas Center
Wild type	CC-125	mt+ nit1 nit2		Chlamydomonas Center
Wild type	CC- 1690	mt+		Chlamydomonas Center
Wild type	CC-1692	mt-		Chlamydomonas Center
Wild type	CC-1175	y8 mt+	yellow in the dark	Chlamydomonas Center
Wild type	CC-1173	y7 mt+	yellow in the dark	Chlamydomonas Center
Wild type	CC- 1170	y5 mt-	yellow in the dark	Chlamydomonas Center
Wild type	CC- 4033	y5 nit+ mt+	yellow in the dark	Chlamydomonas Center
Wild type	CC- 1168	y1a mt+	Yellow in dark	Chlamydomonas Center
Wild type	CC- 1676	msr1 ac20 y6 mt-	yellow in the dark	Chlamydomonas Center

2.2 Experimental Setup

Temperature was selected as independent variable. The value of temperature was 33 °C for *C. reinhardtii,* chosen as data reported in literature. We have two set of samples, one is control and the other is heat stress, control samples were treated at room temperature while high temperature stress of 33 °C was given to stress samples. 250 mL of sample was taken,

To assess the responses of *C. reinhardtii* to heat stress, these mutants were subjected to treatment of high temperature (33 °C) for time interval of 15 days, and on daily basis its biomass and lipid content was estimated. This experiment was designed in Erlenmeyer flasks. The stress treatment was given in shaker incubator while the control samples were placed on constant shaking on (Amerex GYROMAX 747/747R floor model) at room temperature. At the end of the experiment, its carbohydrate, chlorophyll and protein content was estimated.

In another experiment the algae has been analyzed for growth rate and contents of lipid under different concentrations of ammonium nitrate. The experiment was performed in 0, 0.125 mg/L, 0.250 mg/L and 0.50 mg/L of the original nitrogen source concentration. The impact of early nitrogen concentrations on the growth and lipid contents of microalgae was observed for 16 days. Lipid content and biomass was predicted after ›very two days. At the end of the experiment chlorophyll carbohydrates and protein ›tent was measured.

'omass Estimation

'everal ways to evaluate the quantity of algal biomass present in cultures either 'he number of cells or through determination of volume, optical density or

'omass concentration was determined by Gravimetric method. MS lgal culture (50 mL) were taken in pre weighed falcon tubes and rpm for 10 minutes, using a centrifuge model (SIGMA Ostered ›d at 80 °C for 1h. Algal biomass was measured using balance ' 0.1 mg. All measures were carried out in triplicates.

$$\text{Algal biomass} = \text{Weight with pellet} - \text{Empty weight of falcon tub}$$

2.4 Growth Rate

To calculate the growth rate of algae dry weights of algae were used. The specific growth rate, μ, was defined as

$$\mu = \ln(Xm/Xo)/t$$

Where **Xm** was the final concentration of algae (g/L) **Xo** was the initial concentration (g/L) and **t** was the time duration.

2.5 Lipid Extraction

The protocol of Bligh and Dyer (1959) was used for the extraction of lipid. The cells were accumulated by means of centrifugation at 10,000 rpm for 10 min at 4 °C. The cells were then washed once with distilled water and centrifuged again at 10,000 rpm. The wet weight of pellet was estimated, and then dried in oven for 2 h at 80 °C. 2 mL of methanol and 1 mL of chloroform were added for 1 g of algal biomass, and reserved for 18 hours at 25 °C. For 2 minutes the mixture was agitated in vortex. Again 1 mL of chloroform was added and the mixture had been shaken vigorously for 1 minute. After that again 1 mL of distilled water was added and the mixture was blended in a vortex for 2 minutes. The cells were centrifuged for 10 minutes at 2000 rpm and the layers were separated. The lower layer had been separated and the process was repeated again with the pellet. The collected supernatants were permitted to stand for 2 hours. Lower organic layer containing lipids had been transferred to a clean pre-weighed vial (W1). For the removal of evaporation the samples were put in hot air oven at 80 °C for time duration of 50 minutes. The vial weight was again recorded (W2). The lipid content was determined by subtracting W1 from W2 and was indicated as % dry cell weight.

$$\text{Lipid Content} = \text{Weight with lipid (W2)} - \text{Empty weight of falcon tub (W1)}$$

$$\text{Lipid Productivity} = \frac{\text{Lipid content (g/g)} * \text{DCW (g/L)}}{\text{Time}}$$

$$\text{DCW \%} = \frac{\text{Lipid Content g/l}}{\text{Biomass g/l}} * 100$$

2.6 Transesterification

At transesterification step, the obtained lipid was dissolved in 2 mL chloroform and was transferred into pre weighed 1.5 mL glass vial, subsequently 1M 1 mL sulphuric acid. Methanol was added to sample and was maintained for 1 hour at temperature of 100 °C. The samples were naturally cooled and 500 µl distilled water was added and mixed by shaking for 2 minutes.

Finally samples were extracted with n-hexane for 3 times and organic phase was gathered and dried in Concentrator 5301 (Eppendorf, Hamburg · Germany) and obtained methyl ester was weighed.

2.7 Chlorophyll Estimation

A 1mL suspension of mutant strains of *C. reinhardtii* was put in a 15 mL falcon tube and centrifuged for 10 minutes. The supernatant was cast-off and the pellet was washed with distilled water. 1.5 mL Dimethyl sulfoxide (DMSO) has been added and the pellet was put back into suspension. The mixture was then transferred into a tissue grinder which was operated 20 times. This was performed to aid the extraction of chlorophyll. Once grinded, the solution was incubated at 50 °C for 30 minutes. The liquid was then removed from the tissue grinder by pipeting into a 15 mL conical centrifuge tube and the tissue grinder was washed 3X with a total of 8.5 mL of spectroscopic grade acetone. The solution was allowed to stand for a short time period prior to an additional 10 minutes of centrifugation in a swinging bucket clinical centrifuge. The supernatant was then transferred into 1 cm quartz cuvettes and analyzed by way of Spectrophotometry (using a Beckman DU650) at wavelengths between 480 nm, 645nm and 663nm. The results were then recorded in excel.

$$\textbf{Chorophyll a} = 12.7 \times A663 - 2.69 \times A645$$

$$\textbf{Chorophyll b} = 22.9 \times A645 - 4.68 \times A663$$

$$\textbf{Carotenoiods} = OD\ @480 \times 4$$

2.8 Carbohydrates Estimation

Carbohydrates for both stress and control samples were estimated by Phenol–Sulfuric Acid method. Biomass (10 mg) was reconstituted in water (10 mL) to prepare a known sample concentration for each sample (1 mg mL^{-1}). Aliquots of 1 mL sample were reacted with 3 mL of concentrated sulfuric acid (72 wt %) and 1 mL of phenol (5%, w/v) in a water bath. The mixtures were incubated for 5 min at 90 °C in water bath. The absorbance at 490 nm was then measured using a Spectrophotometer. The absorbance measurements were then compared to a standard curve based on glucose.

2.9 Protein Estimation

Total protein content was determined by according to method of Lowery *et al.* (1951). The lowery reagent was prepared by adding solution A, solution B and solution C in 100:1:1 ratio by volume.

Solution A = 2.99 g NaOH + 14.3 Na_2CO_3/ 500 ml

Solution B= 14.2 $CuSO_4$.$5H_2O$/for 100 ml

Solution C= 2.85299 $Na_2C_4H_4O_6$/100 ml

Folin Reagent (instant fresh; 0.1 mL/sample)

5 mL of 2N Folin and Ciocalteu's Phenol Reagent + 6 mL ddl water

This solution is light sensitive. So it should be prepared at the last 5 min of the first sample incubation and kept in a covered container. Standard curve has been prepared accordingly. In distilled water Bovine serum albumin (BSA) powder has been mixed and then diluted to one microgram/micro liter concentration. In four replicates a set of dilutions (0, 1, 2.5, 5, 10, and 20 microgram/well) were prepared with a final volume of 100 micro liter. The samples were diluted very well in such a way that they would fall in the BSA standard range (0-25 microgram / 100 micro liters) accordingly and 100 micro liter fall in every well. The samples were then diluted and moved into the micro plate, 200 micro liter of biuret reagent has been transferred to every well and with repeated pipeting dissolved thoroughly. The preparation of biuret reagent was carried out by dissolving 0.5 milliliter of 1 percent cupric sulfate with 0.5 milliliter of 2 percent sodium

potassium tartrate, and then 50 milliliter of 2 percent sodium carbonate in 0.1 N sodium hydro-oxide has been added. This mixture has been incubated for ten to fifteen minutes at room temperature followed by addition of twenty micro liter of 1.0 N Folin and Ciocalteu's reagent per well. With every addition the samples has been mixed instantly with repeated pipeting. At room temperature the color has been develop for thirty minutes and at 750 nm the absorbance was measured. The water was used as blanked for control. The end product of this reaction has blue color. In these experiments though the plates were read instantly, and the reaction has been found to be stable for one hour. First the proteins are pre- treated with copper ion in alkali solution, and then the aromatic amino acids in the treated sample reduce the phosphomolybdate phosphotungstic acid present in the Folin Reagent. The amount of proteins in the sample can be estimated via reading the absorbance (at 750 nm) of the end product of the Folin reaction against a standard curve of a selected standard protein solution (in our case ; Bovine Serum Albumin- BSA-solution).

2.10 Fourier Transform Infrared Spectroscopy (FTIR)

For FTIR analysis, for every experiment100 mL of algal samples has been got from every replicate flask and dissolved. Then it was centrifuged for 10 minutes at 12000 rpm. The supernatant was discarded and the pellet was oven dried at 40 °C for 30 minutes. The dried biomass was further broken into powder. Infrared analysis for identification of polysaccharides and lipids in algae was carried out at Department of Microbiology, Quaid-i-Azam University, Islamabad, An infrared spectrometer (Bruker Tensor 27, Germany) with software OPUS 6.5 (Bruker, Germany) was used to record the characteristic peak areas of lipid, protein and carbohydrate at 2800–3000 cm^{-1}, 1500–1700 cm^{-1} and 1000–1200 cm^{-1}, respectively. Each sample was analyzed in triplicate.

Results

We evaluated the effect of different nitrogen concentrations and temperatures on the biomass and lipid accumulation of nine different mutant strains of a unicellular green algae *C. reinhardtii*. This study was conducted as batch culturing in the laboratory, cultured species were harvested at their stationary phase and the slurry air dried at room temperature for 24 hours. The mean values from the three replicates were recorded for each of the treatments (low, control and high nitrogen concentrations, and temperature) and the biomass, lipid accumulation, growth rate, total carbohydrate, protein, chlorophyll and carotenoids contents were calculated and displayed (Appendix S2).

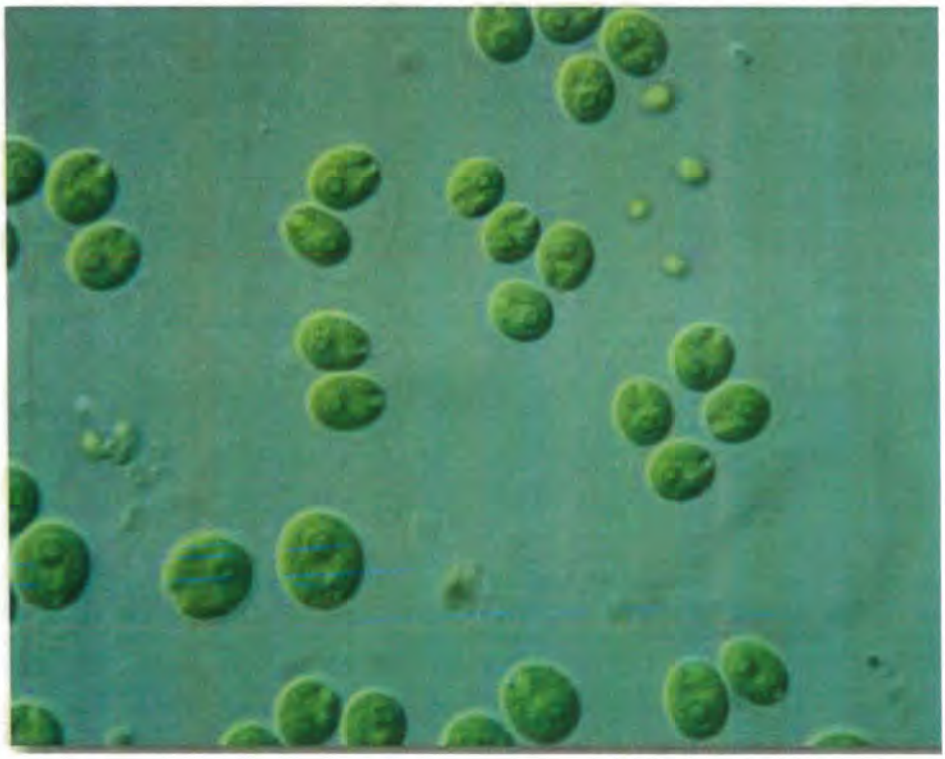


Figure 3.1: *C. reinhardtii*

3.1 Effect of Temperature Treatments on *C. reinhardtii*

Large scale production facilities of microalgae, whether indoor or outdoor face key problem of temperature tolerance. The ability of algae species to respond to changing environmental temperatures rapidly ensures their survival and success for large scale production. Temperature conditions have straightforward implication on growth rate as well as on cell physiology and biochemical compositions. Therefore temperature is a crucial variable to be taken into account for optimizing the design and operation of microalgae culture system whether to produce biomass or high value molecule. We picked this project to examine the effect of temperature on selected strains of *C. reinhardtii*, and we selected 33 °C for high temperature stress for the selected algal mutants while 25 °C (the optimal temperature *C. reinhardtii*) was taken as control temperature. We used yellow-in-dark mutants, for this purpose. Light independent protochlorophyllide reduction leads to chlorophyll formation in dark require both chloroplast and nuclear gene expression in *C. reinhardtii*. Mutations in any of the plastid genes (*chlL*, *chlN*, and *chlB*) or nuclear (*y-1* to *y-10)* genes result in the phenotype of the yellow-in-the-dark or *y* mutants. These mutants respond to different types of stresses by reduction in their growth or color changes. These mutants were grown under autotrophic conditions for ten days and physiological parameters such as growth phenotype, carbohydrates, proteins and chlorophyll content and biomass, lipid accumulation and growth rate was estimated to check the higher temperature tolerance of these mutants.

3.1.1 Physiological Analysis of *C. reinhardtii* After Temperature Stress

We used nine mutant strains to check temperature effects on these yellow-in-dark mutants. Growth of several mutants of *C. reinhardtii* appeared to be affected at temperatures above 33 °C and showed decrease in its growth rate as compared to grown at 25 °C. This difference in the growth was simply detected due to change in color from green to brown in the cells, and as a result the growth rate of microalgal cells was decreases (Fig 3.2). Mutant strains, CC-125 , CC-1175 and CC-1181 appeared to be least affected at 33 °C as compared to their respective control at 25 °C, while CC-125, CC-1690, CC-1692, CC-4033, CC-1173, CC-1168 and CC-735 are more affected at 33 °C as compared to their respective controls at 25 °C. According to our data mutant strains, CC-

125, CC-1175 and CC-1181 stood better with respect to other strains at 33 °C, and these strains can tolerate high temperature stress of 33 °C without any affect on its physiological parameters i.e colour.

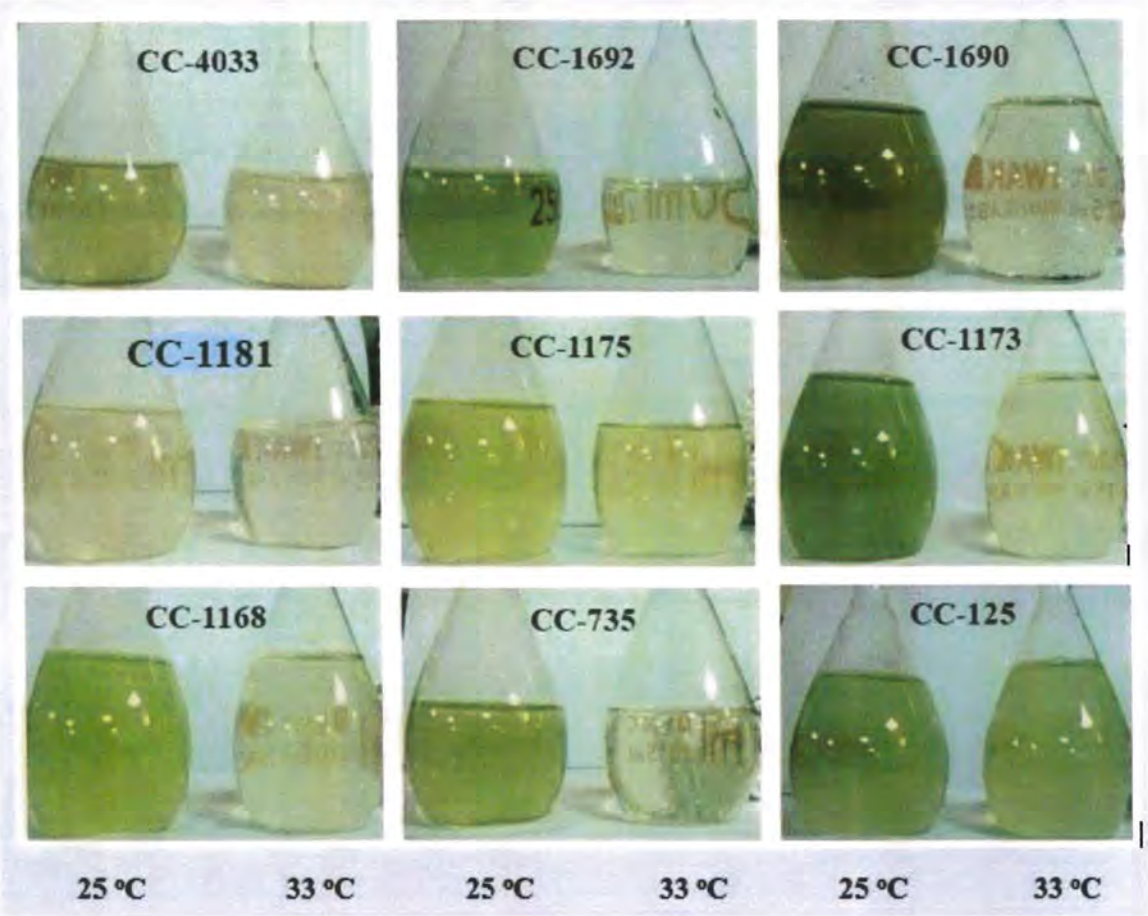


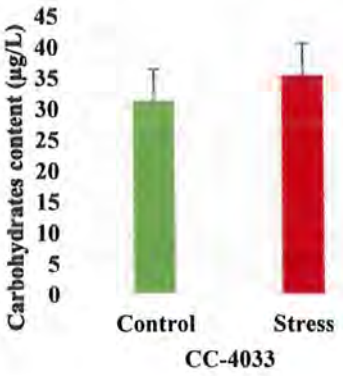
Figure 3.2: Growth Phenotype Analysis to Check the Effect of High Temperature on Mutants of *C. reinhardtii*. Different mutants were grown under high temperature stress for ten days in comparisons to control temperatures This figure shows the phenotypic difference in the growth on selected mutants of *C. reinhardtii* including, CC-4033 , CC-1692, CC-1690, CC-1181, CC-1175, CC-1173, CC-1168, CC-735 and CC-125 growing at 25 °C and 33 °C respectively. The intensity of green color is the indication of growth. The lighter green to colorless is showing the effect of given temperature on the selected mutant strains.

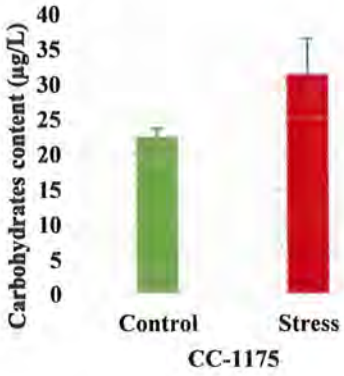
3.1.2 Carbohydrate Contents

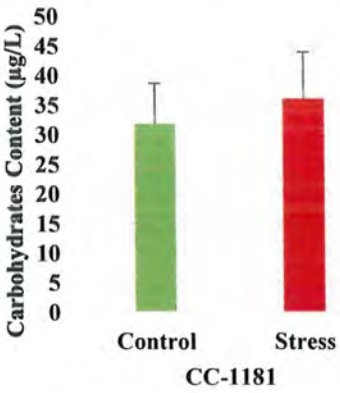
The total carbohydrate contents can be altered by physical factors, such as light density and temperature variability. The mutants of *C. reinhardtii* including, CC-4033, CC-1175, CC-1181 and CC-735 were observed carefully for difference in the carbohydrate contents of algae treated at 33 °C as compared to their respective controls at 25 °C. Mutants of *C. reinhardtii* including, CC-1168, CC-1173, CC-1692, CC-1690 and CC-125 were observed to have less carbohydrate content at 33 °C as compared to their respective controls at 25 °C (Fig. 3.3).

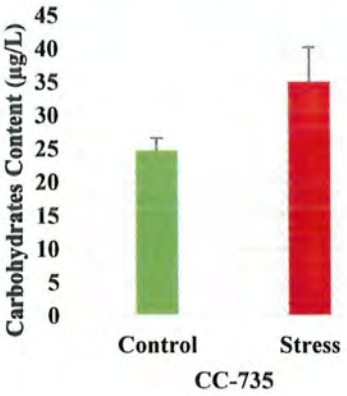
Our data showed that the total carbohydrate contents in the selected strains were highly variable under the selected growth conditions of algae. The highest average total carbohydrate contents was observed in the CC-735 (34.91 ± 5.2 μgl^{-1}), while the lowest value was recorded in CC-4033 (35.32 ± 5.3 μgl^{-1}) with respect to values of their control (24.69 ± 1.8 μgl^{-1} and 31.27 ± 5.1 μgl^{-1}) respectively (Appendix S2).

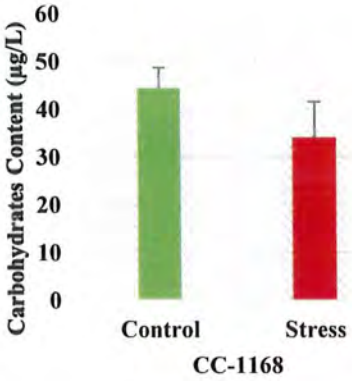
Mutant strains CC-1690, CC-1692, CC-125, CC-1168 and CC-1173 were influenced more as their carbohydrate contents were lower as for their controls. As indicated by our data CC-1181, CC-735, CC-4033 and CC-1175 stood superior to remaining strains in carbohydrate contents at 33 °C.

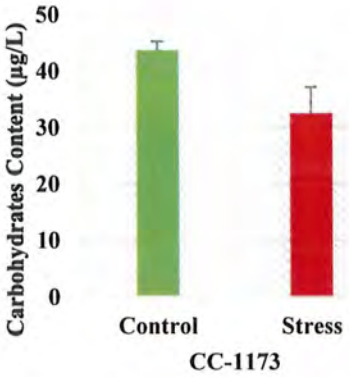


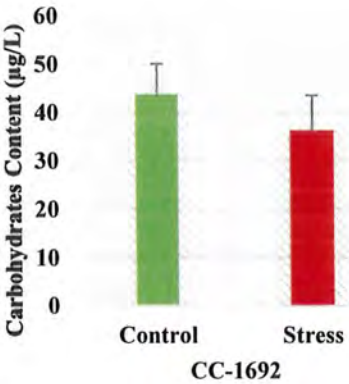


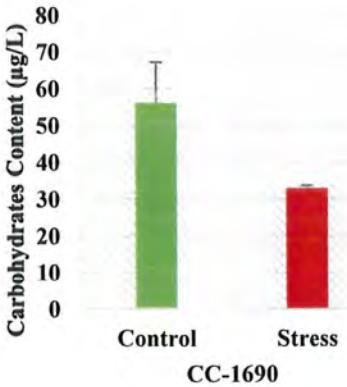

Carbohydrates Content (µg/L)
50
45
40
35
30
25
20
15
10
5
0
Control
Stress
CC-1181


Carbohydrates Content (µg/L)
45
40
35
30
25
20
15
10
5
0
Control
Stress
CC-735


Carbohydrates Content (µg/L)
60
50
40
30
20
10
0
Control
Stress
CC-1168


Carbohydrates Content (µg/L)
50
40
30
20
10
0
Control
Stress
CC-1173


Carbohydrates Content (µg/L)
60
50
40
30
20
10
0
Control
Stress
CC-1692


Carbohydrates Content (µg/L)
80
70
60
50
40
30
20
10
0
Control
Stress
CC-1690

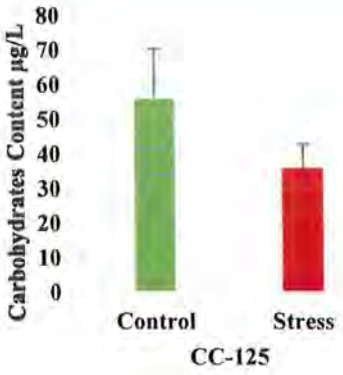
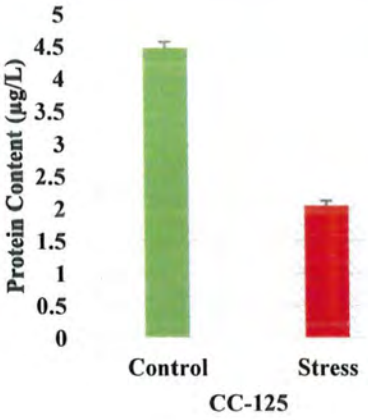
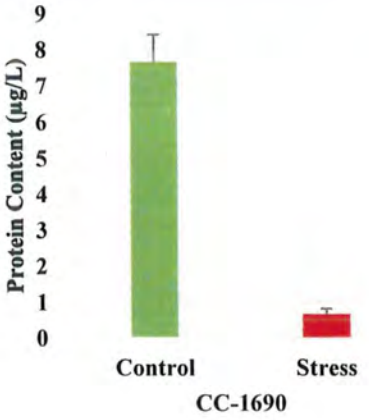


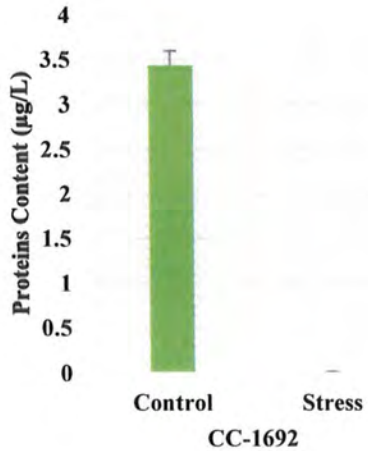
Figure 3.3: Effect of Temperature on Total Carbohydrate Contents of Nine Selected Mutant Strains of *C. reinhardtii.* Green and red colored vertical bars represent the concentration of carbohydrate content along y-axis control and stress respectively. Carbohydrate contents of algae were expressed as micrograms per liter and the values corresponds to the mean of three independent replicates. Values indicates the average of three independent replicates ± s.e

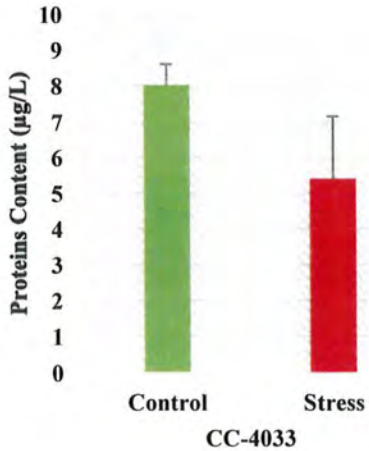
3.1.3 Total Protein Contents

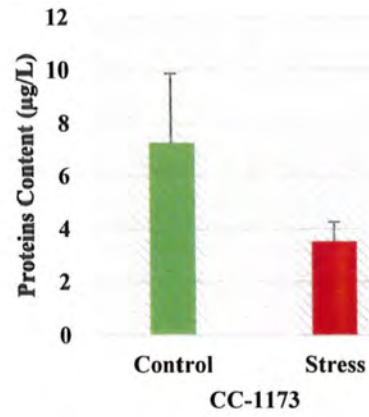
Microalgal system is of great importance for its potential to meet a large impending demand of protein for human and animal health. Microalgae undergoing nutrient limitation and environmental stress change their cellular composition; high temperature can lead to change the protein content. As the temperature increases above the optimum, reduction in protein synthesis takes place, and consequently growth rates decreases. The total protein contents can be altered by physical factors, such as light density and temperature variability. The mutants of *C. reinhardtii* including CC-125, CC-1690, CC-1692, CC-4033, CC-1173, CC-1168, CC-1175, CC-1181, and CC-735 were observed carefully for difference in the protein contents of algae treated at 33 °C and found to have less protein contents as compared to their respective controls at 25 °C (Fig. 3.4). Mutant strains CC-1690, CC-1692 and CC-735 were affected more as their protein contents were much lower with respect to their controls. On the other hand strains CC-4033, CC-1168 and CC-125 were affected least in term of protein contents. According to our data CC-4033, CC-1168 and CC-125 stood better than remaining strains in protein contents at 33 °C.

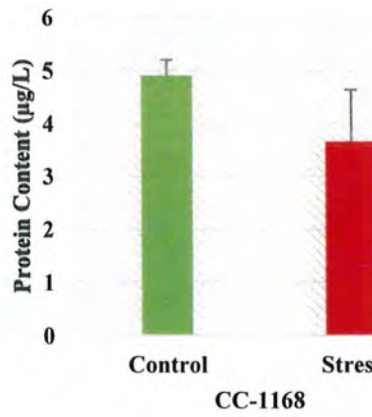

Protein Content (µg/L)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Stress
CC-125


Protein Content (µg/L)
9
8
7
6
5
4
3
2
1
0
Control
Stress
CC-1690


Proteins Content (µg/L)
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Stress
CC-1692


Proteins Content (µg/L)
10
9
8
7
6
5
4
3
2
1
0
Control
Stress
CC-4033


Proteins Content (µg/L)
12
10
8
6
4
2
0
Control
Stress
CC-1173


Protein Content (µg/L)
6
5
4
3
2
1
0
Control
Stress
CC-1168

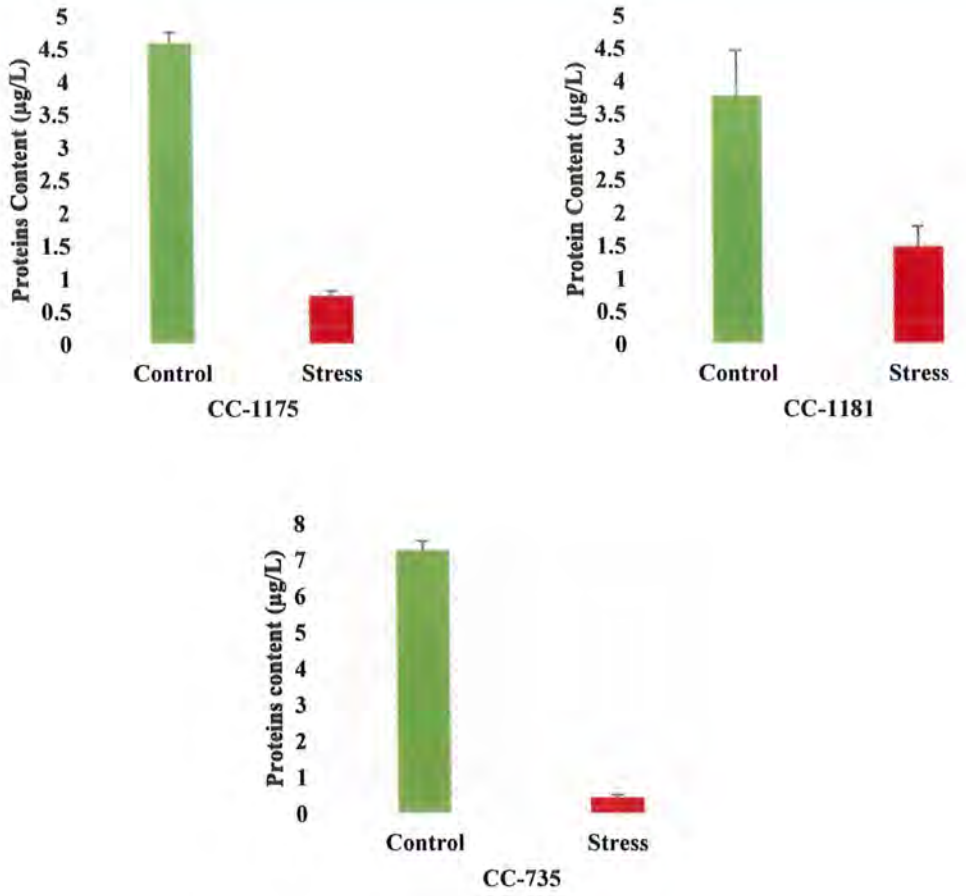


Figure 3.4: Effect of Temperature on Total Protein Contents of Nine Selected Mutant Strains of *C. reinhardtii*. Green and red colored vertical bars represent the concentration of protein content along y-axis control and stress respectively. Protein contents of algae were expressed as micrograms per liter and the values corresponds to the mean of three independent replicates. Values indicate the average of three autonomous replicates ± s.e.

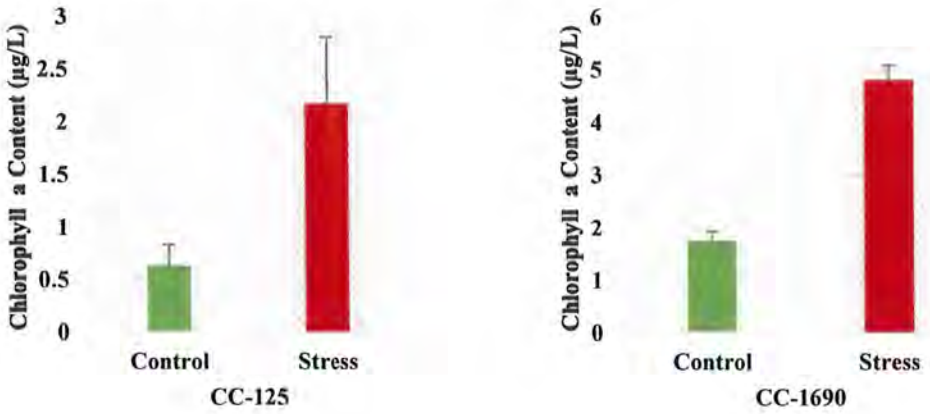
3.1.4 Chlorophyll a Contents

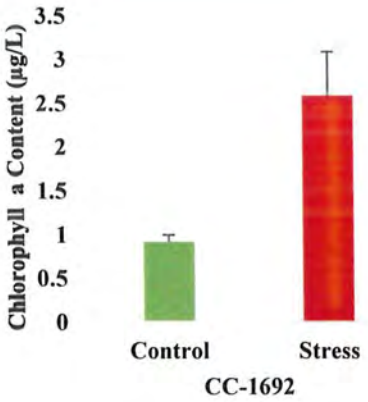
Chlorophyll is a very essential and energetic photosynthetic natural pigment extensively dispersed in species of algae. Concentration of chlorophyll can directly be linked to the algae growth. Increase of chlorophyll as indicator for growth for various microalgal strains. The total chlorophyll contents can be altered by physical factors, such as light

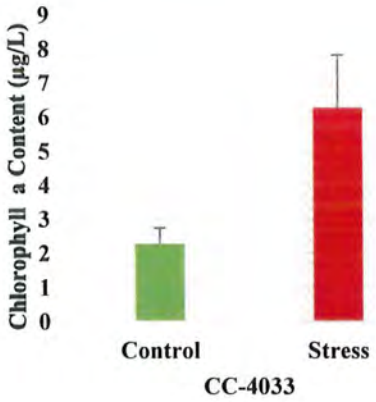
density and temperature variability. Due to the balanced growth, the chlorophyll can be chosen as the representative of growth.

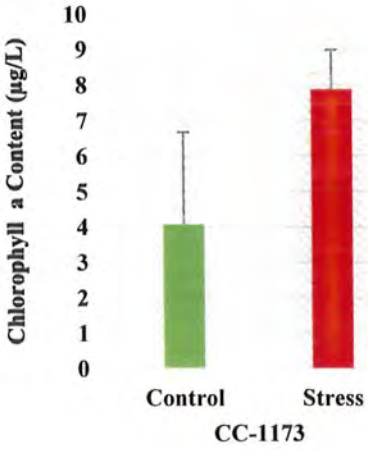
The mutants of *C. reinhardtii* including, CC-125, CC-1690, CC-1692, CC-4033, CC-1173, CC-1168, CC-1175, and CC-735 were observed carefully for difference in the chlorophyll a contents of algae treated at 33 °C and found to have high chlorophyll a contents as compared to their respective controls at 25 °C while mutant of *C. reinhardtii* CC-1181 was observed to have less chlorophyll a content at 33 °C as compared to its respective control at 25 °C (Fig 3.5).

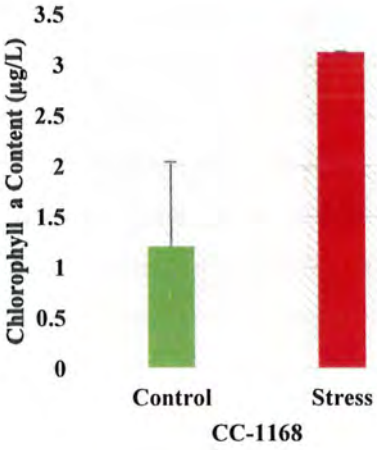
Our data showed that the chlorophyll a content in the selected strains were highly variable under the selected growth conditions of algae. The highest average total chlorophyll a content in the CC-735 (5.444 ± 0.56 μgl^{-1}), while the lowest value was recorded in CC-1175 (1.8152 ± 0.2 μgl^{-1}) with respect to values of their controls (0.59 ± 0.27 and 1.4378 ± 0.19 μgl^{-1}) respectively (Appendix S2). Mutant strains CC-1181 was affected more as its chlorophyll a content was much lower with respect to its control. On the other hand in strains CC-125, CC-1690, CC-1692, CC-735, CC-4033, CC-1168, CC-1173 and CC-1175 there was increase observed in chlorophyll a contents. According to our data CC-125, CC-1690, CC-1692, CC-735, CC-4033, CC-1168, CC-1173 and CC-1175 stood better in chlorophyll a contents at 33 °C.

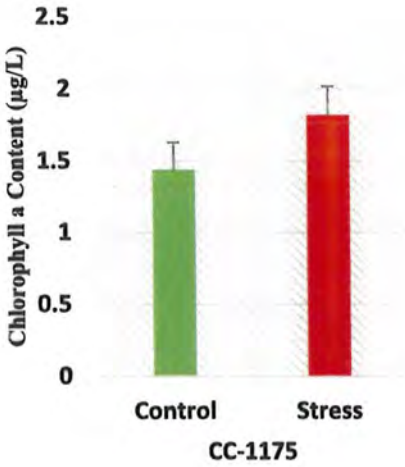


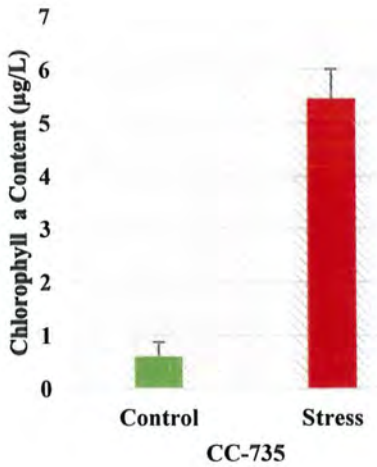

Chlorophyll a Content (µg/L)
3.5
3
2.5
2
1.5
1
0.5
0
Control
Stress
CC-1692


Chlorophyll a Content (µg/L)
9
8
7
6
5
4
3
2
1
0
Control
Stress
CC-4033


Chlorophyll a Content (µg/L)
10
9
8
7
6
5
4
3
2
1
0
Control
Stress
CC-1173


Chlorophyll a Content (µg/L)
3.5
3
2.5
2
1.5
1
0.5
0
Control
Stress
CC-1168


Chlorophyll a Content (µg/L)
2.5
2
1.5
1
0.5
0
Control
Stress
CC-1175


Chlorophyll a Content (µg/L)
7
6
5
4
3
2
1
0
Control
Stress
CC-735

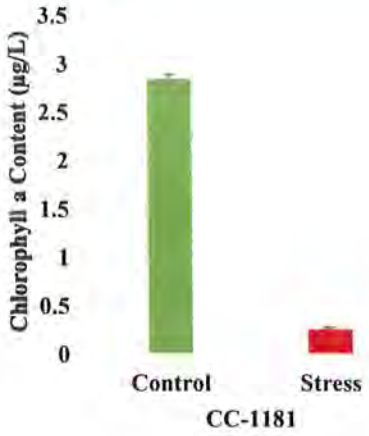


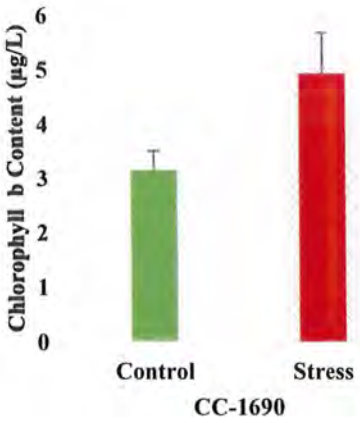
Figure 3.5: Effect of Temperature on Chlorophyll a Contents of Nine Selected Mutant Strains of *C. reinhardtii.* Green and red colored vertical bars represent the concentration of chlorophyll a content along y-axis control and stress respectively. Chlorophyll a contents of algae were expressed as micrograms per liter and the values corresponds to the mean of three independent replicates. Values indicate the average of three autonomous replicates ± s.e.

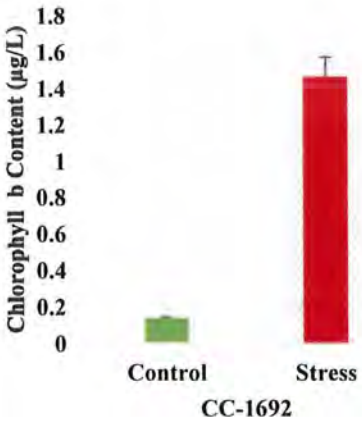
3.1.5 Chlorophyll b Content

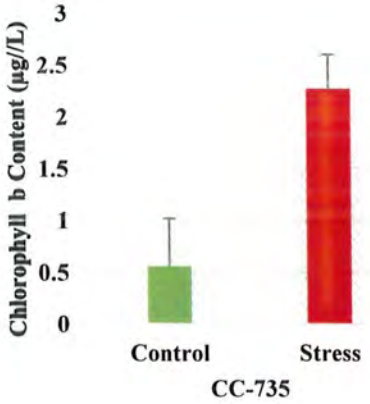
Physical factors, such as light density and temperature variability can alter the total chlorophyll contents. The mutants of *C. reinhardtii* including, CC-1690, CC-1692, and CC-735 were observed carefully for the difference in chlorophyll b contents of algae treated at 33 °C and found to have higher contents as compared to their respective controls at 25 °C. Mutants of *C. reinhardtii* including, CC-125, CC-4033, CC-1175, CC-1181, CC-1168 and CC-1173 were observed to have less chlorophyll b contents at 33 °C as compared to their respective controls at 25 °C (Fig. 3.6).

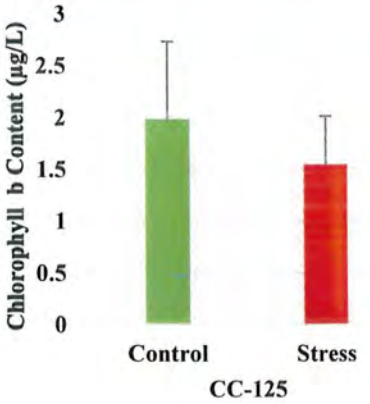
Our data showed that total chlorophyll b contents in the selected strains were highly variable under the selected growth conditions of algae. The highest average total chlorophyll b contents in the CC-735 (2.2659 ± 0.33 μgl^{-1}), while the lowest value was recorded in CC-1692 (1.463 ± 0.11 μgl^{-1}) with respect to values of their control (0.555 ± 0.46 and 0.137 ± 0.00 μgl^{-1}) respectively (Appendix S2). Mutant strains CC-1181 and CC-1175 were affected more as their chlorophyll b content was much lower with respect

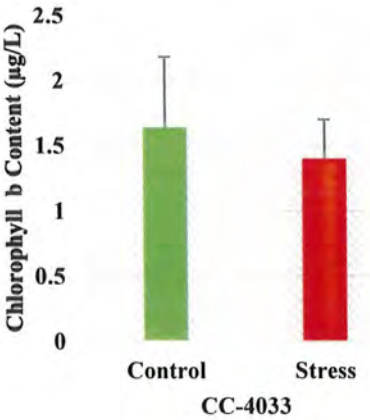
to their controls. On the other hand strains CC-125, CC-4033, CC-1168 and CC-1173 were affected least in term of chlorophyll b contents. While there was an increase observed in chlorophyll b contents of strain CC-1690, CC-1692 and CC-735. According to our data CC-1690, CC-1692 and CC-735 stood better than CC-1181 in chlorophyll b contents at 33 °C.

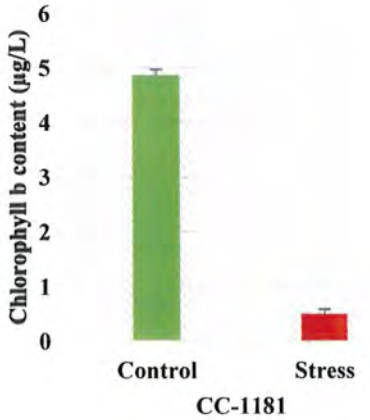


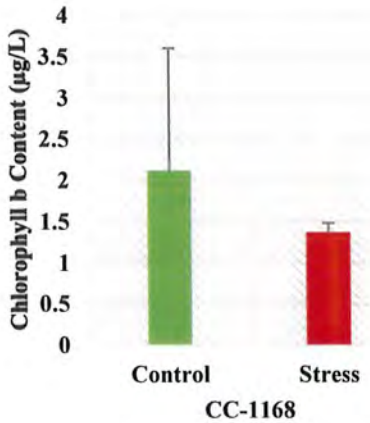


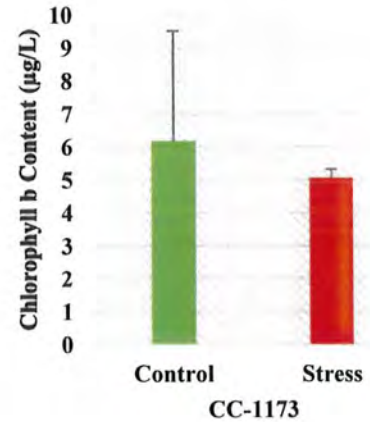

Chlorophyll b Content (µg/L)
3
2.5
2
1.5
1
0.5
0
Control
Stress
CC-735


Chlorophyll b Content (µg/L)
3
2.5
2
1.5
1
0.5
0
Control
Stress
CC-125


Chlorophyll b Content (µg/L)
2.5
2
1.5
1
0.5
0
Control
Stress
CC-4033


Chlorophyll b content (µg/L)
6
5
4
3
2
1
0
Control
Stress
CC-1181


Chlorophyll b Content (µg/L)
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Stress
CC-1168


Chlorophyll b Content (µg/L)
10
9
8
7
6
5
4
3
2
1
0
Control
Stress
CC-1173

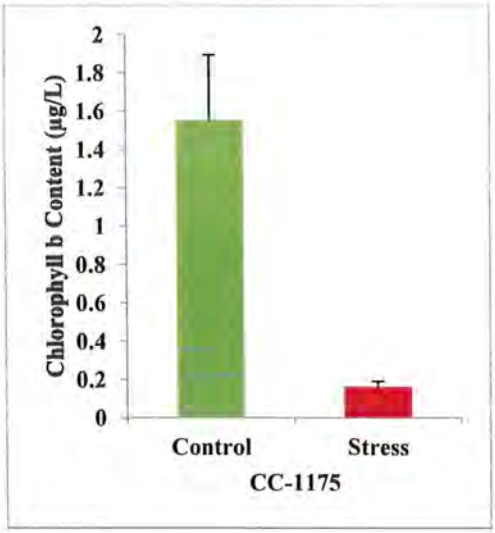
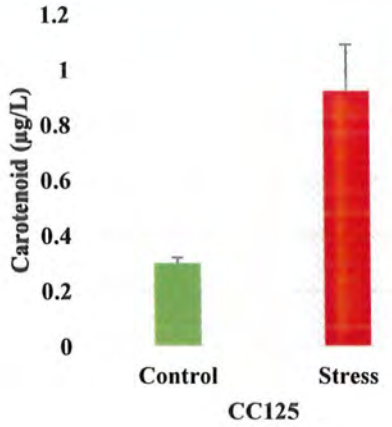


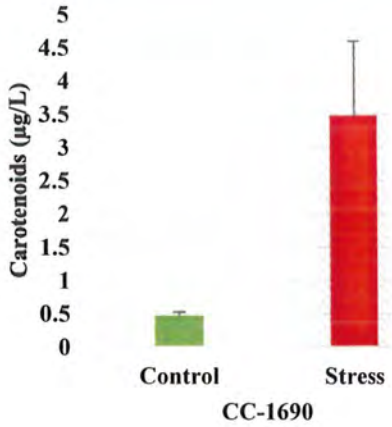
Figure 3.6: Effect of Temperature on Chlorophyll b Contents of Nine Selected Mutant Strains of *C. reinhardtii.* Green and red colored vertical bars represent the concentration of chlorophyll b content along y-axis control and stress respectively. Chlorophyll b contents of algae were expressed as micrograms per liter and the values corresponds to the mean of three independent replicates. Values indicate the average of three autonomous replicates ± s.e.

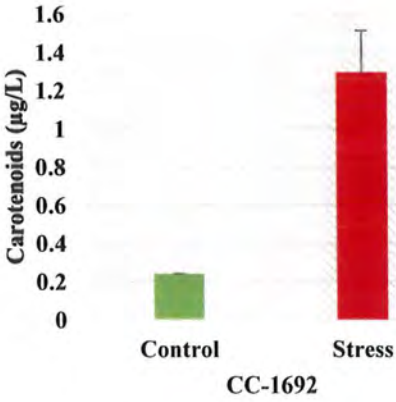
3.1.6 Carotenoids

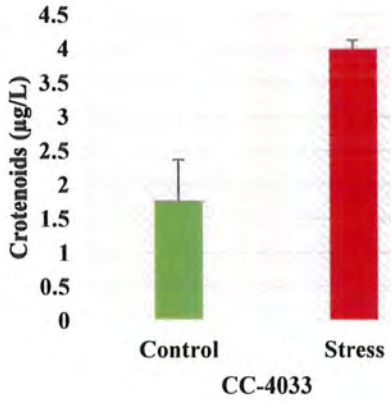
Carotenoids contribute to stabilize the structure and aid in the function of photosynthetic complexes in microalgae. They are involved in scavenging reactive oxygen species and dissipating excess energy. The intrinsic antioxidant activity of carotenoids constitutes the basis for their protective action against oxidative stress; however, not all biological activities claimed for carotenoids relate to their ability to inactivate free radicals and reactive oxygen species. The total carotenoids can be altered by physical factors, such as light density and temperature variability. The mutants of *C. reinhardtii* including, CC-125, CC-1690, CC-1692, CC-4033, CC-1173, CC-1168 and CC-735 were observed carefully for difference in the carotenoids at 33 °C and found to have higher contents as compared to their respective controls at 25 °C. Mutant of *C. reinhardtii* including, CC-1181 and CC-1175 was observed to have less carotenoids at 33 °C as compared to their respective controls at 25 °C.

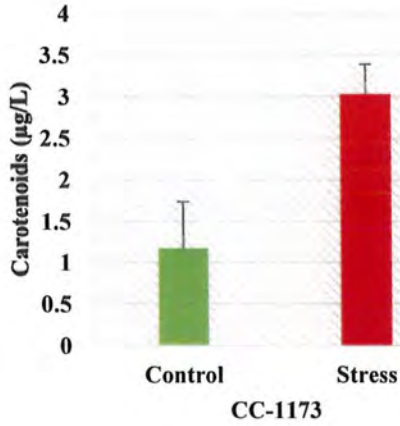
Our data showed that total carotenoids in the selected strains were highly variable under the selected growth conditions of algae. The highest average total carotenoids was observed in the CC-1690 (3.466 ± 1.13 μgl^{-1}), while the lowest value was recorded in CC-125 (0.92 ± 0.17 μgl^{-1}) with respect to values of their controls (0.457 ± 0.06 and 0.3 ± 0.02 μgl^{-1}) respectively (Fig. 3. 7, Appendix S2). Mutant strains CC-1181 was affected more as its carotenoids content was much lower with respect to its control. On the other hand in strains CC-125, CC-1690, CC-1692, CC-735, CC-4033, CC-1168, CC-1173 and CC-1175 there was increase observed in carotenoids contents. According to our data CC-125, CC-1690, CC-1692, CC-735, CC-4033, CC-1168, CC-1173 and CC-1175 stood better in carotenoids contents at 33 °C.

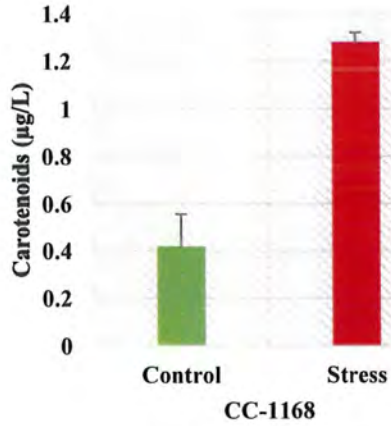

Carotenoid (µg/L)
1.2
1
0.8
0.6
0.4
0.2
0
Control
Stress
CC125


Carotenoids (µg/L)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Stress
CC-1690


Carotenoids (µg/L)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Stress
CC-1692


Crotenoids (µg/L)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Stress
CC-4033


Carotenoids (µg/L)
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Stress
CC-1173


Carotenoids (µg/L)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Stress
CC-1168

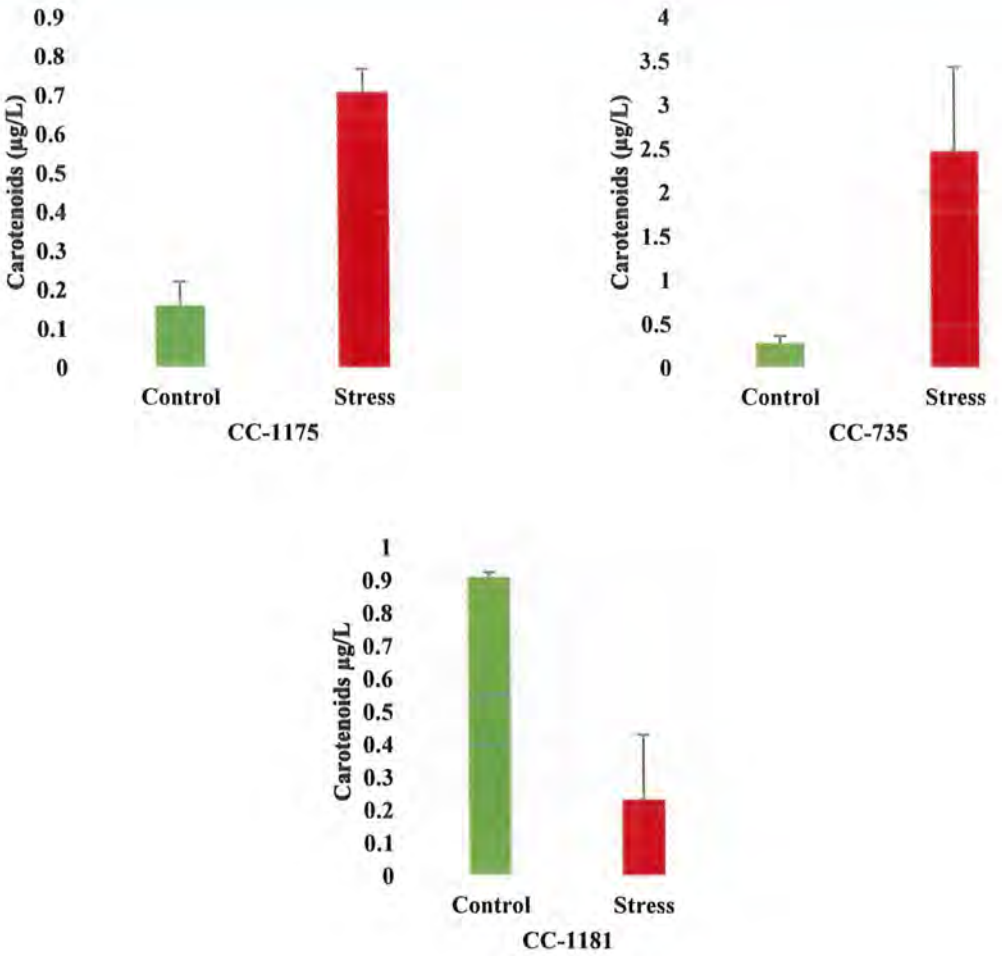


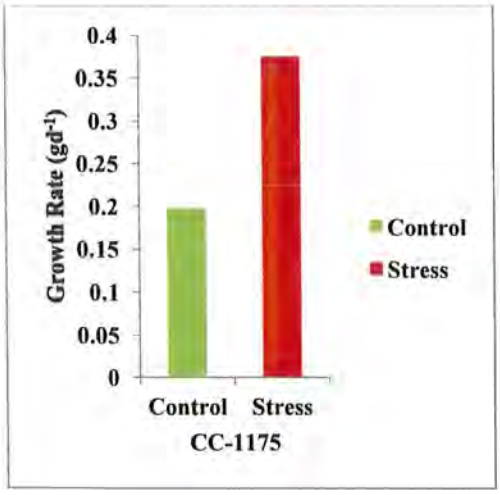
Figure 3.7: Effect of Temperature on Carotenoids Contents of Nine Selected Mutant Strains of *C. reinhardtii.* Green and red colored vertical bars represent the concentration of carotenoids along y-axis control and stress respectively. Carotenoids contents of algae were expressed as micrograms per liter and the values corresponds to the mean of three independent replicates. Values indicate the average of three autonomous replicates ± s.e.

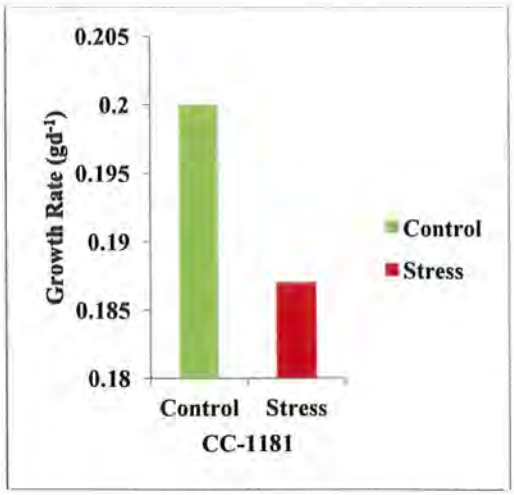
3.1.7 Growth rate

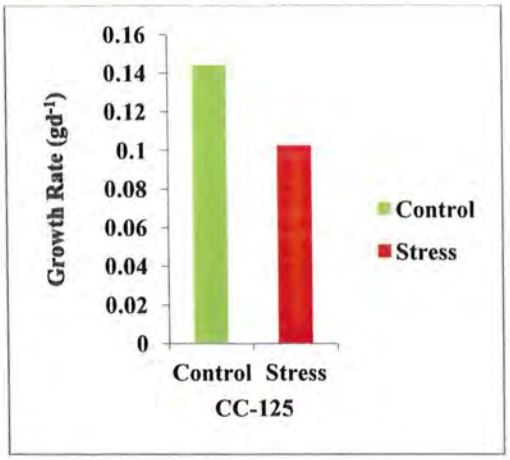
Even though microalgae are capable to stay alive in various temperatures, optimum temperature for growth and development is restricted to a range (20–30 °C). Basically, at optimum range of temperature, increase in temperature promoted the biomass production of microalgae. Increase in temperatures beyond the optimum range leads to cease growth rate, in severe conditions, even kill microalgae cells. The growth rate can be altered by

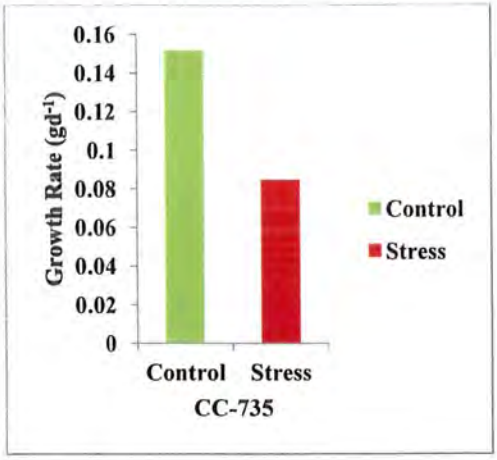
physical factors, such as light density and temperature variability. The mutant of *C. reinhardtii* including CC-1175 was observed carefully for difference in growth rate treated at 33 °C and found to have increase in its growth rate as compared to its respective control at 25 °C. Mutant of *C. reinhardtii* including, CC-1181, CC-125, CC-735, CC-4033, CC-1168, CC-1690, CC-1173 and CC-1692 were observed to have decrease in their growth rates at 33 °C as compared to their respective controls at 25 °C (Fig. 3.8).

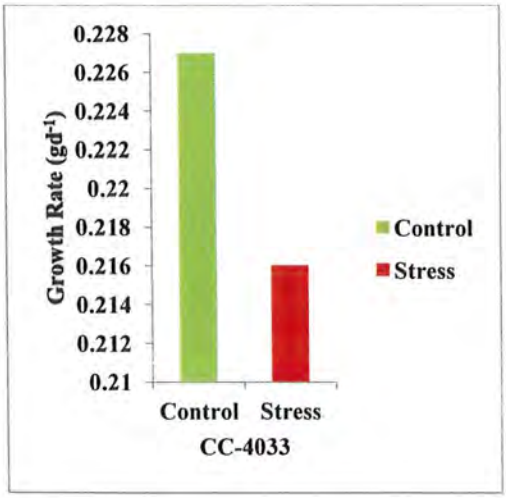
Our data showed that the growth rate in the selected strains were highly variable under the selected growth conditions of algae. The highest growth rate was observed in the CC-1175 (0.375 gd^{-1}), with respect to values of its control (0.198 gd^{-1}) (Appendix S2). Mutant trains CC-1181, CC-1173 and CC-1692 were affected more as their growth rates were much lower with respect to their controls. On the other hand strains CC-125, CC-4033, CC-1168, CC-1690 and CC-735 were affected least in term of their growth rates. While there was an increase observed in growth rate of strain CC-1175. According to our data CC-1175 stood better than all the remaining strains at 33 °C.

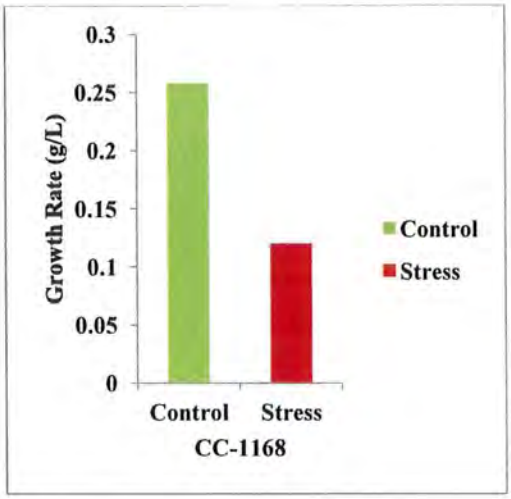


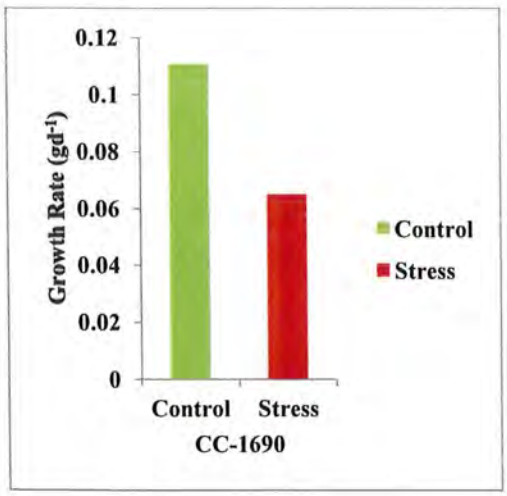


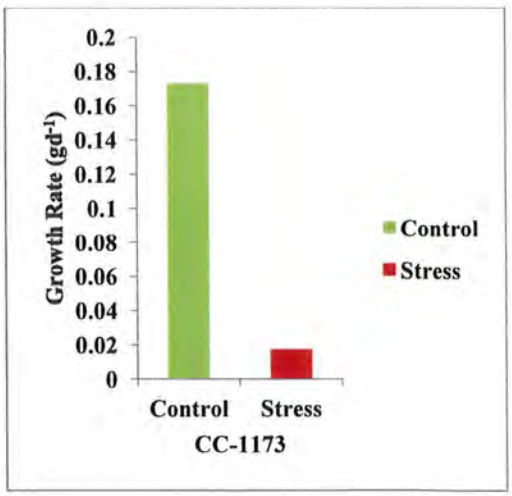

Growth Rate (gd-1)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
Stress
Control Stress
CC-125


Growth Rate (gd-1)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
Stress
Control Stress
CC-735


Growth Rate (gd-1)
0.228
0.226
0.224
0.222
0.22
0.218
0.216
0.214
0.212
0.21
Control
Stress
Control Stress
CC-4033


Growth Rate (g/L)
0.3
0.25
0.2
0.15
0.1
0.05
0
Control
Stress
Control Stress
CC-1168


Growth Rate (gd-1)
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
Stress
Control Stress
CC-1690


Growth Rate (gd-1)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
Stress
Control Stress
CC-1173

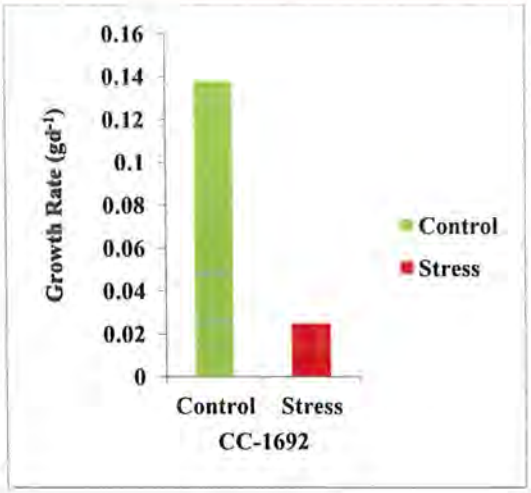


Figure 3.8: Effect of Temperature on Growth Rate of Eleven Selected Mutant Strains of *C. reinhardtii.* Green and red colored vertical bars represent the growth rate along y-axis control and stress respectively. Growth rate expressed as grams per day. Values correspond to the mean of three independent replicates. Values indicate the average of three autonomous replicates ± s.e.

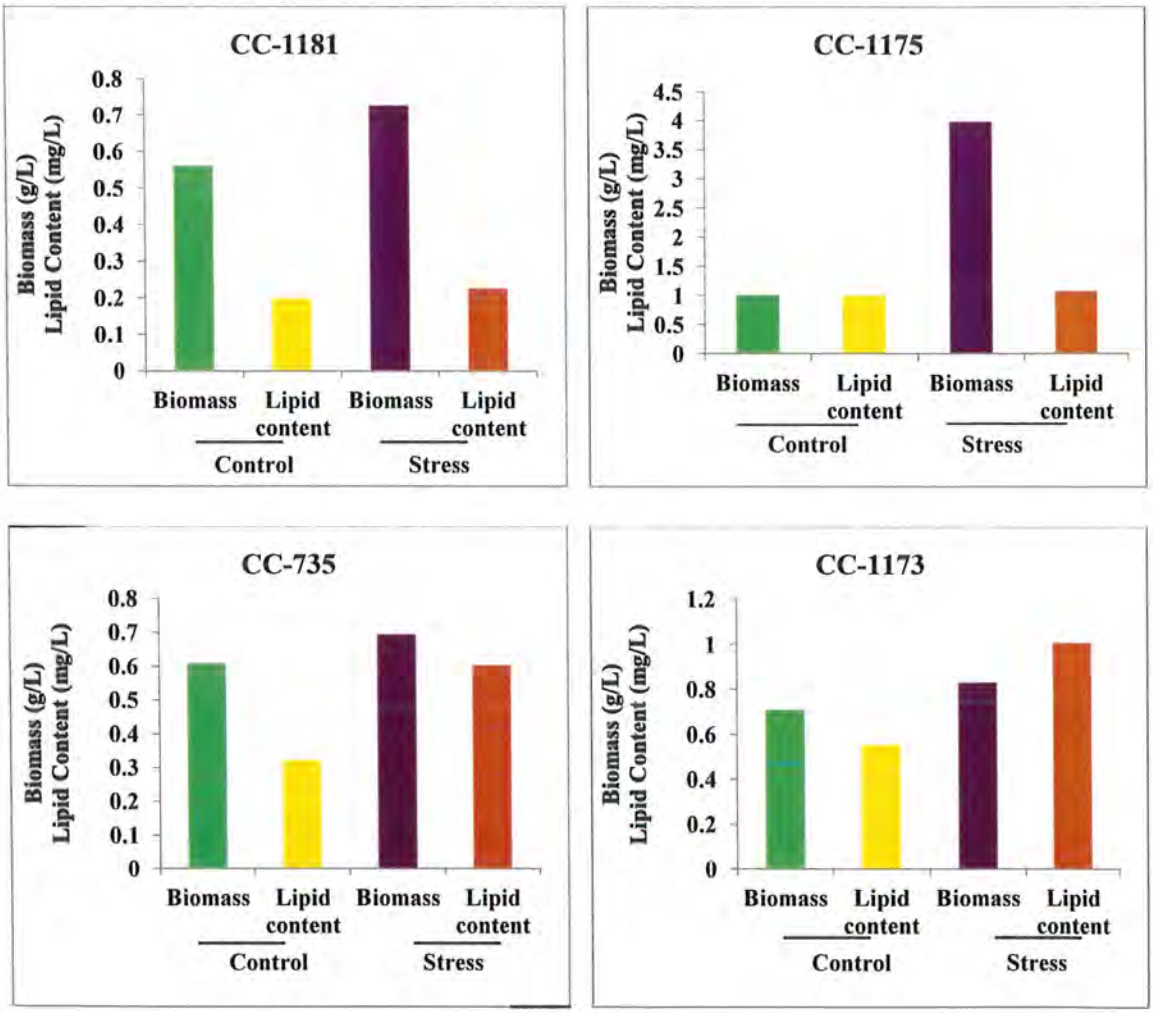
3.1.8 Biomass and Lipid Content

Temperature is able to affect the biomass accumulation and lipid content of microalgae. Numerous microalgae had been originated to increase their lipid content up to 50 % of their dry weight with increasing temperature. Hence, a proper increase in temperature appear to support generation of high quantity (increase in total productivity of lipid) and high quality (high saturation degrees of fatty acids) biodiesels. In order to analyze data more precisely we divided results in following four categories (A, B and C)

A. Strains in Which Both Biomass Accumulation and Lipid Content Increases

The lipid content and biomass accumulation can be altered by physical factors, such as light density and temperature variability. The mutant of *C. reinhardtii* including, CC-1181, CC-735, CC-1690, CC-1173 and CC-1175 were observed carefully for difference in biomass accumulation and lipid contents treated at 33 ^{o}C and found to be increased, as compared to their respective controls at 25 ^{o}C.

Our data showed that the biomass and lipid contents in the selected strains were highly variable under the selected growth conditions of algae. The highest average total biomass in CC-1175 (3.97 g/L), while the lowest value was recorded in CC-1173 that is 0.828 gl^{-1} with respect to values of their control 1.00 g/L and 0.7062 g/L respectively. The highest average total lipid contents was observed in the CC-1173 (1.0048 g/L), while the lowest value was recorded in CC-1181 that is 0.225 gl^{-1} with respect to values of their control 0.5538 g/L and 0.199 g/L respectively (Appendix S2).



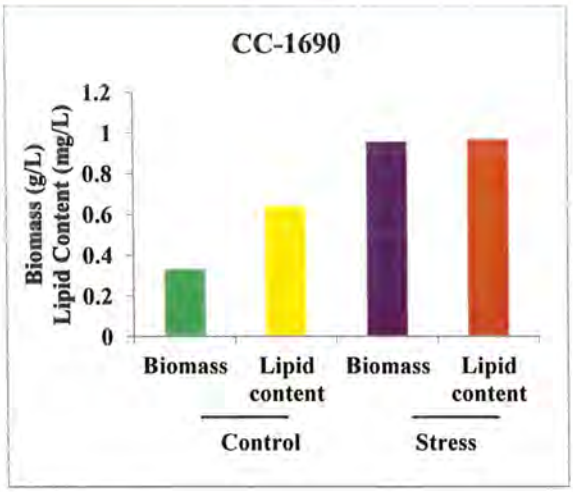


Figure 3.9: Biomass accumulation and Lipid Content in CC-1181, CC-735, CC-1690, CC-1173 and CC-1175 Subjected to High Temperature Stress. Green and purple colored vertical bars represent biomass, while yellow and brown colored vertical bars represent the lipid content along y-axis. The results of the biomass analysis are expressed on g/L. Values correspond to the mean of three independent replicates. Values indicate the average of three autonomous replicates ± s.e.

B. Strain in Which Lipid Content Increases and Biomass Accumulation Decreases

Mutant of *C. reinhardtii* CC-4033 was detected to have raise in lipid content and reduction in biomass accumulation at 33 °C as compared to their respective control at 25 °C. The lipid content was increased in stress (0.6774 g/L) treated at 33 °C, as compared to control (0.5846 g/L) treated at 25 °C. (Appendix S2). While the biomass accumulation found to be decreased from 1.5952 g/L in control to 0.5846 g/L in heat stress.

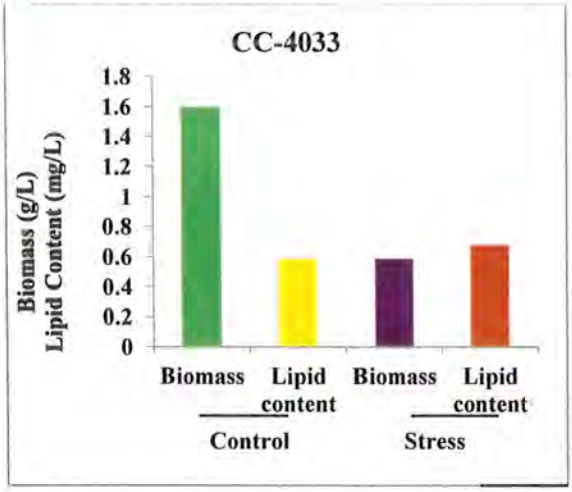
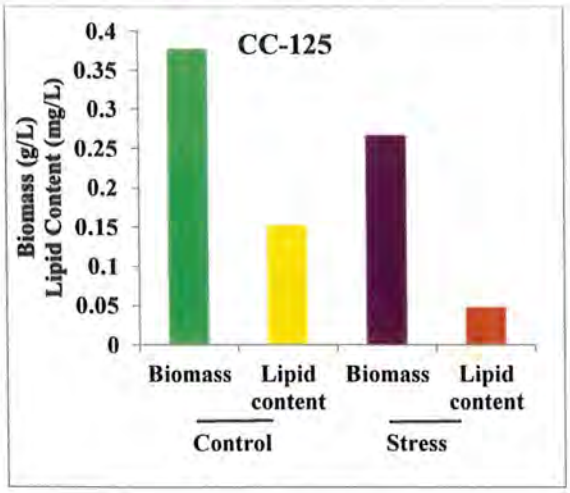
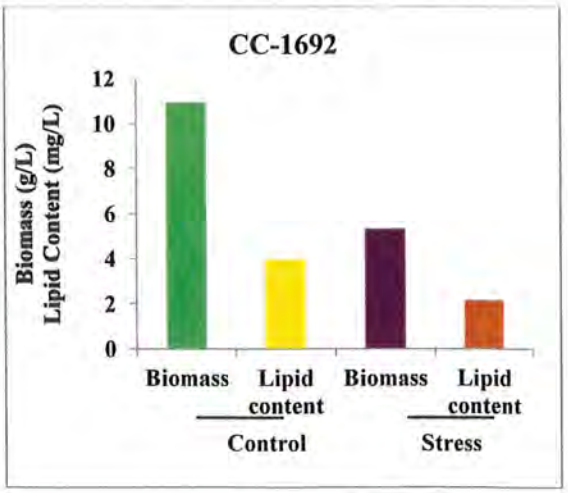


Figure 3.10: Biomass Accumulation and Lipid Content in CC-4033 Subjected to High Temperature Stress. Green and purple colored vertical bars represent biomass, while yellow and brown colored vertical bars represent the lipid content along y-axis. The results of the biomass analysis are expressed on g/L. Values correspond to the mean of three independent replicates. Values indicate the average of three autonomous replicates ± s.e.

C. Strains in Which Both Biomass Accumulation and Lipid Content Decreases

Mutant of *C. reinhardtii* CC-125, CC-1692, and CC-1168 was observed to have decrease in both biomass accumulation and lipid contents at 33 °C as compared to their respective controls at 25 °C.





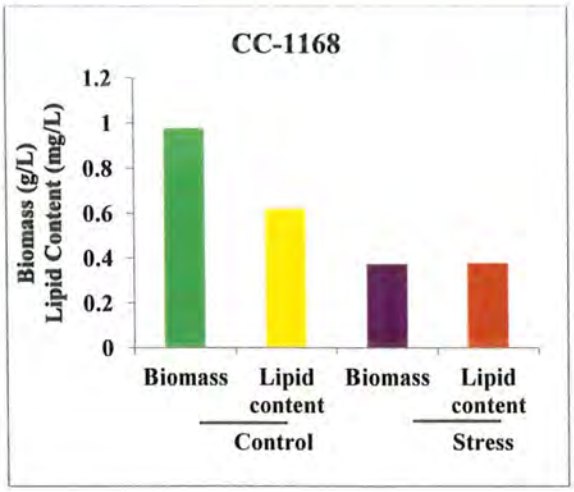


Figure 3.11: Biomass Accumulation and Lipid Content in CC-125, CC-1692 and CC-1168 Subjected to High Temperature Stress. Green and purple colored vertical bars represent biomass, while yellow and brown colored vertical bars represent the lipid content along y-axis. The results of the biomass analysis are expressed on g/L. Values correspond to the mean of three independent replicates. Values indicate the average of three autonomous replicates ± s.e.

3.1.9 FTIR Analysis

On the basis of infrared absorption of functional groups FTIR spectra shows the macromolecular composition of the biomass. Therefore it allows the detection of variations in the relative abundance of organic pool such as carbohydrate, lipid and protein. FTIR spectra have been used as a fingerprint of the biochemical composition of the microalgal cell (Jebsen *et al.*, 2012; Stehfest *et al.*, 2005). There are three main regions that relate to macromolecular pools, according to (Giordano *et al.*, 2001) the lipid band (around 1740 cm^{-1}), the amide I and amide II bands representing proteins (around 1660 and around 1540 cm^{-1}) and the carbohydrate region (1200 – 900 cm^{-1}).

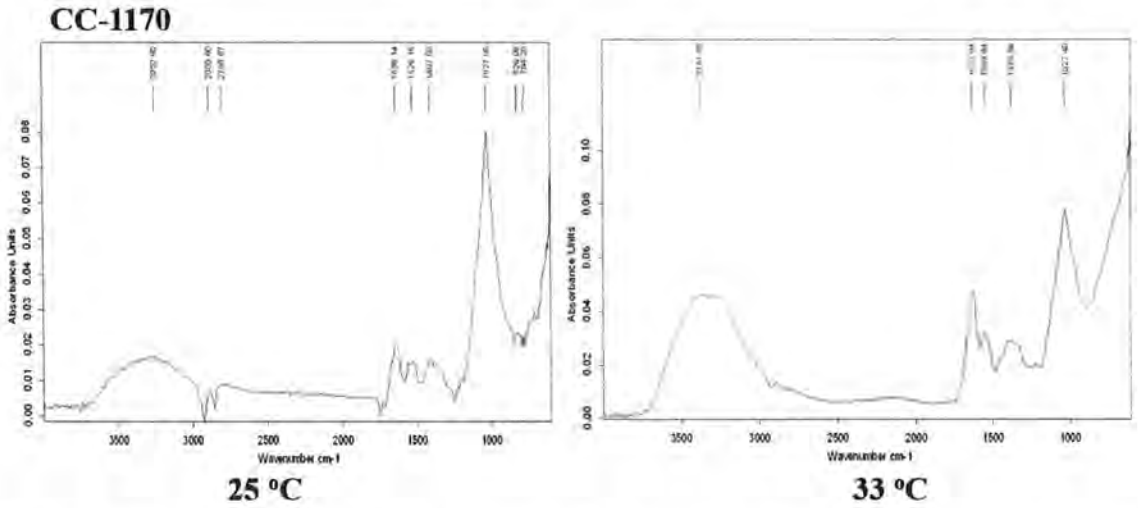


Figure 3.12: FTIR spectrum in CC-1170 Subjected to High Temperature Stress of 33 °C. Each peak was assigned to a functional group. In control at 25 °C nine peaks while in stress at 33 °C six different peaks are observed showing different functional groups which are shown in the table below. In stress absorbance value of functional group has changed to some extent and the intensity of some functional groups has increased while for others has decreased. In the spectrum, the region of CO_2 from the atmosphere is not presented.

Table 3.1

CC-1170					
Wavenumber cm–1		**Bond**		**Functional group**	
Control	**Stress**	**Control**	**Stress**	**Control**	**Stress**
3252.65	3374.95	O–H stretch	O–H stretch, H–bonded	alcohols, phenols	alcohols, phenols
2885.60	1633.94	C–H stretch	N–H bend	Alkanes	1° amines
2795.87	1549.44	H–C=O: C–H stretch	N–O asymmetric stretch	aldehydes	nitro compoun ds
1638.14	1379.96	N–H bend	C–H rock	1° amines	Alkanes
1526.16	1027.40	N–O asymmetric stretch	C–O stretch	nitro compounds	alcohols, carboxyli c acids, esters, ethers
1407.03		C–C stretch		(in–ring) aromatics	
1027.06		C–O stretch		alcohols, carboxylic acids, esters, ethers	
826.08		C–Cl stretch		alkyl halides	
784.20		C–Cl stretch		alkyl halides	

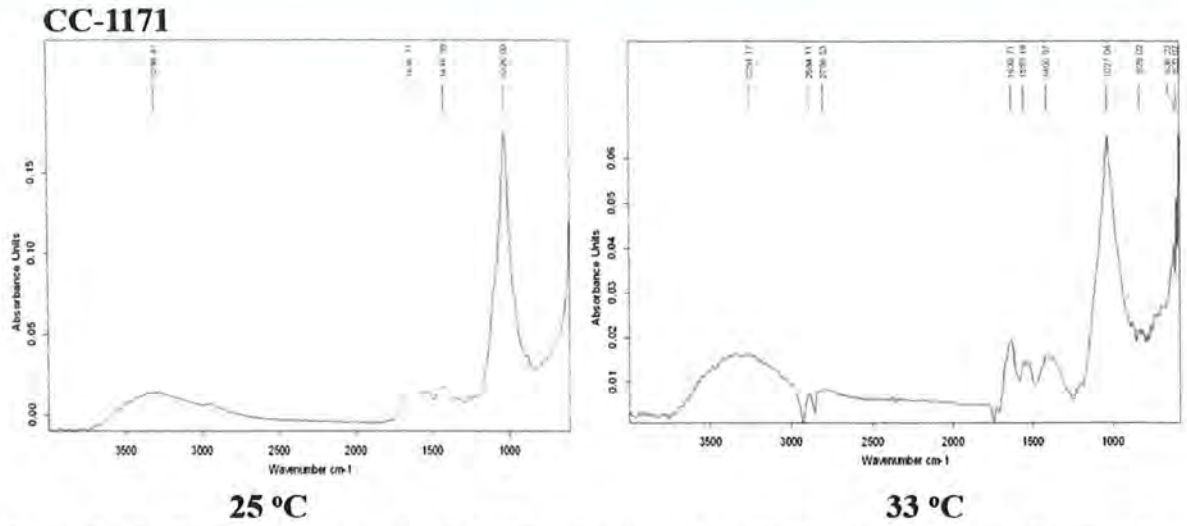


Figure 3.13: FTIR spectrum in CC-1171 Subjected to High Temperature Stress of 33 °C. Each peak was assigned to a functional group. In control at 25 °C four peaks while in stress at 33 °C nine different peaks are observed showing different functional groups which are shown in the table below. In stress absorbance value of functional group has changed to some extent and the intensity of some functional groups has increased while for others has decreased. In the spectrum, the region of CO_2 from the atmosphere is not presented.

Table 3.2

CC-1171					
Wavenumber cm–1		**Bond**		**Functional group**	
Control	**Stress**	**Control**	**Stress**	**Control**	**Stress**
3298.41	3254.71	O–H stretch	O–H stretch	carboxylic acids	carboxylic acids
1636.71	2884.11	N–H bend	C–H stretch	1° amines	Alkanes
1416.35	2796.53	C–C stretch	H–C=O: C–H stretch	(in–ring) aromatics	Aldehydes
1026.00	1638.71	C–O stretch	N–H bend	alcohols, carboxylic acids, esters, ethers	1° amines
	1553.19		N–O asymmetric stretch		nitro compounds
	1406.57		C–C stretch		(in–ring) aromatics
	1027.04		C–O stretch		alcohols, carboxylic acids, esters, ethers
	828.02		C–Cl stretch		alkyl halides
	636.23		–C≡C–H: C–H bend		Alkynes
	620.07		–C≡C–H: C–H bend		Alkynes

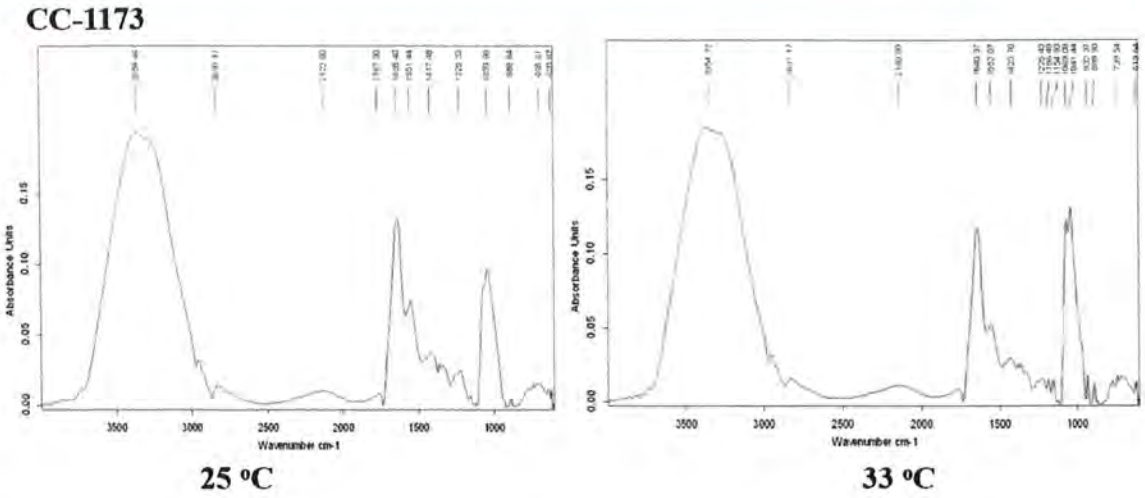


Figure 3.14: FTIR spectrum in CC-1173 Subjected to High Temperature Stress of 33 °C. Each peak was assigned to a functional group. In control at 25 °C and stress at 33 °C twelve different peaks are observed showing different functional groups which are shown in the table below. In stress absorbance value of functional group has changed to some extent and the intensity of some functional groups has increased while for others has decreased. In the spectrum, the region of CO_2 from the atmosphere is not presented.

Table 3.3

CC-1173			
Wavenumber cm–1		**Bond**	**Functional group**
Control	**Stress**		
3359.45	3354.72	N–H stretch	primary, secondary amines, amides
1637.87	1639.97	N–H bond	primary amines
1037.98	1040.72	C–N stretch, C–O stretch	aliphatic amines alcohols, carboxylic acids, esters, ethers

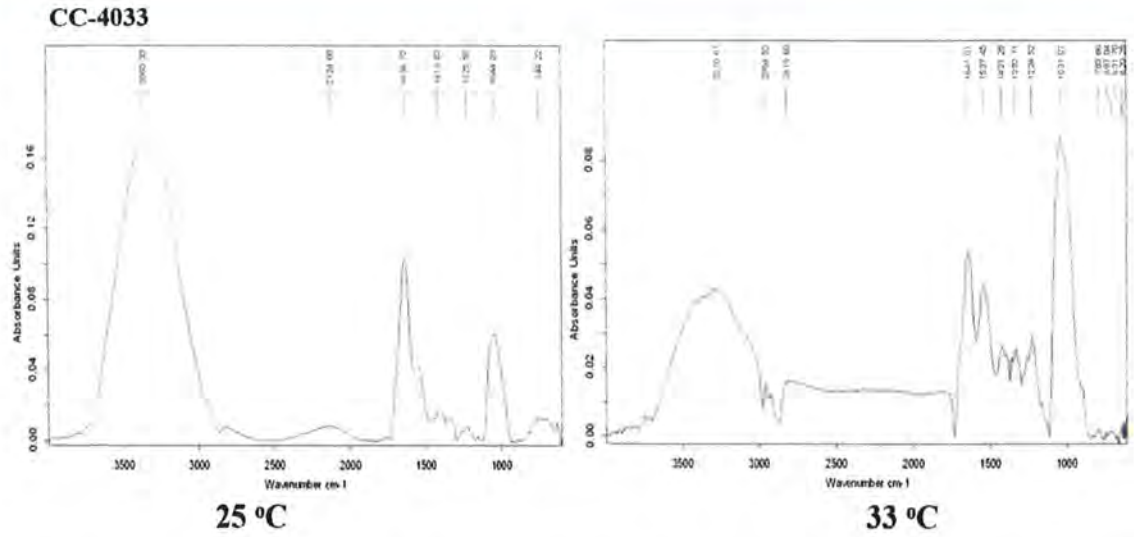


Figure 3.15: FTIR spectrum in CC-4033 Subjected to High Temperature Stress of 33 °C. Each peak was assigned to a functional group. In control at 25 °C seven different peaks while in stress at 33 °C twelve different peaks are observed showing different functional groups which are shown in the table below. In stress absorbance value of functional group has changed to some extent and the intensity of some functional groups has increased while for others has decreased. In the spectrum, the region of CO_2 from the atmosphere is not presented.

Table 3.4

Stress CC-4033					
Wavenumber cm–1		**Bond**		**Functional group**	
Control	**Stress**	**Control**	**Stress**	**Control**	**Stress**
3363.30	3270.81	O–H stretch, H–bonded	N–H stretch	alcohols, phenols	1°, 2° amines, amides
1635.72	2815.67	N–H bend	H–C=O: C–H stretch	primary amines	Aldehydes
1041.46	1640.95	C–N stretch, C–O stretch	N–H bend	aliphatic amines alcohols, carboxylic acids, esters, ethers	primary amines
	1537.41		N–O asymmetric stretch		nitro compounds
	1332.62		N–O symmetric stretch		nitro compounds
	1224.44		C–N stretch		aliphatic amines
	1028.68		C–O stretch		alcohols, carboxylic acids, esters, ethers

3.2 Response to Nitrogen limitation of *C. reinhardtii*

Macronutrients and micronutrients are the nutrients supplied to microalgal cultures. Inadequacy of nutrients might affect structural and physiological variations of microalgal cells, hence decreases the developmental rates and production of biomass. Nitrogen and phosphorus are the two very significant supplements necessary for microalgal growth, and the ratio of nitrogen to phosphorus could control the starvation of nutrients. Nitrogen is involved in the biosynthesis of nucleus acids, proteins, and photosynthetic pigments. Inadequacy of nitrogen reduced the manufacture of photosynthetic proteins and pigments, hence impact the productivity of microalgal biomass.

To analyze the effect of nitrogen (N) stress on *C. reinhardtii* grown under photoautotrophic conditions, we pre-cultured the all the strains under experiments to middle logarithmic phase in MS media with different concentrations of nitrogen. Cells were then grown under photoautotrophic conditions for 14 days. Cultures were examined on every 2nd day for specific analyses.

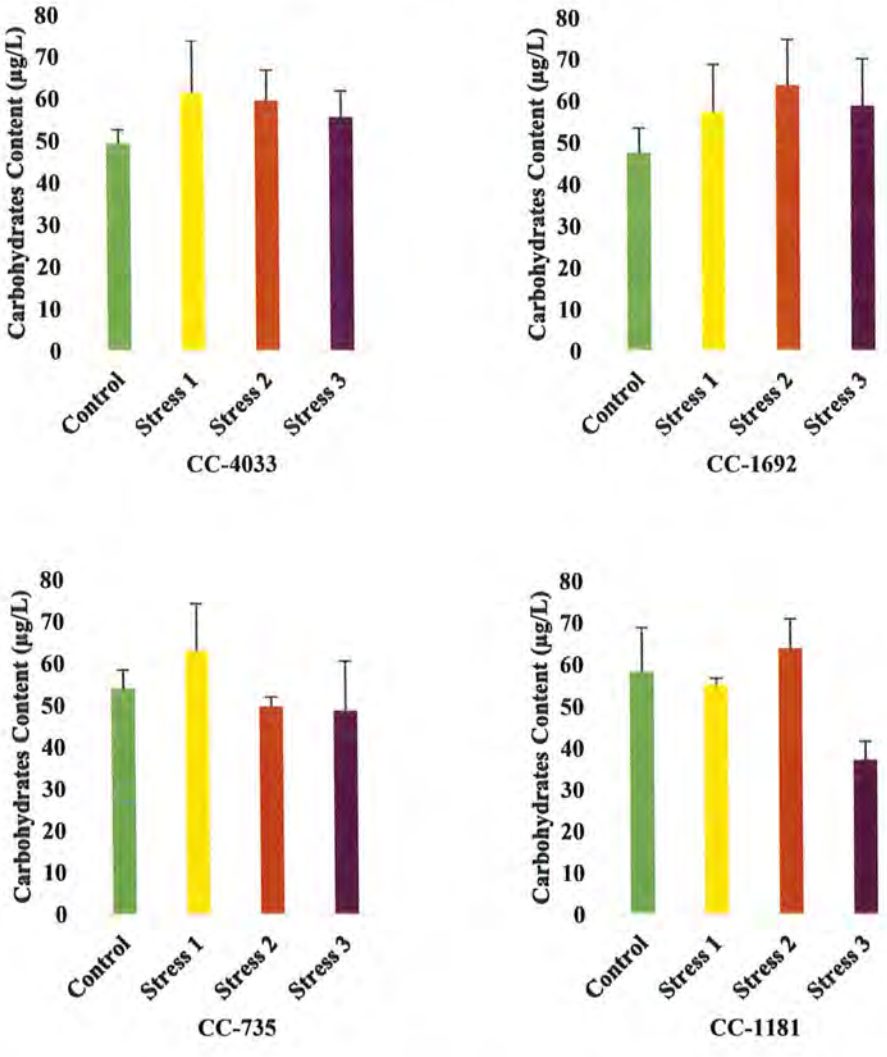
3.2.1 Carbohydrate Content

When microalgae were harvested in condition of nutrient limitation, it changes their metabolic system and biochemical composition. Inadequacy of nitrogen moreover promotes rise in biosynthesis of carbohydrates in several species of microalgae, up to four fold increase in carbohydrate contents.

The mutants of *C. reinhardtii* including CC-735 and CC-1676 were observed carefully for difference in the carbohydrate content at different nitrogen concentrations and seems to have higher content at 0.125 mg/L while CC-1181 appeared to have higher content of total carbohydrate at 0.25 mg/L of nitrogen concentrations as compared to the respective controls grown having normal concentration of nitrogen. Mutants of *C. reinhardtii* CC-4033 and CC-1692 appeared to be got increased in carbohydrate content in all the given concentrations (0.125, 0.25, 0.5 mg/L) of nitrogen as compared to the strains grown in MS media having normal concentration of nitrogen. In CC-1170, CC-1173 and CC-125 carbohydrate content appeared to be got decreased when cultured with media having

higher concentration of nitrogen as compared to the strains grown in MS media having normal concentration of nitrogen (Fig 3.12).

Our data showed that the highest average total carbohydrate content in the CC-1692 ($63.66 \pm 11.0\ \mu gl^{-1}$) under 0.25 mg/L NH_4NO_3 concentration, while the lowest value was recorded in CC-1676 ($49.35 \pm 2.1\ \mu gl^{-1}$) under 0.50 mg/L NH_4NO_3 concentration with respect to values of their controls (47.64 ± 5.8 and $48.16 \pm 10.0\ \mu gl^{-1}$) respectively (Appendix S3). Mutant strains CC-4033 and CC-1692 stood better in carbohydrate contents than the remaining strains as they got increased in all concentrations of nitrogen.



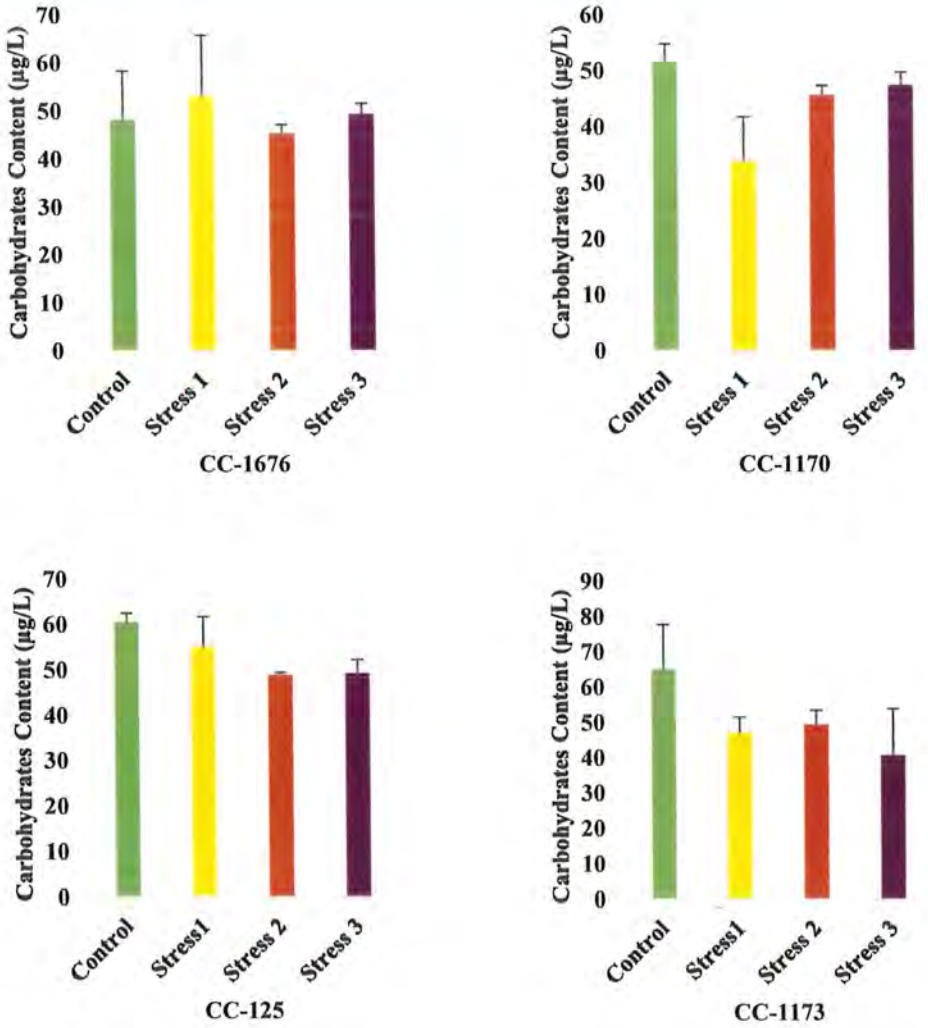


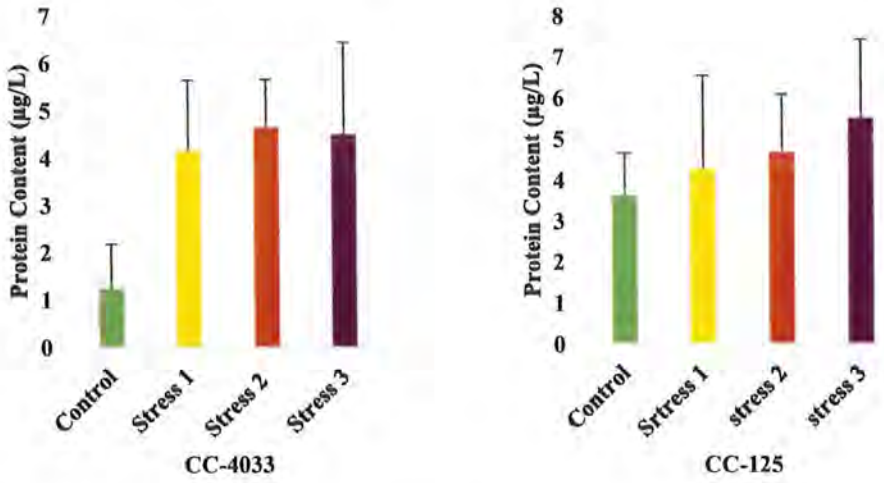
Figure 3.16: Effect of Nitrogen Concentration on Carbohydrate of Eight Selected Mutant Strains of *C. reinhardtii*. Carbohydrate content (μgL^{-1}) of mutants strains of *C. reinhardtii* exposed to different NH_4NO_3 concentrations. Green bars represent treatments without NH_4NO_3, yellow bars 0.125 mg L^{-1}, brown bars represent 0.25 mg L^{-1}, while blue bars represents 0.50 mg L^{-1} of NH_4NO_3. Error bars represent standard deviation for n=3.

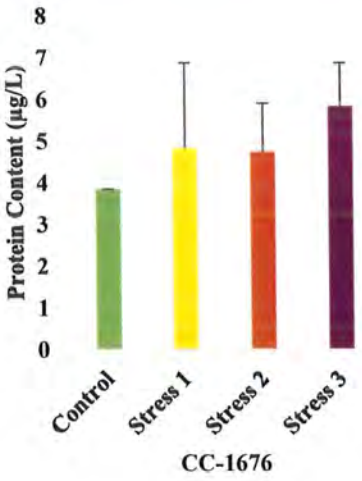
3.2.2 Protein Content

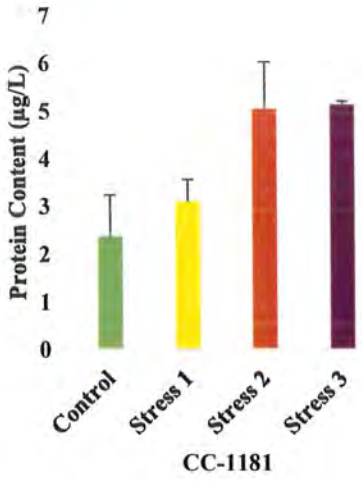
Nitrogen is critical for protein biosynthesis. Inadequacy of nitrogen reduces the production of photosynthetic proteins and pigments, hence impacts the productivity of microalgal biomass. Comprehensive analyses and nutritional studies have demonstrated that these algal proteins are of high quality and comparable to conventional vegetable proteins.

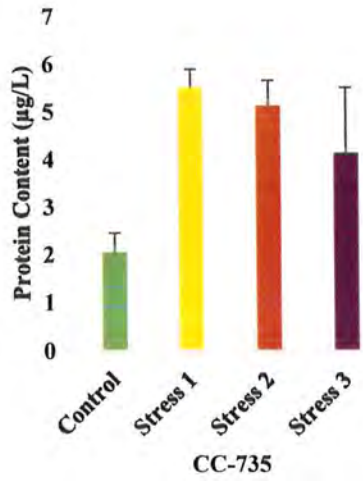
The mutants of *C. reinhardtii* were observed carefully for difference in protein content under different nitrogen concentrations. Strain CC-735 was observed to have higher protein content at 0.125 mg/L of nitrogen, while 0.25 mg/L of nitrogen appeared to be more effective in CC-4033. The remaining mutant strains CC-1173, CC-125, CC-1692, CC-1676, CC-1181 and CC-1170 appeared to achieve high protein content at 0.50 mg/L of nitrogen added to growth media (Fig 3.13).

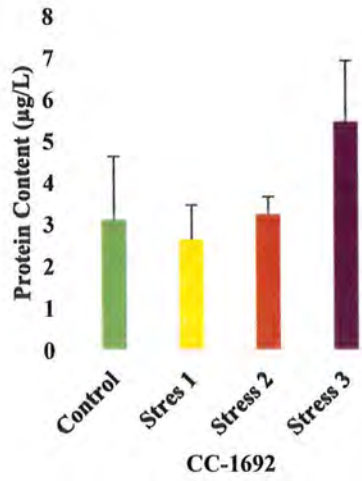
Our data showed that the total protein contents in the selected strains were highly variable under the selected growth conditions of algae. The highest average total protein content in the CC-735 ($5.5066 \pm 0.38\ \mu gl^{-1}$) under 0.125 mg/L NH_4NO_3 concentration, while the lowest value was recorded in CC-1173 ($7.477 \pm 0.58\ \mu gl^{-1}$) under 0.50 mg/L NH_4NO_3 concentration with respect to values of their controls ($2.048 \pm 0.40\ \mu gl^{-1}$ and $7.047 \pm 0.24\ \mu gl^{-1}$) respectively (Appendix S3).




Protein Content (µg/L)
0
1
2
3
4
5
6
7
8
Control
Stress 1
Stress 2
Stress 3
CC-1676


Protein Content (µg/L)
0
1
2
3
4
5
6
7
Control
Stress 1
Stress 2
Stress 3
CC-1181


Protein Content (µg/L)
0
1
2
3
4
5
6
7
Control
Stress 1
Stress 2
Stress 3
CC-735


Protein Content (µg/L)
0
1
2
3
4
5
6
7
8
Control
Stres 1
Stress 2
Stress 3
CC-1692

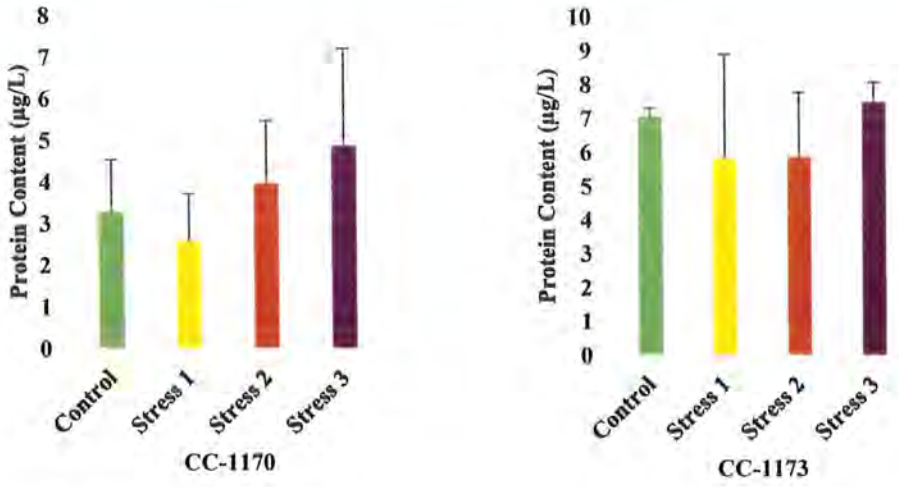


Figure 3.17: Effect of Nitrogen Concentration on Protein Contents of Selected Mutant Strains of *C. reinhardtii*. Protein contents (μgL^{-1}) of mutant strains of *C. reinhardtii* exposed to different NH_4NO_3 concentrations. Green bars represent treatments without NH_4NO_3, yellow bars represent 0.125 mg L^{-1}, brown bars represent 0.25 mg L^{-1}, while blue bars represents 0.50 mg L^{-1} of NH_4NO_3. Error bars represent standard deviation for n=3.

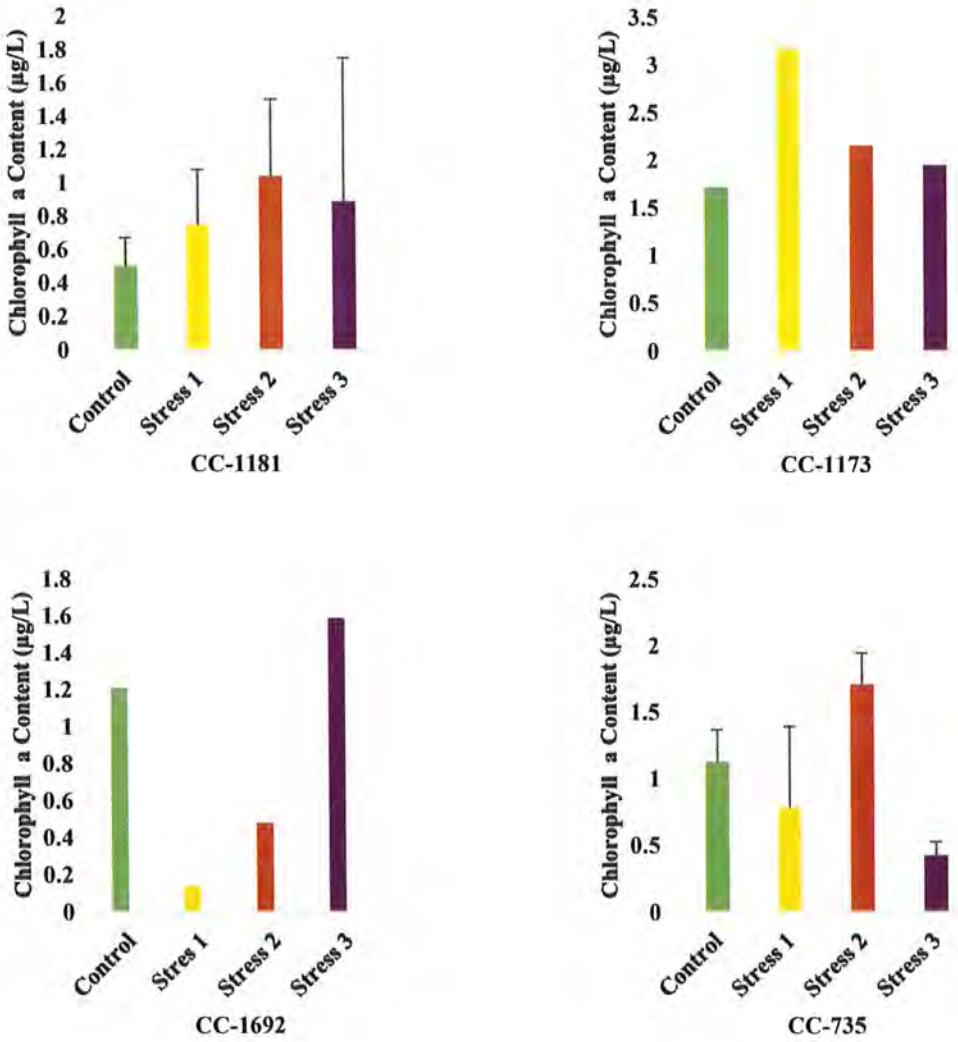
3.2.3 Chlorophyll a Content

The pigment content in microalgae is a specific feature of each species. Chlorophyll a is generally the factor being used as the tropic indicator, mostly for the reason that the association between the content of this pigment and the quantity of algal biomass is quite direct. Spectrophotometric analysis referred as a good and practical tool for chlorophyll evaluation.

The mutants of *C. reinhardtii* were observed carefully for difference in chlorophyll a content under different nitrogen concentrations. The total chlorophyll a contents can be altered by physical factors, such nitrogen limitation, light density and temperature variability. Chlorophyll a content in CC-1173 appeared to have higher value at 0.125 mg/L of nitrogen while 0.25 mg/L of nitrogen concentration appeared to be more effective in CC-735 and CC-1181. On the other hand in strain CC-1690, CC-1676 and CC-1170 there was a maximum increase observed at 0.50 mg/L concentration of nitrogen. In CC-125 and CC-4033 there was no such increase observed under different

nitrogen concentrations as compared to the strains grown in MS media having normal concentration of nitrogen (Fig 3.14).

Our data showed that the total chlorophyll a contents in the selected strains were highly variable under the selected growth conditions of algae. The highest average total chlorophyll a content in the CC-1173 ($3.164 \pm 0.00\ \mu gl^{-1}$) under 0.125 mg/L NH_4NO_3 concentration, while the lowest value was recorded in CC-1676 ($0.42 \pm 0.10\ \mu gl^{-1}$) under 0.50 mg/L NH_4NO_3 concentration with respect to values of their controls ($1.714 \pm 0.00\ \mu gl^{-1}$ and $0.3366 \pm 0.03\ \mu gl^{-1}$) respectively (Appendix S3).



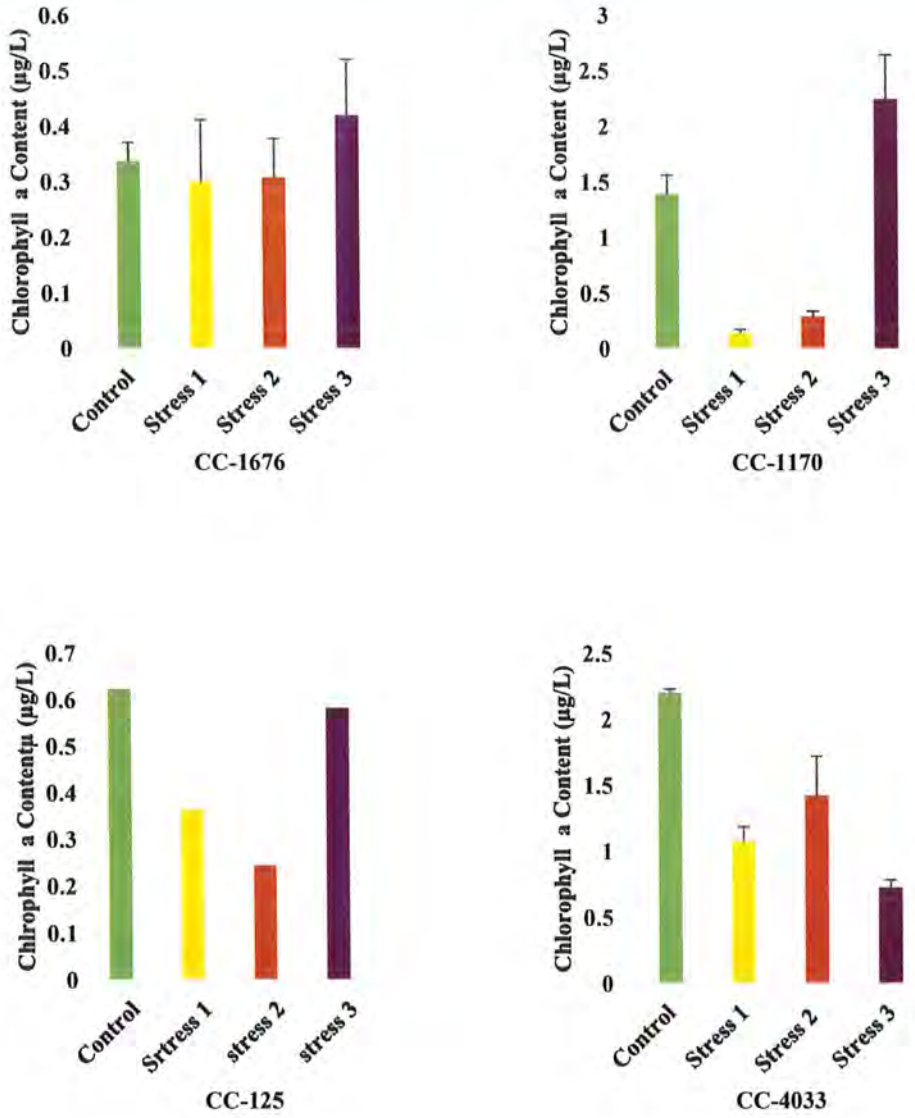
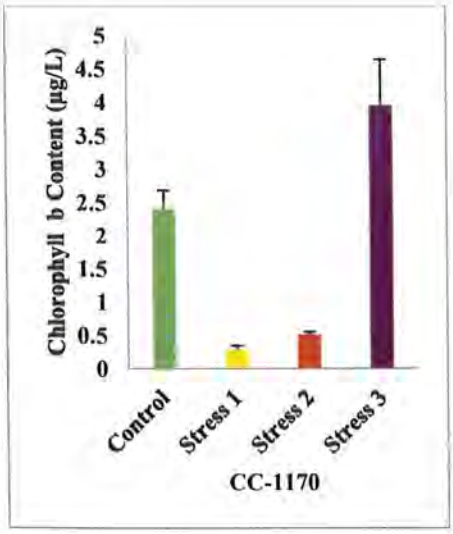


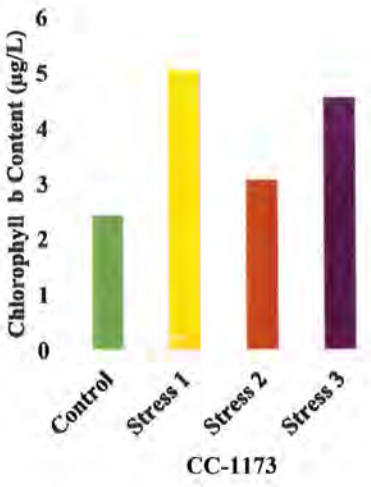
Figure 3.18: Effect of Nitrogen Concentration on Chlorophyll a Content of Eight Selected Mutant Strains of *C. reinhardtii*. Chlorophyll a content (μgL^{-1}) of mutants strains of *C. reinhardtii* exposed to different NH_4NO_3 concentrations. Green bars represent treatments without NH_4NO_3, yellow bars represent 0.125 mg L^{-1}, brown bars represent 0.25 mg L^{-1}, while blue bars represents 0.50 mg L^{-1} of NH_4NO_3. Error bars represent standard deviation for n=3.

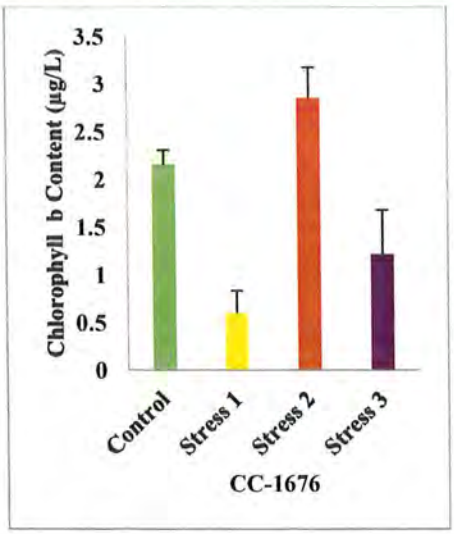
3.2.4 Chlorophyll b Content

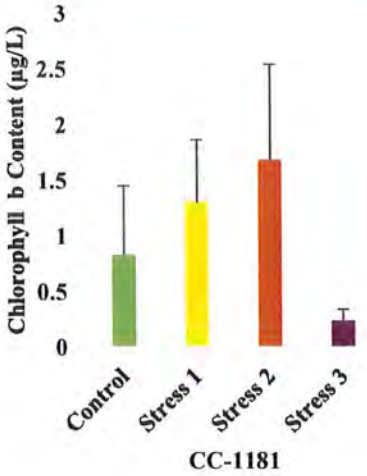
The mutants of *C. reinhardtii* were observed carefully for difference in chlorophyll b content under different nitrogen concentrations. The total chlorophyll b contents can be altered by physical factors, such nitrogen limitation, light density and temperature variability. Chlorophyll b content in CC-1173 appeared to have higher value at 0.125 mg/L of nitrogen while 0.25 mg/L of nitrogen appeared to be more effective in CC-735 and CC-1181 and CC-1676. On the other side in strain CC-125, CC-1692 and CC-1170 there was a maximum increase observed at 0.50 mg/L concentration of nitrogen. In CC-4033 there was no such increase observed under different nitrogen concentrations as compared to the strains grown in MS media having normal concentrations of nitrogen (Fig 3.15).

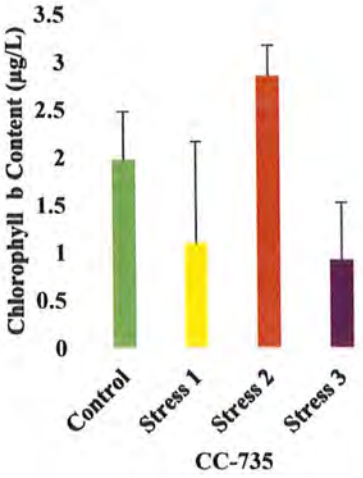
Our data showed that the total chlorophyll b contents in the selected strains were highly variable under the selected growth conditions of algae. The highest average total chlorophyll b content in the CC-1173 (5.042 ± 0.00 μgl^{-1}) under 0.125 mg/L NH_4NO_3 concentration, while the lowest value was recorded in CC-125 (1.1509 ± 0.00 μgl^{-1}) under 0.50 mg/L NH_4NO_3 concentration with respect to values of their controls (2.4311 ± 0.00 μgl^{-1} and 1.092± 0.00 μgl^{-1}) respectively (Appendix S3).

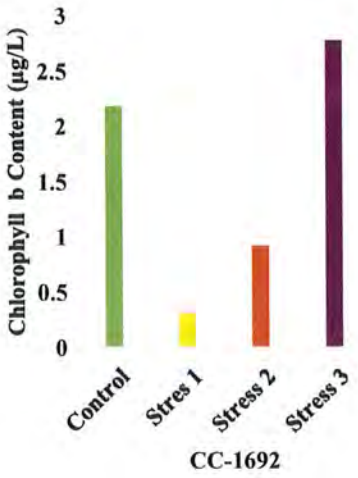





Chlorophyll b Content (µg/L)
3.5
3
2.5
2
1.5
1
0.5
0
Control
Stress 1
Stress 2
Stress 3
CC-1676


Chlorophyll b Content (µg/L)
3
2.5
2
1.5
1
0.5
0
Control
Stress 1
Stress 2
Stress 3
CC-1181


Chlorophyll b Content (µg/L)
3.5
3
2.5
2
1.5
1
0.5
0
Control
Stress 1
Stress 2
Stress 3
CC-735


Chlorophyll b Content (µg/L)
3
2.5
2
1.5
1
0.5
0
Control
Stres 1
Stress 2
Stress 3
CC-1692

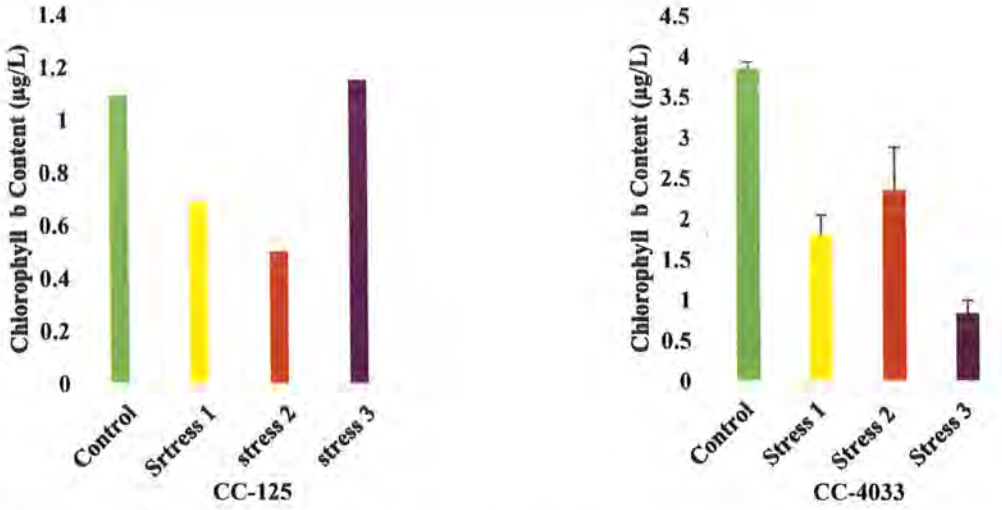


Figure 3.19: Effect of Nitrogen Concentration on Chlorophyll b Contents of Eight Selected Mutant Strains of *C. reinhardtii*. Chlorophyll b content (μgL^{-1}) of mutants strains of *C. reinhardtii* exposed to different NH_4NO_3 concentrations. Green bars represent treatments without NH_4NO_3, yellow bars represent 0.125 mg L^{-1}, brown bars represent 0.25 mg L^{-1}, while blue bars represents 0.50 mg L^{-1} of NH_4NO_3. Error bars represent standard deviation for n=3.

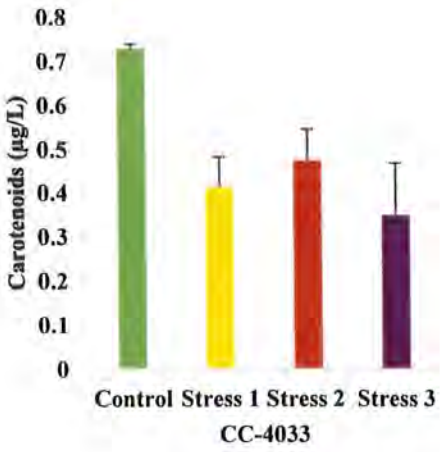
3.2.5 Carotenoids

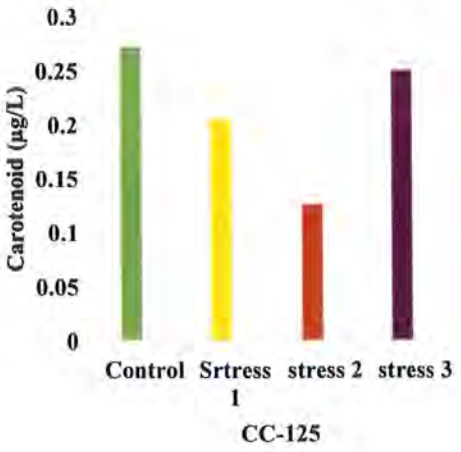
Considering the importance of carotenoids, present work was initiated to investigate on isolation of carotenoids from different mutant strain *C. reinhardtii* and quantity of total carotenoids present in the cell of *C. reinhardtii*. In addition, chemical stress (Nitrogen depletion) will be introduced into mass culture to induce the production of carotenoids. The study was aimed to quantify the percentage of total carotenoids present in a cell of *C. reinhardtii* to promote excessive carotenoids production by introducing chemical stress (Nitrogen depletion) during mass culture and to prepare an optimum medium for growth of *C. reinhardtii*. Carotenoids are sky-scarping in demand in global market owing to its widespread industrial application food processing, pharmaceutical and medicinal purposes. This has stimulated research and development of carotenoids from naturally occurring sources especially microalgae because they are among the fastest growing autotrophs on earth, which utilize commonly available material for growth, high productivity. In microalgae, carotenoids function as accessory pigments in the

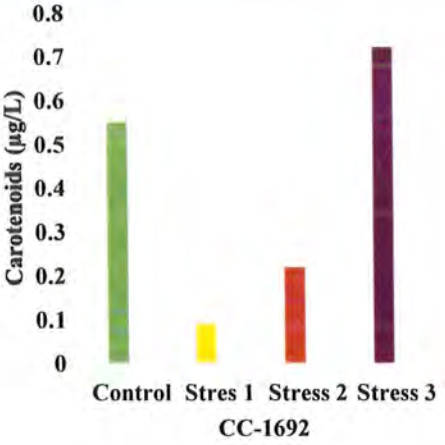
photosystems, as structural components of light harvesting complexes, as well as photo protective agents and also playing role in photoaxis.

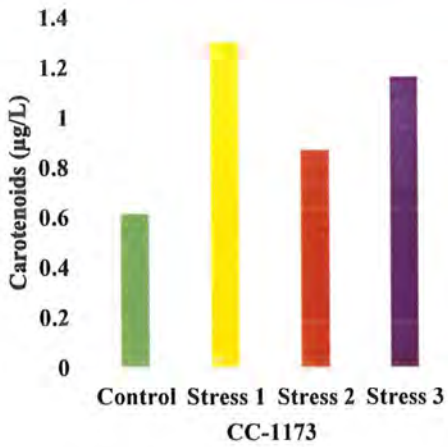
The mutants of *C. reinhardtii* were observed carefully for difference in carotenoids content under different nitrogen concentrations. The total carotenoids content can be altered by physical factors, such nitrogen limitation, light density and temperature variability. Strain CC-1173 appeared to be got increased in carotenoids at 0.125 mg/L concentration of nitrogen, while CC-735, CC-1181 and CC-1676 appeared to have high carotenoids content at 0.25 mg/L. Similarly strain CC-1692 and CC-1170 show a maximum increase at 0.50 mg/L concentration of nitrogen as compared to the strains grown in MS media having normal concentration of nitrogen. However CC-4033 and CC-125 appeared to be got decreased in carotenoids content when cultured with media having higher concentrations of nitrogen as compared to the strains grown in MS media having normal concentrations of nitrogen.

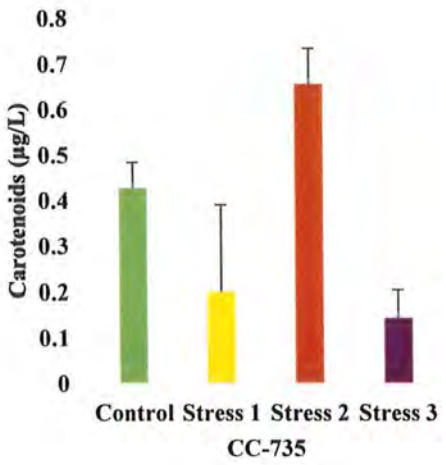
Our data showed that the total carotenoids in the selected strains were highly variable under the selected growth conditions of algae. The highest average total carotenoids in the CC-1173 ($1.303 \pm 0.00\ \mu gl^{-1}$) under 0.125 mg/L NH_4NO_3 concentration (Fig. 3.16), while the lowest value was recorded in CC-1181 that is ($0.185 \pm 0.09\ \mu gl^{-1}$) under 0.125 mg/L NH_4NO_3 concentration with respect to values of their controls ($0.615 \pm 0.00\ \mu gl^{-1}$ and $0.1674 \pm 0.06\ \mu gl^{-1}$) respectively (Appendix S3).

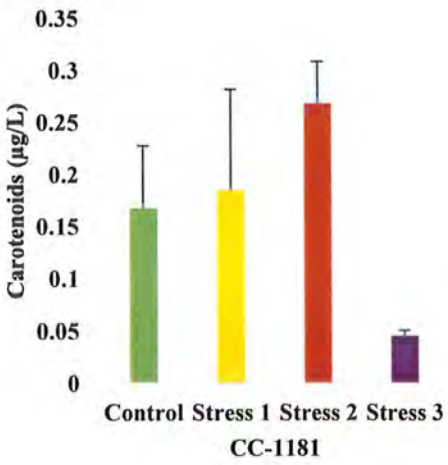


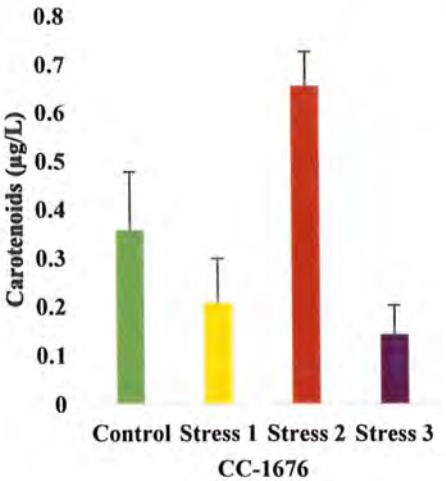



0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Carotenoids (µg/L)
Control Stres 1 Stress 2 Stress 3
CC-1692


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Carotenoids (µg/L)
Control Stress 1 Stress 2 Stress 3
CC-1173


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Carotenoids (µg/L)
Control Stress 1 Stress 2 Stress 3
CC-735


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Carotenoids (µg/L)
Control Stress 1 Stress 2 Stress 3
CC-1181


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Carotenoids (µg/L)
Control Stress 1 Stress 2 Stress 3
CC-1676

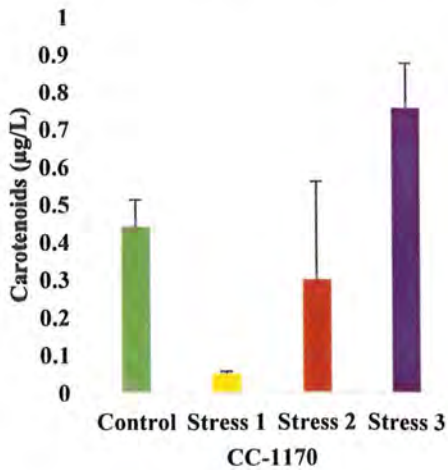

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Carotenoids (µg/L)
Control Stress 1 Stress 2 Stress 3
CC-1170

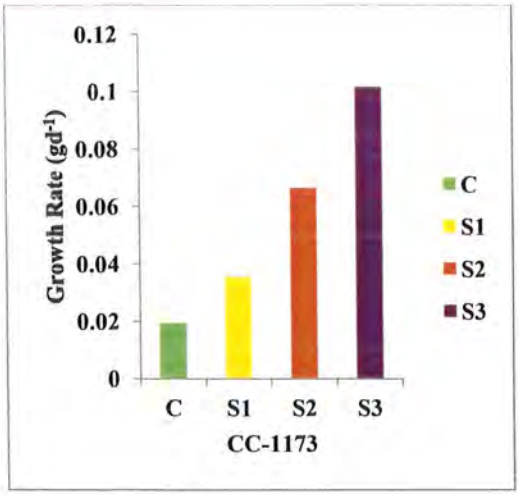
Figure 3.20: Effect Of Nitrogen Concentrations on Carotenoids Contents of Eight Selected Mutant Strains of *C. reinhardtii*. Carotenoids content (μgL^{-1}) of mutants strains of *C. reinhardtii* exposed to different NH_4NO_3 concentrations. Green bars represent treatments without NH_4NO_3, yellow bars represent 0.125 mg L^{-1}, brown bars represent 0.25 mg L^{-1}, while blue bars represents 0.50 mg L^{-1} of NH_4NO_3. Error bars represent standard deviation for n=3.

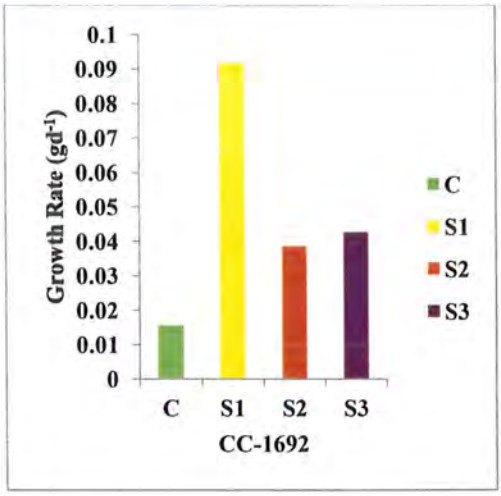
3.2.6 Growth rate

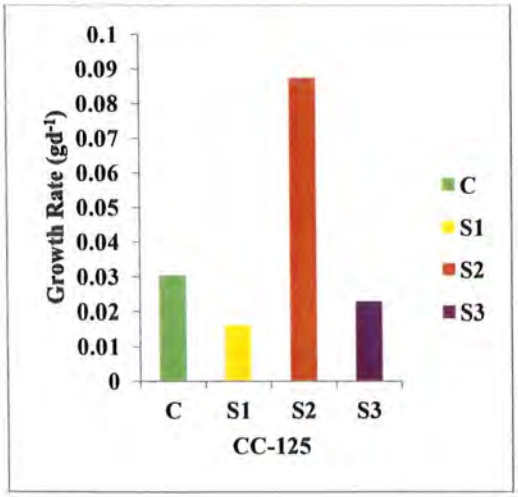
Inadequacy of nitrogen may affect structural and physiological variations in cells of microalgae, hence reduces developmental rates and production of biomass. Microalgae are a promising alternative source of vegetable oil. Due to their simple cellular structure, algae have higher rates of biomass and oil production than conventional crops.

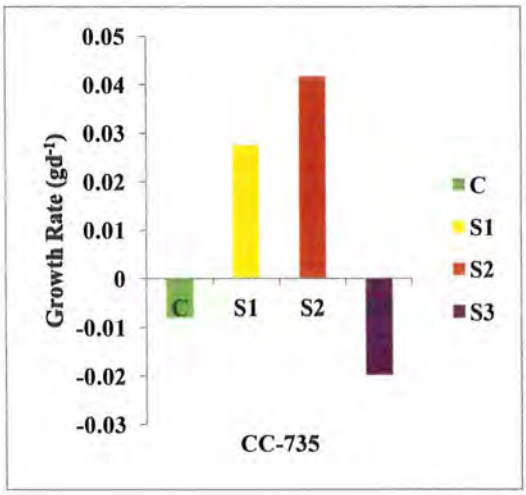
Mutants of *C. reinhardtii* CC-1173 and CC-1692 appeared to be increased in growth rate when cultured with media having higher concentration of nitrogen as compared to the strains grown in MS media having normal concentration of nitrogen while CC-125, CC-735, CC-1181, CC-1676, CC-4033 and CC-1170 appeared to have a gradual increase in growth rate when cultured with media having higher concentration of nitrogen as compared to the strains grown in MS media having normal concentration of nitrogen.

As a result, total growth rate in the selected strains were determined and highly individual differences were defined. The highest average total growth rate in the CC-1173 was 0.1015 gd^{-1} under 0.50 mg/L NH_4NO_3 concentration (Fig 3.17), while the lowest value was recorded in CC-1676 that is 0.100385 gd^{-1} under 0.50 mg/L NH_4NO_3 concentration with respect to values of their control 0.019437 and 0.09944 respectively (Appendix S3).


0.12
0.1
0.08
0.06
0.04
0.02
0
Growth Rate (gd-1)
C
S1
S2
S3
CC-1173


0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Growth Rate (gd-1)
C
S1
S2
S3
CC-1692


0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Growth Rate (gd-1)
C
S1
S2
S3
CC-125


0.05
0.04
0.03
0.02
0.01
0
-0.01
-0.02
-0.03
Growth Rate (gd-1)
C
S1
S2
S3
CC-735

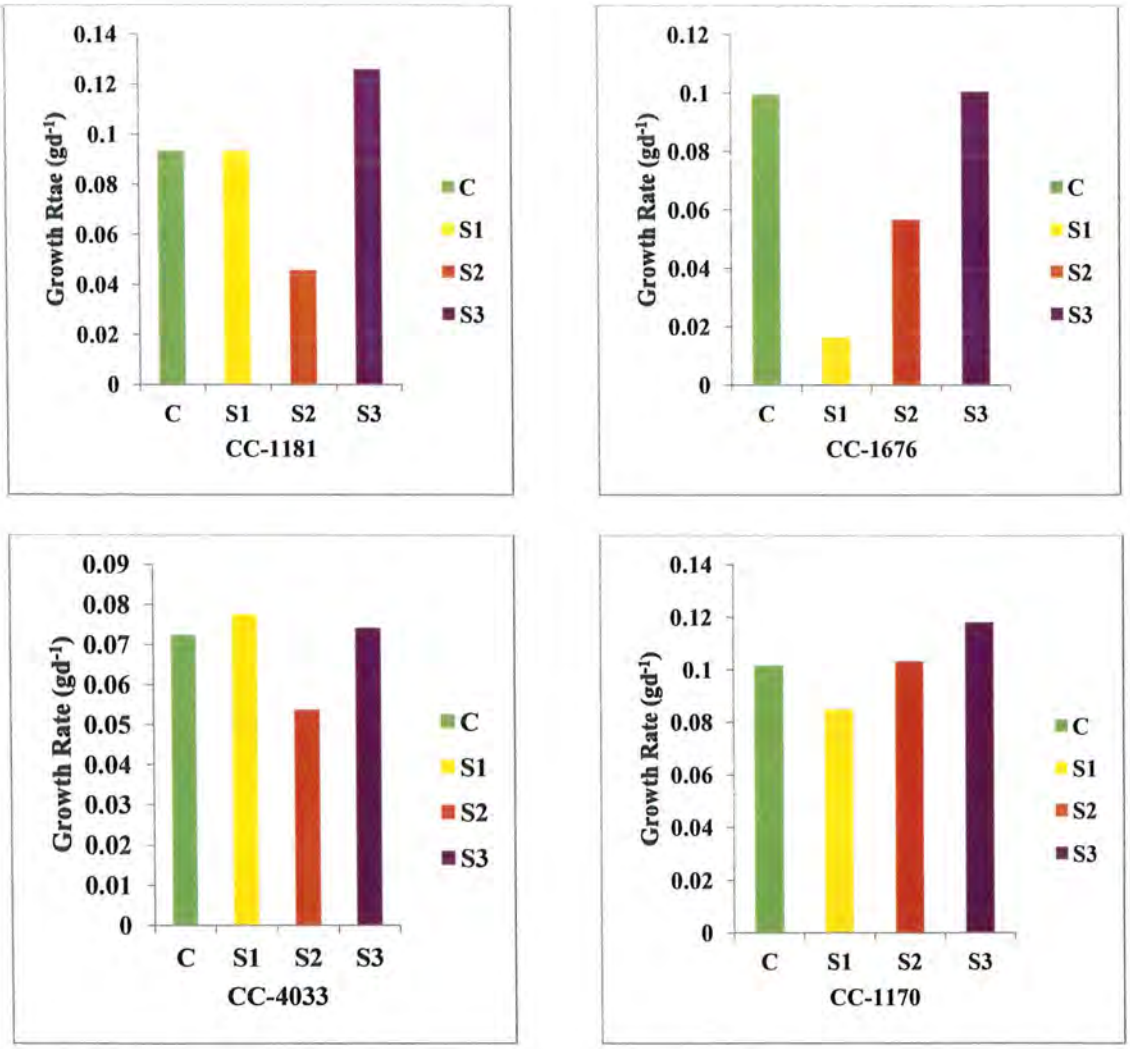


Figure 3.21: Effect of Nitrogen Concentration on Growth Rate of Eight Selected Mutant Strains of *C. reinhardtii*. Growth rate (gd^{-1}) of mutants strains of *C. reinhardtii* exposed to different NH_4NO_3 concentrations. Green bars represent treatments without NH_4NO_3, yellow bars represent 0.125 mg L^{-1}, brown bars represent 0.25 mg L^{-1}, while blue bars represents 0.50 mg L^{-1} of NH_4NO_3.

3.2.7 Biomass and Lipid Content

Nitrogen appears to be a very important and necessary nutrient affecting the metabolism of lipid in microalgal cells. According to some reported data nitrogen limitation leads to increase lipid accumulation in various microalgal species. The production of biomass in microalgae can be greatly motivated by intensity of light, temperature, nutrient availability, salinity and pH.

A. Strains in Which Both Biomass Accumulation and Lipid Content Increases

Our data showed that the biomass and lipid contents in the selected strains were highly variable under the selected growth conditions of algae. Mutants of *C. reinhardtii* CC-1181 and CC-4033 appeared to have increased accumulation in biomass and lipid content when cultured with media having higher concentration of nitrogen as compared to the strains grown in MS media having normal concentration of nitrogen.

As a result, biomass accumulation and lipid content in the selected strains were determined and highly individual differences were defined. The highest average total biomass in CC-4033 (5.866 g/L) under 0.50 mg/L NH_4NO_3 concentration (Fig 3.18), while the lowest value was recorded in CC-4033 (0.7333 g/L) under 0.125 mg/L NH_4NO_3 concentration with respect to values of their control 0.5888 (Appendix S3).

The highest average total lipid contents was observed in biomass in CC-1181 (1.644 g/L) under 0.25 mg/L NH_4NO_3 concentration (Fig 3.18), while the lowest value was recorded in CC-1181 (1.34 g/L) under 0.125 mg/L NH_4NO_3 concentration with respect to values of its control 1.2297 (Appendix S3).

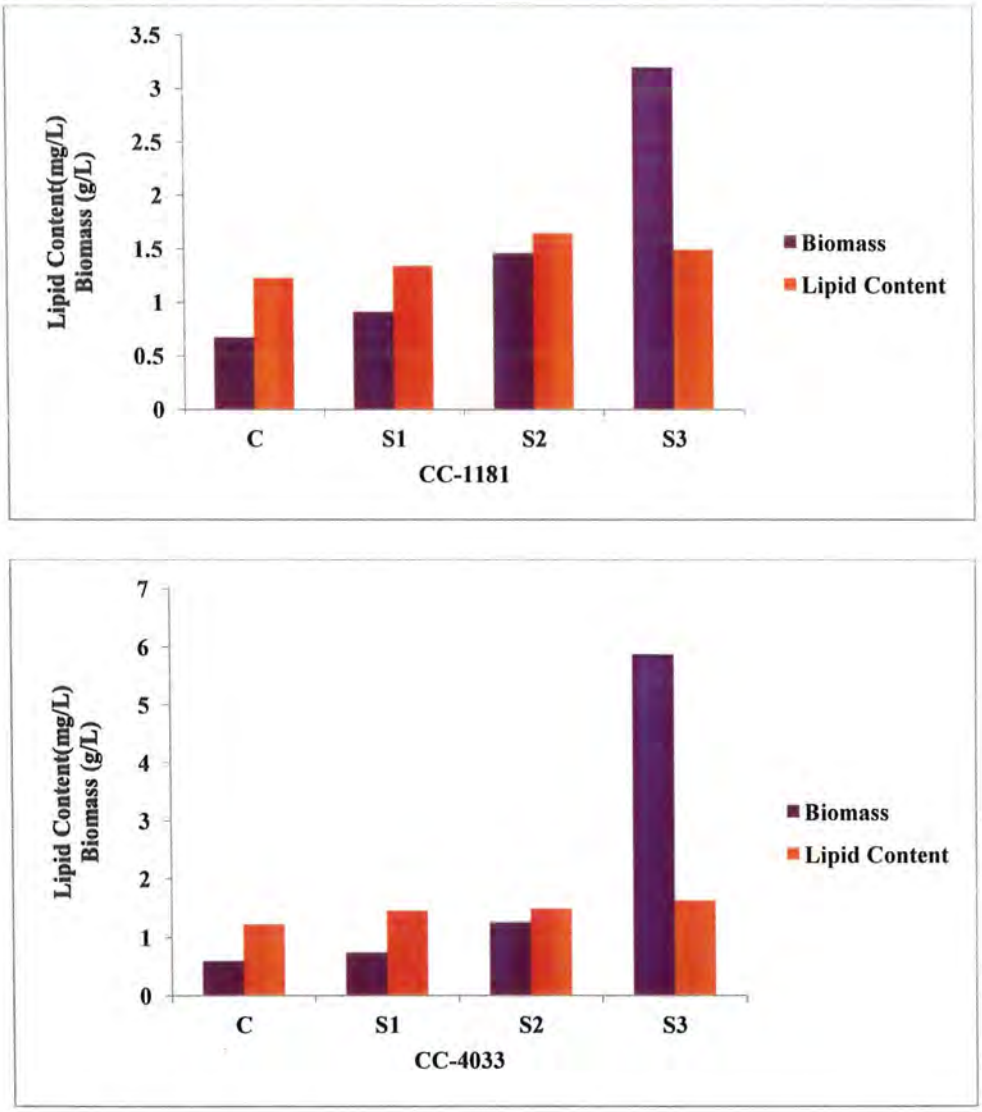


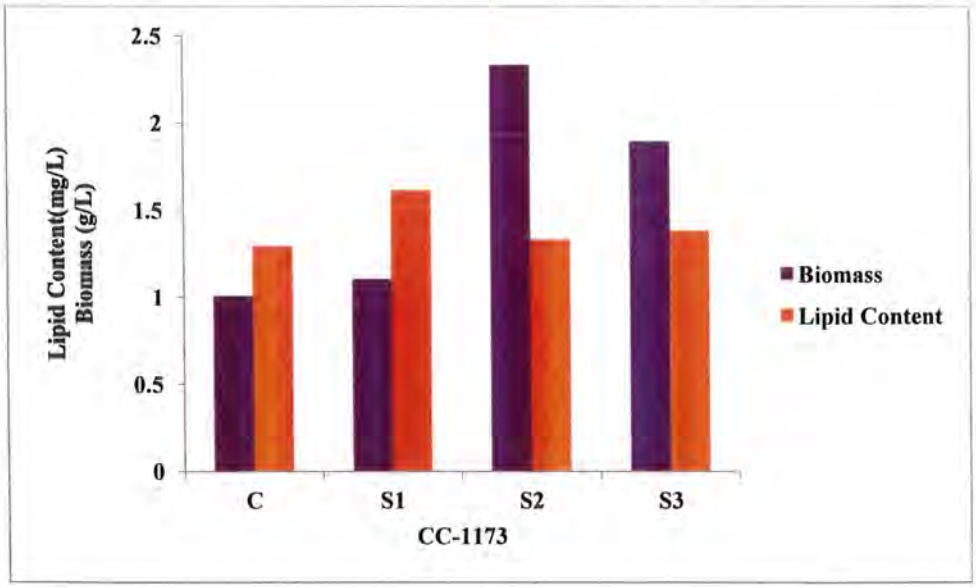
Figure 3.22: Biomass Accumulation and Lipid Content in CC-1181 and CC-4033 Subjected to Nitrogen Stress. Biomass and lipid content (gL^{-1}) of mutants strains of *C. reinhardtii* exposed to different NH_4NO_3 concentrations. Purple bars represent biomass and orange bars represent lipid content at normal (c), stress 1 (0.125 mg L^{-1}), stress 2 (0.25 mg L^{-1}) and stress3 (0.50 mg L^{-1}) of NH_4NO_3 respectively.

B. Strains in Which Biomass Accumulation Increases and Lipid Content Gradually Increases.

Mutants of *C. reinhardtii* CC-1173, CC-125 and CC-1692 appeared to have increase in biomass accumulation and gradual increase in lipid content when cultured with media having higher concentration of nitrogen as compared to the strains grown in MS media having normal concentration of nitrogen.

As a result, biomass accumulation and lipid content in the selected strains were determined and highly individual differences were defined. The highest average total biomass in CC-125 (2.77 g/L) under 0.50 mg/L NH_4NO_3 concentration (Fig 3.19), while the lowest value was recorded in CC-1692 (0.52 g/L) under 0.125 mg/L NH_4NO_3 concentration with respect to values of their controls 0.4841 g/L and 0.4379 g/L respectively (Appendix S3).

The highest average total lipid contents was observed in biomass in CC-1173 (1.5589 g/L) under 0.25 mg/L NH_4NO_3 concentration (Fig 3.19), while the lowest value was recorded in CC-1692 (0.5609 g/L) under 0.25 mg/L NH_4NO_3 concentration with respect to values of their controls 1.1484 g/L and 0.56483 g/L respectively (Appendix S3).



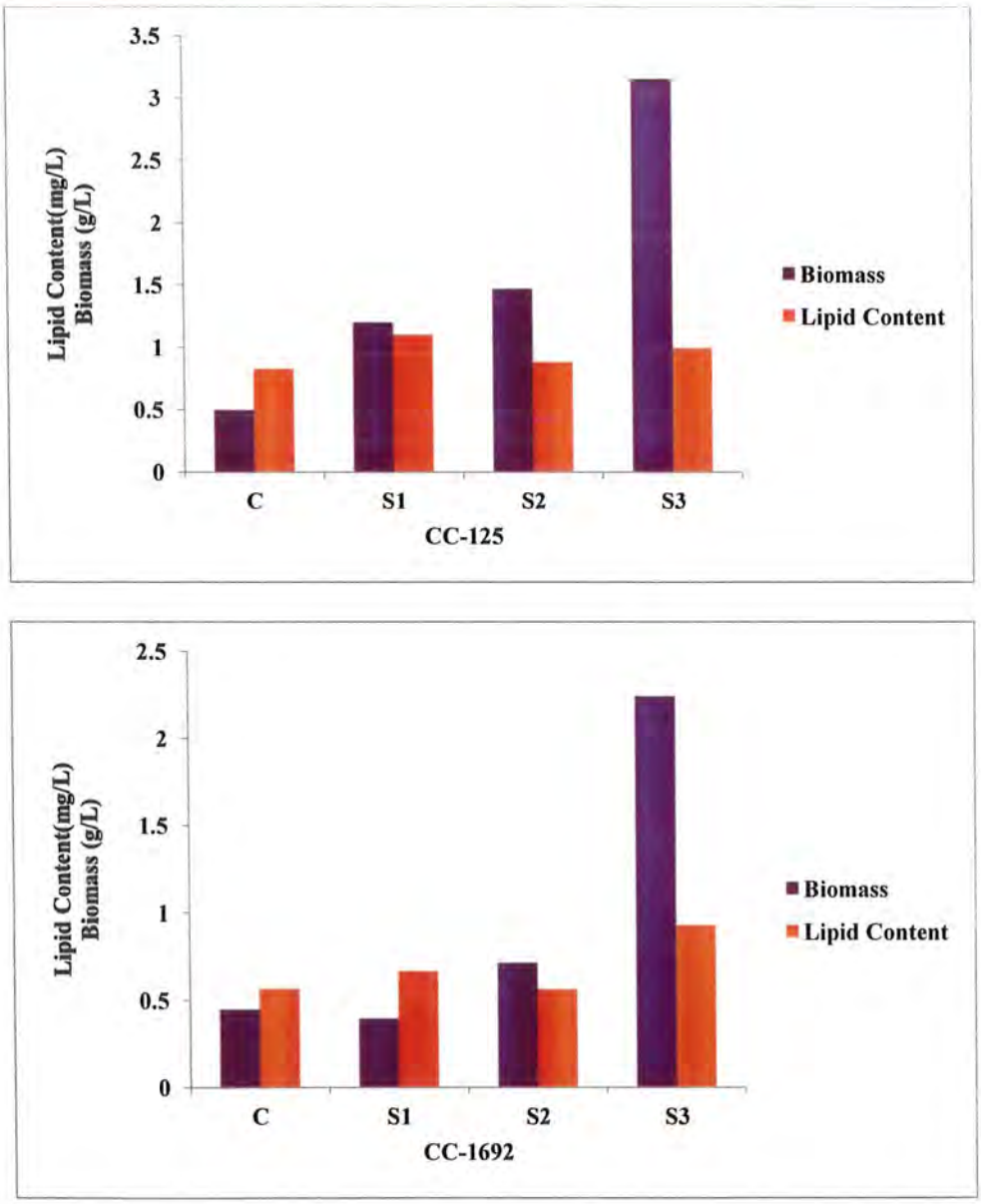


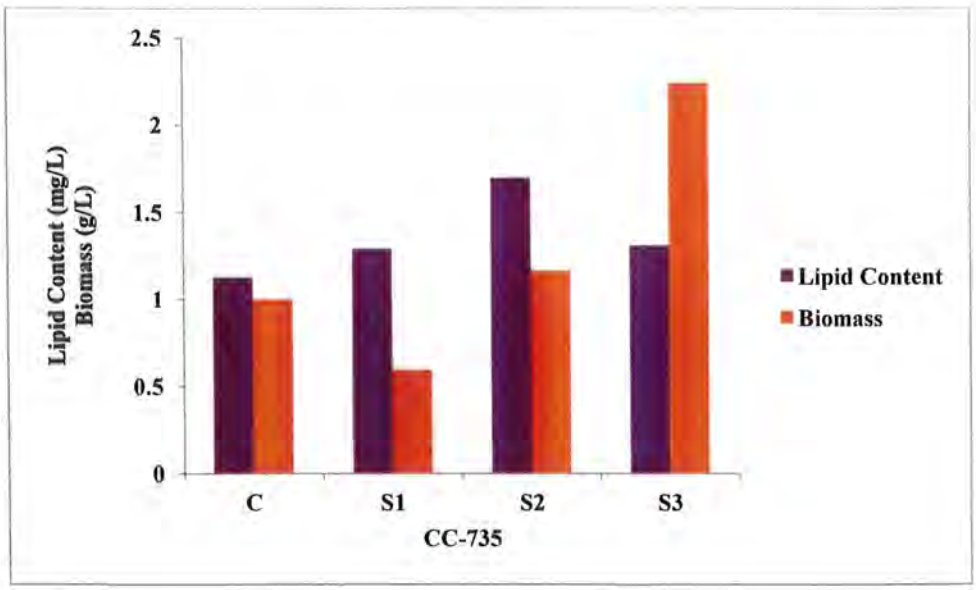
Figure 3.23: Biomass Accumulation and Lipid Content in CC-1173, CC-125 and CC-1692 Subjected to Nitrogen Stress. Biomass and lipid content (gl^{-1}) of mutants strains of *C. reinhardtii* exposed to different NH_4NO_3 concentrations. Purple bars represent biomass and orange bars represent lipid content at normal (c), stress 1 (0.125 mg L^{-1}), stress 2 (0.25 mg L^{-1}) and stress3 (0.50 mg L^{-1}) of NH_4NO_3 respectively.

C. Strains in Which Lipid Content Increases and Biomass Accumulation Gradually Increases

Mutants of *C. reinhardtii* CC-735, and CC-1170 appeared to have increase in lipid content and gradual increase in biomass accumulation when cultured with media having higher concentration of nitrogen as compared to the strains grown in MS media having normal concentration of nitrogen.

As a result, biomass accumulation and lipid content in the selected strains were determined and highly individual differences were defined. The highest average total biomass in CC-1170 (5.225 g/L) under 0.50 mg/L NH_4NO_3 concentration (Fig 3.20), while the lowest value was recorded in CC-735 (0.5955 g/L) under 0.125 mg/L NH_4NO_3 concentration with respect to values of their controls 0.9666 g/L and 1 g/L respectively (Appendix S3).

The highest average total lipid contents was observed in biomass in CC-735 (1.696 g/L) under 0.25 mg/L NH_4NO_3 concentration (Fig 3.20), while the lowest value was recorded in CC-735 (1.288 g/L) under 0.125 mg/L NH_4NO_3 concentration with respect to values of their control 1.12591 g/L (Appendix S3).



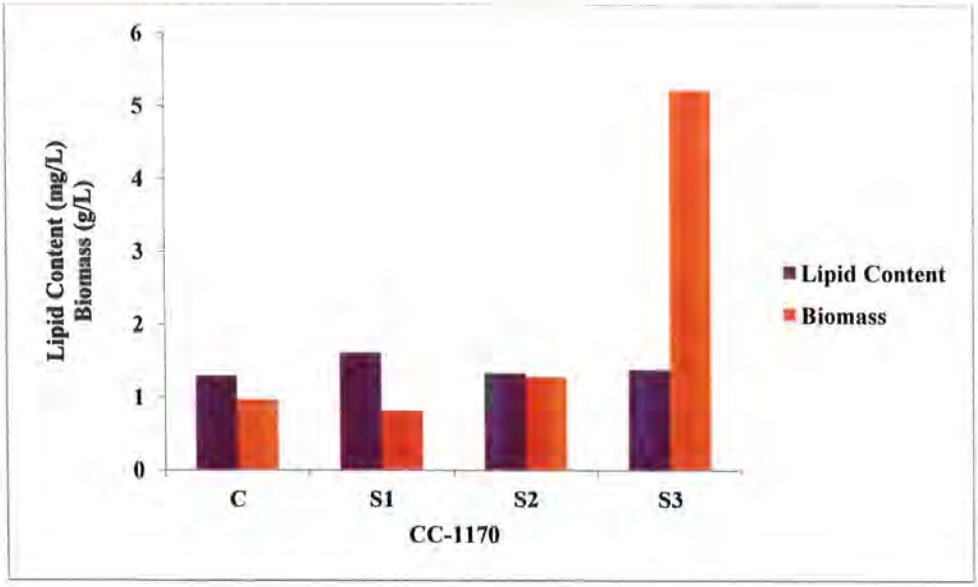


Figure 3.24: Biomass Accumulation and Lipid Content in CC-735 and CC-1170 Subjected to Nitrogen Stress. Biomass and lipid content (gl^{-1}) of mutants strains of *C. reinhardtii* exposed to different NH_4NO_3 concentrations. Purple bars represent biomass and orange bars represent lipid content at normal (c), stress 1 (0.125 mg L^{-1}), stress 2 (0.25 mg L^{-1}) and stress3 (0.50 mg L^{-1}) of NH_4NO_3 respectively.

D. Strains in Which Biomass Accumulation Increases and Lipid content Decreases

Mutant of *C. reinhardtii* CC-1676 appeared to have increase in biomass accumulation and decreases in lipid content when cultured with media having higher concentration of nitrogen as compared to the strains grown in MS media having normal concentration of nitrogen.

As a result, biomass accumulation and lipid content in the selected strains were determined and highly individual differences were defined. The highest average total biomass in CC-1676 (2.805 g/L) under 0.50 mg/L NH_4NO_3 concentration (Fig 3.21), while the lowest value was recorded in CC-1676 (0.4982 g/L) under 0.125 mg/L NH_4NO_3 concentration with respect to values of their control 0.3744 g/L (Appendix S3).

The highest average total lipid contents was observed in biomass in CC-1676 (1.548 g/L) under 0.50 mg/L NH_4NO_3 concentration (Fig 3.21), while the lowest value was recorded

in CC-1676 (1.36 g/L) under 0.25 mg/L NH_4NO_3 concentration with respect to values of their control 1.966 (Appendix S3).

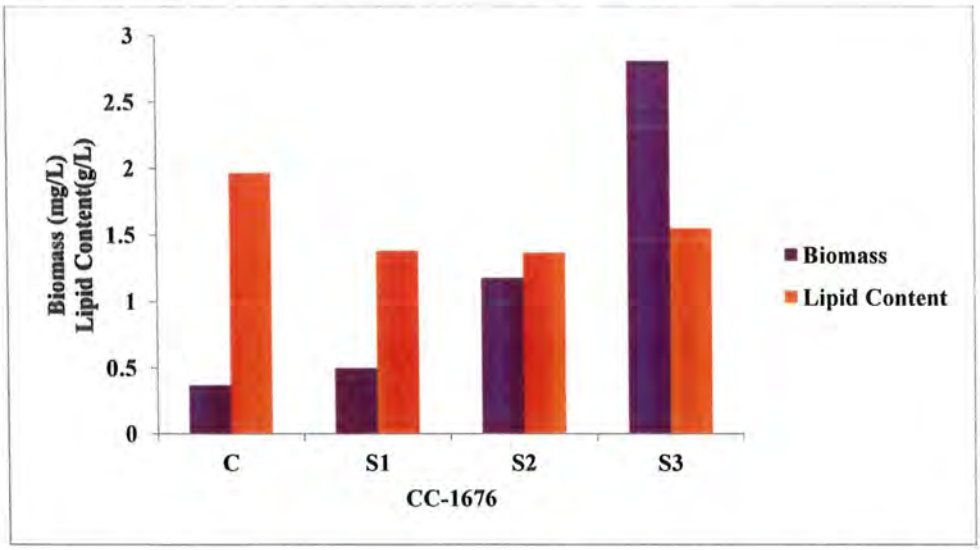


Figure 3.25: Biomass Accumulation and Lipid Content in CC-1676 Subjected to Nitrogen Stress. Biomass and lipid content (gl^{-1}) of mutants strains of *C. reinhardtii* exposed to different NH_4NO_3 concentrations. Purple bars represent biomass and orange bars represent lipid content at normal (c), stress 1 (0.125 mg L^{-1}), stress 2 (0.25 mg L^{-1}) and stress 3 (0.50 mg L^{-1}) of NH_4NO_3 respectively.

Discussion

How to trigger oil synthesis and increase oil content in vegetative cells (plant leaves or microalgae) is a hot topic in many industrial and academic labs across the globe. Microalgal biomass and lipid content plays an important role for economical production of biofuel. The algal species *C. reinhardtii* cannot grow in absence of source of nitrogen and its developmental rate is related directly to nitrate concentration in the medium. In *Nannochloropsis* sp, with low nitrate concentration decrease in algal biomass concentration has been reported by Hanhua and Gao (2005). It has also been reported that under nitrogen stress conditions, carbon metabolites were accumulated by algal cells as lipids (Yeesang and Cheirsilp, 2011). According to some earlier reports, in nitrogen deprived conditions, macromolecules containing nitrogen and carbon store compounds such as carbohydrates and fats are increased (Banerjee *et al.*, 2002; Dayananda *et al.*, 2006).

Previous studies have shown that nitrogen inadequacy results in an increase in the cell volume, also in *C. reinhardtii* (Donk *et al.*, 1997) and *S. subspicatus* (Sterner *et al.*, 1993). In contrast, other algae including *Ankistrodesmus* and *Stephanodiscus* have earlier shown a decrease in cell volume in response to inadequacy of nitrogen (Kilham *et al.*, 1997; Lynn *et al.*, 2000). Majority of previous investigations of nutrient limitation inducing lipid production, assessed nitrogen limitation alone with development in the medium having very low concentrations of nitrogen (Tornabene *et al.*, 1983).

The most promising renewable source of biofuel is considered microalgae. However, for economically feasible production of algal biofuels, different studies have underlined the requirement of low-cost culture systems, by use of sunlight as major energy source for biomass production (Griffiths and Harrison, 2009; Hu *et al.*, 2008; Rodolfi *et al.*, 2009; Scott *et al.*, 2010; Wijffels and Barbosa, 2010). Therefore, accumulation of metabolites in Chlamydomonas and Coccomyxa cells subjected to nitrogen starvation was investigated in harshly photoautotrophic conditions, as a result that the information gained might be functional for understanding the life of strains production (Morowvat *et al.*, 2010).

C. reinhardtii, when subjected to a variety of stress conditions like nitrogen deprivation, high salinity, sulfur deficiency or exposure to high light, it accumulates both starch and TAGs (Ball *et al.*, 1990; Dean *et al.*, 2010; Doebbe *et al.*, 2010; Fan *et al.*, 2011; Klein, 1987; Kropat *et al.*, 2011; Li *et al.*, 2010; Matthew *et al.*, 2009; Moellering and Benning, 2010; Siaut *et al.*, 2011; Work *et al.*, 2010).

The nitrogen deficiency is necessary to induce a significant lipid production (Spoehr and Milner, 1949), but such culture conditions strongly affect the growth rate, and thus the net productivity (Rodolfi *et al.*, 2009). Looking for a species which can maintain a high productivity under nitrogen-limiting conditions is thus a key challenge. The present study shows that in strain CC- 1173, CC-1181 and CC-735 there was a maximum increase of 1.5 folds and 1.3 folds respectively in lipid content at 0.250 mg/L concentration of NH_4NO_3 in comparison with control at 0 mg/L concentration. In strain CC-125 and CC-1170 there was a maximum increase of 1.3 folds and 1.5 folds in lipid content at 0.125 mg/L concentration of NH_4NO_3 respectively as compared to control with 0 mg/L. similarly in strain CC-1692 and CC-4033 there was a maximum increase of 1.6 folds and 1.3 folds at 0.50 mg/L concentration of NH_4NO_3 respectively. While in CC-1676 there is no such increase found in lipid content under different concentrations of nitrogen. The reaction of NH_4NO_3 concentration on *C. reinhardtii* growth and lipid content is summarized in Table S3 below.

The present study shows that in strain CC-1690, CC-4033, CC-1173, CC-1181, CC-735 and CC-1175 there was a maximum increase of 1.5, 1.1, 1.8, 1.1, 1.8 and 1 fold respectively in lipid content under high temperature stress of 33 °C, while in strain CC-125, CC-1692, CC-1168, CC-1170 and CC-1171 there was no such increase found in the lipid content at 33 °C. The effect of high temperature on *C. reinhardtii* growth and lipid content is summarized in Table S2 below.

In many species of marine microalgae high growth temperatures have been shown to favor the formation of saturated FAs (Mortensen *et al.*, 1988; Renaud *et al.*, 1995; Thompson *et al.*, 1992). It has been reported that small but significant decreases has been found in the lipid production at higher growth temperatures in three species (*Chaetoceros sp.*, *Rhodomonas sp.* and *prymnesiophyte* NT19), (Renaud *et al.*, 2002) which follows the

trend reported earlier for *Chaetoceros calcitrans* and *C. simplex* (Thompson *et al.*, 1992) and *Nitzschia* sp. (Renaud *et al.*, 1995). Yet, these results has been in disagreement with reports of higher production of lipid, been observed at high temperatures for several species of microalgae (Aaronson, 1973; De Oliveira *et al.*, 1999; Tomaselli *et al.*, 1988; Tornabene *et al.*, 1983). It has been recommended that the effect of high temperature was due to termination of growth at intense temperatures by permanent damage to plant enzymes (Opute, 1974).

It has been reported that nitrogen starvation conditions were significantly increasing the contents of lipid of many microalgae (Illman *et al.*, 2000). In *N. oculata* and *C. vulgaris*, it has been reported that under nitrogen stress (of $NaNO_3$) the growth rate was not specifically affected and a threefold increase was observed in the content of lipid. On the other hand a gradual reduction in growth rate has been observed in *N. oculata* and the content of lipids were increased by two folds (Converti *et al.*, 2009). The present study shows that in strain CC- 1173, CC-1692, CC-1181, CC-1676 and CC-1170 there was a maximum increase of 5.2, 2.7, 1.3,1 and 0.1 folds respectively in growth rate at 0.50 mg/L concentration of NH_4NO_3. In strain CC-125 and CC-735 there was a maximum increase of 1.8 folds in growth rate at 0.25 mg/L concentration of NH_4NO_3 as compared to control with 0 mg/L. Similarly in strain CC-4033 there is a maximum increase of 0.04 folds in growth rate at 0.125 mg/L concentration of NH_4NO_3 as compared to control under nitrogen stress.

Under high temperature stress of 33 °C there was no such increase found in growth rate of different mutants of *C. reinhardtii* except CC-1175 in which there was a maximum increase of 1.8 folds.

It has been verified that the reduction of light intensity plays a significant role in reducing the growth rate of microorganisms (Danesi *et al.*, 2004). The nutrients present in the culturing medium also play an important role in growth of microalgae. An example is the growth of microalgae in nitrogen limited conditions which results in reduction in the density of cell (Dean *et al.*, 2010; Nigam *et al.*, 2011). The data shows that decreasing the intensity of light and inadequacy of nitrogen reduces the growth in *Scenedesmus*

dimorphus. Yet, the anti-proliferative effect was more distinct in cells cultured in limitation of light.

The present study shows that in strain CC-1676, CC- 125, CC-735, CC-1181, CC-1692, CC-4033 and CC-1170 there was a maximum increase of 7.5, 5.7, 2.2, 4.7, 4.7, 10 and 5.4 folds respectively in biomass at 0.50 mg/L concentration of NH_4NO_3. Similarly In strain CC-1173 there was a maximum increase of 2.2 folds in biomass at 0.25 mg/L concentration of NH_4NO_3 as compared to control with 0 mg/ concentration of NH_4NO_3.

There are a 2.9, 1.2, 1.3, 1.1, 1.5 and 3.9 folds increase in biomass accumulation of strains CC-1690, CC-1173, CC-1181, CC-735, CC-1171 and CC-1175 Under high temperature stress of 33 °C. While in strain CC-125, CC-1692, CC-4033, CC-1168 and CC-1170 there was no such increase observed.

The variability in carbohydrate content with change in culture temperature has been previously described for temperature microalgal species cultured within the range 10–25 °C (Thompson *et al.*, 1992) Those workers reported percentages of carbohydrate fluctuating by 3% over the experimental temperature range, which was similar to the findings of the present study.

Interestingly, in spite of these species-specific variations, both *Coccomyxa* sp. C-169 and *C. reinhardtii* CC-125 shows same trends in the production of starch and in the reduction of chlorophyll content trigger by nitrogen limitation. Giving the significant variance in habitat and phylogeny among these algal species, these observations suggests that some metabolic responses to N starvation might be shared by a wide range of green microalgae.(Msanne *et al.*, 2012).

The present study shows that in strains CC-1692 and CC-1181 there is a maximum increase of 1 fold in carbohydrate content under nitrogen stress of 0.25 mg/L NH_4NO_3 as compared to control having 0 concentration of NH_4NO_3, Similarly in strains CC-735, CC-1676 and CC-4033 there was a maximum increase of 1.16, 1.1 and 1.3 folds respectively in carbohydrate content at 0.125 mg/L of NH_4NO_3. While in strain CC-1173, CC-125 and CC-1170 there is no increase found in the content of carbohydrate under nitrogen stress as compared to control at 0 mg/L concentration of NH_4NO_3.

The present study shows that in strain CC-4033, CC-1175, CC-1181 and CC-735 there was a maximum increase of 1.1, 1.3, 1.1 and 1.4 folds respectively in carbohydrate content at high temperature of 33 °C as compared to control samples at 25 °C while in strains CC-125, CC-1690, CC-1173 and CC-1168 there is no such increase in the content of carbohydrates at 33 °C as compared to control at 25 °C.

In the algal samples the quantification of total protein concentration shows that in response to nitrogen starvation a minimal change has been observed in protein content. An examples are the cells of *C. reinhardtii* and *S. subspicatus* which when grown in low nitrogen medium, shows an 18% and 15% reduction in total protein content. On the other hand the total protein contents were reduced by 11% and 9%, respectively in intermediate-N medium in *C. reinhardtii* cells and *S. subspicatus* cells. These observations are against to the observation of some previous reports. In marine diatom *Chaetoceros muellerii* the total cellular protein had been extensively decreased following nitrogen limitation (Giordano *et al.*, 2001). while, for some microalgae, such as *Chlorella emersonii* and *Chlorella protothecoides*, low nitrogen treatment do not significantly decrease the content of protein recommending that on cellular protein the effect of N-starvation might also be dependent on species.(Illman *et al.*, 2000)

The present study shows that in strain CC-1173, CC- 125, CC-1692, CC-1181, CC-1676 and CC-1170 there was a maximum increase of 1, 1.5, 1.7, 2.1, 1.5 and 1.4 folds respectively in protein content at 0.50 mg/L concentration of NH_4NO_3 as compared to control with 0 mg/L. similarly in strain CC-735 and CC-4033 there was a maximum increase of 2.6 and 3.7 folds at 0.125 mg/L and 0.25 mg/L concentration of NH_4NO_3 respectively as compared to control with 0 mg/L. There was no such increase recorded in protein content at 33 °C as compared to strains at 25 °C.

The availability of nitrogen and the intensity of light are the significant features that regulate the growth, biochemical composition of microalgae and the content of chlorophyll. And hence, the availability of nitrogen and the intensity of light are important for development, reproduction and photosynthetic activity of microalgae. Therefore, it has been estimated that in nitrogen deficiency in the medium extensively decrease the production of chlorophyll in *Scenedesmus dimorphus*. Same reports were

observed by using microalgae *Chlorella minutissima*, which in three days of nitrogen starvation shows an increase in the content of lipid and condensed chloroplast, which suggests that nitrogen starvation leads to chloroplast damage (Wang *et al.*, 2011).

The present study shows that in strain CC-1692, CC-1676 and CC-1170 there was a maximum increase of 1.3, 1.2 and 1.6 folds respectively in chlorophyll a content at 0.50 mg/L concentration of NH_4NO_3 as compared to control with 0 mg/L. Similarly in strain CC-735 and CC-1181 there was a maximum increase of 1.5 and 2 folds respectively at 0.25 mg/L concentration of NH_4NO_3 While in strain CC-1173 there was a maximum increase of 1.8 folds at 0.125 mg/L concentration of NH_4NO_3 as compared to control with 0 mg/L. On the other hand in strains CC-125 and CC-4033 there is no such increase found in the chlorophyll a content under different concentrations of NH_4NO_3.

The present study shows that in strain CC-125, CC-1690, CC-1692, CC-1173, CC-1168, CC-1175 and CC-735 there is a maximum increase of 3.4, 2.7, 2.8, 1.9, 2.5, 1.2 and 9.1 folds respectively in chlorophyll a content at 33 °C as compared to control strains at 25 °C. While in strain CC-4033 and CC-1181 there is no such increase in chlorophyll a content at 33 °C.

The present study shows that in strain CC-125, CC-1692 and CC-1170 there is a maximum increase of 1, 1.2 and 1.6 respectively in chlorophyll b content at 0.50 mg/L concentration of NH_4NO_3 as compared to control with 0 mg/L. Similarly in strain CC-735, CC-1181 and CC-1676 there was a maximum increase of 1.4, 2 and 1.3 folds respectively at 0.25 mg/L concentration of NH_4NO_3 and in strain CC-1173 there is a maximum increase of 2 folds at 0.125 mg/L concentration of NH_4NO_3 as compared to control with 0 mg/L. while in strain CC-4033 there is no such increase found in the chlorophyll b content.

The present study shows that in strain CC-1690, CC-1692 and CC-735 there was a maximum increase of 1.5, 1 and 4 folds in chlorophyll b content at 33 °C as compared to control strains at 25 °C. While in strain CC-125, CC-4033, CC-1173, CC-1168, CC-1175 and CC-1181 there was no such increase recorded in chlorophyll b content at 33 °C as compared to control strains at 25 °C.

The present study shows that in strain CC-1692 and CC-1170 there was a maximum increase of 1.3 and 1.7 folds in carotenoids at 0.50 mg/L concentration of NH_4NO_3 as compared to control with 0 mg/L. similarly in strain CC-735, CC-1181and CC-1676 there was a maximum increase of 1.5, 1.6 and 1.8 folds respectively at 0.25 mg/L concentration of NH_4NO_3 and in strain CC-1173 there was a maximum increase of 2 folds at 0.125 mg/L concentration of NH_4NO_3 as compared to control with 0 mg/L. While in strains CC-125 and CC-4033 there was no such increase found in the carotenoids.

The present study shows that in strain CC-125, CC-1690, CC-1692, CC-4033, CC-1173, CC-1168, CC-1175 and CC-735 there was a maximum increase of 3, 7.7, 5.3, 2.2, 2.5, 3, 4.4 and 8.8 folds in carotenoids at 33 °C as compared to control strains at 25 °C. While in strain CC-1181 there is no such increase in carotenoids at 33 °C as compared to control strains at 25 °C.

In control of CC-1170 nine and stress five different peaks are observed in FTIR at different positions. At 3252.65 O-H stretch is present which detect alcohols or phenols. In stress this signal has shifted from 3252.65 to 3374.95 and the intensity is increased by 0.04 folds from 0.05 to 0.01. In control at 1638.14 N–H bend detecting 1-amine is present while in stress the signal has shifted from 1638.14 to 1633.94 and the intensity is increased by 0.02 folds from 0.04 to 0.02. In control at 1027.06 C–O stretch detecting alcohols, carboxylic acids, esters, ethers is observed while in stress at 1027.40 the intensity is slightly reduced by 0.0.1 fold.

In control of CC-1171 four and in stress ten different peaks has observed in FTIR at different positions. At 3298.41 O-H stretch is present which detects carboxylic acids. In stress this signal has shifted from 3298.41 to 3254.71 and the intensity is reduced by 0.01 folds from 0.02 to 0.00. In control at 1636.71 N-H bonds detecting 1amines is present while in stress the signal is shifted from 1636.71 to 1638.71 and the intensity is reduced very slightly in stress. Similarly in control at 1026.00 C-O stretch detecting alcohols, carboxylic acids, esters, ethers is observed while in stress at 1027.04 the intensity is decreased by 0.1 folds from 0.14 to 0.04.

In control of CC-1173 and stress three different peaks has observed in FTIR at different positions. At 3359.45 N-H stretch is present which detects primary, secondary amines or

amides. In stress the signal is shifted from 3359.45 to 3354.72 and the intensity is very slightly reduced. In control at 1637.87 N-H bond is present which detects primary amines and in stress the intensity is slightly reduced in comparison to control. Similarly in control at 1037.98 C–N stretch or C–O stretch is present which detects aliphatic amines or alcohols, carboxylic acids, esters, ethers has been observed while in stress the intensity is very slightly reduced.

In control of CC-4033 seven and in stress twelve different peaks has observed in FTIR at different positions. At 3363.30 O-H stretch is present which detects alcohols, phenols. In stress this signal has shifted from 3363.30 to 3270.81 having N-H stretch and detects 1, 2 amines or amides and the intensities are reduced by 0.13 folds from 0.15 to 0.03. In control at 1635.72 N -H bond detecting 1amines is present while in stress the signal is shifted from 1635.72 to 1640.95 and the intensity is reduced by 0.05 folds from 0.10 to 0.05. Similarly at 1041.46 C-N stretch and C-O stretch detecting aliphatic amines or alcohols, carboxylic acids, esters, ethers has observed while in stress the signal is shifted from 1041.46 to 1028.68 and the intensity is slightly reduced.

An informative results are been acquired in this research which are helpful for further researchers. An initial step towards the development of further investigations has been characterized by these results regarding the modulation of the production of unicellular photosynthetic microalgal biomass as a source for feedstock production.

Conclusion

Algae are more promising feed stocks due to their wide spread availability and higher oil yields. The cultivation of the Yellow in Dark mutants of *C. reinhardtii* was feasible under high temperature and nitrogen sources (ammonium nitrate). The best results as biomass obtained was about 0.9g/L at 33 ^{o}C and 5.8 g/L in the presence of 0.75g/L concentration of ammonium nitrate and the lipid concentration of 1.6g/L of algae. The increasing temperature and nitrogen concentrations in the medium can reduce the price of inputs and would be an economic improvement for large scale cultivation. The addition of nitrogen at different concentrations in the medium, serve to accelerate physiological and biochemical activities of the fresh water green algae *C. reinhardtii*.

References

Aaronson S (1973). Effect of incubation temperature on the macromolecular and lipid content of the phytoflagellate *ochromonas danica*. Journal of Phycology 9(1):111-113.

Alonso DL, Belarbi E-H, Fernandez-Sevilla JM, Rodriguez-Ruiz J, Grima EM (2000). Acyl lipid composition variation related to culture age and nitrogen concentration in continuous culture of the microalga *Phaeodactylum tricornutum*. Phytochemistry 54(5):461-471.

Apt KE, Behrens PW (1999). Commercial developments in microalgal biotechnology. Journal of Phycology 35(2):215-226.

Aslan S, Kapdan IK (2006). Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. Ecological Engineering 28(1):64-70.

Awang R, YuenMay C (2007). Effect of hydroxylated compounds on properties and emission of palm biodiesel. American Journal of Applied Sciences 4(2):99-101.

Azov Y (1982). Effect of pH on inorganic carbon uptake in algal cultures. Applied and environmental microbiology 43(6):1300-1306.

Bajguz A (2009). Brassinosteroid enhanced the level of abscisic acid in *Chlorella vulgaris* subjected to short-term heat stress. Journal of plant physiology 166(8):882-886.

Ball SG, Dirick L, Decq A, Martiat J-C, Matagne R (1990). Physiology of starch storage in the monocellular alga *Chlamydomonas reinhardtii*. Plant Science 66(1):1-9.

Bandarra Nm, Pereira Pa, Batista I, Vilela Mh (2003). fatty acids, sterols and α-tocopherol in *isochrysis galbana*. Journal of Food Lipids 10(1):25-34.

Banerjee A, Sharma R, Chisti Y, Banerjee U (2002). *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Critical reviews in biotechnology 22(3):245-279.

Ben-Amotz A, Avron M (1990). The biotechnology of cultivating the halotolerant alga *Dunaliella*. Trends in biotechnology 8:121-126.

Borkhsenious ON, Mason CB, Moroney JV (1998). The intracellular localization of ribulose-1, 5-bisphosphate carboxylase/oxygenase in *Chlamydomonas reinhardtii*. Plant Physiology 116(4):1585-1591.

Borowitzka MA (1999). Commercial production of microalgae: ponds, tanks, tubes and fermenters. Journal of biotechnology 70(1):313-321.

Boussiba S, Vonshak A, Cohen Z, Avissar Y, Richmond A (1987). Lipid and biomass production by the halotolerant microalga *Nannochloropsis salina*. Biomass 12(1):37-47.

Boyce DG, Lewis MR, Worm B (2010). Global phytoplankton decline over the past century. Nature 466(7306):591-596.

Brown LM, Zeiler KG (1993). Aquatic biomass and carbon dioxide trapping. Energy conversion and management 34(9):1005-1013.

Brown MR, Dunstan GA, Norwood S, Miller KA (1996). Effects of harvest stage and light on the biochemical composition of the diatom *thalassiosira pseudonana*. Journal of Phycology 32(1):64-73.

Brueggeman AJ, Gangadharaiah DS, Cserhati MF, Casero D, Weeks DP, Ladunga I (2012). Activation of the carbon concentrating mechanism by CO_2 deprivation coincides with massive transcriptional restructuring in *Chlamydomonas reinhardtii*. The Plant Cell 24(5):1860-1875.

Burlew JS (1953). Algal culture. From Laboratory to Pilot Plant, Carnegie Inst. Washington Publ 600(1).

Carvalho AP, Malcata FX (2005). Optimization of ω-3 fatty acid production by microalgae: crossover effects of CO_2 and light intensity under batch and continuous cultivation modes. Marine biotechnology 7(4):381-388.

Chen M, Tang H, Ma H, Holland TC, Ng KS, Salley SO (2011). Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource technology 102(2):1649-1655.

Chenl Y, Celia DGE (1994). Effects of pH on the growth and carbon uptake of marine phytoplankton. Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 3755:83-94.

Chisti Y (2007). Biodiesel from microalgae. Biotechnology advances 25(3):294-306.

Chisti Y (2008). Biodiesel from microalgae beats bioethanol. Trends in biotechnology 26(3):126-131.

Christov C, Pouneva I, Bozhkova M, Toncheva T, Fournadzieva S, Zafirova T (2001). Influence of temperature and methyl jasmonate on *Scenedesmus incrassulatus*. Biologia plantarum 44(3):367-371.

Cohen Z, Norman H, Heimer Y (1995). Plants in Human Nutrition. World Rev. Nutr. Diet. 77: 1.

Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M (2009). Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chemical Engineering and Processing: Process Intensification 48(6):1146-1151.

Cuhel RL, Ortner PB, Lean DR (1984). Night synthesis of protein by algae1. Limnology and Oceanography 29(4):731-744.

Cvengros J, Cvengrosova Z (2004). Used frying oils and fats and their utilization in the production of methyl esters of higher fatty acids. Biomass and Bioenergy 27(2):173-181.

Danesi E, Rangel-Yagui C, Carvalho J, Sato S (2004). Effect of reducing the light intensity on the growth and production of chlorophyll by *Spirulina platensis*. Biomass and Bioenergy 26(4):329-335.

Dayananda C, Sarada R, Shamala T, Ravishankar G (2006). Influence of nitrogen sources on growth, hydrocarbon and fatty acid production by *Botryococcus braunii*. Asian Journal of Plant Sciences. 5: 799-804.

Dean AP, Sigee DC, Estrada B, Pittman JK (2010). Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. Bioresource technology 101(12):4499-4507.

Demon A, De Bruin M, Wolterbeek HT (1989). The influence of pre-treatment, temperature and calcium ions on trace element uptake by an alga (Scenedesmus pannonicus subsp. Berlin) and fungus (Aureobasidium pullulans). Environmental monitoring and assessment 13(1):21-33.

Demirbas A (2009). Progress and recent trends in biodiesel fuels. Energy conversion and management 50(1):14-34.

Delucchi M, Model ALE (2003). Lifecycle Emissions From Transportation Fuels, Motor Vehicles, Transportation Modes, Electricity Use, Heating and Cooking Fuels, and Materials. University of California-Davis. Institute for Transportation Studies, University of California at Davis, Davis, CA:88.

De Oliveira M, Monteiro M, Robbs P, Leite S (1999). Growth and chemical composition of *Spirulina maxima* and *Spirulina platensis* biomass at different temperatures. Aquaculture international 7(4):261-275.

De Morais MG, Costa JAV (2007). Carbon dioxide fixation by *Chlorella kessleri, C. vulgaris*, *Scenedesmus obliquus* and *Spirulina* sp. cultivated in flasks and vertical tubular photobioreactors. Biotechnology letters 29(9):1349-1352.

Doebbe A, Keck M, La Russa M, Mussgnug JH, Hankamer B, Tekçe E, Niehaus K, Kruse O (2010). The interplay of proton, electron, and metabolite supply for photosynthetic H_2 production in *Chlamydomonas reinhardtii.* Journal of Biological Chemistry 285(39):30247-30260.

Dent RM, Han M, Niyogi KK (2001). Functional genomics of plant photosynthesis in the fast lane using *Chlamydomonas reinhardtii.* Trends in plant science 6(8):364-371.

Donk Ev, Lurling M, Hessen D, Lokhorst G (1997). Altered cell wall morphology in nutrient-deficient phytoplankton and its impact on grazers. Limnology and Oceanography 42(2):357-364.

Dote Y, Sawayama S, Inoue S, Minowa T, Yokoyama S-y (1994). Recovery of liquid fuel from hydrocarbon-rich microalgae by thermochemical liquefaction. Fuel 73(12):1855-1857.

Durrett TP, Benning C, Ohlrogge J (2008). Plant triacylglycerols as feedstocks for the production of biofuels. The Plant Journal 54(4):593-607.

Fabregas J, Abalde J, Herrero C, Cabezas B, Veiga M (1984). Growth of the marine microalga *Tetraselmis suecica* in batch cultures with different salinities and nutrient concentrations. Aquaculture 42(3):207-215.

Fan J, Andre C, Xu C (2011). A chloroplast pathway for the de novo biosynthesis of triacylglycerol in *Chlamydomonas reinhardtii.* FEBS letters 585(12):1985-1991.

Fang W, Si Y, Douglass S, Casero D, Merchant SS, Pellegrini M, Ladunga I, Liu P, Spalding MH (2012). Transcriptome-wide changes in *Chlamydomonas reinhardtii*

gene expression regulated by carbon dioxide and the CO_2-concentrating mechanism regulator CIA5/CCM1. The Plant Cell 24(5):1876-1893.

Feofilova E, Sergeeva YE, Ivashechkin A (2010). Biodiesel-fuel: Content, production, producers, contemporary biotechnology (Review). Applied Biochemistry and Microbiology 46(4):369-378.

Field CB, Behrenfeld MJ, Randerson JT, Falkowski P (1998). Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281(5374):237-240.

Forjan E, Garbayo I, Henriques M, Rocha J, Vega JM, Vílchez C (2011). UV-A mediated modulation of photosynthetic efficiency, xanthophyll cycle and fatty acid production of Nannochloropsis. Marine biotechnology 13(3):366-375.

Freedman B, Pryde EH, Mounts TL (1984). Variables affecting the yields of fatty esters from transesterified vegetable oils. Journal of the American Oil Chemists Society 61(10):1638-1643.

Fujita RM, Wheeler PA, Edwards RL (1988). Metabolic regulation of ammonium uptake by *ulva rigida* (chlorophyta): a compartmental analysis of the rateâ€limiting step for uptake1. Journal of Phycology 24(4):560-566.

Fukuzawa H, Miura K, Ishizaki K, Kucho K-i, Saito T, Kohinata T, Ohyama K (2001). Ccm1, a regulatory gene controlling the induction of a carbon-concentrating mechanism in *Chlamydomonas reinhardtii* by sensing CO_2 availability. Proceedings of the National Academy of Sciences 98(9):5347-5352.

Fulton L (2004). Biomass and agriculture sustainability, markets and policies. International Energy Agency (IEA) biofuels study of interim report:105-112.

Ghaly AE, Dave D, Brooks MS, Budge S (2010). Production of biodiesel by enzymatic transesterification: review. American journal of biochemistry and biotechnology 6(2):54-76.

Giordano M, Kansiz M, Heraud P, Beardall J, Wood B, McNaughton D (2001). Fourier transform infrared spectroscopy as a novel tool to investigate changes in intracellular macromolecular pools in the marine microalga *Chaetoceros muellerii* (Bacillariophyceae). Journal of Phycology 37(2):271-279.

Goes JI, Handa N, Taguchi S, Hama T, Saito H (1995). Impact of UV radiation on the production patterns and composition of dissolved free and combined amino acids in marine phytoplankton. Journal of plankton research 17(6):1337-1362.

Govorunova E, Sineshchekov O (2005). Chemotaxis in the green flagellate alga Chlamydomonas. Biochemistry (Moscow) 70(7):717-725.

Griffiths MJ, Harrison ST (2009). Lipid productivity as a key characteristic for choosing algal species for biodiesel production. Journal of Applied Phycology 21(5):493-507.

Grossman AR (2000). *Chlamydomonas reinhardtii* and photosynthesis: genetics to genomics. Current opinion in plant biology 3(2):132-137.

Grossman AR, Catalanotti C, Yang W, Dubini A, Magneschi L, Subramanian V, Posewitz MC, Seibert M (2011). Multiple facets of anoxic metabolism and hydrogen production in the unicellular green alga *Chlamydomonas reinhardtii*. New phytologist 190(2):279-288.

Grossman AR, Croft M, Gladyshev VN, Merchant SS, Posewitz MC, Prochnik S, Spalding MH (2007). Novel metabolism in Chlamydomonas through the lens of genomics. Current opinion in plant biology 10(2):190-198.

Guckert JB, Cooksey KE (1990). Triglyceride accumulation and fatty acid profile changes in Chlorella (chlorophyta) during high ph-induced cell cycle inhibition. Journal of Phycology 26(1):72-79.

Guschina IA, Harwood JL (2006). Lipids and lipid metabolism in eukaryotic algae. Progress in lipid research 45(2):160-186.

Gutman BL, Niyogi KK (2004). Chlamydomonas and Arabidopsis. A dynamic duo. Plant Physiology 135(2):607-610.

Hansen PJ (2002). Effect of high pH on the growth and survival of marine phytoplankton: implications for species succession. Aquatic microbial ecology 28(3):279-288.

Harris EH (2001). Chlamydomonas as a model organism. Annual review of plant biology 52(1):363-406.

Harris G (2012). Phytoplankton ecology: structure, function and fluctuation. Springer Science & Business Media.

Ho SH, Chen WM, Chang J-S (2010). *Scenedesmus obliquus* CNW-N as a potential candidate for CO_2 mitigation and biodiesel production. Bioresource technology 101(22):8725-8730.

Holm-Hansen O, Nishida K, Moses V, Calvin M (1959). Effects of mineral salts on short-term incorporation of carbon dioxide in Chlorella. Journal of Experimental Botany 10(1):109-124.

Hossain ABMS, Salleh A, Boyce AN, Chowdhury P, Naqiuddin M (2008). Biodiesel fuel production from algae as renewable energy. American journal of biochemistry and biotechnology 4(3):250-254.

Hsieh C-H, Wu W-T (2009). Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresource technology 100(17):3921-3926.

Hu H, Gao K (2006). Response of growth and fatty acid compositions of Nannochloropsis sp. to environmental factors under elevated CO_2 concentration. Biotechnology letters 28(13):987-992.

Hu Q (2004). 5 Environmental Effects on Cell Composition. Handbook of microalgal culture: biotechnology and applied phycology:83.

Hu Q (2006). PSA abstracts. Journal of Phycology 42:1-48.

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. The Plant Journal 54(4):621-639.

Huntley ME, Redalje DG (2007). CO_2 mitigation and renewable oil from photosynthetic microbes: a new appraisal. Mitigation and adaptation strategies for global change 12(4):573-608.

Illman A, Scragg A, Shales S (2000). Increase in Chlorella strains calorific values when grown in low nitrogen medium. Enzyme and microbial technology 27(8):631-635.

James GO, Hocart CH, Hillier W, Chen H, Kordbacheh F, Price GD, Djordjevic MA (2011). Fatty acid profiling of *Chlamydomonas reinhardtii* under nitrogen deprivation. Bioresource technology 102(3):3343-3351.

Jebsen C, Norici A, Wagner H, Palmucci M, Giordano M, Wilhelm C (2012). FTIR spectra of algal species can be used as physiological fingerprints to assess their actual growth potential. Physiologia Plantarum 146(4):427-438.

Jiang H, Gao K (2004). Effects of lowering temperature during culture on the production of polyunsaturated fatty acids in the marine diatom *Phaeodactylum tricornutum* (bacillariophyceae). Journal of Phycology 40(4):651-654.

Jiang Y, Chen F (1999). Effects of salinity on cell growth and docosahexaenoic acid content of the heterotrophic marine microalga *Crypthecodinium cohnii*. Journal of Industrial Microbiology and Biotechnology 23(6):508-513.

Joh T, Yoshida T, Yoshimoto M, Miyamoto T, Hatano S (1993). Composition and positional distribution of fatty acids in polar lipids from *Chlorella ellipsoidea* differing in chilling susceptibility and frost hardiness. Physiologia Plantarum 89(2):285-290.

Kalacheva GS, Zhila NO, Volova TG, Gladyshev MI (2002). The effect of temperature on the lipid composition of the green alga Botryococcus. Microbiology 71(3):286-293.

Khotimchenko SV, Yakovleva IM (2005). Lipid composition of the red alga *Tichocarpus crinitus* exposed to different levels of photon irradiance. Phytochemistry 66(1):73-79.

Khozin-Goldberg I, Cohen Z (2011). Unraveling algal lipid metabolism: Recent advances in gene identification. Biochimie 93(1):91-100.

Khrebtukova I, Spreitzer RJ (1996). Elimination of the Chlamydomonas gene family that encodes the small subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase. Proceedings of the National Academy of Sciences 93(24):13689-13693.

Kilham S, Kreeger D, Goulden C, Lynn S (1997). Effects of nutrient limitation on biochemical constituents of *Ankistrodesmus falcatus*. Freshwater Biology 38(3):591-596.

Klein U (1987). Intracellular carbon partitioning in *Chlamydomonas reinhardtii*. Plant Physiology 85(4):892-897.

Kolber Z, Zehr J, Falkowski P (1988). Effects of growth irradiance and nitrogen limitation on photosynthetic energy conversion in photosystem II. Plant Physiology 88(3):923-929.

Konopka A, Brock TD (1978). Effect of temperature on blue-green algae (cyanobacteria) in Lake Mendota. Applied and environmental microbiology 36(4):572-576.

Kropat J, Hong-Hermesdorf A, Casero D, Ent P, Castruita M, Pellegrini M, Merchant SS, Malasarn D (2011). A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. The Plant Journal 66(5):770-780.

Kumar D, Murthy G (2011). Pretreatments and enzymatic hydrolysis of grass straws for ethanol production in the Pacific Northwest US. Biological engineering 3:97-110.

Lee J-H, Lee J-S, Shin C-S, Park S-C, Kim S-W (2000). Effects of NO and SO_2 on growth of highly-CO_2-tolerant microalgae. Journal of microbiology and biotechnology 10(3):338-343.

Levine RP (1968). Genetic dissection of photosynthesis. Science 162(3855):768-771.

Li Y, Han D, Hu G, Sommerfeld M, Hu Q (2010). Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*. Biotechnology and bioengineering 107(2):258-268.

Li Y, Han D, Sommerfeld M, Hu Q (2011). Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp.(Chlorophyceae) under nitrogen-limited conditions. Bioresource technology 102(1):123-129.

Li Y, Horsman M, Wang B, Wu N, Lan CQ (2008). Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Applied microbiology and biotechnology 81(4):629-636.

Liang Y, Beardall J, Heraud P (2006). Effects of nitrogen source and UV radiation on the growth, chlorophyll fluorescence and fatty acid composition of *Phaeodactylum tricornutum* and *Chaetoceros muelleri* (Bacillariophyceae). Journal of Photochemistry and Photobiology B: Biology 82(3):161-172.

Liu Z-Y, Wang G-C, Zhou B-C (2008). Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresource technology 99(11):4717-4722.

Lynn SG, Kilham SS, Kreeger DA, Interlandi SJ (2000). Effect Of Nutrient Availability On The Biochemical And Elemental Stoichiometry In The Freshwater Diatom

St*ephanodiscus Minutulus* (Bacillariophyceae). Journal of Phycology 36(3):510-522.

Matthew T, Zhou W, Rupprecht J, Lim L, Thomas-Hall SR, Doebbe A, Kruse O, Hankamer B, Marx UC, Smith SM (2009). The metabolome of *Chlamydomonas reinhardtii* following induction of anaerobic H_2 production by sulfur depletion. Journal of Biological Chemistry 284(35):23415-23425.

Mayo AW (1997). Effects of temperature and pH on the kinetic growth of unialga *Chlorella vulgaris* cultures containing bacteria. Water environment research 69(1) :64-72.

McLarnon-Riches CJ, Rolph CE, Greenway DL, Robinson PK (1998). Effects of environmental factors and metals on *Selenastrum capricornutum* lipids. Phytochemistry 49(5):1241-1247.

Merchant SS, Kropat J, Liu B, Shaw J, Warakanont J (2012). TAG, You're it! Chlamydomonas as a reference organism for understanding algal triacylglycerol accumulation. Current opinion in Biotechnology 23(3):352-363.

Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, Terry A, Salamov A, Fritz-Laylin LK, Marechal-Drouard L (2007). The Chlamydomonas genome reveals the evolution of key animal and plant functions. Science 318(5848):245-250.

Metting Jr FB (1996). Biodiversity and application of microalgae. Journal of industrial microbiology 17(5-6):477-489.

Miao X, Wu Q (2006). Biodiesel production from heterotrophic microalgal oil. Bioresource technology 97(6):841-846.

Milne TA, Evans RJ, Nagle N (1990). Catalytic conversion of microalgae and vegetable oils to premium gasoline, with shape-selective zeolites. Biomass 21(3):219-232.

Minowa T, Yokoyama S-y, Kishimoto M, Okakura T (1995). Oil production from algal cells of *Dunaliella tertiolecta* by direct thermochemical liquefaction. Fuel 74(12):1735-1738.

Mock T, Kroon BM (2002). Photosynthetic energy conversion under extreme conditions—II: the significance of lipids under light limited growth in Antarctic sea ice diatoms. Phytochemistry 61(1):53-60.

Moellering ER, Benning C (2010). RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. Eukaryotic cell 9(1):97-106.

Morowvat MH, Rasoul-Amini S, Ghasemi Y (2010). Chlamydomonas as a "new" organism for biodiesel production. Bioresource technology 101(6):2059-2062.

Morris I, Glover HE, Yentsch CS (1974). Products of photosynthesis by marine phytoplankton: the effect of environmental factors on the relative rates of protein synthesis. Marine Biology 27(1):1-9.

Mortensen SH, Børsheim KY, Rainuzzo J, Knutsen G (1988). Fatty acid and elemental composition of the marine diatom *Chaetoceros gracilis Schutt*. Effects of silicate deprivation, temperature and light intensity. Journal of experimental marine biology and ecology 122(2):173-185.

Msanne J, Xu D, Konda AR, Casas-Mollano JA, Awada T, Cahoon EB, Cerutti H (2012). Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169. Phytochemistry 75:50-59.

Najafi G, Ghobadian B, Yusaf T, Rahimi H (2007). Combustion analysis of a CI engine performance using waste cooking biodiesel fuel with an artificial neural network aid. American Journal of Applied Sciences 4(10):756-764.

Napolitano GE (1994). The relationship of lipids with light and chlorophyll measurements in freshwater algae and periphyton1. Journal of Phycology 30(6):943-950.

Nichols NN, Bothast, RJ.(2008). Production of ethanol from grain. Genetic Improvement of Bioenergy Crops. Springer New York 75-88

Nigam S, Rai MP, Sharma R (2011). Effect of nitrogen on growth and lipid content of *Chlorella pyrenoidosa*. American journal of biochemistry and biotechnology 7(3):124-129.

Nishida I, Murata N (1996). Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. Annual review of plant biology 47(1):541-568.

Ogbonna JC, Masui H, Tanaka H (1997). Sequential heterotrophic/autotrophic cultivation–An efficient method of producing Chlorella biomass for health food and animal feed. Journal of Applied Phycology 9(4):359-366.

Opute F (1974). Studies on fat accumulation in Nitzschia palea Kutz. Annals of Botany 38(4):889-902.

Orcutt DM, Patterson GW (1974). Effect of light intensity upon lipid composition of *Nitzschia closterium* (Cylindrotheca fusiformis). Lipids 9(12):1000-1003.

Patterson GW (1970). Effect of culture temperature on fatty acid composition of *Chlorella sorokiniana*. Lipids 5(7):597-600.

Pasqualino JC, Montane D, Salvado J (2006). Synergic effects of biodiesel in the biodegradability of fossil-derived fuels. Biomass and Bioenergy 30(10):874-879.

Pereira MG, Mudge SM (2004). Cleaning oiled shores: laboratory experiments testing the potential use of vegetable oil biodiesels. Chemosphere 54(3):297-304.

Pienkos PT, Darzins AL (2009). The promise and challenges of microalgal□derived biofuels. Biofuels, Bioproducts and Biorefining 3(4):431-440.

Powell N, Shilton AN, Pratt S, Chisti Y (2008). Factors influencing luxury uptake of phosphorus by microalgae in waste stabilization ponds. Environmental science & technology 42(16):5958-5962.

Pratoomyot J, Srivilas P, Noiraksar T (2005). Fatty acids composition of 10 microalgal species. Songklanakarin Journal of Science Technology 27(6):1179-1187.

Praveenkumar R, Shameera K, Mahalakshmi G, Akbarsha MA, Thajuddin N (2012). Influence of nutrient deprivations on lipid accumulation in a dominant indigenous microalga *Chlorella* sp., BUM11008: Evaluation for biodiesel production. Biomass and Bioenergy 37:60-66.

Pruder GD, Bolton ET (1979). The role of CO_2 enrichment of aerating gas in the growth of an estuarine diatom. Aquaculture 17(1):1-15.

Radakovits R, Jinkerson RE, Darzins A, Posewitz MC (2010). Genetic engineering of algae for enhanced biofuel production. Eukaryotic cell 9(4):486-501.

Raja R, Hemaiswarya S, Kumar NA, Sridhar S, Rengasamy R (2008). A perspective on the biotechnological potential of microalgae. Critical Reviews in Microbiology 34(2):77-88.

Ramos MJ, Fernandez CM, Casas A, Rodriguez L, Perez A (2009). Influence of fatty acid composition of raw materials on biodiesel properties. Bioresource technology 100(1):261-268.

Rao AR, Dayananda C, Sarada R, Shamala TR, Ravishankar GA (2007). Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents. Bioresource technology 98(3):560-564.

Raven JA, Geider RJ (1988). Temperature and algal growth. New phytologist:441-461.

Renaud S, Zhou H, Parry D, Thinh L-V, Woo K (1995). Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae *Isochrysis* sp., *Nitzschia closterium*, *Nitzschia paleacea*, and commercial species *Isochrysis* sp.(clone T. ISO). Journal of Applied Phycology 7(6):595-602.

Renaud SM, Parry DL (1994). Microalgae for use in tropical aquaculture II: Effect of salinity on growth, gross chemical composition and fatty acid composition of three species of marine microalgae. Journal of Applied Phycology 6(3):347-356.

Renaud SM, Thinh L-V, Lambrinidis G, Parry DL (2002). Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. Aquaculture 211(1):195-214.

Renaud SM, Thinh L-V, Parry DL (1999). The gross chemical composition and fatty acid composition of 18 species of tropical Australian microalgae for possible use in mariculture. Aquaculture 170(2):147-159.

Richardson B, Orcutt DM, Schwertner HA, Martinez CL, Wickline HE (1969). Effects of nitrogen limitation on the growth and composition of unicellular algae in continuous culture. Applied microbiology 18(2):245-250.

Richmond A (2008). Handbook of microalgal culture: biotechnology and applied phycology. John Wiley & Sons.

Rochaix J-D (2002). Chlamydomonas, a model system for studying the assembly and dynamics of photosynthetic complexes. FEBS letters 529(1):34-38.

Rochaix J (1995). *Chlamydomonas reinhardtii* as the photosynthetic yeast. Annual review of genetics 29(1):209-230.

Rodolfi L, Chini Zittelli G, Bassi N, Padovani G, Biondi N, Bonini G, Tredici MR (2009). Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and bioengineering 102(1):100-112.

Rosenberg JN, Oyler GA, Wilkinson L, Betenbaugh MJ (2008). A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. Current opinion in Biotechnology 19(5):430-436.

Sato N, Hagio M, Wada H, Tsuzuki M (2000). Environmental effects on acidic lipids of thylakoid membranes. Biochemical Society Transactions 28(6):912-914.

Sawayama S, Inoue S, Dote Y, Yokoyama S-Y (1995). CO_2 fixation and oil production through microalga. Energy conversion and management 36(6):729-731.

Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussgnug JH, Posten C, Kruse O, Hankamer B (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy research 1(1):20-43.

Scott SA, Davey MP, Dennis JS, Horst I, Howe CJ, Lea-Smith DJ, Smith AG (2010). Biodiesel from algae: challenges and prospects. Current opinion in Biotechnology 21(3):277-286.

Shapiro J (1973). Carbon-Dioxide And Ph-Effect On Species Succession Of Algae. Science 182(4109):307-307.

Sheehan J, Dunahay T, Benemann J, Roessler P (1998). A look back at the US Department of Energy's aquatic species program: biodiesel from algae. National Renewable Energy Laboratory Golden.

Shelef G, Azov Y, Moraine R, Oron G (1980). Microalgae and wastewater treatment. Saudi Journal of Biological Sciences 19 (3): 257–275

Shifrin NS, Chisholm SW (1981). Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light and dark cycles. Journal of Phycology 17(4):374-384.

Siaut M, Cuine S, Cagnon C, Fessler B, Nguyen M, Carrier P, Beyly A, Beisson F, Triantaphylides C, Li-Beisson Y (2011). Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common

laboratory strains and relationship with starch reserves. BMC biotechnology 11(1):7.

Siegenthaler P-A, Murata N (2006). Lipids in photosynthesis: structure, function and genetics. Advances in Photosynthesis and Respiration 6:1-10

Smith A, Morris I (1980). Pathways of carbon assimilation in phytoplankton from the Antarctic Ocean. Limnology and Oceanography 25(5):865-872.

Solovchenko A, Khozin-Goldberg I, Didi-Cohen S, Cohen Z, Merzlyak M (2008). Effects of light and nitrogen starvation on the content and composition of carotenoids of the green microalga *Parietochloris incisa*. Russian journal of plant physiology 55(4):455-462.

Somerville C (1995). Direct tests of the role of membrane lipid composition in low-temperature-induced photoinhibition and chilling sensitivity in plants and cyanobacteria. Proceedings of the National Academy of Sciences 92(14):6215-6218.

Spalding MH (2008). Microalgal carbon-dioxide-concentrating mechanisms: Chlamydomonas inorganic carbon transporters. Journal of Experimental Botany 59(7):1463-1473.

Spoehr H, Milner HW (1949). The chemical composition of Chlorella; effect of environmental conditions. Plant Physiology 24(1):120.

Spreitzer RJ, Mets L (1981). Photosynthesis-deficient mutants of *Chlamydomonas reinhardii* with associated light-sensitive phenotypes. Plant Physiology 67(3):565-569.

Srinivas R, Ochs C (2012). Effect of UV-A Irradiance on Lipid Accumulation in *Nannochloropsis oculata*. Photochemistry and photobiology 88(3):684-689.

Stehfest K, Toepel J, Wilhelm C (2005). The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae. Plant Physiology and Biochemistry 43(7):717-726.

Stephenson AL, Dennis JS, Howe CJ, Scott SA, Smith AG (2010). Influence of nitrogen-limitation regime on the production by *Chlorella vulgaris* of lipids for biodiesel feedstocks. Biofuels 1(1):47-58.

Sterner RW, Grover JP (1998). Algal growth in warm temperate reservoirs: kinetic examination of nitrogen, temperature, light, and other nutrients. Water Research 32(12):3539-3548.

Sterner RW, Hagemeier DD, Smith WL, Smith RF (1993). Phytoplankton nutrient limitation and food quality for Daphnia. Limnology and Oceanography 38(4):857-871.

Sushchik N, Kalacheva G, Zhila N, Gladyshev M, Volova T (2003). A temperature dependence of the intra-and extracellular fatty-acid composition of green algae and cyanobacterium. Russian journal of plant physiology 50(3):374-380.

Sydney EB, Sturm W, de Carvalho JC, Thomaz-Soccol V, Larroche C, Pandey A, Soccol CR (2010). Potential carbon dioxide fixation by industrially important microalgae. Bioresource technology 101(15):5892-5896.

Takagi M, Yoshida T (2006). Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae Dunaliella cells. Journal of bioscience and bioengineering 101(3):223-226.

Takeshita T (2011). Competitiveness, role, and impact of microalgal biodiesel in the global energy future. Applied Energy 88(10):3481-3491.

Tatsuzawa H, Takizawa E (1995). Changes in lipid and fatty acid composition of *Pavlova lutheri*. Phytochemistry 40(2):397-400.

te Beest M, Le Roux JJ, Richardson DM, Brysting AK, Suda J, Kubesova M, Pysek P (2011). The more the better? The role of polyploidy in facilitating plant invasions. Annals of Botany 109(1):19-45.

Thompson GA (1996). Lipids and membrane function in green algae. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism 1302(1):17-45.

Thompson PA, Guo Mx, Harrison PJ (1992). Effects of variation in temperature. On the biochemical composition of eight species of marine phytoplankton. Journal of Phycology 28(4):481-488.

Tomaselli L, Giovannetti L, Sacchi A, Bocci F (1988). Effects of temperature on growth and biochemical composition in *Spirulina platensis* strain M2. Algal biotechnology :305-314.

Tornabene T, Holzer G, Lien S, Burris N (1983). Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*. Enzyme and microbial technology 5(6):435-440.

Tsukahara K, Sawayama S (2005). Liquid fuel production using microalgae. Jornal of the Japan Petroleum Institue 48(5):251.

Vasudevan PT, Briggs M (2008). Biodiesel production current state of the art and challenges. Journal of Industrial Microbiology & Biotechnology 35(5):421-430.

Vazquez-Duhalt R, Arredondo-Vega BO (1991). Haloadaptation of the green alga *Botryococcus braunii* (race A). Phytochemistry 30(9):2919-2925.

Vergara JJ, Bird KT, Niell FX (1995). Nitrogen assimilation following NH sub (4) super (+) pulses in the red alga *Gracilariopsis lemaneiformis*: Effect on C metabolism. Marine ecology progress series. Oldendorf 122(1):253-263.

Wang B, Li Y, Wu N, Lan CQ (2008). CO_2 bio-mitigation using microalgae. Applied microbiology and biotechnology 79(5):707-718.

Wang Y, Duanmu D, Spalding MH (2011). Carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*: inorganic carbon transport and CO_2 recapture. Photosynthesis research 109(1-3):115-122.

Wang Y, Spalding MH (2006). An inorganic carbon transport system responsible for acclimation specific to air levels of CO_2 in *Chlamydomonas reinhardtii*. Proceedings of the National Academy of Sciences 103(26):10110-10115.

Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U (2009). Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. Eukaryotic cell 8(12):1856-1868.

Weiss-Schneeweiss H, Emadzade K, Jang T-S, Schneeweiss G (2013). Evolutionary consequences, constraints and potential of polyploidy in plants. Cytogenetic and genome research 140(2-4):137-150.

Wen Z-Y, Chen F (2001). Optimization of nitrogen sources for heterotrophic production of eicosapentaenoic acid by the diatom *Nitzschia laevis*. Enzyme and microbial technology 29(6):341-347.

Widjaja A, Chien C-C, Ju Y-H (2009). Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. Journal of the Taiwan Institute of Chemical Engineers 40(1):13-20.

Wijffels RH, Barbosa MJ (2010). An outlook on microalgal biofuels. Science (Washington) 329(5993):796-799.

Work VH, Radakovits R, Jinkerson RE, Meuser JE, Elliott LG, Vinyard DJ, Laurens LM, Dismukes GC, Posewitz MC (2010). Increased lipid accumulation in the *Chlamydomonas reinhardtii* sta7-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains. Eukaryotic cell 9(8):1251-1261.

Xin L, Hong-ying H, Ke G, Ying-xue S (2010). Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp. Bioresource technology 101(14):5494-5500.

Xu X-Q, Beardall J (1997). Effect of salinity on fatty acid composition of a green microalga from an antarctic hypersaline lake. Phytochemistry 45(4):655-658.

Yeesang C, Cheirsilp B (2011). Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Bioresource technology 102(3):3034-3040.

Yeh KL, Chang JS (2011). Nitrogen starvation strategies and photobioreactor design for enhancing lipid content and lipid production of a newly isolated microalga *Chlorella vulgaris* ESP-31: Implications for biofuels. Biotechnology journal 6(11):1358-1366.

Yun Y-S, Park J (2001). Attenuation of monochromatic and polychromatic lights in *Chlorella vulgaris* suspensions. Applied microbiology and biotechnology 55(6):765-770.

Zhila NO, Kalacheva GS, Volova TG (2011). Effect of salinity on the biochemical composition of the alga *Botryococcus braunii* KA¼tz IPPAS H-252. Journal of Applied Phycology 23(1):47-52.

Zhu C, Lee Y, Chao T (1997). Effects of temperature and growth phase on lipid and biochemical composition of *Isochrysis galbana* TK1. Journal of Applied Phycology 9(5):451-457.

Appendices

Table S1: Murashige and Skoog medium Composition

Major salts (macronutrients)	Concentrations
Ammonium nitrate (NH_4NO_3)	1,650 mg/l
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	440 mg/l
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	370 mg/l
Potassium phosphate (KH_2PO_4)	170 mg/l
Potassium nitrate (KNO_3)	1,900 mg/l

Minor salts (micronutrients)	Concentrations
Boric acid (H_3BO_3)	6.2 mg/l
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.025 mg/l
Cupric sulphate ($CuSO_4 \cdot 5H_2O$)	0.025 mg/l
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	27.8 mg/l
Manganese sulphate ($MnSO_4 \cdot 4H_2O$)	22.3 mg/l
Potassium iodide (KI)	0.83 mg/l
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.25 mg/l
Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	8.6 mg/l
$Na_2EDTA \cdot 2H_2O$	37.2 mg/l
VITAMINS AND ORGANICS	**CONCENTRATIONS**
Inositol	100 mg/l
Niacin	0.5 mg/l
Pyridoxine· HCl	0.5 mg/l
Thiamine · HCl	0.1 mg/l
Glycine	2 mg/l

Table S2: Effect of High Temperature on Biomass Production, Lipid Content of Selected Mutants of *Chlamydomonas reinhardtii* along with Growth Rate, Carbohydrate, Protein and Chlorophyll Content

Lipid Content	Carbohydrates		Protein Content		Chlorophyll a		Chlorophyll b		Carotenoids	
Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress	Control	Stress
0.04788	55.74±14.3	35.70±6.9	4.46±0.09	2.03±0.08	0.62±0.2	2.16±0.6	1.98±0.7	1.53±0.4	0.3±0.02	0.92±0.17
0.9676	56.01±11.2	32.89±0.8	7.63±0.7	0.64±0.16	1.73±0.19	4.79±0.28	3.15±0.35	4.92±0.76	0.45±0.06	3.466±1.13
2.1468	43.775 ± 6.2	36.23±7.2	3.43±0.16	0.01±0.00	0.91±0.07	2.57±0.5	0.137±0.00	1.463±0.11	0.24±0.00	1.29±0.22
0.6774	31.27±5.1	35.32±5.3	8.02±0.59	5.39±1.7	2.27±0.46	6.27±1.55	1.637±0.54	1.39±0.30	1.76±0.6	3.98±0.14
1.0048	43.58±1.5	32.36±4.6	7.25±2.6	3.51±0.73	4.068±2.6	7.87±1.12	6.188±3.3	5.059±0.26	1.166±0.57	3.026±0.36
0.3778	44.31±4.3	33.97±7.5	4.90±0.2	3.64±0.9	1.2±0.84	3.11±0.01	2.117±1.48	1.3 6±0.11	0.41±0.14	1.28±0.04
4.591	22.41±1.2	31.27±5.1	4.57±0.1	0.72±0.08	1.437±0.19	1.81±0.2	1.55±0.34	0.158±0.03	0.16±0.06	0.706±0.06
0.225	31.81±6.7	35.98±7.8	3.75±0.71	1.46±0.32	2.84±0.04	0.250±0.17	4.868±0.09	0.49±0.089	0.90±0.015	0.23±0.2
0.6024	24.69±1.8	34.91±5.2	7.25±0.2	0.42±0.09	0.593±0.27	5.44±0.56	0.555±0.46	2.26±0.33	0.27±0.08	2.466±0.96

Strains	Biomass		Growth Rate		
	Control	Stress	Control	Stress	Control
CC-125	0.3774	0.2664	0.1441	0.1024	0.1525
CC-1690	0.3292	0.956	0.1106	0.0649	0.64
CC-1692	10.923	5.3153	0.138	0.0247	3.99
CC-4033	1.5952	0.5846	0.227	0.216	0.5846
CC-1173	0.7062	0.828	0.1729	0.01717	0.5538
CC-1168	0.9754	0.3726	0.2581	0.1196	0.6196
CC-1175	18.11	72.033	0.198	0.375	4.269
CC-1181	0.5606	0.7266	0.2	0.187	0.199
CC-735	0.60758	0.6926	0.1516	0.0843	0.3224

Table S3: Effect of Nitrogen Concentration on Biomass Production, Lipid Content of Selected Mutants of *Chlamydomonas reinhardtii* along with Growth Rate, Carbohydrate, Protein and Chlorophyll Content

Chlorophyll a			Chlorophyll b				Carotenoids			
Stress1	Stress2	Stress3	Control	Stress1	Stress2	Stress3	Control	Stress1	Stress2	Stress3
3.164±0.00	2.151±0.00	1.944±0.00	2.431±0.00	5.042±0.00	3.073±0.00	4.545±0.00	0.615±0.00	1.303±0.00	0.870±0.00	1.162±0.00
0.364±0.00	0.245±0.00	0.582±0.00	1.092±0.00	0.690±0.00	0.502±0.00	1.150±0.00	0.271±0.00	0.205±0.00	0.125±0.00	0.250±0.00
0.139±0.00	0.479±0.00	1.586±0.00	2.179±0.00	0.307±0.00	0.918±0.00	2.775±0.00	0.550±0.00	0.090±0.00	0.218±0.00	0.721±0.00
0.7829±0.6	1.702±0.23	0.4201±0.1	1.9762±0.5	1.104±1.06	2.852±0.32	0.9249±0.6	0.427±0.05	0.200±0.19	0.65630.07	0.143±0.06
0.748±0.33	1.041±0.46	0.887±0.86	0.828±0.26	1.302±0.56	1.683±0.86	0.2386±0.1	0.1674±0.06	0.1849±0.09	0.2684±0.04	0.0456±0.005
0.301±0.11	0.307±0.07	0.420±0.10	2.155±0.14	0.600±0.23	2.852±0.32	1.217±0.46	0.358±0.12	0.208±0.09	0.656±0.07	0.143±0.06
1.073±0.11	1.4201±0.3	0.719±0.06	3.839±0.09	1.803±0.24	2.352±0.53	0.834±0.16	0.728±0.01	0.413±0.07	0.474±0.07	0.347±0.12
0.142±0.03	0.289±0.05	2.2459±0.4	2.398±0.28	0.283±0.06	0.509±0.04	3.940±0.70	0.441±0.07	0.05±0.005	0.299±0.26	0.756±0.12

Lipid Content			Carbohydrates				Proteins				
Stress1	Stress2	Stress3	Control	Stress1	Stress2	Stress3	Control	Stress1	Stress2	Stress3	Control
1.052208	1.55589	1.067875	64.93±12.7	47.04±4.36	49.34±3.92	40.62±13.1	7.047±0.24	5.805±3.07	5.855±1.91	7.477±0.58	1.714±0.00
1.100356	0.88184	0.99309	60.52±1.9	55.02±6.6	48.90±0.4	49.25±2.9	3.607±1.03	4.260±2.2	4.666±1.4	5.493±1.9	0.623±0.00
0.66602	0.560951	0.928135	47.64±5.8	57.34±11.3	63.66±11.0	58.74±11.3	3.11±1.52	2.639±0.82	3.242±0.41	5.471±1.46	1.207±0.00
1.2888	1.6962	1.3073	54.051±4.3	62.95±11.2	49.70±2.22	48.57±11.9	2.048±0.40	5.506±0.38	5.131±0.52	4.134±1.37	1.123±0.24
1.34072	1.6444	1.4925	58.15±10.6	55.18±1.4	63.75±7.08	37.038±4.4	2.368±0.86	3.095±0.46	5.032±0.99	5.124±0.08	0.498±0.17
1.379615	1.366658	1.54812	48.16±10.0	53.04±12.5	45.29±1.8	49.358±2.1	3.8378±0.9	4.821±2.0	4.728±1.1	5.818±1.04	0.336±0.03
1.446281	1.479613	1.6222	49.367±3.2	61.48±12.2	59.45±7.27	55.62±6.14	1.239±0.93	4.145±1.49	4.640±1.01	4.486±1.95	2.199±0.03
1.612898	1.331457	1.3814	51.54±3.15	33.761±7.9	45.688±1.6	47.44±2.2	3.262±1.26	2.589±1.12	3.961±1.5	4.850±2.3	1.390±0.17

Strains	Biomass				Growth Rate				
	Control	Stress1	Stress2	Stress3	Control	Stress1	Stress2	Stress3	Control
CC-1173	0.98199	1.07798	2.222	2.0419	0.01943	0.03539	0.06634	0.10159	1.4849
CC-125	0.48421	1.07376	1.41354	2.77463	0.030363	0.01632	0.08749	0.02277	0.828085
CC-1692	0.43799	0.52443	0.6811	2.07331	0.01556	0.091818	0.038508	0.04258	0.564835
CC-735	1.001767	0.59443	1.16287	2.24064	0	0.0275	0.041713	-0.01975	1.125912
CC-1181	0.67043	0.9111	1.45931	3.19619	0.093412	0.09341	0.04571	0.1259	1.22977
CC-1676	0.37044	0.4982	1.17398	2.80552	0.09944	0.016408	0.05663	0.10038	1.96665
CC-4033	0.5888	0.7333	1.25176	5.8666	0.07226	0.07754	0.0537	0.074055	1.227763
CC-1170	0.9666	0.8204	1.28154	5.22594	0.10149	0.084864	0.10317	0.11788	1.29443

idra thesis

RIGINALITY REPORT

17%	9%	13%	0%
MILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

RIMARY SOURCES

1	Mata, T.M.. "Microalgae for biodiesel production and other applications: A review", Renewable and Sustainable Energy Reviews, 201001 Publication	1%
2	thescipub.com Internet Source	1%
3	www.wpi.edu Internet Source	1%
4	web.itu.edu.tr Internet Source	1%
5	Christian Jebsen. "The FTIR spectra of algal species can be used as physiological fingerprints to assess their actual growth potential", Physiologia Plantarum, 04/2012 Publication	1%
6	Renaud, S.M.. "Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures", Aquaculture,	1%