ASSESSMENT OF MICROALGAL ACCase AND rbcL GENE EXPRESSION AS A FUNCTION OF NUTRIENT AND METAL STRESS

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2017

I declare that the thesis herewith submitted for the degree of Doctor of Philosophy: Biotechnology at the Durban University of Technology is the result of my own work and has not been previously submitted for a degree at any other University.

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This____ day_____ of 2017, at the Durban University of Technology

DEDICATION

This thesis is dedicated to my beloved family specially my beloved brother

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LIST OF ABBREVIATIONS

ACCase – acetyl co-A carboxylase
ANOVA – analysis of variance
ATP – adenosine triphosphate
Auto – autotrophic cultivation mode
BG11 – blue-green medium
BSA – bovine serum albumin
Ca – calcium
Chl – chlorophyll
Co – cobalt
CO_2 – carbon dioxide
Cu – copper
DCW – dry cell weight
EDTA – Ethylenediaminetetraacetic acid
ETR – electron transport rate
Fe – iron
G3P – glyceraldehyde-3-phosphate
IR – infrared spectroscopy
K – potassium
Mg – magnesium
N – nitrogen
Na – sodium
NADH – nicotinamide adenine dinucleotide
NaOH – sodium hydroxide
OII – optimised individual iron concentration
OIM – optimised individual magnesium concentration
ON – optimum nitrogen concentration
ONP – optimum nitrogen and phosphorus concentration
OP – optimum phosphorus concentration
P – phosphorous
PAM – Pulse Amplitude Modulation Flourometry
PAR – photosynthetically active radiation
PCR – polymerase chain reaction
PGA – 3-phosphoglyceraldehyde
PO_4 – phosphate
PPFD – photosynthetically active photon flux density
PS II – photosystem II
qPCR – quantitative polymerase chain reaction
rETR – relative electron transport rate
RT-qPCR – reverse transcriptase quantitative polymerase chain reaction
RuBisCO – Ribulose-1,5-bisphosphate carboxylase/oxygenase
Tm- melting temperature
UK – United Kingdom
USA – United States of America
UV-vis – Ultraviolet-visible

LIST OF MATHEMATICAL SYMBOLS

Beta – β

Degrees Celsius – ^{o}C

Grams – g

Grams per litre – gL^{-1}

Gram per dry cell weight – $g^{-1}DCW$

Grams per litre per day – $gL^{-1}d^{-1}$

Hours – h

Intrinsic fluorescence – F_o

Litre – L

Maximum efficiency of PS II – Fv/Fm

Maximal fluorescence yield – F_m

Maximum fluorescence in a light adapted sample – F′m

Microgram per millilitre - $\mu g\ mL^{-1}$

Micro litre – µL

Micrometre – µm

Watts – W

Micromole – µmol

Milligram – mg

Milligrams per litre – mgL^{-1}

Milligrams per litre per day – $mgL^{-1}d^{-1}$

Millilitre – mL

Minutes – min

Molar – M

Nano meter – nm

Percent – %

Per day – d^{-1}

Percentage by weight – wt. %

PS II operating efficiency – F′q/F′m

Rotations per minute – rpm

Seconds – s

Standard deviation – SD

Variable fluorescence – F_v

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ABSTRACT

Microalgae are considered to be a potential feedstock for biodiesel production. However, the main concern with regard to the large scale microalgal biodiesel production process is its competence and economic viability. The commercial realization of microalgal biodiesel production requires substantial impetus towards development of efficient strategies to improve lipid yields upstream. Nitrogen (N) and phosphorus (P) stress during cultivation are the widely used lipid accumulation strategies for microalgae. However, these individual nutrient stress strategies are associated with compromised biomass productivity which hampers overall lipid productivity. Lipid enhancement strategies based on light, temperature and CO_2 are associated with technological barriers for scale up and incur additional cost.

Thus, the main aim of this study was to develop an integrated, easily applicable and scalable lipid enhancement strategy based on nutrients and metals such as N, P, iron (Fe), magnesium (Mg), calcium (Ca) and EDTA stress for selected indigenous microalgal strains. The effect of metal concentrations individually and in combination on microalgal lipids and biomass production is a scarcely exploited area. In this study, a novel approach involving individual as well as combined metals and EDTA stress under N and P limited conditions for lipid enhancement in microalgae was investigated. Microalgal growth physiology, photosynthetic performance, biochemical composition (lipid, carbohydrate and protein) and expression of selected key genes involved in photosynthesis (*rbcL*) and fatty acid biosynthesis (*accD*) were studied both under selected individual and combined stress conditions.

Out of seven microalgal isolates obtained during the initial isolation and screening process, two strains were selected for lipid enhancement study based on their growth rates, biomass yields, lipid content and lipid productivities. The strains were later identified as *Acutodesmus obliquus* and *Chlorella sorokiniana* based on both morphological characteristics and phylogenetical analysis. The selected strains were thereafter subjected to different cultivation conditions involving varying metal, EDTA and nutrient stress conditions. A significant increase in lipid productivity was observed when the concentrations of Fe, Mg and EDTA were increased and Ca was decreased to degree in the N and P stress BG11 medium. For *A. obliquus*, a highest lipid productivity of 80.23 $mgL^{-1}d^{-1}$ was achieved with the developed strategy under limited N (750 mg L^{-1}) condition which was 2.18 fold higher than BG11 medium and 1.89 fold higher than N limited condition alone. Similarly, for *C. sorokiniana*, highest lipid productivity of 77.03 $mgL^{-1}d^{-1}$ was achieved with the developed strategy under limited N (500 mgL^{-1}) and P (10 mgL^{-1}) which was 2.67 fold higher than BG11 medium and 2.35 fold higher than N and P limited condition alone. For both the microalgal strains, Fe was the most significant trace metal affecting their lipid productivity. These above observations were further confirmed through photosynthetic performance analysis and gene expression studies. At mid log phase, 6.38 and 5.15 fold increases in the expression levels of *rbcL* gene were observed under combined stress (OCMS+OE) as compared to the control (BG11) condition in *A. obliquus* and *C. sorokiniana* respectively. This also resulted in an increased expression level of *accD* gene involved in lipid biosynthesis to 10.25 fold and 9.79 fold in *A. obliquus* and *C. sorokiniana* respectively at late log phase. The results from expression studies of *rbcL* and *accD* genes were in compliance with biomass yields, photosynthetic performance, protein yield and lipid productivities for both the strains under different cultivating conditions.

The universal applicability of the above strategy was confirmed by applying it to five other microalgae strains isolated in this study which resulted in considerable increase in their overall lipid productivity under optimized conditions. Attempts were made to scale up the lab scale study to open circular pond (3000L) cultivation for *A. obliquus*. Results showed a 2.08 fold increase in lipid productivity under optimized conditions compared to the control, which emphasizes the scalability of the developed strategy even under uncontrolled conditions.

In conclusion, the developed combined metal and EDTA stress strategy not only assisted in alleviating the biomass productivity but also enhanced the lipid accumulation which resulted in overall increased lipid productivity under N and P limited condition. Furthermore, the improved carbohydrate and protein productivities observed with the developed lipid enhancement strategy make it suitable for biorefinery approach with multiple products. An improvement in lipid profile and high biodiesel conversion were also observed with this universally applicable and scalable lipid enhancement strategy confirming their potential applicability during large scale cultivation for biodiesel production.

PREFACE

Publications

Journal articles

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CHAPTER ONE

INTRODUCTION

Fossil oil depletion, rising prices and its contribution to greenhouse gas (GHG) emissions has garnered interest in alternative renewable forms of energy including biodiesel. Biodiesel can be produced from edible plant oil and non-edible plant oils, as well from microalgal lipids. However, many oil plant crops cannot meet the current global transportation fuel demand and have been argued to be unsustainable as they compete for nutrients; water and arable land with food crops, and also disrupt the biodiversity (Singh et al., 2014, Benemann, 2013, Chisti, 2007).

Microalgal traits such as its fast growth rates, high lipid accumulation, suitable fatty acid compositions and adaptability towards a wide range of climatic and environmental conditions makes it an appropriate feedstock for biodiesel production. Microalgae are a versatile and ubiquitous group of organisms that are naturally found in the ocean, freshwater bodies, on rocks, soils and even on trees (Sharma et al., 2011). They are diverse in terms of their morphological, cytological, molecular and reproductive characteristics. Microalgae have efficient physiological mechanisms that utilize solar energy and atmospheric CO_2 to synthesize valuable products such as lipids, carbohydrate, pigments etc. Microalgae have higher photosynthetic efficiencies, faster growth rate and higher lipid productivities than terrestrial plants (Demirbas and Fatih Demirbas, 2011, Abomohra et al., 2016). Many microalgal species are known to accumulate substantial quantities of lipid under stress conditions (Mata et al., 2010, Quinn and Davis, 2015). Despite the remarkable advantages of microalgae over other biodiesel feedstocks, commercial algal biofuels production is still not

economically viable due to the high cultivation cost for algal biomass generation and challenges in scaling up of harvesting, lipid extraction and biodiesel conversion technologies (Singh et al., 2014, Abomohra et al., 2016). One possible solution for improving the economics of microalgal cultivation is to select a suitable strain and to enhance its overall lipid productivity by altering the cultivation conditions.

Selecting a fast-growing and hyper lipid accumulating microalgal strain, which is acclimatized to the local climatic conditions, is essential for the success of microalgal cultivation for commercial scale biodiesel production (Le Chevanton et al., 2013, Nascimento et al., 2012). This requires systematic screening processes based on their physiological, biochemical and genetic characterization.

Enhancement of the lipid accumulation in microalgae could improve the economics of the biodiesel production process. A numbers of factors are known to influence the lipid accumulation in microalgae, such as nitrogen starvation or limitation, phosphate limitation, high salinity, CO_2 concentration, light intensity, and temperature etc. (Abd El Baky et al., 2012, Cao et al., 2014, Jiang et al., 2012, Atta et al., 2013). Under the stress conditions, microalgae tend to accumulate energy in dense forms such as lipids and carbohydrates. At large scale, microalgae can be cultivated in closed photobioreactors or open cultivation systems. Closed photobioreactors include several types such as tubular photobioreactors, flat plate photobioreactors etc. Open cultivation systems include raceway ponds, open tanks etc. (Rawat et al., 2013). The effects of environmental factors such as light intensity, CO_2 concentration and temperature on microalgal lipid production were studied mostly at laboratory scale under controlled environments. However, controlling these conditions at commercial scale are impractical and energy intensive thus incurring additional cost (Rawat

et al., 2013). Alteration of nutrient concentrations in the cultivation medium could be the viable approach to lipid enhancement which is easy, scalable and can be applied to various types of microalgal cultivation systems. Among these, nitrogen (N) and phosphorous (P) limitations are widely applied strategies for lipid enhancement; however, they are associated with compromised biomass production which ultimately affects the overall lipid productivity (Chu et al., 2014, Adams et al., 2013). Therefore, there is need for alternate and novel strategies to be developed which can be coupled with N and P limitation to improve overall lipid productivity.

Trace metals such as iron (Fe), magnesium (Mg), copper (Cu), calcium (Ca), manganese (Mn) are very important for the cellular metabolic mechanism of microalgae *viz*, photosynthesis, cell division, respiration, intra cellular transportation, protein and lipid biosynthesis and alterations of their concentrations are reported to improve biomass and lipid yields in microalgae (Fan et al., 2014, Gao et al., 2013, Cao et al., 2014). A few studies have reported on the effect of individual metals (Fe, Mg, Cu, and Ca) on the microalgal lipid accumulation (Sibi et al., 2014, Abd El Baky et al., 2012, Ren et al., 2014). However, the combined effect of these metals on microalgal lipid yield has not yet been thoroughly investigated. Very few studies report the actual role of these nutrients and metals on microalgal growth, photosynthetic performance and lipid biosynthesis at physiological as well as molecular levels. Varying the metal concentrations of nutrient stress media could assist the microalgae cell to overcome the challenges of lower biomass production under N and P limited conditions and may result in increased lipid productivity.

The influence of stress conditions on the molecular mechanism of photosynthesis and lipid biosynthesis is not well understood both at biochemical and molecular level (Fan et al.,

2014, Blatti et al., 2012). It is essential to systematically understand the cellular response to the stress conditions from different level including biochemical and genetic response to provide theoretical and experimental bases for improvement in growth and production of metabolites (lipid, carbohydrate and protein) (Fan et al., 2016, Miller et al., 2010). Progress in molecular biology tools have allowed the researchers to understand the function of genes involves in basic metabolism of microalgae including photosynthesis and fatty acid biosynthesis (Shin et al., 2015, El Amrani et al., 2015). Expression studies of these key genes could assist in understanding the molecular mechanism under various stress conditions and may assist in paving the path for future genetic engineering studies. Reverse transcriptase PCR (RT-qPCR) and transcriptome analysis are the currently employed as tool for analysis of the gene expression at transcriptomics (RNA) level (Yu et al., 2016). RT-qPCR coupled with transcriptome analysis of microalgae could give a better understanding of the changes in gene expression levels of key genes involves in basic cell metabolism including lipid biosynthesis pathways in response to different environmental and cultivation conditions (Liang and Jiang, 2013, Fan et al., 2016, El Amrani et al., 2015). However, there are very few studies conducted on the gene expression analysis of microalgae under different nutrient and metals stress conditions.

Considering the research gaps identified and advancement in technology, the aim of this study was to develop an easily applicable and scalable lipid enhancement strategy for economical biodiesel production from indigenous microalgae. The research was focused on developing lipid enhancement strategy combining macronutrient and metal stress and further to assess their impact on growth, photosynthesis, biochemical composition and expression of key genes responsible for carbon fixation and lipid biosynthesis in microalgae. The findings

of the study will give an efficient cultivation strategy for production of microalgal biomass with substantial lipid accumulation. The results will aid in understanding the role of stress factors towards improving lipid yields in microalgae. The results will also provide further knowledge towards genetic engineering of microalgae to enhance the biomass and lipid production. The outcomes of this research will contribute to the current global body of knowledge in the search for alternative renewable fuel production processes that can compete economically and quantitatively with fossil fuel.

1.1 Aim

The aim of this study was to develop an integrated lipid enhancement strategy based on combined nutrient and metal stress (N, P, Mg, Fe, Ca and EDTA) and to investigate its effect on biochemical composition, photosynthetic performance and expression of key genes involved in photosynthesis and lipid biosynthesis pathways.

1.2 Objectives

1. Isolation, screening and phylogenetic analysis of indigenous lipid producing microalgae
2. Evaluation of biomass and lipid production of microalgae under selected nutrient and metal stressed conditions
3. Gene expression analysis of key genes involved in biosynthesis of fatty acid (ACCase) and carbon fixation (rbcL) under selected stress conditions
4. Elucidation of the effects of nutrient and metal stress on microalgal carbohydrate, lipid, protein content and the suitability of lipids for biodiesel production

CHAPTER TWO

LITERATURE REVIEW

SINGH, P., KUMARI, S., GULDHE, A., MISRA, R., RAWAT, I. & BUX, F. 2016. Trends and novel strategies for enhancing lipid accumulation and quality in microalgae. *Renewable and sustainable energy reviews* 55(16) 1-15.

2.1 Microalgae as a biodiesel feedstock

Microalgae have attracted significant interest from researchers as a biodiesel feedstock due to its high growth rate, hyper lipid accumulation ability and environmental benefits (Zhu et al., 2013). Microalgae have shown lipid accumulation between 20-70% lipids per dry cell weight (Amaro et al., 2011). Microalgal lipids are constituted of saturated, monounsaturated and polyunsaturated fatty acids and include C14:0, C16:0, C18:1, C18:2 and C18:3 as major contributing fatty acids (Song et al., 2013) which are considered to be suitable for biodiesel production. Production of value added by-products and utilization of lipid-extracted biomass (animal feed, aquaculture and biomethane production) have further strengthened the case for microalgae as a sustainable feedstock for biodiesel production.

The microalgae biodiesel production process involves multiple steps, which includes, bioprospecting of potential microalgal strains, cultivation of microalgae for biomass production, harvesting of biomass, extraction of lipids from biomass and conversion of lipids

to biodiesel. Therefore, despite many advantages, commercial realization of microalgal biodiesel is still a challenge owing to its high production cost (Singh et al., 2014, Pulz and Gross, 2004, Sharma et al., 2011, Wobbe and Remacle, 2014). More focus needs to be given on screening of locally adapted hyper lipid producing microalgal strain, ensuring high lipid accumulation, cost effective harvesting technology, efficient lipid extraction and biodiesel conversion processes for economical biodiesel production from microalgae.

Selection of robust hyper lipid producing microalgal strain and enhancement of microalgal lipid content is the first and amongst the most important steps in microalgae based biodiesel production. Different microalgae strains with varying adaptability and lipid accumulation capabilities were isolated from different geographical locations thus far by various researchers. Nutrient limitation and induction of stress under controlled cultivation conditions have been the norm to improve lipid content in microalgae (Singh et al., 2014, Klok et al., 2013, Jiang et al., 2012). The nutrient limitation strategies however, are associated with lower biomass productivity which is attributed to the arrest of proteins that are involved in transmitting signals for cell division (Miller et al., 2010, Guo et al., 2014, Peccia et al., 2013). The abiotic factors that influence growth and lipid accumulation in microalgae include light, CO_2, temperature, salinity and pH (Lv et al., 2010, Roleda et al., 2013). Controlling these factors at large scale cultivation may pose technical difficulties or add to biomass production cost. Recently, novel strategies have been explored to overcome the challenges of conventional approaches and to achieve maximum possible outcomes in terms of lipid yields, sustainability and cost effectiveness (Li et al., 2012, Park et al., 2013). These strategies include a combination of stress factors, co-culturing with other microorganisms, addition of phytohormones and chemical additives (Salama el et al., 2014,

Vila et al., 2012, Le Chevanton et al., 2013). These lipid enhancement strategies however need to be thoroughly investigated for their effect on growth physiology, photosynthesis and molecular mechanisms of microalgae in order to develop efficient, cost effective, scalable microalgal cultivation technologies.

2.2 Bioprospecting of oleaginous microalgal strains

Microalgae exist in a variety of forms from unicellular to multi-cellular and in diverse environments from normal freshwater, brackish and marine water to extreme environments such as hyper saline, hot water-springs, and extreme cold. Microalgae have a very large biodiversity with over one million species which includes Diatoms (Bacillariophyceae) (≈1, 00,000 species) followed by green algae (Chlorophyceae) (≈8,000 species), blue-green algae (Cyanophyceae) (≈2,000 species) and golden algae (≈1,000 species) (Pereira et al., 2016, Kirrolia et al., 2013, Mutanda et al., 2011). However, limited numbers of microalgal strains have been conserved worldwide in culture collections and very few (≈ 10% of total collection) have been investigated for their biochemical composition (Cobos et al., 2017, Duong et al., 2012). The environmental conditions, such as temperature, light, pH and salinity affect the physiology of microalgae and provide distinctive features according to the habitats (Gouveia and Oliveira, 2009). This vast variety is a key of success for microalgal biofuels production and for various other applications. Microalgal biomass can be used for different types of renewable biofuels including methane, bio-crude oil and biodiesel. The composition of microalgal biomass depends on the type and habitat of the microalgal strain. Fresh water microalgae are seen as ideal sources of biodiesel as they have high

photosynthetic efficiency and the ability to produce high quantities of lipids (Mondal et al., 2017, Karpagam et al., 2015). However, the biochemical composition varies from species to species.

In the bioprospecting process, high lipid and biomass yield, fast growth rate, robustness towards environmental stress are the key characteristics for the selection of microalgal strain for biodiesel production. Local algal strains isolated from natural habitats and collected during different seasons are expected to be the best adapted to those specific local conditions; therefore, indigenous strains would be the best for large-scale cultivation.

The idea of bioprospecting microalgae from local habitats for biofuels or high-value products was started in 1978 by National Renewable Energy Laboratory (NREL). The NREL launched the Aquatic Species Program (ASP) and collected over 3000 microalgae strains from the west, northwest, and south-eastern regions of the continental US, as well as Hawaii to systematically screen for high oil content strains grown under normal or severe conditions (Sheehan et al., 1998). Table 2.1 represents the lipid content of microalgae strains from different geographical regions. Do Nascimento et al. (2012) have isolated 34 microalgal strains from Mar del Plata, Buenos Aires, Argentina and screened all microalgal strains for biodiesel production. In their study, *S. obliquus* was selected on the basis of high lipid content, short doubling period and suitable fatty acid profile. Ahn et al. (2012) have isolated *Chlorella* sp. from sea ice Spitsbergen, Norway and investigated it as a potential candidate for biodiesel production. In their study, isolated *Chlorella* strains have shown a suitable lipid profiles for biodiesel production. Similarly, several microalgal strains have been isolated by researchers from different parts of the world each having some distinctive feature, however very few have been selected as potential candidates for biodiesel production. South Africa is

rich in biodiversity but very little research on the isolation and identification of microalgal strains suitable for mass cultivation and biodiesel production are reported. Most of the previous studies were based on ecological aspects (Anandraj et al., 2008, Thomas et al., 2005, van der Molen and Perissinotto, 2011, Perissinotto et al., 2010). Thus, there is immense scope for bioprospecting of hyper lipid accumulating microalgal strains in South Africa for biodiesel production.

Table 2.1: Lipid content of microalgal strains from different geographical location for biodiesel production

Microalgae	**Geographical location**	**Lipid content (%)**	**Reference**
Ankistrodesmus sp.	Amazon river Peru	27-35	(Cobos et al., 2017)
Chlorella lewinii	Amazon river Peru	22.7	(Cobos et al., 2017)
Monoraphidium sp.	Nisargruna biogas plants, India	34.3	(Tale et al., 2014)
Scenedesmus sp.	Nisargruna biogas plants, India	28.3	(Tale et al., 2014)
Tetradesmus sp.	Nanay River, Peru	13.5	(Cobos et al., 2017)
Poterioochromonas sp.	Vietnam	28	(Thao et al., 2017)
Chlorella vulgaris	Vietnam	10	(Thao et al., 2017)
Nannochloropsis sp.	Hainan, Province, China	3	(Yu et al., 2007)
Acutodesmus dimorphus	Ankleshwar, Gujrat, India	27.3	(Chokshi et al., 2015)
Botryococcus braunii	Nayok province, Thailand	12	(Ruangsomboon, 2012)
Scenedesmus obliquus	Wonju, South Korea	21-58	(Abou-Shanab et al., 2011a)
Micractinium pusillum	Wonju, South Korea	24	(Abou-Shanab et al., 2011a)
Chlorella ellipsoidea	Wonju, South Korea	32	(Abou-Shanab et al., 2011a)
Chlamydomonas mexicana	Wonju, South Korea.	29	(Abou-Shanab et al., 2011b)
Chlorococcum *humicola*	Penang Island, Malaysia	29.16	(Hena et al., 2015)
Anabaena sp	Penang Island, Malaysia	7.24	(Hena et al., 2015)

2.3 Photosynthesis and lipid biosynthesis in microalgae

2.3.1 Photosynthesis

Eukaryotic microalgae predominantly follow photoautotrophic nutrition mode whereby energy is acquired from the process of photosynthesis (Sayre, 2010). Studying microalgal photosynthesis is important in order to develop the strategies for cultivation of microalgae, CO_2 sequestration and biomass production for biodiesel production (Sayre, 2010, Jiang et al., 2012, van Kooten and Snel, 1990). The photosynthetic efficiency of microalgae is higher than that of terrestrial plants (Cuellar-Bermudez et al., 2015). Peccia et al. (2013) reported the photosynthetic efficiency of microalgae to range from 6 to 10 % as compared to 1 to 2 % by the terrestrial plants. The oil accumulation in the microalgal cell is governed by photosynthetic efficiency during stress condition (Kirrolia et al., 2013). All photoautotrophic organisms (algae and plant) have the capability to capture light energy and convert it in to the chemical energy in presence of $CO_{2,}$ inorganic nutrients, and water. Chlorophyll, contained within the chloroplast is responsible for photosynthesis (van Kooten and Snel, 1990).

Photosynthesis is a complex process that involves two stages of reactions (Jiang et al., 2012). First is light dependent reaction where water molecules are split into oxygen, protons, and electrons in the presence of light (Cheung et al., 2012). This step provides the energy sources like ATP and NADPH for the next steps of the photosynthesis. The second stage is light independent reactions, also known as dark reaction which involves the conversion of atmospheric CO_2 to carbohydrates (Seo et al., 2015). Carbohydrates produced are used as precursors for other metabolic pathways such as lipid and protein biosynthesis.

In microalgal photosynthesis, light is captured by the proteins known as the light harvesting complex (LHC) (Berteotti et al., 2016). LHC associates with chlorophyll and plays dual role of capturing light as well as dispersal of surplus energy. Photosystem I (PS I) and photosystem II (PS II) are the reaction centers where light energy is converted to chemical energy. Light energy excitation is utilized in PS II for the photolysis of water to generate protons, electrons and oxygen (Berteotti et al., 2016). Electrons are transported to NADPH via electron transport chain consisting of plastoquinone (PQ), cytochrome b6 f (Cyt b6 f), photosystem I (PSI), and ferredoxin (Fd) (Parmar et al., 2011, Brand and Becker, 1984, Mekhalfi et al., 2014). Protons are also released in the thylakoid by PS II which generates a proton gradient which is used for ATP synthesis assisted by ATP synthase. NADPH and ATP synthesized in the process are utilized in the Calvin cycle and other metabolic pathways to produce carbohydrates and lipids.

The Calvin cycle is responsible for CO_2 fixation and its conversion into carbohydrates in a three step process (Figure 2.1). In the first step, CO_2 reacts with 5 carbon compound ribulose-1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate (3PGA). This reaction is catalyzed by enzyme, Rubisco (ribulose-bisphosphate carboxylase) (Parry et al., 2008, Andersson and Backlund, 2008) which is considered to be the key enzyme involved in the process of photosynthesis in microalgae. Stress conditions can alter the activity of this enzyme which can affect the rate of photosynthesis reaction. Numerous studies have been conducted on assessing the effect of different stress condition on the activity of RuBisCO enzyme both in plants as well as microalgae (Petrou et al., 2014, Negi et al., 2015). In the second step, 3PGA is phosphorylated to form 1, 3-bisphosphoglycerate with the aid of ATP and phosphoglycerate kinase. The 1, 3-bisphosphoglycerate is reduced to glyceraldehyde-3-

phosphate (G3P) with the aid of NADPH and glyceraldehyde-3-phosphate dehydrogenase. ATP and NADPH utilized for this reduction are supplied by the light reaction of photosynthesis (Li et al., 2014). The third and final step is regeneration of RuBP which enters into another cycle of CO2 fixation. G3P is converted into carbohydrates by subsequent biochemical pathways.

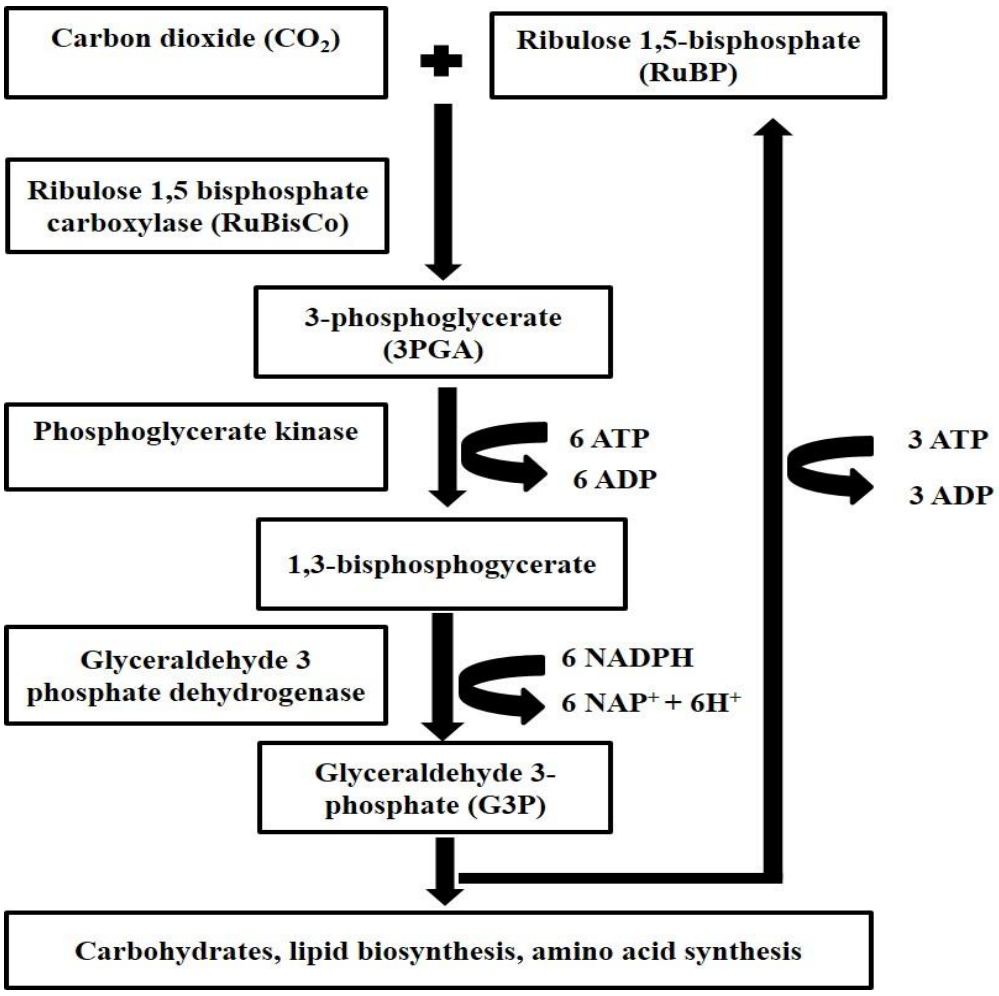


Figure 2.1: The Calvin cycle in microalgae

2.3.2 Lipid biosynthesis

Arabidopsis sp. is considered to be a model organism for understanding the enzymes involved in lipid synthesis of all photosynthetic organisms (Misra et al., 2012, Yu et al., 2011, Ohlrogge and Browse, 1995). Based on the sequence homology of genes involved in lipid metabolism of microalgae and *Arabidopsis,* it was generally assumed that microalgae have similar lipid metabolic pathway to that of higher plants (Misra et al., 2012). However, after the whole genome sequencing of *Chlamydomonas*, it was established that microalgae have a simple lipid metabolic pathway compared to higher plants (Merchant et al., 2012). Biosynthesis of fatty acids in microalgae occur in the chloroplast and are regulated by an enzyme complex acyl carrier protein (ACP) fatty acid synthase (FAS) type 2 (Harwood and Guschina, 2009, Liang and Jiang, 2013). Figure 2.2 depicts the fatty acid biosyhntesis in microalgae.

FAS enzyme is a polypeptide chain with multiple domains, each having a distinct enzyme activity required for fatty acid biosynthesis. The first step in fatty acid biosynthesis is the formation of malonyl-CoA from acetyl CoA, catalysed by acetyl-CoA carboxylase (ACCase) (Huerlimann and Heimann, 2013, Salie and Thelen, 2016, Hu et al., 2008). In the chloroplast, photosynthesis provides an endogenous source of acetyl CoA, and more than one pathway may contribute in maintaining the acetyl CoA pool. ACCase is considered as the main enzyme that catalyses the first step reaction in lipid biosynthesis. It has three major functional sites that promote the formation of malonyl–CoA; the main carbon donor for fatty acid biosynthesis. This is considered as a rate limiting step in lipid synthesis which involves two stages: a) The transfer of CO_2 (HCO_3^-) by biotin carboxylase portion of ACCase to a biotin prosthetic group that is attached to the E-amino group of lysine residue and b) the

transfer of activated CO_2 from biotin to acetyl CoA (Figure 2.2). Production of Acetyl-CoA and its conversion to fatty acid, however, depends on factors such as tissue type, light/dark condition, developmental stages and species (Thelen and Ohlrogge, 2002, Salie and Thelen, 2016).

Biosynthesis of fatty acid is a multistep step reaction which involves condensation, reduction, dehydration and reduction. A malonyl group is first transferred from CoA to a protein cofactor acyl carrier protein (ACP) which is involved in the whole process until the formation of a 16 to 18-carbon product, ready to be transferred either to the glycerolipids or exported from the plastid (Castro et al., 2016). The next step is a condensation reaction. At this stage, the malonyl group of malonyl-ACP undergoes a series of condensation reactions with acetyl-CoA releasing CO_2, that helps to drive the reaction forward. At least three groups of condensing enzymes (commonly called 3-ketoacyl-ACP synthase) are involved in the synthesis of 18-carbon fatty acids (Harwood and Guschina, 2009). The initial condensation reaction leads to the formation of a four carbon product, 3-ketoacyl-ACP, catalysed by 3-ketoacyl ACP synthase III (KASIII) followed by another condensation step catalysed by enzymes is KASI (Dehesh et al., 1998) (Figure 2.2). This reaction cycle is completed by a reduction reaction catalysed by enoyl-ACP reductase. Each cycle of these four reaction steps lengthen the fatty acid precursor chain by 2-carbons, a process known as elongation leading to the formation of saturated 16:0-ACP and 18:0-ACP. Unsaturated fatty acids are formed when a double bond is introduced by the enzyme stearoyl ACP desaturase. Elongation of a fatty acid chain takes place in the plastid and continues until the acyl group is removed from ACP, either as a result of hydrolysis of acyl-ACP by acyl-ACP thioesterase, releasing free

fatty acid or by the transfer of fatty acid from ACP to glycerol-3-phosphate or to monoacylglycerol-3-phosphate by one of the two acyltransferases (Hu et al., 2008).

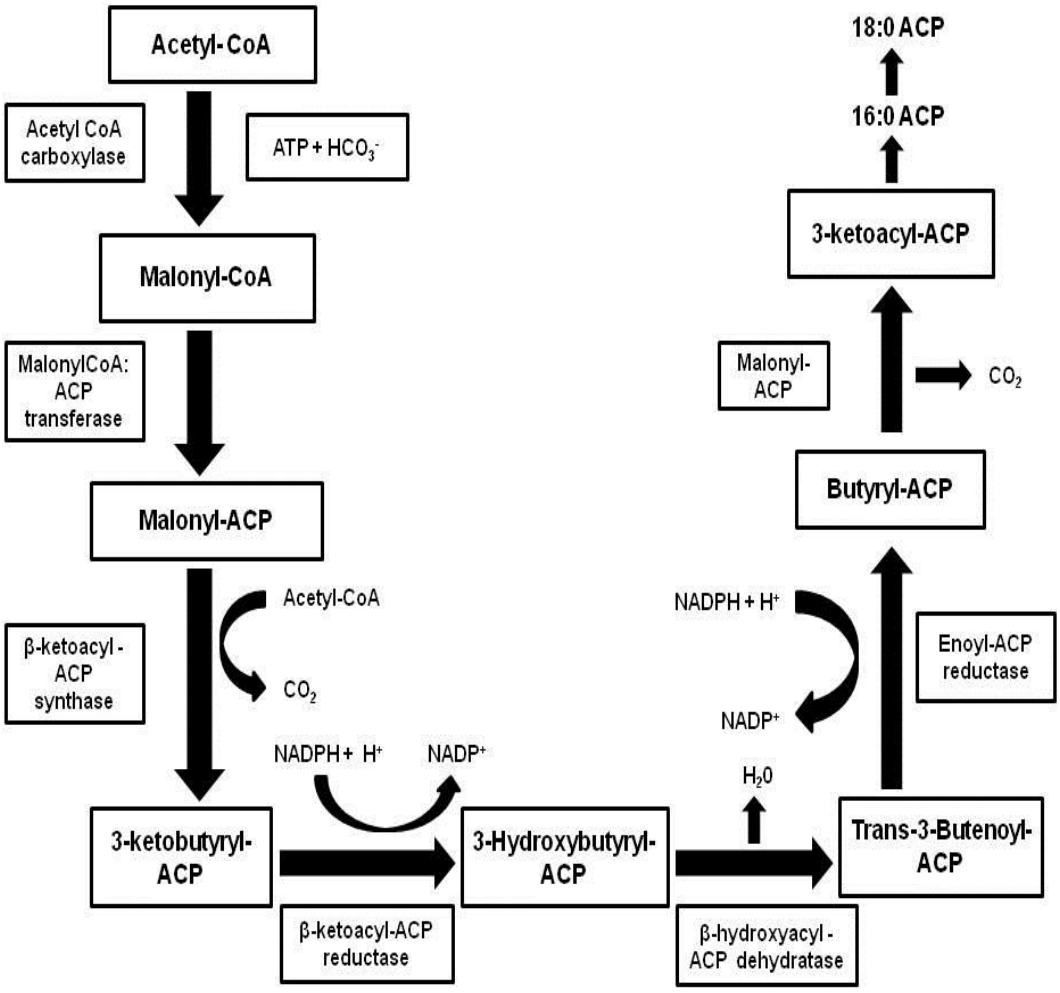


Figure 2.2: Fatty acid biosynthesis pathways in microalgae

2.4 Factors affecting lipid accumulation in microalgae

Microalgae are able to survive in extreme environments as they can alter their metabolism according to change in environmental conditions. Under unfavorable conditions, microalgae have the tendency to accumulate lipids to protect cells from photo-oxidation (Zhang et al., 2013, Jiang et al., 2012). Lipid enhancement strategies involving alteration of the nutrient regime and cultivation conditions are widely studied. Factors such as nutrient stress, light, temperature, CO_2, salinity, etc. have been explored by several researchers to enhance lipid accumulation in microalgae (Merchant et al., 2012, Sharma et al., 2012).

2.4.1 Effect of nutrient regimes on lipid accumulation

Nutrients such as N, Fe, P, Mg, Ca, Cu, etc. are very important for cellular mechanism *viz*, photosynthesis, cell division, respiration, intracellular transportation, protein synthesis etc. (Zhang et al., 2014, Klok et al., 2013). Under nutrient limited (stress) conditions, microalgae tend to accumulate energy in the form of polysaccharides, and/or lipids. This defence mechanism of the microalgal cell under stressed conditions has been exploited widely for the production of neutral lipids, carotenoid, polysaccharides and many other metabolites (Lamers et al., 2012, Stehfest et al., 2005, Yu et al., 2015).

2.4.1.1 Nitrogen

Nitrogen is an important nutrient for growth of microalgae as it enables cell division and is a major component of proteins (Lopez Garcia de Lomana et al., 2015). Nitrogen is generally provided in the culture media as nitrates, urea and ammonium salts; however, uptake and utilization of these N forms by microalgae may vary (Xin et al., 2010, Cao et al.,

2014). Nitrogen deprivation conditions could lead to reduced cell division and thus accumulation of photosynthetic carbon into storage molecules such as lipids (Amaro et al., 2011). Reduced cell division shifts the lipid biosynthetic pathways to synthesize more neutral lipids rather than synthesizing membrane lipids required for cell wall formation for new cells during cell division (Converti et al., 2009, Ji et al., 2013). Subsequent accumulation of NADH due to the slower photosynthetic rate inhibits enzyme citrate synthase and prevents acetyl CoA from entering into the TCA cycle. Elevated concentrations of acetyl CoA activate acetyl CoA carboxylase, which converts acetyl CoA to malonyl CoA. This irreversible conversion reaction is the rate limiting step in fatty acid biosynthesis which leads to enhanced lipid accumulation in microalgal cells (Praveenkumar et al., 2012). Biomass yield is directly proportional to N concentration in culture media while lipid accumulation of microalgal cells is inversely proportional to N concentration. Nitrogen starvation is the most widely used strategy to improve lipid accumulation. Researchers have been optimizing the N concentration in culture media for microalgae to obtain high biomass yield with substantial lipid accumulation (Han et al., 2015, Peccia et al., 2013).

Converti et al. (2009) studied the effect of N limitation on lipid yields of *N. oculata* and *C. vulgaris* (Table 2.2). Under N limited condition, lipid yield was increased to 15.31% and 16.41% in *N. oculata* and *C. vulgaris* respectively. Similarly Gao et al. (2013) reported an increase in lipid content of *C. muelleri* from 21% to 46% when the microalgae was grown under N deprivation condition. In their study, though N deprivation has resulted in high lipid accumulation but the biomass productivity of the strains was decreased drastically from 20 $mgL^{-1}d^{-1}$ to 8 $mgL^{-1}d^{-1}$. Adams et al. (2013) have studied effect of the N limitation on the biomass and lipid productivity of the microalgae, *C. vulgaris*, *C. sorokiniana*, *C. oleofaciens,*

N. oleoabundans, S. dimorphus, and S. naegleii. In their study, they observed a low to high N stress have resulted in a decrease in the biomass yield by 20-55%; however, there was an increase in overall lipid content of the cells. Most of these studies were focused on lipid enhancement of microalgae, however low biomass productivity under low N concentration is a major drawback of this strategy which can hamper the overall lipid yield considerable during large scale cultivation.

2.4.1.2 Phosphorus

Phosphorus plays an important role in various metabolic processes such as signalling pathways, energy generation, and photosynthesis (Chu et al., 2014, Liang et al., 2012). It is a major component of phospholipids which is a cell wall constituent of microalgae. Phosphorus is supplied in nutrient medium as phosphate. Phosphate limitation in medium is known to increase the lipid content in a microalgal cells. This behaviour is attributed to the breakdown of phospholipids from cell wall into neutral lipids in order to obtain required phosphate for other metabolic processes (Qi et al., 2013, Sharma et al., 2012).

Xin et al. (2010) reported a lipid accumulation of 53% in *Scenedesmus* sp. under P limitation (0.1mgL^{-1}) (Table 2.2). Chu et al. (2013) studied the effect of P limitation on lipid yield, biomass productivity as well as fatty acid composition of *C. vulgaris*. In their study, they did not observe any significant impact of P limitation on the lipid content of the *C. vulgaris.* The lipid content of 37.73% under P limited condition was almost similar to the lipid content observed in control experiment (37.60%). However, limitation of P in the culture medium has drastically affected the biomass productivity of *C. vulgaris* in their study. The biomass productivity was decreased from 100 mg$L^{-1}d^{-1}$ to 38.25 mg$L^{-1}d^{-1}$ under P limitation. Furthermore, in their study, under the P limited conditions, the fatty acid

composition of *C. vulgaris* showed higher percentage of saturated and mono-unsaturated fatty acids as compared to the control. The above discussed studies showed that P limitation has varying response in terms of lipid accumulation in different microalgal strains. Microalgal cells store P as polyphosphate under nutrient sufficient condition. These polyphosphate molecules are utilized by cell during nutrient deficient condition (Qi et al., 2013, Liang et al., 2012, Chu et al., 2014). Although the N and P limitation strategies are successful in terms of enhancing lipid content in microalgae; low biomass production under nutrient deprivation is the main limitation as it affects the overall lipid productivity.

2.4.1.3 Metals

Metals such as Fe, Mg, Cu, Ca, Mn and Co also affect growth physiology of microalgae. These metals are indispensable part of many metabolic pathways of microalgae *viz*, photosynthesis, cell division, respiration, intra cellular transportation, protein and lipid biosynthesis (Wan et al., 2014, Ren et al., 2014). Dou et al. (2013), have studied the effect of Fe, Zn, Mn, Cu, and Co on the growth and lipid accumulation of microalgae *Nannochloropsis oculata*. In their study, six times increase in the concentration of Fe and Zn have resulted in an increase in biomass yield of *N. oculata.* Zinc plays role in photosynthesis as it is an important metal of enzyme carbonic anhydrase. Similarly, Fe is one of the most important metals in photosystem (I) and photosystem (II), and is also integral part of various enzymes and co-enzymes in lipid biosynthesis pathway (Abd El Baky et al., 2012). Thus supplementation of Fe improves both biomass and lipid accumulation. Liu et al. (2008) have reported 3-7 folds increase in lipid accumulation as compare to the control in *C. vulgaris* under higher Fe concentration condition. Abd El Baky et al. (2012) cultivated *S. obliquus* in N-9 medium with varying concentrations of $FeCl_3$ and lipid yields were found to be increased

with an increase in $FeCl_3$ concentration. In their study, maximum lipid yield of 28.12% was observed in culture with $FeCl_3$ concentration of 20 mgL^{-1}.

Similarly, Mg was also found to influence the lipid accumulation in microalgae (Sirin et al., 2013). Gorain et al. (2013) observed an improvement in lipid content (1.44 fold rise) with the supplementation of Mg (100 mgL^{-1}) in the media. Magnesium promotes the activity of acetyl-CoA, the enzyme that regulates the first committing step of microalgal lipid biosynthesis and is required for chloroplast pyruvate dehydrogenase complex, which provides acetyl-CoA and NADH for fatty acid synthesis (Ren et al., 2014, Plank et al., 2001). Therefore, an increase in Mg supply to the media could enhance the activity of these enzymes, leading to an enhanced lipid accumulation under nutrient stress conditions.

Calcium regulates cyclic electron flow and signal transduction during stress condition in microalgae (Chen et al., 2015). Ren et al. (2014), reported an increase in the lipid content of *Scenedesmus* sp. from 10% to 47% with the increase in Ca concentration in the medium from 0 to 9.8×10^{-4} gL^{-1}. Copper is reported to enhance lipid accumulation and fatty acid content in *C. vulgaris* by increasing oxidative stress (Sibi et al., 2014). Sibi et al. (2014) observed an increase in the fatty acid content of *C. vulgaris, C. protothecoides* and *C. pyrenoidosa* with increase in Cu concentration upto 4 mgL^{-1}.

Though, metals have great influence on the growth and physiology of microalgae, optimization of the metal concentration in the medium is necessary as very high concentration can be toxic microalgae (Dou et al., 2013, Shaul, 2002, Ren et al., 2014). Most of the previous studies have focused on the effect of individual metals on the lipid content of microalgae. None of the previous studies were really focused on understanding the regulatory mechanism of these metals on the metabolic pathways of microalgae.

2.4.1.4 Ethylenediaminetetraacetic acid (EDTA)

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent widely used in microalgae growth media. EDTA, a Lewis acid with six binding sites, reacts with metals and forms a stable ion structure. EDTA has been extensively studied in phytoremediation (Chen et al., 2004, Luo et al., 2005). In a study by Ren et al. (2014) it was reported that the total lipid content and lipid productivity of *Scenedesmus* sp. was increased by 28.2% and 29.7%, respectively with increasing EDTA from 0 to 1.0×10^{-3} g L^{-1}. EDTA added to the medium can enhance the solubility of metals, and thereby facilitating the uptake of metals into the microalgal cells. Hence, EDTA has the potential to enhance the microalgal lipid production. Due to the comparatively small amount of additional EDTA required and inexpensive nature of the chemical, this approach could be easily adapted at large scale.

Nutrient regime alterations are the preferred choice for the enhancement of lipid accumulation, because of its easy applicability at both lab and large scale cultivation. However, the main challenge for this strategy is the trade-off between biomass and lipid yields. Nutrients like N have significant influence on both the biomass generation as well as the lipid accumulation in the cell. Increase in the N concentration in the medium directly affects the biomass and inversely affects the lipid yields (Jiang et al., 2012). Nutrients such as N, Fe and P has been widely studied for microalgal lipid enhancement, however other nutrients also need to be investigated. The further in depth studies on actual physiological role of these nutrient stresses on the microalgae are required in order to develop efficient lipid enhancement approaches.

Table 2.2: Effect of nutrients and cultivation condition on microalgal lipids in previous studies

	Microalgal strain	Stress levels	Lipid content	Lipid productivity ($mgL^{-1}d^{-1}$)	Reference
Nutrient stress					
N	*C. vulgaris*	375 mgL^{-1}	16.51%	20.30	(Converti et al., 2009)
	N. oculata	75 mgL^{-1}	15.51%	16.41	(Converti et al., 2009)
	C. ellipsoideum	2.9 mML^{-1}	40.5%	236.9	(Li et al., 2013)
	S. deserticola	5.8mML^{-1}	54.4%	216.6	(Li et al., 2013)
P	*Scenedesmus sp.*	0.1 mgL^{-1}	53%	-	(Xin et al., 2010)
	Chlorella sp.	32µM	23.6%	15.67	(Liang et al., 2012)
	C. vulgaris	-	37.73	19.50	(Chu et al., 2013)
Fe	*S. obliquus*	20 mgL^{-1}	28.12%	-	(Abd El Baky et al., 2012)
	C. vulgaris	1.2×10^{-5}mol L^{-}	56.6%	-	(Liu et al., 2008)
Cultivation conditions					
Light	*Nannochloropsis*	100 µmol m^{-2} s^{-1}	31.3%	-	(Atta et al., 2013)
Temperature	*N. oculata*	20-25 ºC	7.42-14.9%	-	(Converti et al., 2009)
CO_2	*S. obliquus*	12% CO_2	-	33.14%	(Abd El Baky et al., 2012)
	Nannochloropsis	15% CO_2	-	1.43 g L^{-1}	(Jiang et al., 2011)
Salinity	*D. salina*	1.0M	67%	-	(Venkata Mohan and Devi, 2014)

2.4.2 Effect of cultivation conditions on lipid accumulation

Cultivation conditions such as light, temperature and salinity have known to influence microalgal growth (Ho et al., 2014, Rao et al., 2007). Table 2.2 represents the effect of different cultivation conditions on microalgal lipids in previous studies. Light is important for photosynthesis in autotrophic microalgae and variation in light intensity and photoperiod

could alter the lipid biosynthesis (Choi et al., 2015, Xu et al., 2016). Cultures grown at high light intensity tend to accumulate lipids. The electron acceptor pool ($NADP^+$) is depleted under high light intensity (Wong, 2016). Increased fatty acid biosynthesis in turns uses NADPH and produces $NADP^+$ and serves as protection mechanism which makes $NADP^+$ available for photosynthesis. At low light intensities, more chloroplast membrane synthesis occurs which requires sufficient supply of polar membrane lipids (Wahidin et al., 2013, Choi et al., 2015). Wahidin et al. (2013) studied the effect of light intensity and photoperiod on lipid accumulation in *Nannochloropsis* sp. (marine microalga) and noticed an increase in lipid accumulation of upto 31.3% after 8 day cultivation under 100 $\mu molm^{-2}s^{-1}$ light intensity and photoperiod of 18 h light: 6 h dark cycle. However, a gradual decrease in cell density and growth rate was noticed when the photoperiod cycles were extended to 24 h. It is also reported that during low light intensities, microalgae tend to produce more polar lipids due to the increase in chloroplast membrane synthesis. A stepwise increase in light intensity however, tends to accumulate more neutral lipids without affecting the biomass yield (Breuer et al., 2013). Although light intensity has shown a significant role in microalgal biomass and lipid accumulation, the approach is expensive and is energy intensive to scale up.

Similarly, supply of CO_2 is crucial in autotrophic cultivation of microalgae. Microalgae fix CO_2 via photosynthesis to form various organic metabolites. CO_2 can be supplied by aeration of the microalgae culture which also serves the purpose of proper culture mixing. It was observed that an increased concentration of CO_2 supply resulted in an increase in both biomass and lipid productivities (Amaro et al., 2011). Jiang et al. (2011) observed an increase in biomass productivity ($0.39\text{-}1.43 gL^{-1}$) and growth rate ($0.33\text{-}0.52 d^{-1}$) of *Nannochloropsis* sp. under increased CO_2 (15%) supplementation.

Microalgae can tolerate a wide range of temperatures depending upon the strain. Most of the algal strains can grow well within the temperature range of 25 to 30°C (Roleda et al., 2013). It has been well documented that an increase in temperature increases the saturated fatty acids composition in microalgae while decrease in temperature increases the unsaturated fatty acid composition of total cellular lipids (Pan et al., 2011, Converti et al., 2009). The temperature effect on microalgal species can however be purely strain specific. The effect of temperature on lipid yields of *N. oculata* and *C. vulgaris* were studied by Converti et al. (2009) who have reported that an increase in temperature from 20 to 25°C have resulted in two-fold increase in lipid yield (7.90 to 14.92%) for *N. oculata*, while an increase in temperature from 25 to 30°C decreased the lipid yield from 14.71 to 5.90% for *C. vulgaris*.

Similarly, an increase in NaCl (salt stress) in the medium can lead to an increase in lipid content of microalgae (Venkata Mohan and Devi, 2014). An increased NaCl concentration from 0.5 to 1.0 M improved lipid content upto 67% in *D. salina.* High salinity in the culture medium creates oxidative stress to the microalgal cells which may induces lipid accumulation. An increase in the expression levels of antioxidative enzymes was reported in *C. reinhardtii* upon NaCl stress (Yoshida et al., 2004). Increase in salt concentration can also have qualitative effect on microalgal lipids. Salinity stress also could facilitate the maintenances of saturated fatty acid in higher amounts in microalgal species. *B. braunii* grown under high salinity concentrations of 34 mM and 85 mM, showed 1.7–2.25 fold increase in the relative proportion of palmitic acid and 2 fold increases in oleic acid (Rao et al., 2007). Considering the impact of salinity on quality and quantity of lipids, this approach can be used to enhance lipid accumulation in microalgae however this strategy could be feasible only for halotolerant strains.

Cultivation conditions such as temperature, pH, light intensity, CO_2 supply are easy to maintain in the lab, but maintaining them at large scale is challenging and cost intensive (Atta et al., 2013). Controlling temperature and light is unfeasible in the open cultivation system and cost intensive in the closed cultivation system (Christenson and Sims, 2011, Converti et al., 2009). The supply of CO_2 to both, closed or open cultivation systems is unfeasible because of technical and economic challenges. The choice of the cultivation system is also a long discussed topic. Table 2.2 depicts effect of conventional approaches on lipid content and lipid productivity of microalgae. Each system has its pros and cons, however the microalgal strain and economics of the process makes the choice easier. Further, in depth investigation is required on the parameters discussed above, in order to decide on the economically feasible, easy and scalable strategy for the enhancement of lipid accumulation in microalgae (Table 2.2).

2.4.3 Combined nutrients and abiotic stress

Recent reports have shown the potential of combining different stress factors to improve the microalgal lipid productivity. To obtain maximum yields, it is imperative to have knowledge on the synergistic effects of factors as well as significance of each factor with regard to lipid accumulation (Sun et al., 2014, Cao et al., 2014). Breuer et al. (2013) investigated the effects of light, pH, and temperature on triacylglycerol (TAG) accretion under N deficient conditions and found pH and temperature to be major influencing factors for TAG accumulation. The highest TAG content (40%) was obtained at pH 7 and 27.6 °C, which was independent of light variation. Cao et al. (2014) observed high lipid accumulation in *C. minutissima* as a result of combined influence of Fe, sodium chloride. Ji et al. (2013) studied the effect of variation in temperature, light intensity and photoperiod on

Desmodesmus sp. and observed high biomass production at a combination of temperature: 30 ºC, light intensity: 98 µmolm^{-2}s^{-1} and photoperiod: 14:10 (L: D Light: Dark photoperiod). Similarly, Pan et al. (2011) have subjected four thermo-tolerant *Desmodesmus* strains to N starvation under different temperature regimes. Two of the isolated strains showed lipid production of 50% at 45 ºC. The combined stress strategies have shown that microalgae are responsive towards the nutrient stress and cultivation conditions in terms of biomass growth or metabolite production. Thus in appropriate combined stress strategy one factor compensates for the negative effect of the other factor. However, most of these studies have been carried out at lab scale, thus evaluation of these strategies for their scalability is crucial. It is important that combined strategy should be efficient in terms of lipid enhancement as well as each factor should be easily scaled up without incurring extra cost.

2.5 Expression analysis of functional genes with response to different cultivation conditions

Several researchers have described the lipid biosynthesis pathway in microalgae (Harwood and Guschina, 2009, Khozin-Goldberg and Cohen, 2011, Cao et al., 2014) which involves multistep reaction that is catalysed by an enzyme complex (acyl carrier protein, fatty acid synthase) (Harwood and Guschina, 2009). It is important to understand the mechanism and behaviour of these enzymes in different environmental conditions. Progress in molecular biology tools allowed the researchers to understand the function of major genes involves in basic metabolism of microalgae including photosynthesis and fatty acid biosynthesis (Shin et al., 2015, El Amrani et al., 2015). Gene expression analysis is a process to quantify the

activity level of a particular gene within the cell. Gene expression analysis is mainly carried out by measuring the mRNA level in the cell in response to certain regulatory factors that can influence the gene expression. Reverse transcriptase quantitative PCR (RT-qPCR) is the widely used technique for gene expression studies (Taylor et al., 2010). In RT-qPCR method, the levels of mRNA are quantitated by reverse transcription of the RNA to cDNA followed by quantitative PCR (qPCR) using functional gene specific primers (Taylor et al., 2010).

Transcriptome analysis is another tool for the quantification of gene expression by RNA-sequencing coupled with bioinformatics (Külahoglu and Bräutigam, 2014). This is also called whole-transcriptome shotgun sequencing, which involves the use of next generation high throughput sequencing technologies such as Miseq-Illumina platform for characterizing the RNA content and composition of a given sample. For RNA sequencing, mRNA is converted in to cDNA to from an RNA-sequencing library. The obtained RNA libraries are then subjected to meta transcriptome analysis. The sequence information obtained from the transcripts during sequencing will thereafter assembled using *de novo* assembly and compared against references transcripts obtained from the gene bank. This approach would assist in understanding the response of multiple genes involved in different metabolic processes and enable us to get a comprehensive understanding of the molecular level response. However, there are very few studies available on the gene expression analysis of microalgae under different nutrient and metals stress conditions.

2.5.1 Gene expression analysis of microalgal strains by reverse transcriptase quantitative PCR

Under favourable (optimum nutrients and cultivation parameters) conditions, microalgal growth is endorsed by increased translation and transcription processes which

results in high growth rates and biomass production (Merchant et al., 2012, Liu et al., 2013). Under nutrient depletion conditions, growth is inhibited as most of the anabolic machinery is generally retarded. Fan et al. (2014) investigated the effect of nutrient stress on the expression of various functional genes encoding the key enzymes involved in lipid synthesis of *C. pyrenoidosa*. In their study, N stress resulted in an increase in the expression of genes related to lipid biosynthesis such *accD* gene by 38.9 fold, me gene by 4 fold and dgat gene by 24 fold. It was also observed that the N stress resulted in 4 fold decrease in the *rbcL* gene expression. Few other studies have also conducted to elucidate the effect of nutrients on the gene expression of microalgae (Wan et al., 2014, Li et al., 2015, Jusoh et al., 2015). Table 2.3 represents effect of environmental condition on gene expression of functional genes related to lipid biosynthesis. Most of the studies are either focused on N stress or single nutrient or abiotic stress. Thus there is need to study the various other factors and nutrients individually and in combination which can affect the growth and lipid metabolism in microalgae.

Table 2.3: Effect of various stress conditions on the expression analysis of the functional genes related to lipid synthesis

Microalgae	**Cultivation Condition**	**Targeted enzymes**	**Selected genes**	**Effect on relative gene expression**	**Lipid content/fatty acid content/lipid productivity**	**Reference**
C. pyrenoidosa	N Deficient	Rubisco	*rbcL*	4 times decrease		(Fan et al., 2014)
C. pyrenoidosa	N Deficient	PEPC	pepc	Slight increase	50.4% 34.42 $mgL^{-1}d^{-1}$	
C. pyrenoidosa	N Deficient	PEPC	pepc	Slight increase		
C. pyrenoidosa	N Deficient	Malic	me	4.04 fold increase		
C. pyrenoidosa	N Deficient	ACCase	accA	3.5 folds increase		
C. pyrenoidosa	N Deficient	ACCase	*accD*	38.9 folds increase		
C. pyrenoidosa	N Deficient	DGAT	dgat	24.5 fold increase		
C. pyrenoidosa	Phosphorus	Malic	Me	8.43 fold increase	19.6 $mgL^{-1}d^{-1}$	(Fan et al., 2014)
C. pyrenoidosa	Heterotrophic	PEPC	pepc	3.1 fold increase		
C. pyrenoidosa	Continuous Light	ACCase	*accD*	2.5 fold increase	34.7%	
C. pyrenoidosa	Heterotrophy to phototrophy	DGTA	dgta	47.6 fold increase		
C. sorokiniana	Mixotrophic	ACCase	*accD*	Increase in expression level	51%	(Wan et al., 2014)
C. reinhardtii	Nitrogen stress	DGTA	DGTT1	Increase in expression level	Increases in saturated fatty acids	(Msanne et al., 2012)

2.5.2 Gene expression analysis of microalgal strains by transcriptome analysis

The transcriptome analysis represents the complete set of transcripts in a cell and their quantity for a specific developmental stage or physiological condition. Transcriptome analysis with next-generation sequencing (NGS) enables the capture and annotation of any of the coding or non-coding RNA, to help researchers to obtain a deeper understanding of the genetic information and metabolic pathways of microalgae. To date, little is known about the transcriptional regulation of lipid biosynthesis pathways in microalgae (Rismani-Yazdi et al., 2011). Lv et al. (2013) used transcriptome analysis to identify putative transcriptional factors required for lipid metabolism during the lipid accumulation phase in *C. reinhardtii*. They observed, upregulation in 100 genes during the lipid accumulation phase (late log phase), though most of the genes were not identified due to the limited information available for microalgal metabolism. Recently, Yu et al. (2016) have also conducted transcriptome analysis to identify the genes involved in the lipid biosynthesis in *C. minutissima* UTEX2341. There are very few reports on the effect of the nutrient stress on the lipid metabolism and other metabolic pathways at transcriptome level. Bochenek et al. (2013), observed upregulation of genes involved in lipid and carbohydrate metabolism in microalga *Emiliania huxleyi,* under sulphate deficiency. In a similar study by Lopez Garcia de Lomana et al. (2015) on effect of N stress on the *C. reinhardtii* upregulation in the genes related to lipid accumulation was observed.

These expression analyses give insight about the actual mechanism of lipid accumulation in the microalgae (Fan et al., 2014). Thus the expression analysis could assist in improving the existing stress strategies and also to develop novel strategies for better lipid yields in microalgae. Expression analysis also reveals the genes involved in enhancing lipid

biosynthesis and growth. This knowledge can be exploited for various metabolic and genetic engineering studies in microalgae for promoting growth and metabolites production.

2.6 Motivation of research and knowledge gaps

To cater to the need of renewable transportation fuels colossal amounts of biodiesel is required. Economical production of biodiesel from microalgae can be achieved by ensuring high lipid accumulation in microalgal cells. Conventional approaches such as altering nutrient regime and cultivation conditions are either expensive or associated with overall low lipid productivity. To address these challenges, several novel approaches have been investigated in the recent past. Strategies like combined stress can be efficiently applied to commercial scale microalgal cultivation system. This novel approach has shown promising results for successfully enhancing biomass and lipid productivity. However, there are very few studies available and most of these reports are based on laboratory scale investigations and did not elucidate the role and extent of individual factors on lipid productivity and are yet to be proven for universal applicability for all microalgal strains. Effect of these combined stress strategies at molecular level have not been reported in previous studies. Large scale trials for these techniques are still required to validate their application. The advancements and novel approaches for lipid enhancement in microalgae will certainly lead towards the economical and sustainable biodiesel production.

The knowledge gaps in the context of the research are presented as follows

1. Previously reported lipid enhancement studies were mostly focused on macronutrient stress and have the limitation of low lipid productivity

2. Metals are not studied in detail, few studies report the effect of selected individual metals on lipid content, and there is limited information about the actual mechanism.
3. Combined lipid enhancement strategy with macronutrients and metals have not been investigated thoroughly to alleviate the challenges of macronutrient stress alone
4. Studies on effect of nutrients and metals on growth physiology, photosynthetic performance and expression of key genes at molecular level are scarce
5. Lipid enhancement strategies have not been tested at scaled-up cultivation systems
6. Effect of lipid enhancement strategy on protein and carbohydrate composition have been reported by only few studies

CHAPTER THREE

ISOLATION, SCREENING AND IDENTIFICATION OF INDIGENOUS LIPID PRODUCING MICROALGAE

SINGH, P., GUPTA, S.K., GULDHE, A., RAWAT, I. & BUX, F. 2015. Microalgal isolation and basic culture techniques. *Handbook of Marine Microalgae: Biotechnology Advances*, (Ed) KIM SE-KWON, Academic press, Elsevier. Chapter 4: pages 45-53.

3.1 Introduction

Selection of a fast growing and hyper lipid accumulating indigenous microalgae is imperative for economically viable biodiesel production from microalgae (Mutanda et al., 2011, Rawat et al., 2013). Indigenous microalgal strains have higher adaptive abilities towards local environmental conditions; therefore stands better chance of thriving at large scale outdoor cultivation. Isolation and screening from appropriate aquatic environments is the primary step in microalgal biodiesel production process (Duong et al., 2012, Grimi et al., 2014). Bioprospecting of microalgae involves multistep process and comprises of 1) sampling, 2) isolation of single cultures, 3) determining optimum nutrient requirements and cultivation parameters, 4) screening of microalgal species based on target application, and 5) developing suitable cultivation techniques. A selection criterion is mainly based on

downstream applications where biomass production and lipid accumulation are the two key criteria for the selection of microalgal strain for biodiesel production.

South Africa is one of the most biodiverse regions in the world; however, the microalgae culture collection is very limited. There are very few microalgal culture collection facilities available such as South African national phycology culture collection started in 2016 (https://sanpcc.org.za/). However, the above culture collection bank is not primarily targeting the hyper lipid producing strains. Thus, there is immense scope to explore the South African environment for screening for microalgae with potential lipid accumulation capabilities.

The aim of work presented in this chapter was to isolate and screen indigenous microalgal species as potential biodiesel feedstock from freshwater bodies in KwaZulu-Natal, South Africa. The microalgae strains were isolated and preliminarily identified based on morphotaxonomic characteristics. The growth rate, biomass and lipid content of isolated strains were determined. Potential microalgal strains were selected based on the overall lipid productivity for biodiesel production. The selected microalgal strains were further identified by molecular characterization.

3.2 Materials and methods

3.2.1 Collection and isolation of microalgal strains

Freshwater samples were collected by the Institute for Water and Wastewater Technology, Durban University of Technology for their culture collection, from freshwater

bodies in and around Durban, KwaZulu-Natal, South Africa. These samples were used for the isolation and screening of microalgal strains for this study. BG11 medium, the commonly used isolation medium for cyanobacteria (Cyanophyta) and green microalgal (Chlorophyta) (Lee et al., 2014, Mahmoud et al., 2015, Karthikeyan et al., 2007) was used for isolating the microalgae strains. The collected samples were first diluted from 10^{-1} to 10^{-9} in the sterilized BG11. A 100 µL of the diluted samples from 10^{-4} to 10^{-6} dilutions were transferred to the sterilized agar plates containing BG11 medium using spread plate methods. The inoculated plates were incubated at 25 °C, at a photon flux of approximately 120 µmol $m^{-2}s^{-1}$ with a 16:8 h light dark cycle (Ramanna et al., 2014). The microalgal colonies obtained were thereafter streaked onto new agar plates containing BG11. The streak plate method was repeated until axenic unialgal cultures were obtained. Stock cultures of isolated pure strains were maintained on the agar slants containing BG11 medium through monthly sub culturing. Isolated microalgal strains were also grown in 100 mL liquid BG11 medium in 250 mL conical flask by incubating at 25 °C, at a photon flux of approximately 120 µmol $m^{-2}s^{-1}$ with a 16:8 h light dark cycle. Preliminary identification of the collected microalgal strains was done using light microscopy (Axiolab, Zeiss, Germany) on the basis of morphological characteristics. Microscopic observations of all isolates were performed periodically to ensure the purity of the culture. Figure 3.1, depicts the overall steps involve in the screening and selection of the microalgal strain for biodiesel production.

3.2.2 Growth and biomass measurement

The microalgal isolates obtained from the Chlorophyta division (green microalgae) were grown at 25 °C, at a photon flux of approximately 120 µmol $m^{-2}s^{-1}$, with a 16:8 h light dark cycle on an orbital shaker (110 rpm) in 500 mL BG11 medium in 1 L conical flasks. The

microalgal cells with the initial concentration of 0.1 gL^{-1} were used as the inoculum for all subsequent experiment runs. Each experimental flask was inoculated with 10% of the total working volume. All the experiments were performed in triplicate. Microalgal growth was assessed by optical density measurements at 680 nm using a UV/VIS–spectrophotometer (Spectroquant R Pharo 300, Merck Germany) (Lee et al., 2014).

Biomass was estimated gravimetrically at initial, middle and late log phases of the growth. Cells were harvested by centrifugation, washed with deionized water and dried overnight at 105°C for dry weight measurement (Abou-Shanab et al., 2011b). Biomass productivity ($mgL^{-1}d^{-1}$) was calculated as the dry biomass produced at late log phase using the formula (Eq. 3.1)

$$\text{Biomass productivity} = \frac{\text{Amount of biomass at late log}}{\text{Number of days}} \quad \text{(Eq. 3.1)}$$

Where, amount of biomass = mgL^{-1}

The specific growth (μ) of the culture was calculated based on Eq. 2

$$\mu = \frac{1}{t_m} \ln(X_f / X_i) \quad \text{(Eq. 3.2)}$$

Where X_f is biomass at late log phase of the culture, X_i biomass at initial log phase and t_m is total time from initial log to late log phase.

3.2.3 Lipid extraction

Lipids were extracted from dried biomass harvested at late log phase from all the isolates using microwave assisted solvent extraction method to calculate the lipid content and lipid productivity (Guldhe et al., 2014). Biomass was harvested by centrifugation followed by

freeze drying. For freeze drying, the wet biomass was frozen overnight at −84°C and thereafter lyophilized using a freeze dryer (Mini lyotrap, LTE scientific Ltd. United Kingdom). The dried biomass (500 mg) was added to a 20 ml mixture of chloroform and ethanol (1:1, v/v) and subjected to cell disruption by microwave (Milestone S.R.L., Italy, output power 1200 W) at 100 °C for 10 min at 1000 W (Guldhe et al., 2014). The treated samples were then filtered to remove biomass residues. Organic solvent collected after filtration was washed with water to remove impurities by centrifuging for 5 minutes at 4000 rpm. The organic layer was collected and oven dried at 70°C for lipid recovery. The lipids obtained were measured gravimetrically and the percentage lipid content was determined based on lipid recovered from known weight of dry biomass. Lipid productivity in $mgL^{-1}d^{-1}$ was calculated according to the following equation

$$\text{Lipid productivity} = \text{Biomass productivity} \times \frac{\text{Lipid conent}}{100} \quad \text{(Eq. 3.3)}$$

Where, lipid content is in percentage per DCW and biomass productivity is in $mgL^{-1}d^{-1}$.

3.2.4 Molecular identification and phylogenetic analysis

3.2.4.1 Genomic DNA extraction

An aliquot (50 mL) of cultured cells was harvested at mid log phase (day 7) by centrifugation and the cell pellets were washed twice using sterile deionized water in a sterile centrifuge tube. The genomic DNA of the selected microalgal strains was extracted using ZR plant/seed DNA kit (Zymo research Inc. Bohemia, New York, USA) following the manufacturer's instruction. Extracted DNA was quantified by Nano-Drop 1000

spectrophotometer (Coleman Technologies Inc., USA). Qualitative analysis of genomic DNA was done using 1% agarose gel electrophoresis.

3.2.4.2 Polymerase chain reaction and sequencing

Amplification of genomic DNA was done by using the D1R forward (ACCCGCTGAATTTAAGCATA) and D2C reverse (CCTTGGTCCGTGTTTCAAGA) primers (Godhe et al., 2002). The optimised PCR conditions includes: 5 min of initial denaturation at 95 °C, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 60 s with a final extension of 10 min at 72 °C. The PCR products were separated by electrophoresis on 1% agarose gel.

The positive amplicons were sequenced at Inqaba Biotechnical Industries (Pty) Ltd., South Africa and the obtained sequences were analysed using BLAST Programme (NCBI BLAST, USA). The nucleotides sequences obtained, were thereafter aligned in CLUSTAL X and edited using BioEdit and exported to MEGA5.10 for evolutionary analysis. The phylogenetic tree was constructed from the alignment and bootstrap analysis using 1000 replicates by neighbour-joining method (Saitou and Nei, 1987). The nucleotide sequences were submitted to GenBank at National Center for Biotechnology Information (NCBI).

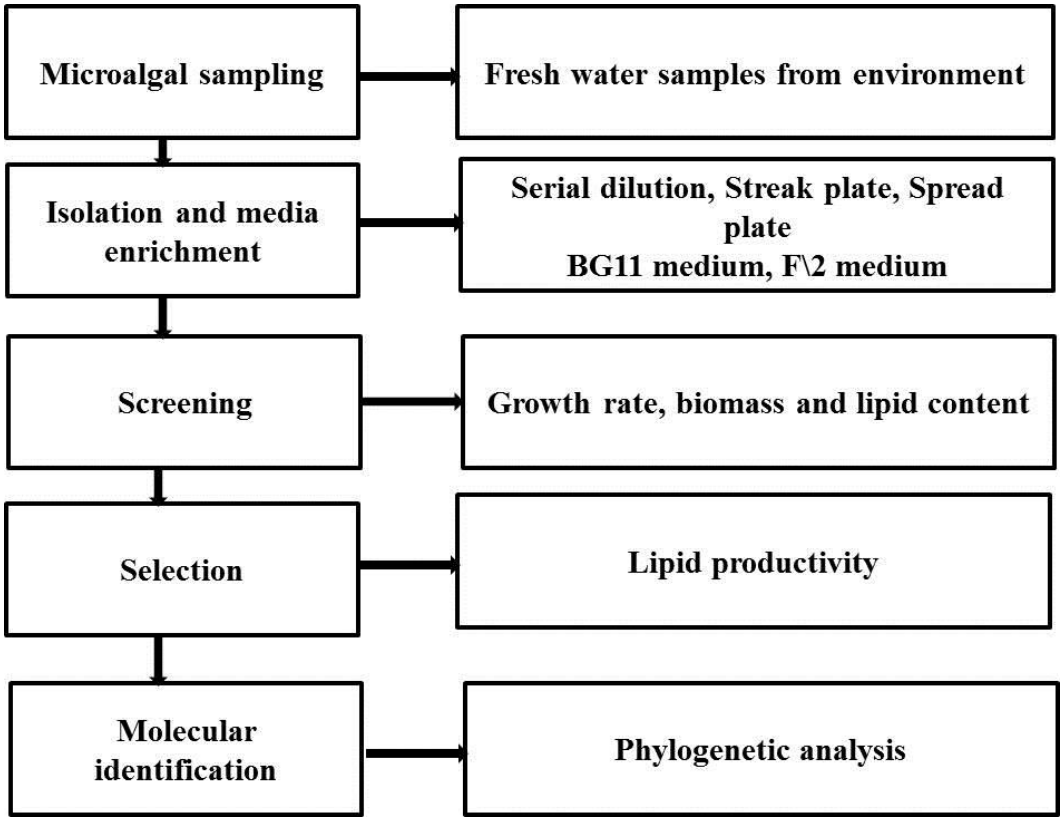


Figure 3.1: Schematic representation of method for isolation and selection of microalgae for biodiesel production

3.3 Results and discussion

3.3.1 Isolation and preliminary identification of microalgal strains

A total of 12 microalgal strains were isolated from the collected samples using spread and streak plate technique (Figure 3.2).

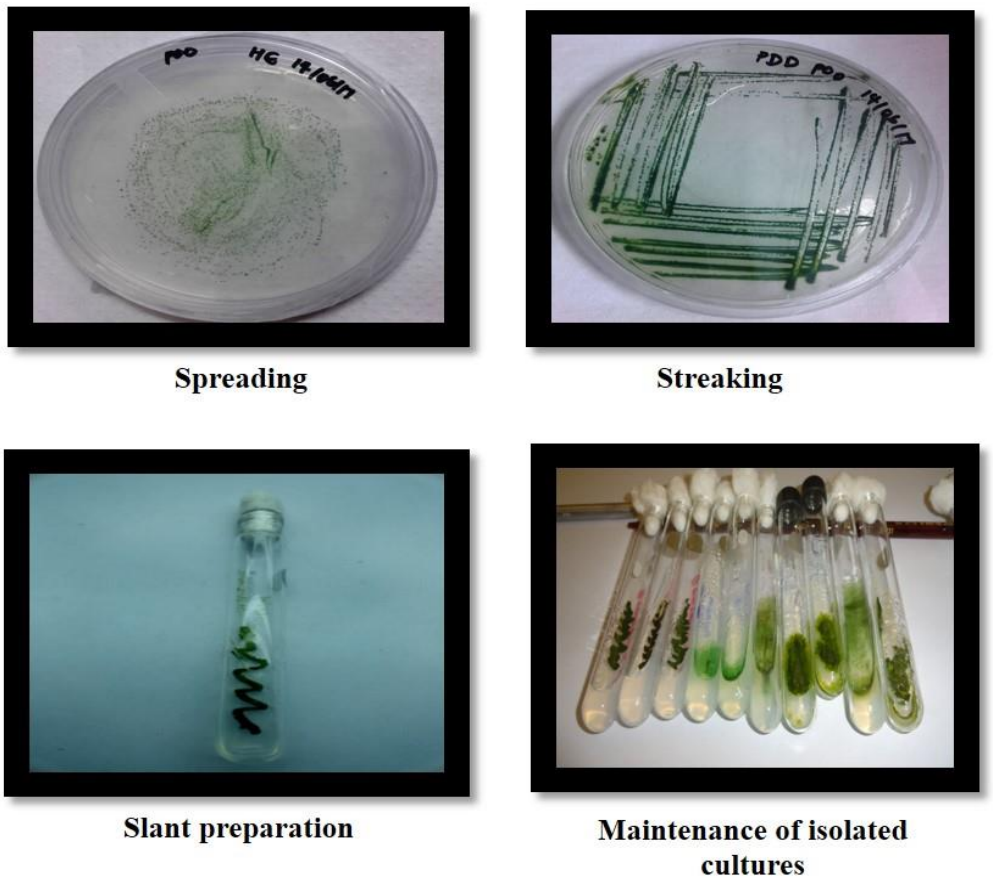


Figure 3.2: Isolation and purification of microalgal strain

Based on preliminary observations, seven microalgal strains were identified as Chlorophyta (KZN01, KZN02, KZN03, KZN04, KZN05, KZN06 and KZN07) and the remaining five as Cyanophyta (KZN08, KZN09, KZN10, KZN11 and KZN12). They were identified on the basis of their morphotaxonomic characteristics as described in table 3.1. The microphotograph of isolated green microalgal strains are presented in figure 3.3 and micrographs of isolated cyanobacteria presented in figure 3.4. Cyanobacteria are known to produce high value products such as polyketides, amides, alkaloids, indoles and bioactive compounds (Abed et al., 2009). Isolated cyanobacterial strains such as *Oscillatoria* sp.,

Spirulina sp., and *Anabeana* sp. are reported to accumulate a very low amount of lipids, thus considered not suitable for biodiesel production (Griffiths and Harrison, 2009). Green microalgal strains are identified as the suitable candidates for biodiesel production due to their high biomass and lipid productivity (Thompson, 1996, Kirrolia et al., 2013, Griffiths and Harrison, 2009). Thus out of 12 isolates, 7 green microalgal strains were selected for further screening and lipid enhancement studies (Table 3.1, Figure 3.3).

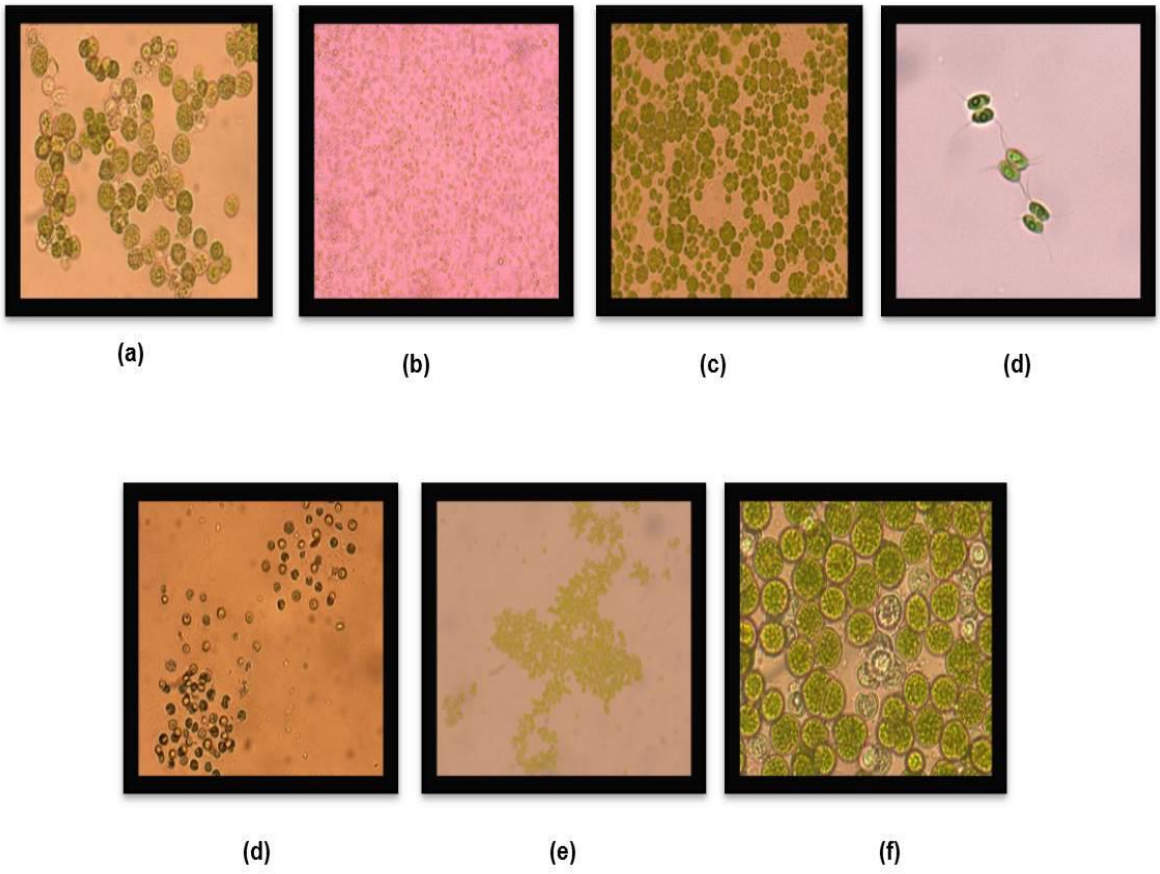


Figure 3.3: Microphotograph of isolated green microalgal strain (a) *Chlorella* sp. KZN01 (b) *Nannochloropsis* sp. KZN02 (c) *Gloeocystis* KZN03 (d) *Scenedesmus* sp. KZN04 (e) *Chlorococcum* sp. KZN05 (f) *Chlorella* sp. KZN06 (g) *Chlorella* sp. KZN07

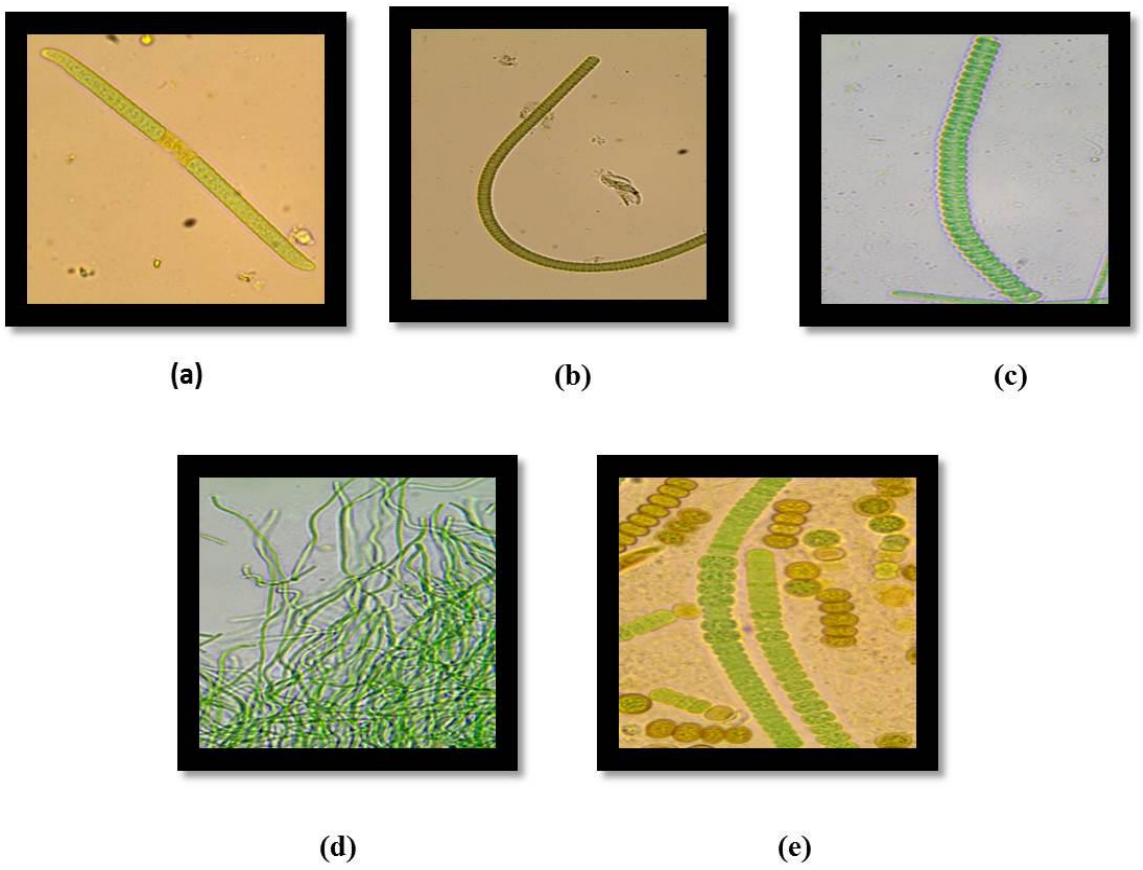


Figure 3.4: Microphotograph of isolated cyanobacteria (a) *Oscillatoria* sp. KZN08 (b) *Oscillatoria* sp. KZN09 (c) *Spirulina* sp. KZN10 (d) *Phormidium* sp. KZN11 (e) *Anabeana* sp. KZN12

Table 3.1: Identification of isolated microalgal strain

Isolated strains	Morphotaxonomic description	Genus	Reference
Green algae			
Isolate KZN01	Unicellular green algae, spherical in shape, diameter 2-5 µm	*Chlorella* sp.	(Prescott, 1964)
Isolate KZN02	Unicellular algae with 1-2µm length and 1-2 µm width	*Nannochloropsis* sp.	(Prescott, 1964)
Isolate KZN03	Colonies free floating, ovoid, with lamellate sheath	*Gloeocystis* sp.	(Prescott, 1964)
Isolate KZN04	Colonies 2-8 cells, cells 3-7 µm broad, 9-18.5 µm µ long. Spines 6.5-15 µm long	*Scenedesmus* sp.	(Prescott, 1964)
Isolate KZN05	Cells spherical, 9.35-13.37 µm broad cell wall, smooth chloroplasts parietal	*Chlorococcum* sp.	(Prescott, 1964)
Isolate KZN06	Cells ellipsoidal, 7-8 µm in diameter 9-9.5 µm in long	*Chlorella* sp.	(Prescott, 1964)
Isolate KZN07	Cells spherical, chloroplast a parietal cup shape, cells 5-8.5 µm in diameter.	*Chlorella* sp.	(Prescott, 1964)
Cyanobacteria			
Isolate KZN08	Thallus pale blue-green, thin, membranous, expanded; trichome slightly bent, 1-2 µm broad, sheath thin, cell 2.5-5 µm long	*Oscillatoria* sp.	(Prescott, 1964)
Isolate KZN09	Trichome single 5-6µ m broad, nearly straight, not attenuated at the apices; cells 1-2 µm long,	*Oscillatoria* sp.	(Prescott, 1964)
Isolate KZN10	Trichome 1 µm broad, green, very regularly coiled; spiral close to each other, spirals 2-2.7 µm broad.	*Spirulina* sp.	(Prescott, 1964)
Isolate KZN11	Densely entangled, 1-2 µm broad, pale blue-green; sheath thin, cell longer than broad 2.5-5 µm long	*Phormidium* sp.	(Prescott, 1964)
Isolate KZN12	Cells up to 350 µm long and 5-5.56 µm broad, at the apex 4µm broad; cells barrel shaped, 4.48-8 µm long; heterocyst almost spherical, 6.4-8.4 µm broad	*Anabeana* sp.	(Prescott, 1964)

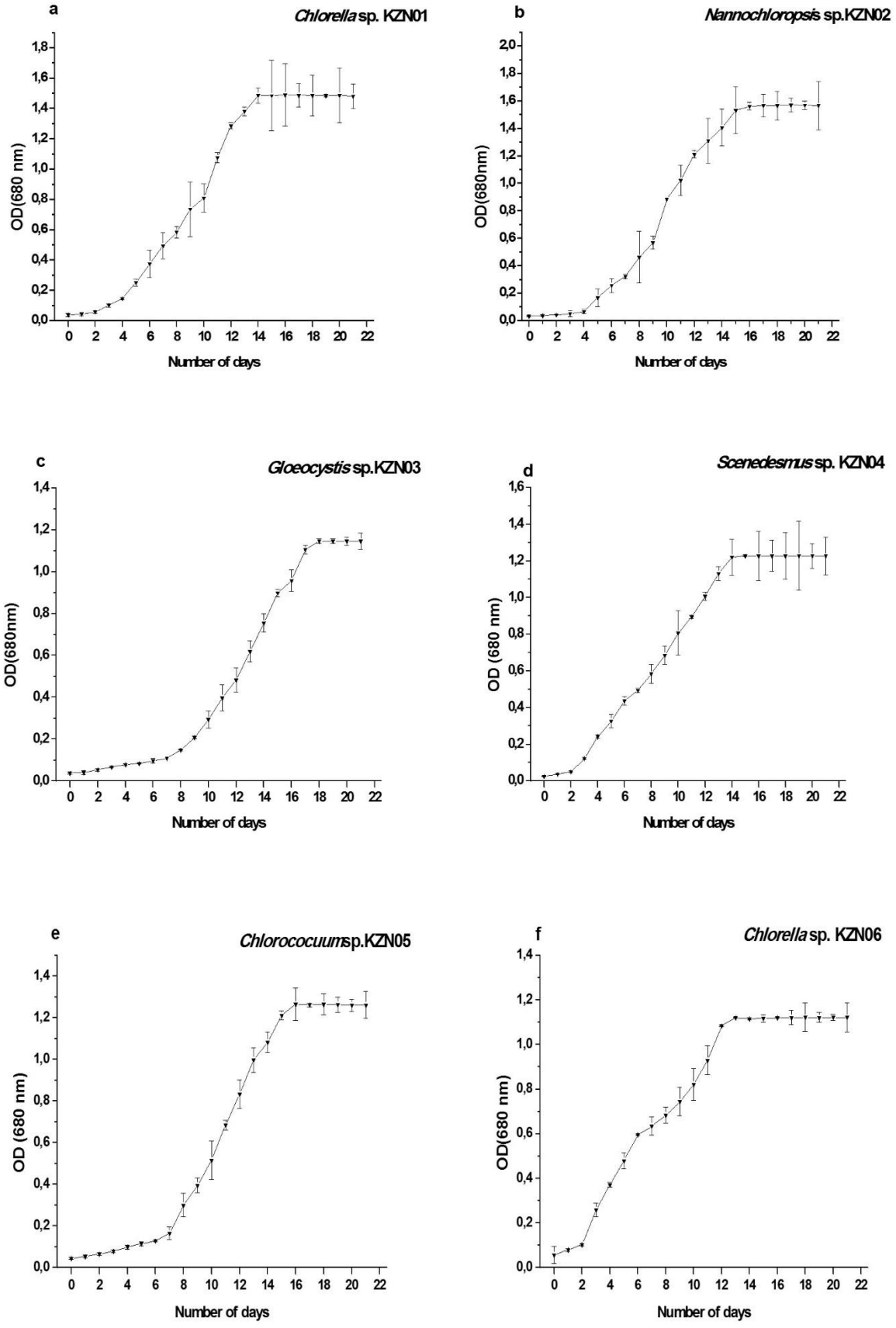
3.3.2 Screening of potential microalgal strain

Screening and selection of microalgal strain for biodiesel production was done on the basis of growth rate, biomass production, lipid content and lipid productivity (Table 3.2, Figure 3.4, and Figure 3.5).

3.3.2.1 Growth rate and biomass production of isolated microalgal strains

The growth curve analysis for all the seven microalgal strains were performed to determine the various phases of microalgal growth (Figure 3.5a to Figure 3.5g). Based on the growth curve obtained, the cultivation period of all microalgal strains can be divided into lag, log and stationary phase. All the strains displayed 2-4 days of lag phase except *Gloeocystis* sp. KZN03, where, the initial lag phase extended up to 6 days. For most strains, the log phase was between day 4 and day 14. However, for *Gloeocystis* sp. KZN03, *Nannochloropsis* sp. KZN02 and *Chlorococcum* sp. KZN05 the log phase lasted up to the day 16 (Figure 3.5a to Figure 3.5g). Hena et al. (2015), have observed that, microalgal strains with extended lag phase shows very slow growth. In their study *Botrococccus brunii* and *Gloeocystis* sp. have showed longest lag and log phases. Moreover, individual growth curve analysis is important to determine the harvesting time of the biomass from the culture as it is known that the lipid accumulation in microalgae occurs mostly in late log phase (Khatoon et al., 2017).

Growth rate represents cell proliferation in microalgae. Higher growth rates are associated with high biomass production (Lee et al., 2014). Table 3.2 represents, growth rate of all seven microalgal strains used in this study. Among these, *Chlorella* sp. KZN01 showed the highest growth rate of 0.64 d^{-1} followed by *Scenedesmus* sp. KZN04 and *Nannochloropsis* sp. KZN02 which exhibited growth rates of 0.56 d^{-1} and 0.54 d^{-1} respectively.


a
Chlorella sp. KZN01
OD(680 nm)
Number of days
b
Nannochloropsis sp.KZN02
OD(680nm)
Number of days
c
Gloeocystis sp.KZN03
OD(680nm)
Number of days
d
Scenedesmus sp. KZN04
OD (680 nm)
Number of days
e
Chlorococuum sp.KZN05
OD (680 nm)
Number of days
f
Chlorella sp. KZN06
OD(680 nm)
Number of days

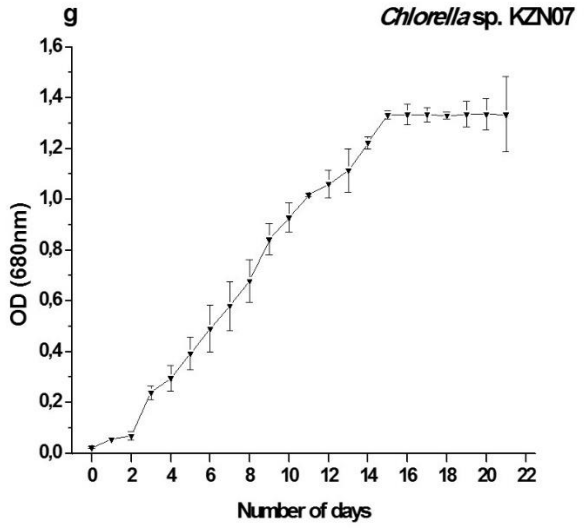


Figure 3.5: Growth curve analysis of isolated microalgal strains: (a) *Chlorella* sp. KZN01 (b) *Nannochloropsis* sp. KZN02 (c) *Gloeocystis* sp. KZN03 (d) *Scenedesmus* sp. KZN04 (e) *Chlorococcum* sp. KZN05 (f) *Chlorella sp.* KZN06 and (g) *Chlorella sp.* KZN07

Biomass was calculated at late log phase for all the strains based on their growth curve analysis. Among the three *Chlorella* isolates, *Chlorella* sp. KZN01 produced the highest biomass of 2.32 gL^{-1} followed by *Chlorella* sp. KZN07 which produced a biomass of 1.5 gL^{-1} (Table 3.2). *Scenedesmus* sp. KZN04 and *Nannochloropsis* sp. KZN02 have also shown decent amount of biomass yields of 1.98 gL^{-1} and 1.92 gL^{-1} respectively (Table 3.2). In a study, Abou-Shanab et al. (2011a) isolated eight microalgal strain from fresh water lake from Yonsei University, Wonju, South Korea for biodiesel production and the highest biomass yield of 1.75 gL^{-1} was obtained in *S. obliquus.* In this study, the highest biomass productivity of 165±1.67 $mgL^{-1}d^{-1}$ was observed in *Chlorella* sp. KZN01 (Figure 3.6) followed by *Scenedesmus* sp. KZN04 which showed biomass productivity of 141 $mgL^{-1}d^{-1}$.

Lowest biomass productivity was observed in *Gloeocystis* sp. KZN03, which also coincided with the growth curve analysis.

Table 3.2: Growth rate, biomass yield and lipid content of isolated microalgal strains

Microalgal strain	**Growth rate (d^{-1})**	**Biomass yield (gL^{-1})**	**Lipid content (%)**
Chlorella sp. KZN01	0.64±0.04	2.32±0.023	19.59±0.31
Nannochloropsis sp. KZN02	0.54±0.05	1.92±0.013	15.40±0.20
Gloeocystis sp. KZN03	0.14±0.08	1.2±0.007	9.99±0.02
Scenedesmus sp. KZN04	0.56±0.04	1.98±0.010	26.03±0.72
Chlorococcum sp. KZN05	0.52±0.06	1.23±0.036	17.91±0.28
Chlorella sp. KZN06	0.52±0.07	1.32±0.030	16.35±0.47
Chlorella sp. KZN07	0.34±0.06	1.49±0.040	18.39±0.47

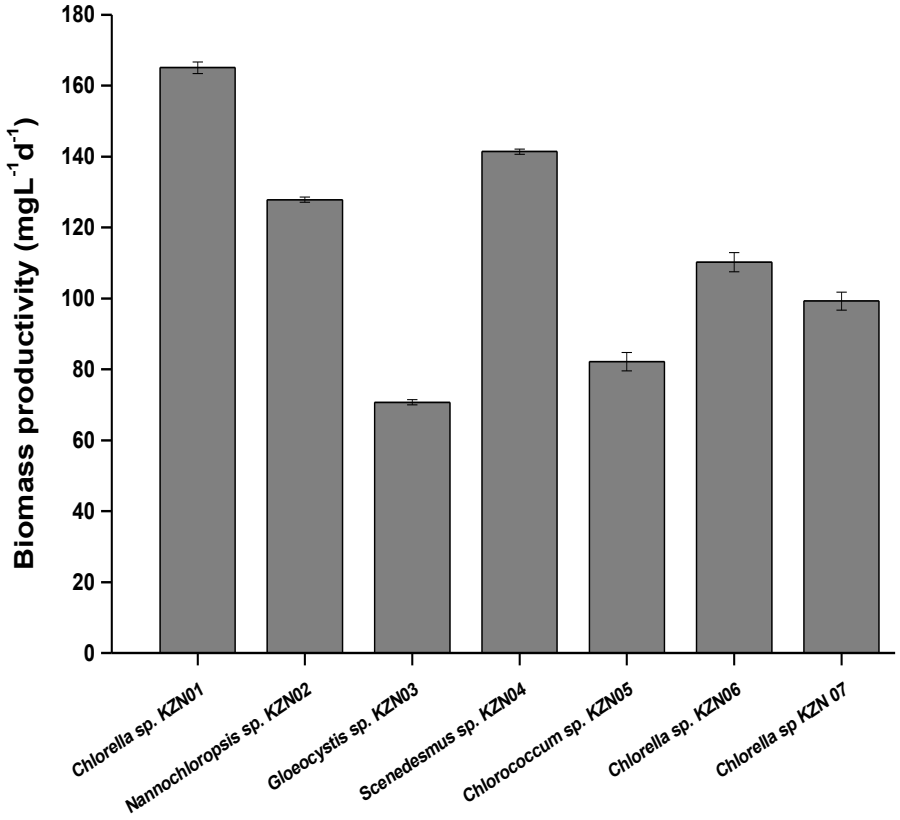


Figure 3.6: Biomass productivity of isolated microalgal strains

3.3.2.2 Lipid content and lipid productivity of microalgal strains

Microalgal lipids are considered to be viable feedstocks for biodiesel synthesis. Fresh water microalgae are reported to accumulate 10-30% of lipids in the cells (Abou-Shanab et al., 2011b, Mahmoud et al., 2015). In this study, *Scenedesmus* sp. KZN04 displayed the highest lipid content of 26% followed by *Chlorella* sp. KZN01 (19.59%) and *Chlorella* KZN07 (18.39%) (Table 3.2). Lowest lipid content of 9.99% was observed in *Gloeocystis* sp. KZN03. Rosenberg et al. (2014) have also reported similar lipid content for *C. sorokiniana*

(18%) and *C. vulgaris* (17.5%). Furthermore, the lipid content obtained in this study for *Scenedesmus* sp. KZN04 (26%) was significantly higher than the lipid content (16.73%) of *Scenedesmus* sp. reported in the previous study by Nascimento et al. (2012). Lipid content of *Gloeocystis* sp. KZN03 (9.99%) was found to be lowest among the isolated green microalgal strains. Lipid content of *Gloeocystis* sp. KZN03 from this study was in compliance with the lipid content of *Gloeocystis* sp. (9.88%) previously reported by Hena et al. (2015). Though these microalgal strains belong to same order, they have shown different abilities to accumulate lipid. This could be as result of number of factors including the geographical adaptations of the microalgae strains to certain environmental stress conditions. This emphasizes the importance of screening for potential indigenous microalgae strains that can naturally accumulate high amount of lipids in their cells.

Lipid productivity was considered as the most appropriate criteria for selection of microalgae for biodiesel production as it is calculated based on both lipid content and biomass productivity. Lipid productivity of all the seven microalgal strains assessed in this study are displayed in Figure 3.5. The highest lipid productivity of 36.82 $mgL^{-1}d^{-1}$ was observed in *Scenedesmus* sp. KZN04 followed by *Chlorella* sp. KZN01 which showed lipid productivity of 32.34 $mgL^{-1}d^{-1}$. Previous studies have also reported *Scenedesmus* sp. and *Chlorella* sp. as promising microalgal strains for biodiesel production (Arias-Peñaranda et al., 2013, Chu et al., 2014, Sacristán de Alva et al., 2013, Cao et al., 2014, Li et al., 2015). Song et al. (2013) evaluated 10 microalgal strains for biodiesel production. They have also selected *Scenedesmus* sp. and *Chlorella* sp. as the strains with the most potential for microalgal biodiesel production based on their lipid productivity. Species such as *Chlorella* and *Scenedesmus* are the most common fresh water green microalgal strains found in pond, pools,

rivers and lakes and also in wastewater treatment plants (Sacristán de Alva et al., 2013, Zhang et al., 2014). *Chlorella* sp. have been widely used commercially as a food and feed supplement due to their rapid growth and capability to survive in extreme environment. *Scenedesmus* sp. is also one of most common green microalgae, which are recently investigated for production of various types of biofuels such as biohydrogen, biodiesel and bioethanol (Florin et al., 2001, Ho et al., 2013). Other than fuel properties, *Scenedesmus* sp. is also reported to remove nutrients from different kind of wastewater (Sacristán de Alva et al., 2013). Therefore, based on the literature evidence and experimental results from this study, *Chlorella* sp. KZN01 and *Scenedesmus* sp. KZN04 were selected for further evaluation involving lipid enhancement studies.

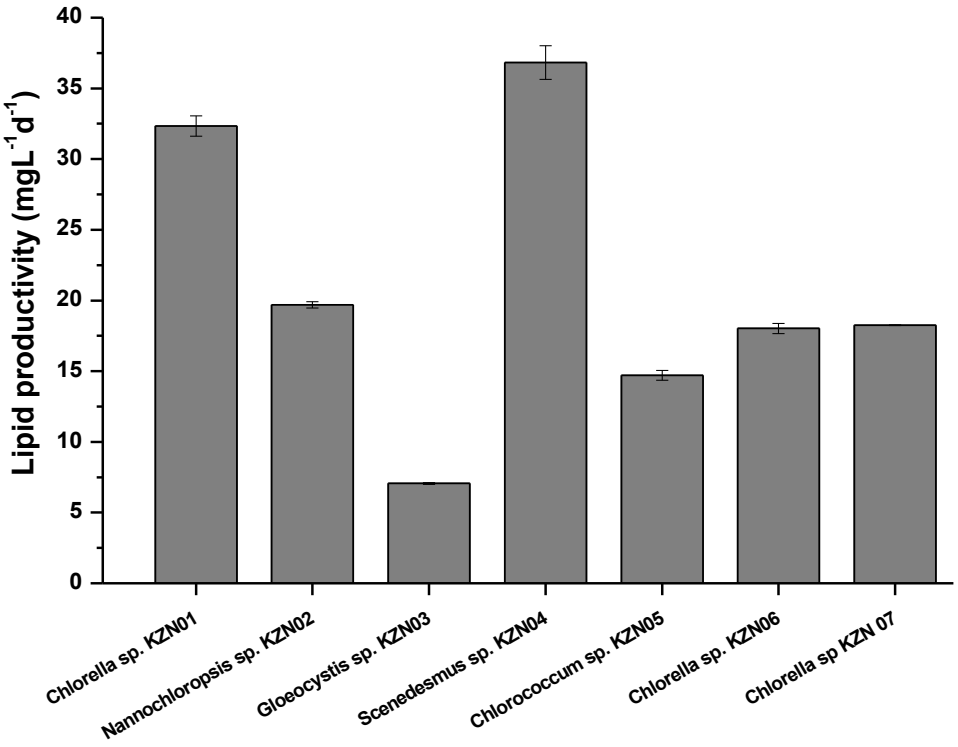


Figure 3.7: Lipid productivity of isolated microalgal strains

3.3.3 Molecular identification and phylogenetic analysis of selected strains

Though microalgae can be identified on the basis of their morphological structures, the species of the same genus generally have similar cellular features and can only be distinguished by genetic analysis (Rosenberg et al., 2014). In a previous study by Blanc et al. (2012), confirmed the identification of *Coccomyxa subellipsoidea* by comparative genomics, which was initially identified as *Chlorella variabilis* due to similarity in the physiological behaviour. Therefore, in this study the two microalgae strains that showed the highest lipid productivity were further identified based on their 18S and 28S rRNA analysis.

The sequencing and BLAST analysis of the *Chlorella* sp. KZN01 and *Scenedesmus* sp. KZN04 have shown 99% similarity with *Chlorella sorokiniana* JF834706.1 and 96% similarity to *Acutodesmus obliquus* JX401411 respectively. The phylogenetic tree obtained from the partial sequencing of 18S and 28S rRNA is displayed in Figure 3.8. T The accession number provided by NCBI for *C. sorokiniana* and *A. obliquus* was KT338813.1 and KJ956694 respectively.

In this study, *C. sorokiniana* (KT338813.1) have shown close similarity to the *C. sorokiniana* JF834706.1 and *C. sorokiniana* KP201573.1 (Figure 3.8). All *Chlorella* species have similar morphological characteristics such as round cell, small cell size, and similar chloroplast structure and generally misidentified as different species (Heeg and Wolf, 2015). In this study isolated *Chlorella* sp. KZN01 have shown 99% similarity with *C. sorokiniana* JF834706.1 and distant relationship with *C. vulgaris* KF746940.1. Phylogenetic analysis of *A. obliquus* KJ956694 revealed a close relationship with *A. obliquus* X401411 with 96% similarity. Overall, the identification of native microalgae strains presented a good correspondence between morphological characters and sequence-based phylogenetic analysis.

Therefore based on both morphological and molecular analysis, the two strains selected for this study were confirmed as *C. sorokiniana* KT338813.1 and *A. obliquus* KJ956694.

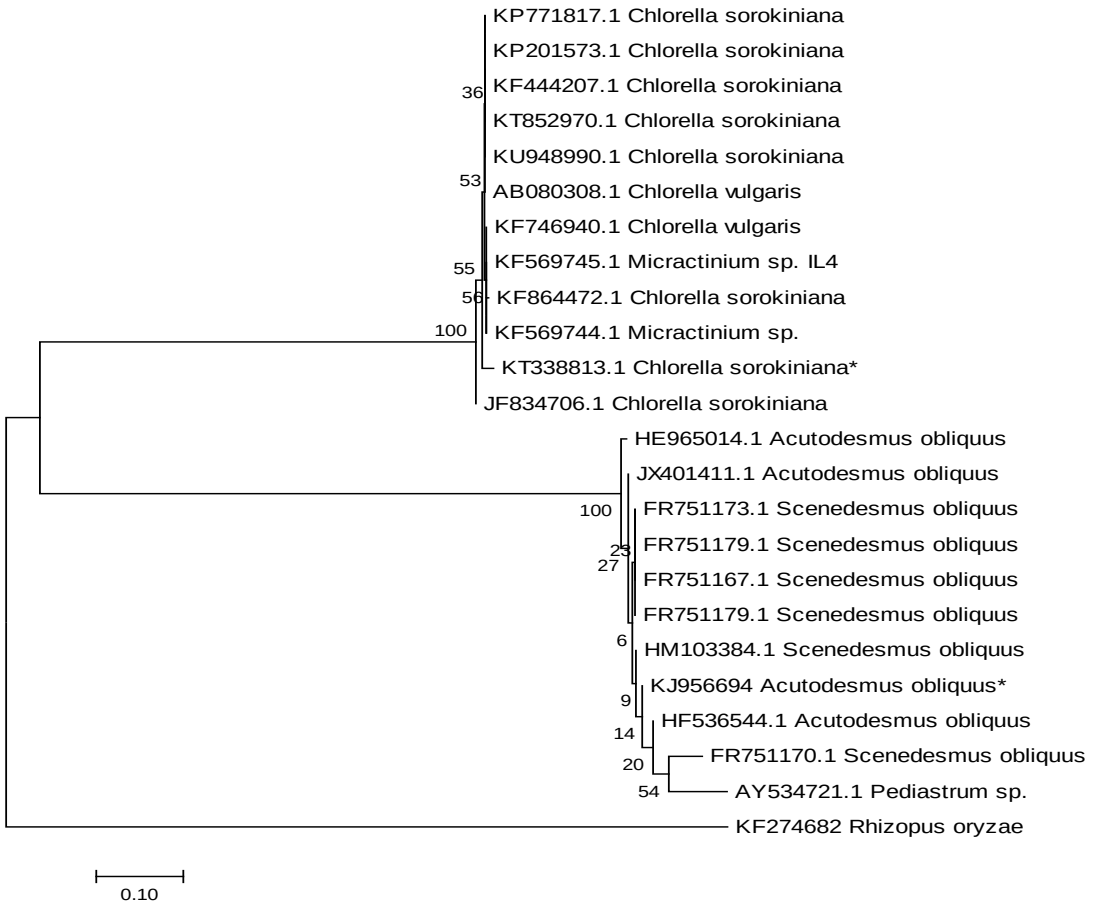


Figure 3.8: Phylogenetic tree depicts results of Neighbour-joining analysis (mega5.10) and *Rhizopus oryzae* was used as out groups in the analysis. **Chlorella sorokiniana* KT338813.1 and *Acutodesmus obliquus* KJ956694* is isolated in present study

3.4 Conclusion

Seven microalgal strains with varying growth rate and lipid contents were isolated and morphologically characterized. Among the strains screened, *Chlorella* sp. KZN01 exhibited the highest biomass productivity of 165 $mgL^{-1}d^{-1}$ followed by *Scenedesmus* sp. KZN04 with a biomass productivity of 141 $mgL^{-1}d^{-1}$. *Scenedesmus* sp. KZN04 have also shown the highest lipid productivity of 36.82 $mgL^{-1}d^{-1}$ with an overall high lipid content of 26.03% followed by *Chlorella* sp. KZN01 with a lipid productivity of 32.34 $mgL^{-1}d^{-1}$. Based on growth pattern analysis 14 day cultivation period was determined for both selected microalgal strains. The *Chlorella* sp. KZN01 is later identified as *C. sorokiniana* and *Scenedesmus* sp. KZN04 as *Acutodesmus obliquus* based on phylogenetic analysis. The above two strains were selected for further investigation for developing lipid enhancement strategy for improved biodiesel production.

CHAPTER FOUR

EVALUATION OF BIOMASS AND LIPID PRODUCTION OF MICROALGAE UNDER SELECTED NUTRIENT AND METAL STRESSED CONDITIONS

SINGH, P., GULDHE, A., KUMARI, S., RAWAT, I. & BUX, F. 2016 Combined metals and EDTA control: An integrated and scalable lipid enhancement strategy to alleviate biomass constraints in microalgae under nitrogen limited conditions. *Energy conservation and Management*. 115 100-109.

4.1 Introduction

Ensuring high lipid accumulation in microalgal biomass could aid in making the biodiesel production process economically viable (Chen et al., 2011, Negi et al., 2015). A number of factors could influence lipid accumulation in microalgae, such as nitrogen stress (Jiang et al., 2012, Pancha et al., 2014), phosphate stress (Chu et al., 2013) high salinity, carbon source concentration, light intensity, and temperature (Breuer et al., 2013, Takeshita et al., 2014, Venkata Mohan and Devi, 2014). Each individual stress conditions, however, are associated with their own challenges such as low lipid productivity, compromized biomass yield, technological barriers and high cost (Rawat et al., 2013).

To develop an efficient strategy for maximum lipid yields during large scale cultivation, it is imperative to understand the basic mechanism of the synergistic as well as individual effects of different factors on microalgal lipid accumulation (Cao et al., 2014, Sun et al., 2014, Wei et al., 2013, Breuer et al., 2013). Recent reports have shown the potential of combining different nutrient and abiotic stress to enhance lipid productivity in microalgae (Breuer et al., 2013, Wei et al., 2013). Ji et al. (2013) studied the effect of variation in temperature, light intensity and photoperiod on *Desmodesmus* sp. and observed higher biomass production at a combination of temperature: 30ºC, light intensity: 98 $\mu molm^{-2}s^{-1}$ and photoperiod: 14:10 (L: D Light: Dark photoperiod).

Alteration of nutrient concentration could be the easily applicable lipid enhancement strategy for large scale cultivation systems. Trace metals such as Fe, Mg, Cu, Ca, Mn are very important for the cellular mechanism of microalgae *viz*, photosynthesis, cell division, respiration, intra cellular transportation, protein and lipid biosynthesis are reported to improve biomass and lipid yields in microalgae (Wan et al., 2014, Sibi et al., 2016, Ren et al., 2014, Gorain et al., 2013). EDTA is a common chelating agent used in microalgae growth media (Ren et al., 2014). However, potential of using these trace metals and EDTA for enhancing lipid productivity in microalgae is still a scarcely exploited area. Very few studies have reported the use of Fe, Mg, Ca and EDTA individually for enhancing biomass and lipid accumulation (Ren et al., 2014, Huang et al., 2014, Abd El Baky et al., 2012). To the best of our knowledge none of the studies have coupled metals and EDTA stress strategy with N and P limitation and elucidated the effect of combined strategy on growth physiology, photosynthetic performance and lipid productivity.

Thus, this chapter focuses on developing an integrated strategy to enhance lipid accumulation for achieving the possible ceiling lipid productivity in *A. obliquus* and *C. sorokiniana* under N and P limitation. Concentrations of nutrients (N and P), metals (Fe, Mg and Ca), and EDTA were optimized separately for *A. obliquus* and *C. sorokiniana* to develop integrated lipid enhancement strategy. Response surface methodology (RSM) experimental design was used to optimize combined metal stress. RSM results not only give optimized levels of selected metals but also give its extent of influence on the lipid productivity. Effect of individual and combined stress on biomass, lipid content and lipid productivity was investigated. The photosynthetic performance of microalgae under individual and combined stress conditions was also examined for better understanding of role of these metals in tackling the drawbacks of N and P limitations alone.

4.2 Material and methods

4.2.1 Microalgal strain

The microalgae strains *A. obliquus* and *C. sorokiniana* were selected on the basis of high lipid productivity (Chapter 3) and used for this study. The cultures were grown at 25 ºC, at a photon flux of approximately 120 µmol m^{-2} s^{-1}, with a 16:8 h light dark cycle in an orbital shaker (110 rpm) using BG11 medium. Similar cultivation conditions were maintained for all the subsequent experiments. The microalgae cells with the initial concentration of 0.12 gL^{-1} were used as inoculum for all the experiments. Each experimental flask was inoculated with 10% of the total working volume.

4.2.2 Preliminary optimization of individual nutrients, metals and EDTA stress in *A. obliquus and C. sorokiniana*

A. obliquus and *C. sorokiniana* were grown on BG11 medium containing different concentrations of sodium nitrate which was the N source and di-potassium-ortho-phosphate which was the P source (Table 4.1). The optimum N and P concentrations were chosen on the basis of lipid productivity and all further experiments were carried out using the optimised N and P levels for that particular strain. To analyse the effect of individual metals and EDTA on the lipid productivity of selected strains, a preliminary experiments were conducted using BG11 medium with different concentrations of metals and EDTA (Table 4.1). In this study, ferric ammonium citrate was used as Fe source, magnesium sulphate heptahydrate as Mg source and calcium chloride dehydrate as a Ca source. All the experiments were performed in 1 L conical flasks with culture volume of 500 mL. All the experiments have been performed in triplicate.

Table 4.1: Various concentration of selected nutrients, metals and EDTA used in the study

Variables	**Concentration (mgL^{-1})**
Nutrients	
Nitrogen	0, 250, 500, 750, 1000, 1250, 1500, 1800
Phosphorus	0, 10, 20, 30, 40, 50, 60
Metals	
Fe	0, 3, 6, 9, 12, 15
Mg	0, 25, 50, 75, 100 125, 150
Ca	0, 9, 18, 27, 36, 45
EDTA	0, 1, 2, 3, 4, 5

4.2.3 Experimental design for combined metal stress optimization in *A. obliquus and C. sorokiniana*

The effect of the combination of metal stress on the selected microalgal strains under N and P optimized conditions in BG11 medium was studied by applying the Box–Behnken design (Minitab Statistical Software) of response surface methodology (RSM). The experimental design consisted of three metal stress factors: ferric ammonium citrate (Fe source), magnesium sulphate heptahydrate (Mg source) and calcium chloride dehydrate (Ca source). Ranges for the experimental design for three metal stresses were selected based on preliminary experimental results with individual metals. The three levels of variables for the selected factors are depicted in table 4.2a and table 4.2b for *A. obliquus* and *C. sorokiniana* respectively.

In the experimental design for each microalgal strain, 13 experiments with varying metal concentrations and three replicates at the centre points were carried out making a total of 15 experiments. Nitrogen and phosphorus concentrations were kept at optimized levels for respective strains for all the experiments. The N concentration for *A. obliquus* was kept at 750 mgL^{-1} and P concentration was kept at 40 mgL^{-1} in all experiments in the design. The N concentration for *C. sorokiniana* was kept at 500 mgL^{-1} and P concentration was kept at 10 mgL^{-1} in all experiments in the design. In addition to the experiments in the design, control experiments were also conducted for *A. obliquus* with N limited (ON) conditions (N =750 mgL^{-1}); for *C. sorokiniana* with N (N=500 mgL^{-1}) and P (P=10 mgL^{-1}) limited conditions (ONP). In both these control experiments the concentrations of the selected metal ions and other nutrients were unchanged from BG11 medium. Both strains were also subjected to an experimental condition without any of the selected metal ions (ON-M) in the nutrient

medium. All the experiments were performed in 1 L conical flasks with culture volume of 500 mL. Lipid productivity was analysed as the response for the experimental design. The effect of independent factors on the dependent factors in both strains was analysed using the quadratic equation:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_2X_3 + a_{23}X_1X_3 + e \quad \text{(Eq. 4.1)}$$

Where, Y is the response (lipid productivity), a_0 is offset term; a_1, a_2 and a_3 are linear coefficients; a_{11}, a_{22} and a_{33} are the squared term coefficients; a_{12}, a_{13} and a_{23} are the interaction coefficients. X_1, X_2 and X_3 are Fe, Mg and Ca concentrations and e is error.

Table 4.2a: Factors and levels for experimental design of nutrient stress optimization for *A. obliquus*

Factors	**A (Ferric ammonium citrate mgL^{-1})**	**B (Magnesium sulphate heptahydrate mgL^{-1})**	**C (Calcium chloride dehydrate mgL^{-1})**
Level 1	-1 (6)	-1 (75)	-1 (18)
Level 2	0 (9)	0 (100)	0 (27)
Level 3	1 (12)	1 (125)	1 (36)

Table 4.2b: Factors and levels for experimental design of nutrient stress optimization for *C. sorokiniana*

Factors	**A (Ferric ammonium citrate mgL^{-1})**	**B (Magnesium sulphate heptahydrate mgL^{-1})**	**C (Calcium chloride dehydrate mgL^{-1})**
Level 1	-1 (9)	-1 (100)	-1 (18)
Level 2	0 (12)	0 (125)	0 (27)
Level 3	1 (15)	1 (150)	1 (36)

4.2.4 Analytic methods

4.2.4.1 Growth and biomass determination

Microalgal growth was monitored daily using spectrophotometric method as described in Chapter 3, section 3.2.2. Biomass was estimated gravimetrically at initial, middle and late log phases of growth. Biomass productivity ($mgL^{-1}d^{-1}$) was calculated at late log phase gravimetrically as describes in Chapter 3, section 3.2.2.

4.2.4.2 PAM and chlorophyll analysis

Pulse Amplitude Modulation (PAM) fluorometry was used to determine the photosynthetic performance of *A. obliquus* and *C. sorokiniana* under different stress conditions. Fluorescence measurements were obtained non-invasively using a Dual-PAM 100 chlorophyll fluorometer (Heinz Walz Gmbh, Effeltrich, Germany). Microalgal cultures were adapted to dark conditions for approximately 20 minutes as Photosystem II (PS II) reaction centres are open and require to be closed before analysis. Rapid photosynthetic light curves were then generated by providing a sequence of increasing actinic irradiance in 15 discrete increments using Dual PAM software v1.9. Cultures were exposed to a Saturation Pulse (SP) for 10 s of actinic light, before a blue light, (0.6 s at 10,000 mol photons $m^{-2}s^{-1}$) was applied to determine the Electron Transport Rate (ETR) for each irradiance level. Relative electron transport rates were recorded as per Ramanna et al. (2014).

$$rETR = F'_q / F'_m \times \text{PPFD} \qquad \text{(Eq. 4.2)}$$

Where $F'_q = (F'_m - F')$, F'_m is maximum fluorescence in a light adapted sample and F' is dark fluorescence yield. F'_q / F'_m is the Photosystem II (PS II) operating efficiency and

provides estimations of the efficiency of light usage absorbed by PS II. This parameter provides an estimate of the quantum yield of linear electron flux through PS II at a particular photosynthetically active photon flux density (PPFD).

Maximum quantum efficiency of photosystem II charge separation (F_v/F_m) was calculated in accordance with Ramanna et al. (2014):

$$F_v/F_m = F_m - F_o/ F_m \quad \text{(Eq. 4.3)}$$

Where F_m is the maximum fluorescence in a dark adapted sample and F_o is the minimum fluorescence in a dark adapted sample, which results in the variable fluorescence F_v.

Chlorophyll content was determined by the methanol extraction method by Bajguz and Piotrowska-Niczyporuk (2013). Microalgal cells were mixed with 99.9% methanol and kept in dark. For the extraction of chlorophyll, the homogenised mixture of microalgal cells and methanol was heated at 70 °C for 30 min and centrifuged (1,000 g, 10 min). The supernatant was used for the chlorophyll determination. The total chlorophyll was quantified by measuring absorbance at 652.4 and 665.2 nm. The chlorophyll content was determined using the following formula

$$\text{Total Chlorophyll} = 1.44 \cdot A^{665.2} + 24.93 \cdot A^{652.4} \quad \text{(Eq. 4.4)}$$

4.2.4.3 Lipid analysis

Lipids were extracted from the biomass obtained for each experiment as described in chapter 3, section 3.2.3.

4.2.4.4 Statistical analysis

Data analysis for the experimental design was done using the Minitab Statistical Software. All experiments were performed in triplicates (n=3). Data is represented as mean value ± SD (standard deviation). Significance of the results was tested at 0.05 levels by comparing mean values using one-way analysis of variance (ANOVA).

4.3 Result and discussion

4.3.1 Preliminary optimization of macronutrients (N and P)

4.3.1.1 Effect of N concentration on the biomass and lipid productivity of *A. obliquus* and *C. sorokiniana*

Variations in N concentration of the culture medium have displayed significant impacts on both biomass and lipid productivity of *A. obliquus* as well as *C. sorokiniana*. An increase in the N concentration from 0-1800 mgL^{-1} resulted in an increase in biomass productivity from 24.39 $mgL^{-1}d^{-1}$ at 0 mgL^{-1} N to 145.1 $mgL^{-1}d^{-1}$ at 1800 mgL^{-1} N for *A. obliquus* and from 31.07 $mgL^{-1}d^{-1}$ at 0 mgL^{-1} N to 158.28 $mgL^{-1}d^{-1}$ at 1800 mgL^{-1} N for *C. sorokiniana* (Figure 4.1a and 4.1b). In contrast to biomass production, the decrease in N concentration has resulted in an increase in lipid accumulation (Table 4.3a). This may be due to the fact that N is an important nutrient for microalgal growth, as it is involved in vital metabolic processes, protein synthesis, and cell division etc. Limitation of N can therefore result in the reduction of cell growth, leading to low biomass production and storage of energy in the form of lipids (Allen et al., 2015, Liang et al., 2012). Limitation of N in the

medium although improved the lipid accumulation, it also resulted in reduced biomass which affected the overall lipid productivity. Highest lipid productivity of 42.5±0.79 $mgL^{-1}d^{-1}$ was obtained at 750 mgL^{-1} of N for *A. obliquus.* However, biomass productivity for *A. obliquus* was decreased to 109.29 $mgL^{-1}d^{-1}$ at 750 mgL^{-1} from 141.07 $mgL^{-1}d^{-1}$ in BG11 medium. While, in case of *C. sorokiniana* highest lipid productivity of 33.32±0.15 $mgL^{-1}d^{-1}$ was observed at 500 mgL^{-1} of N (Figure 4.1a and 4.1b). However, biomass productivity for *C. sorokiniana* was decreased to 103.15 $mgL^{-1}d^{-1}$ at 500 mgL^{-1} from 156.07 $mgL^{-1}d^{-1}$ in BG11 medium. Similarly, Griffiths et al. (2014) reported a lipid productivity of 33 $mgL^{-1}d^{-1}$ under N concentration of 420 mgL^{-1} in *Chlorella vulgaris*.

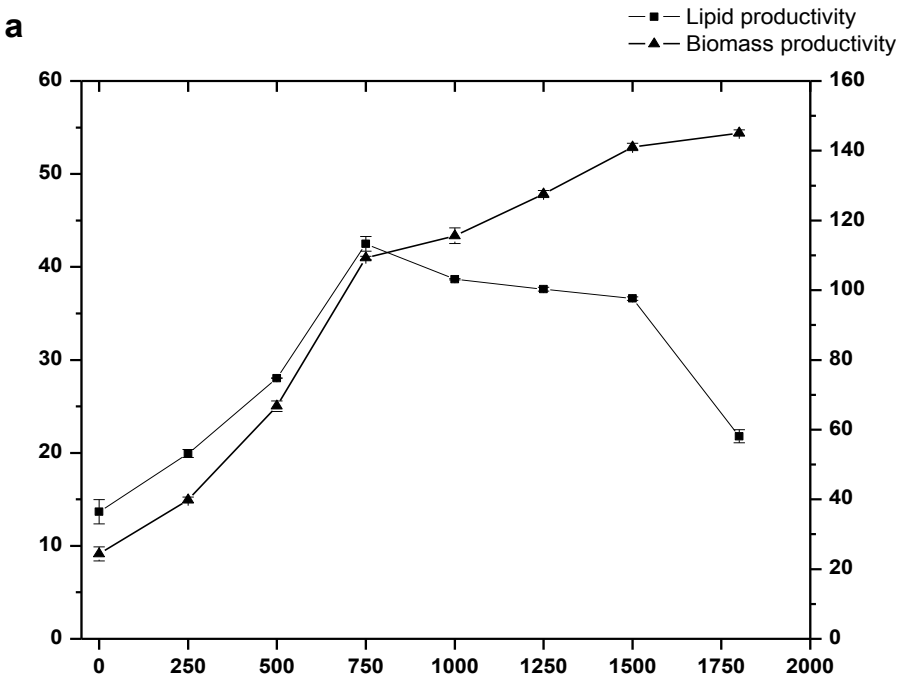


Figure 4.1a: Effect various N concentration on biomass productivity and lipid productivity *A. obliquus*

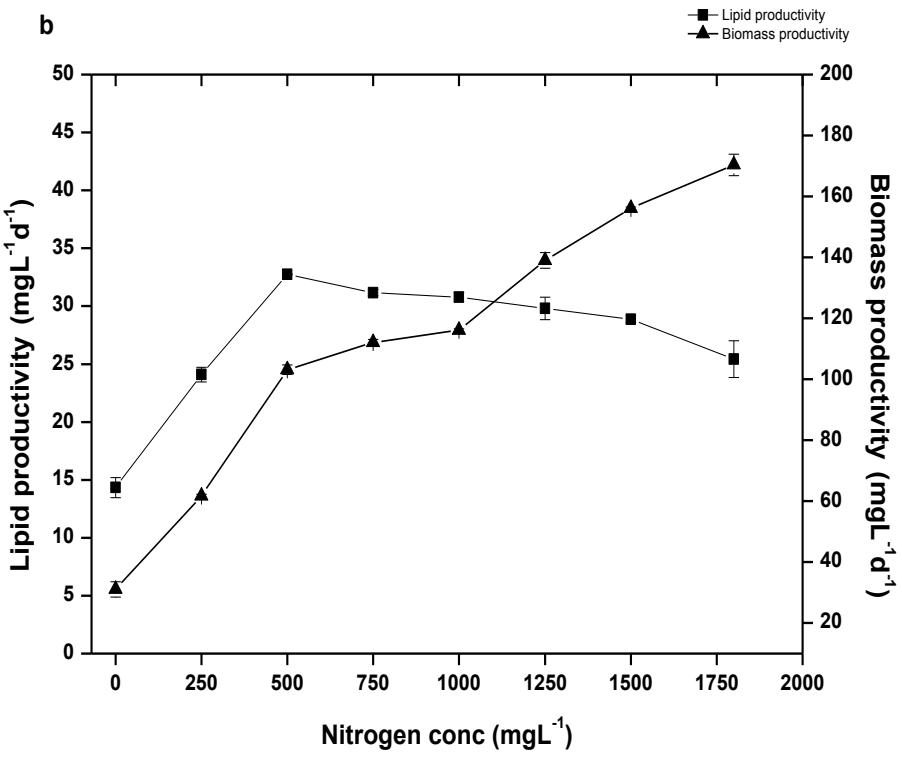


Figure 4.1b: Effect of various N concentrations on biomass productivity and lipid productivity *C. sorokiniana*

Table 4.3a: Effect of various N concentrations on the lipid content of *A. obliquus* and *C. sorokiniana*

N concentrations (mgL^{-1})	***A. obliquus* Lipid content (%)**	***C. sorokiniana* Lipid content (%)**
0	55.07±0.13	46.16±0.91
250	49.60±0.53	39.00±1.33
500	42.03±0.90	31.75±1.06
750	38.89±0.81	27.80±0.51
1000	33.45±0.61	26.53±0.24
1250	29.48±0.07	21.40±0.85
1500	25.95±0.25	18.50±0.14
1800	15.02±0.53	14.94±1.24

4.3.1.2 Effect of P concentrations on the biomass and lipid productivity of *A. obliquus* and *C. sorokiniana*

Variations in the P concentration in the medium have also shown noticeable influence on biomass and lipid productivity of *A. obliquus* and *C. sorokiniana.* Figure 4.2a and 4.2b represents the effect of P on biomass and lipid productivity of *A. obliquus* and *C. sorokiniana* respectively. Increase in P concentration (0-40 mgL^{-1}) initially resulted in an increase in the biomass productivity for both strains, but a further increase in P concentration has resulted in decreased biomass productivity (Figure 4.2a and 4.2b). Highest biomass productivity of 141 $mgL^{-1}d^{-1}$ for *A. obliquus* and 156.07 $mgL^{-1}d^{-1}$ for *C. sorokiniana* was obtained when P concentration was increased to 40 mgL^{-1}. This may be due to the fact that P plays crucial role in microalgal growth and signalling pathways thus increase in the P concentration up to certain levels resulted in increased biomass production. Microalgal growth is highly dependent on balanced N/P ratio in the medium, high concentration of the P can hamper this balance which eventually affects the microalgal growth.

Similar to N, P stress also led to accumulation of lipid in microalgae. In this study, an increase in P concentrations in the medium has resulted in decrease in the lipid content in both the strains (Table 4.3b). Highest lipid productivity of 36.17 $mgL^{-1}d^{-1}$ was obtained at 40 mgL^{-1} of P for *A. obliquus* (Figure 4.1a). For *C. sorokiniana*, the highest lipid productivity of 30.83 $mgL^{-1}d^{-1}$ was obtained at 10 mgL^{-1} P (Figure 4.1b). On the basis of lipid productivity, 40 mgL^{-1} for *A. obliquus* and 10 mgL^{-1} for *C. sorokiniana* was selected as optimum P concentrations and kept as constants for further experiments. Under N and P limited conditions, even though lipid productivity was improved in both strains as compared to

standard BG 11 medium, biomass productivity was hampered which needs to be improved to get highest possible lipid productivities.

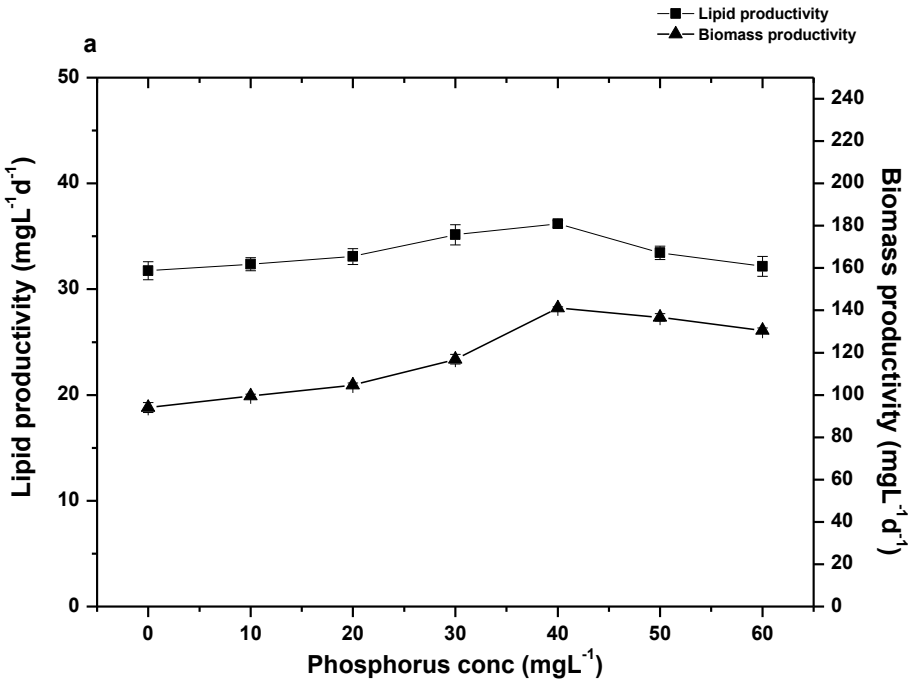


Figure 4.2a: Effect of various P concentrations on biomass productivity and lipid productivity *A. obliquus*

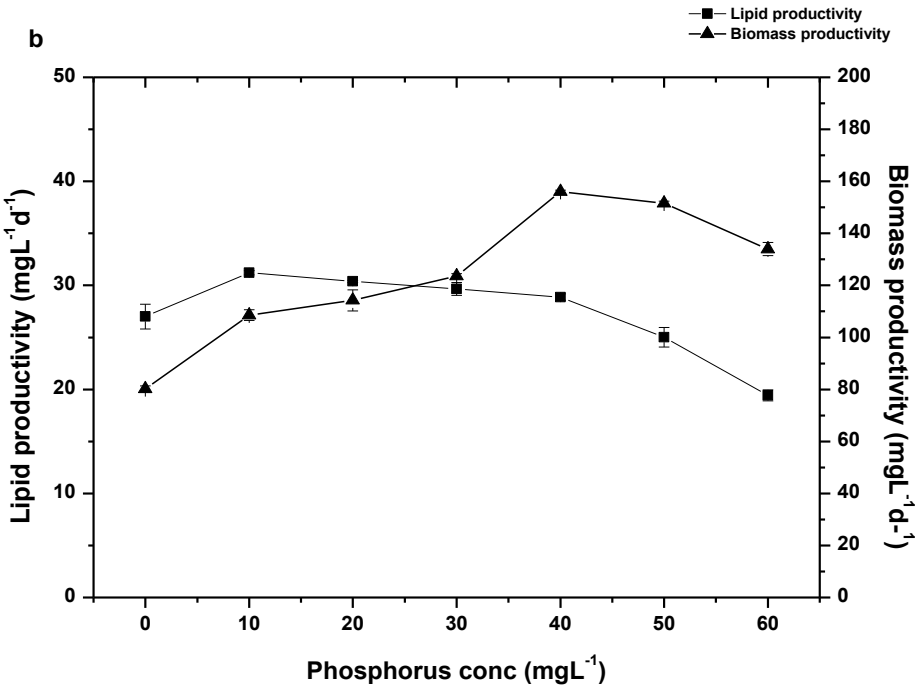


Figure 4.2b: Effect of various P concentrations on biomass productivity and lipid productivity *C. sorokiniana*

Table 4.3b: Effect of various P concentrations on the lipid content of *A. obliquus* and *C. sorokiniana*

P concentration (mgL^{-1})	***A. obliquus*** **Lipid content (%)**	***C. sorokiniana*** **Lipid content (%)**
0	33.77±0.22	33.75±1.06
10	32.51±0.38	28.75±0.35
20	31.62±0.80	26.35±0.49
30	30.06±0.26	24.00±0.71
40	25.64±0.12	18.50±0.14
50	24.47±0.18	16.40±0.57
60	24.64±0.89	14.53±0.68

4.3.2 Effect of metals and EDTA concentration on the biomass and lipid productivity of *A. obliquus* and *C. sorokiniana*

4.3.2.1 Effect of Fe concentrations on the biomass and lipid productivity of *A. obliquus* and *C. sorokiniana*

Biomass productivity of *A. obliquus* was increased from 62 $mgL^{-1}d^{-1}$ to 126 $mgL^{-1}d^{-1}$ with an increase in Fe concentration in the media from 0 to12 mgL^{-1}, where the highest lipid productivity of 49.48 $mgL^{-1}d^{-1}$ was achieved at 9 mgL^{-1} Fe (OII for *A. obliquus*). A further increase in Fe concentration to 15 mgL^{-1} showed a slight decrease in biomass production (Figure 4.3a). For *C. sorokiniana* the biomass productivity increased with increase in Fe concentrations from 0 to 15 mgL^{-1}. An increase in Fe concentration from 0 to 15 mgL^{-1} (OII for *C. sorokiniana*) in the media resulted in increase in lipid productivity from 17.98 $mgL^{-1}d^{-1}$ to 43.76 $mgL^{-1}d^{-1}$ and biomass productivity was also found to be increased from 60.71 $mgL^{-1}d^{-1}$ to 116.07 $mgL^{-1}d^{-1}$ in *C. sorokiniana* (Figure 4.3b). Iron plays a significant role in activity of enzymes involved in photosystem II and lipid biosynthesis. Thus, increase in Fe concentration may have resulted in enhanced photosynthesis in microalgae leading to an overall increase in both biomass and lipid production.

It was observed that increase in Fe concentration in the medium not only influenced the biomass, but it also influenced the lipid accumulation in both strains. An increase in Fe concentration from 0-9 mgL^{-1} has resulted in an increase in the lipid content of *A. obliquus* from 37.18% to 41.25% (Table 4.4a). A further increase in the Fe concentration to 15 mgL^{-1} however, has affected the lipid accumulation in *A. obliquus.* Similarly, an increase in Fe concentration from 0-15 mgL^{-1} has resulted in an increase in the lipid content of *C. sorokiniana* from 30.12% to 37.63% (Table 4.4a). Similarly, Abd El Baky et al. (2012) also

observed an increase in the lipid content of *S. obliquus* with increase in the $FeCl_3$. In their study, maximum lipid yield of 28.12% was observed in culture with $FeCl_3$ concentration of 20 mgL^{-1}. Other studies have also shown the positive effect of Fe on the lipid accumulation of microalgae (Yeesang and Cheirsilp, 2011, Concas et al., 2014).

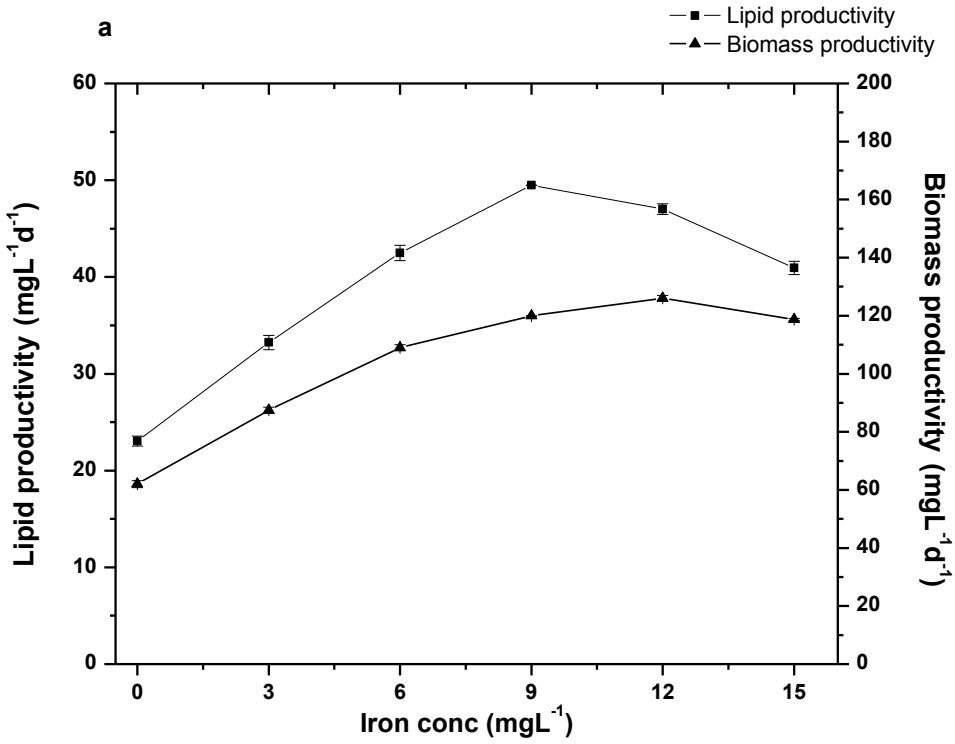


Figure 4.3a: Effect of Fe concentration biomass and lipid productivity of *A. obliquus* under N limited condition

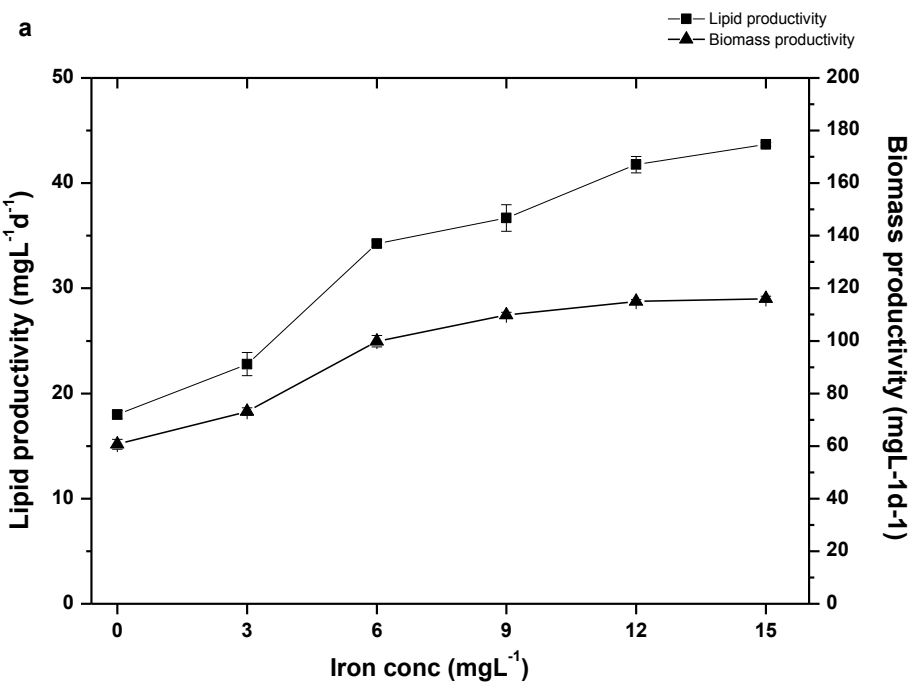


Figure 4.3b: Effect of Fe concentration biomass and lipid productivity of *C. sorokiniana* under N and P limited condition

Table 4.4a: Effect of variation in the Fe concentration on the lipid on *A. obliquus* and *C. sorokiniana*

Fe Concentration (mgL^{-1})	***A. obliquus*** **Lipid content (%)**	***C. sorokiniana*** **Lipid content (%)**
0	37.18±0.43	30.12±0.06
3	38.01±0.66	31.88±0.94
6	39.00±1.08	34.25±0.35
9	41.25±0.03	35.58±0.56
12	37.33±0.26	37.12±0.44
15	34.49±0.57	37.63±0.32

4.3.2.2 Effect of Mg concentrations on biomass and lipid productivity of *A. obliquus* and *C. sorokiniana*

An increase in Mg concentration from 0 to 125 mgL^{-1} in the media has resulted in an increase in the biomass and lipid productivity of *A. obliquus*. The biomass productivity of *A. obliquus* increased from 90.87 to 129.07 $mgL^{-1}d^{-1}$ and lipid productivity from 27.33 to 47.73 $mgL^{-1}d^{-1}$ with an increase in Mg concentration from 0 to 125 mgL^{-1} in the medium (Figure 4.4a). Similar results were observed for *C. sorokiniana*, addition of Mg in the culture medium from 0 to 150 mgL^{-1} resulted in increased biomass productivity from 74.93 to 106.54 $mgL^{-1}d^{-1}$ and lipid productivity from 19.29 to 39.27 $mgL^{-1}d^{-1}$ (Figure 4.4b). Magnesium is one of the essential nutrients for growth of the microalgae (Wang et al., 2014). It is a core constituent of chlorophyll molecules thus regulates photosynthesis and also aids in the activation of many enzymes related to growth and protein synthesis in microalgae (Zhang et al., 2010, Shaul, 2002, Wang et al., 2014, Gorain et al., 2013). Huang et al. (2014) reported 1.25 folds increase in the cell density of *Monoraphidium* sp. FXY-10 with supplementation of 100 µM Mg into the medium. Similar to Fe, Mg has also influenced the lipid content of both microalgal strains. Increase in the Mg concentration from 0 to 150 mgL^{-1} has resulted in increase in lipid content of both strains. The lipid content of *A. obliquus* and *C. sorokiniana* was found to be 36.92% and 35.69% at 150 mgL^{-1} of Mg respectively (Table 4.4b).

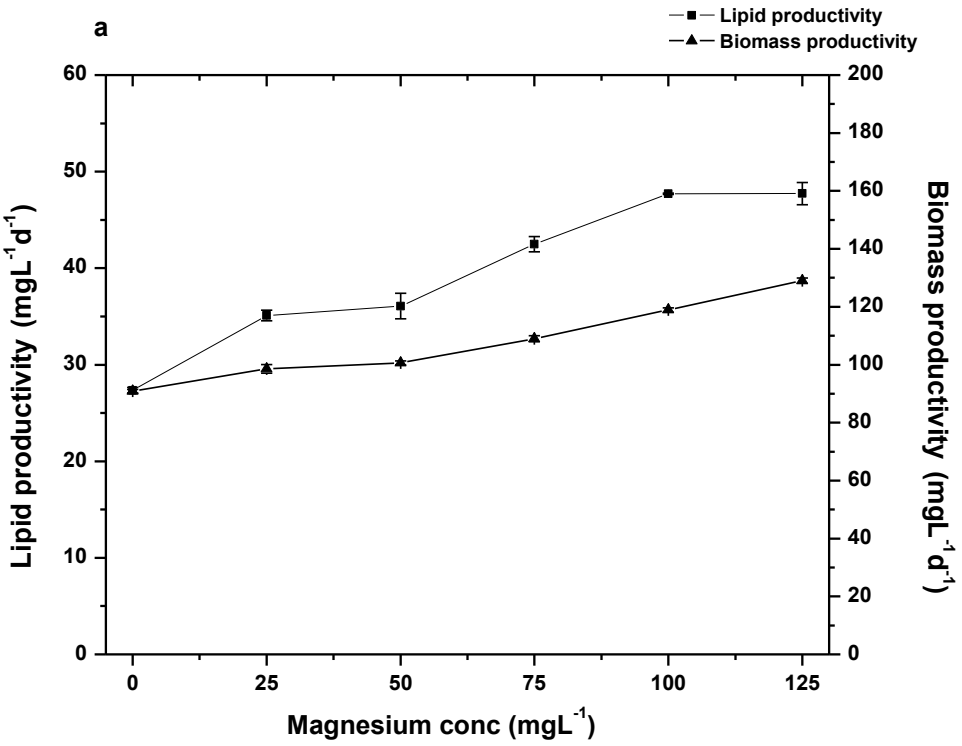


Figure 4.4a: Effect of Mg concentration biomass and lipid productivity of *A. obliquus* under N limited condition

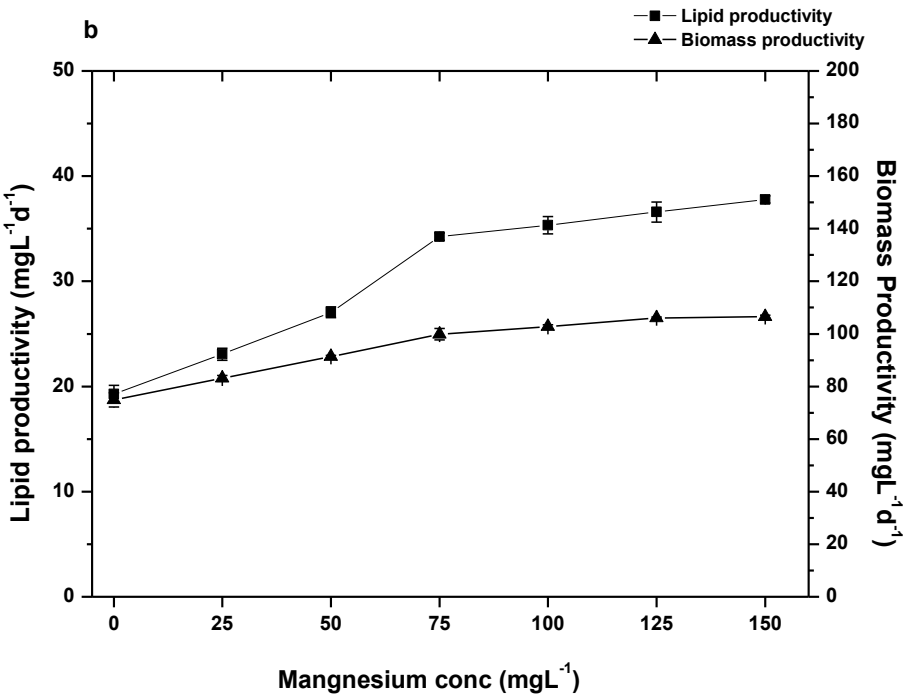


Figure 4.4b: Effect of Mg concentration biomass and lipid productivity of *C. sorokiniana under* N and P limited condition

Table 4.4b: Effect of variation in the Mg concentration on the lipid on *A. obliquus* and *C. sorokiniana*

Mg Concentration (mgL^{-1})	***A. obliquus* Lipid content (%)**	***C. sorokiniana* Lipid content (%)**
0	30.08±0.48	25.75±0.52
25	35.63±0.74	27.76±0.62
50	35.86±1.23	29.62±0.43
75	39.00±1.08	34.25±0.35
100	40.08±0.30	34.36±0.63
125	36.98±1.15	34.50±0.92
150	36.92±0.80	35.69±0.65

4.3.2.3 Effect of Ca concentrations on biomass and lipid productivity of *A. obliquus* and *C. sorokiniana*

In this study, increase in Ca concentration from 0 to 36 $mgL^{-1}d^{-1}$ resulted in an increase in biomass productivity of *A. obliquus* from 80.71 to 109 $mgL^{-1}d^{-1}$, however a further increase in Ca concentration to 45 mgL^{-1} have reduced biomass productivity to 107.14 $mgL^{-1}d^{-1}$. The lipid productivity of *A. obliquus* was initially increased from 29.47 to 48.33 $mgL^{-1}d^{-1}$ with an increase in the concentration of Ca in the medium from 0 to 18 mgL^{-1} and thereafter decreased to 39.63 mg $L^{-1}d^{-1}$ with further increase in the Ca supplementation (Fig. 4.5a). In *C. sorokiniana*, the biomass productivity increased from 61.9 $mgL^{-1}d^{-1}$ to 101.88 $mgL^{-1}d^{-1}$ with increase in Ca concentration from 0 to 45 mgL^{-1}, however, the highest lipid productivity of 36.28 $mgL^{-1}d^{-1}$ was observed at a concentration of 27 mgL^{-1}, a further increase in Ca concentration, however led to a decrease in lipid productivity (Fig. 4.5b).

In microalgal cells, calcium is likely to be involved in the cell growth and lipid accumulation through mechanisms such as formation of cell membrane and cytoskeleton and maintenance of membrane stability (Burstrom, 1968). Calcium is also reported to play role in photosynthetic activity of microalgae (Hepler, 2005, Cardol et al., 2011). In the present study, effect of the Ca concentration on the lipid accumulation was different for each strain. In *A. obliquus*, the lipid content was initially increased from 36.51% to 47.99% with the increase in calcium concentration from 0 to 18 mgL^{-1} and then decreased with further increase in Ca concentration up to 45 mgL^{-1} (Table 4.4c). While, in *C. sorokiniana* the lipid content was decreased from 40.34% to 30.98% with increase in the Ca concentration from 0 to 45 mgL^{-1}. Previous studies also showed the variation in the impact of Ca on the lipid accumulation of different microalgal strains. Ren et al. (2014) studied the effects of Ca concentration on the

lipid accumulation of *Scenedesmus* sp. and observed an increase in lipid content from 10.47% to 47.45% with an increase in Ca concentration from 0 to 9.8×10^{-4} gL^{-1}. However, further increase in calcium concentration did not influence lipid accumulation in their study. In contrast, Gorain et al. (2013) reported an increase in lipid content of *Chlorella vulgaris*, under Ca starved conditions.

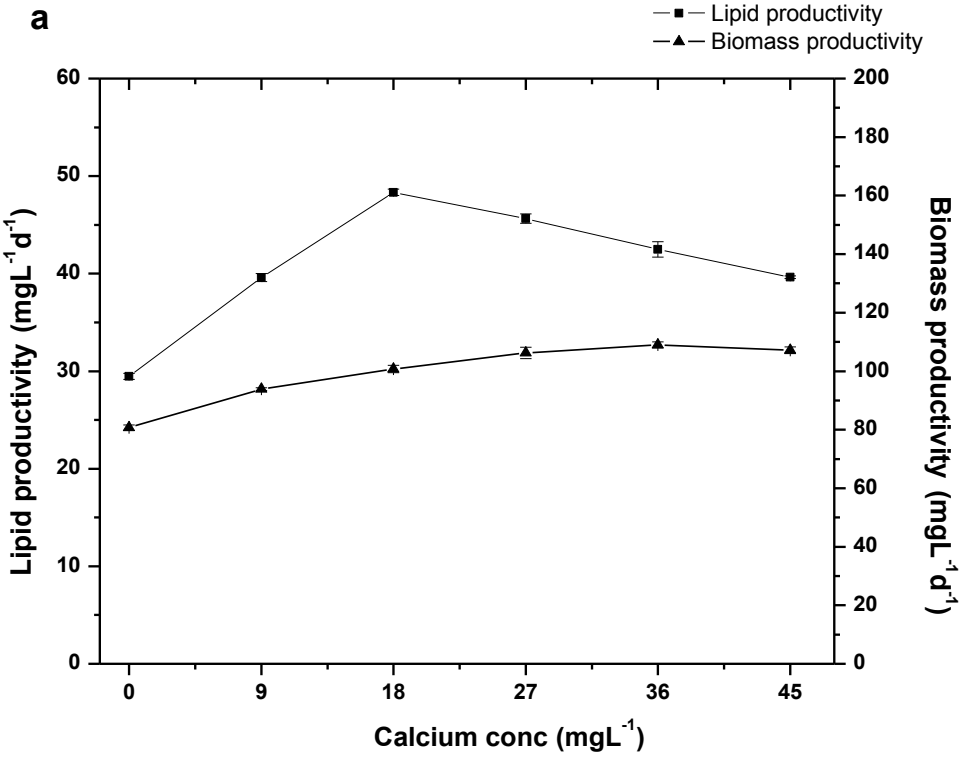


Figure 4.5a: Effect of Ca on concentrations on biomass and lipid productivity of *A. obliquus* N limited condition

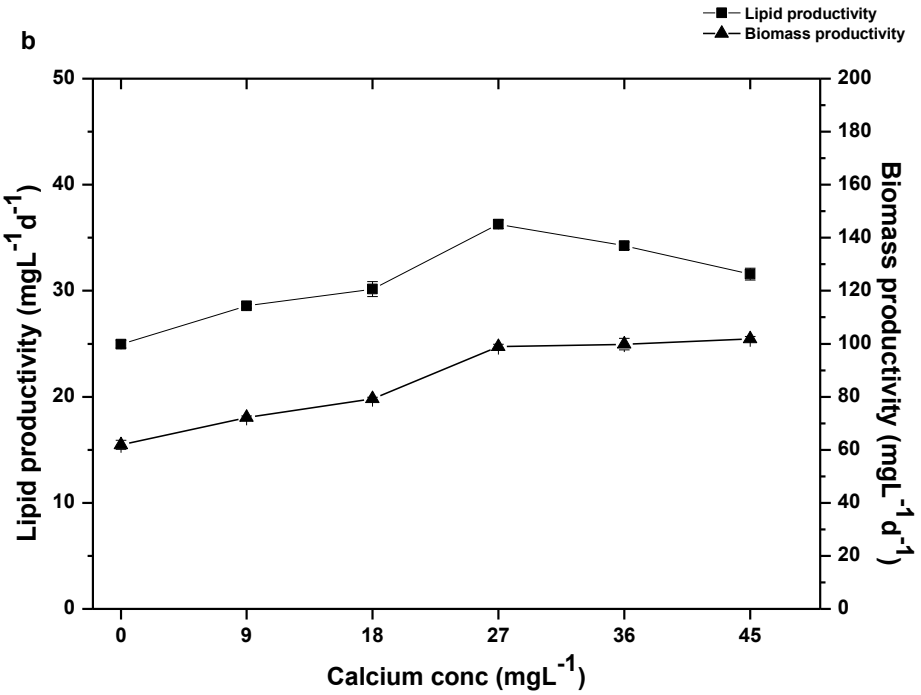


Figure 4.5b: Effect of Ca on concentrations on biomass and lipid productivity of *C. sorokiniana* under N and P limited condition

Table 4.4c: Effect of variation in the Ca concentration on the lipid on *A. obliquus* and *C. sorokiniana*

Ca Concentrations (mgL^{-1})	***A. obliquus* Lipid content (%)**	***C. sorokiniana* Lipid content (%)**
0	36.51±0.72	40.34±0.60
9	42.19±0.34	39.54±0.15
18	47.99±0.29	38.05±0.75
27	42.96±0.99	36.67±0.75
36	39.00±1.08	34.25±0.35
45	36.99±0.32	30.98±0.56

4.3.2.4 Effect of EDTA concentrations on the biomass and lipid productivity of *A. obliquus* and *C. sorokiniana*

Preliminary experiments were conducted to investigate the effect of EDTA on lipid productivity of *A. obliquus* and *C. sorokiniana* under N and P limited conditions. In *A. obliquus*, an increase in EDTA concentration from 1 to 2 mgL^{-1} resulted in an increase in lipid productivity form 34.37 $mgL^{-1}d^{-1}$ to 46.62 $mgL^{-1}d^{-1}$, while, a further increase in EDTA concentration did not affect the lipid productivity (Figure 4.6a). A similar trend was also observed in *C. sorokiniana* where an increase in EDTA concentration from 1 to 3 mgL^{-1} resulted in increased lipid productivity from 28.95 to 35.97 $mgL^{-1}d^{-1}$ thereafter the lipid productivity remained unchanged with any further increase in EDTA (Figure 4.6b). The increased EDTA concentration through its chelating property could be possibly facilitating the uptake of metals by microalgae (Xu et al., 2011, Chen et al., 2004). Ren et al. (2014) reported an increase in the total lipid content (28.2%) of *S. obliquus* with an increase in EDTA concentration (0-1 mgL^{-1}).

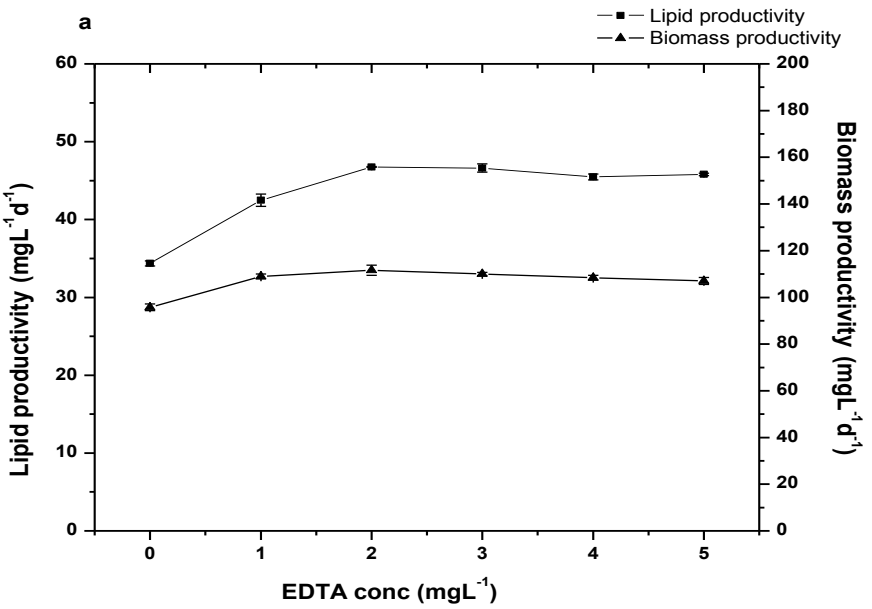


Figure 4.6a: Effect of EDTA concentrations on biomass and lipid productivity of *A. obliquus* under N limited condition

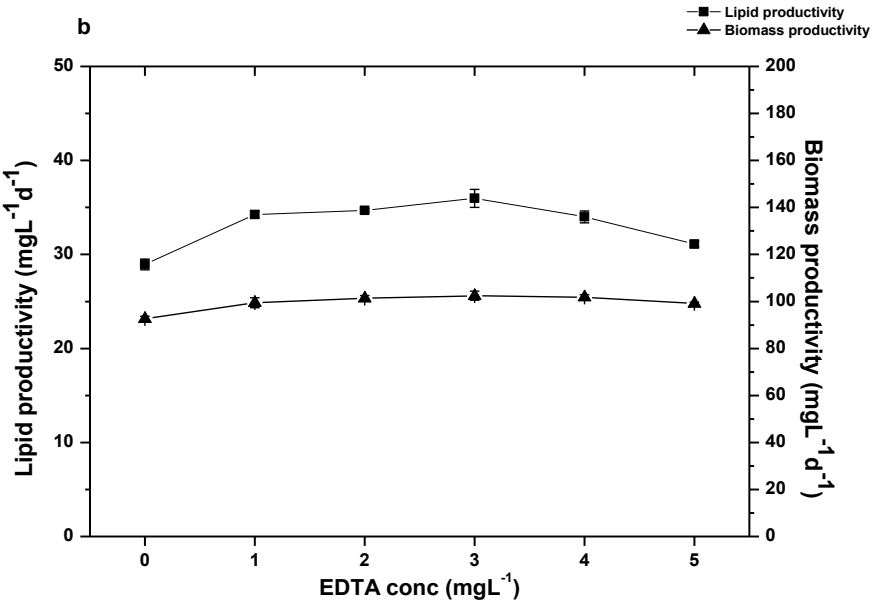


Figure 4.6b: Effect of EDTA concentrations on biomass and lipid productivity of *C. sorokiniana* under N and P limited condition

Table 4.4d: Effect of variation in the EDTA concentration on the lipid on A. *obliquus* and *C. sorokiniana*

EDTA concentration (mgL^{-1})	***A. obliquus* Lipid content (%)**	***C. sorokiniana* Lipid content (%)**
0	35.91±0.68	31.26±0.65
1	39.00±1.08	34.25±0.35
2	41.90±0.84	34.23±0.56
3	42.39±0.74	35.11±0.8
4	41.95±0.75	33.41±0.36
5	42.79±0.47	31.36±0.42

4.3.3 Effect of combined metal stress on biomass and lipid productivity

The effect of combination of selected metals, on the lipid productivity of *A. obliquus* under N limited condition and *C. sorokiniana* under N and P limited conditions was investigated. The RSM result was further compared with the results of individual stress experiments. Individual optimum nitrogen stress has been denoted as ON, individual P stress as OP, combined N and P optimum stress as ONP, individual optimum Fe concentration as OII, individual optimum Mg concentration as OIM, individual optimum Ca concentration as OIC and optimum EDTA as OE.

4.3.3.1 Effect of combined metal stress on the biomass and lipid productivity of *A. obliquus* under N limited conditions using RSM

For all the experimental runs (Exp. no. 1-15), lipid content, lipid productivity and biomass productivity were calculated at the late log phase (Table 4.5). Lipid productivity was taken as the response to analyse the combined effect of the chosen metals on *A. obliquus*. The

highest lipid productivity of 73.23 $mgL^{-1}d^{-1}$ under N limited condition (750 mgL^{-1}) was obtained with a combination of Fe 9 mgL^{-1}, Mg 100 mgL^{-1}, and Ca 27 mgL^{-1} (Exp. 10) (OCMS). In control experiment (ON) with N concentration of 750 mgL^{-1} the lipid productivity was 42.5 $mgL^{-1}d^{-1}$. The lipid productivity was increased by 1.72 folds with the combined metal stress under N limited condition (OCMS) as compared to control (ON) (Figure 4.7). Moreover, the lipid content of *A. obliquus* with optimum combined metal stress was 50.01%, while that with N limitation alone was 38.89% and with the normal BG11 medium was 25.95% (Table 4.5). Biomass productivity of *A. obliquus* with the combined metal stress was higher (146.43 $mgL^{-1}d^{-1}$) than N limitation alone (109.29 mg $L^{-1}d^{-1}$) and was comparable to normal BG11 medium (141.07 mg $L^{-1}d^{-1}$).

With the results obtained from Box-Behnken design and data analysis, a quadratic polynomial equation was derived based on multiple regression analysis. The equation with actual variables was:

$$R1 = 72.8 + 7.2\ Fe\ (A) + 4.66\ Mg\ (B) - 1.29\ Ca\ (C) - 22.04\ Fe^2\ (A^2) - 5.79\ Mg^2\ (B^2) - 11.2\ Ca^2\ (C^2) + 0.11\ Fe \times Mg\ (AB) + 0.98\ Fe \times Ca\ (AC) - 2.08\ Mg \times Ca\ (BC) \quad \text{(Eq. 4.5)}$$

Where, R1 is the observed response i.e., lipid productivity of *A. obliquus* grown with chosen metal stress conditions under optimized N concentration in BG11 growth medium. A, B and C are the coded values of independent factors Fe, Mg and Ca concentrations respectively.

Table 4.5: Box-Behnken model results of combined effect of metal stress under N limited condition in *A. obliquus*

Ex. No.	Iron (A)	Magnesium (B)	Calcium (C)	Lipid productivity ($mgL^{-1}d^{-1}$)	Biomass productivity ($mgL^{-1}d^{-1}$)	Lipid content (%)
1	9	75	18	51.62	113.57	45.45
2	12	125	27	54.64	160.71	34.00
3	9	75	36	52.07	125.71	41.42
4	9	125	18	63.71	159.29	40.00
5	6	125	27	46.00	131.43	35.00
6	9	100	27	72.16	144.29	50.01
7	6	100	36	27.69	108.57	25.50
8	9	100	27	73.02	143.57	50.86
9	6	75	27	35.52	92.86	38.25
10	9	100	27	73.23	146.43	50.01
11	12	100	18	49.47	115.71	42.75
12	9	125	36	55.83	142.86	39.08
13	6	100	18	31.07	107.14	29.00
14	12	100	36	50.00	125.00	40.00
15	12	75	27	43.73	110.00	39.75
BG 11	6	75	36	36.60	141.00	25.95
ON	6	75	36	42.5	109.29	38.89
ON-M	0	0	0	7.88	18.64	42.25

*ON-optimum N concentration, ON-M- optimum N concentration without selected metals

The statistical significance of the designs was determined by the F-test for ANOVA. The statistical relation between the response and selected factors at 95% confidence level showed that the regression analysis is statistically significant since the p value for the model was 0.002 (< 0.05) (Table 4.6). The value of the determination coefficient R^2 = 97.28% indicates that only 2.72% of the total variations in the model cannot be explained, thus the model fits quite well (Table 4.6). In this experimental design, factors Fe (A) and Mg (B) were

found to be significant (Table 4.6). From table 4.6 it can be concluded that the metal with most notable impact was Fe (p = 0.004) followed by Mg (p = 0.021) and then Ca (p = 0.4).

For validation of the RSM experimental design, triplicate experiments were performed at optimal combined metal stress conditions (9 mgL^{-1} of Fe, 100 mgL^{-1} of Mg, and 27 mgL^{-1} of Ca). A lipid productivity of 72.47±1.14 mg $L^{-1}d^{-1}$ was obtained which is in compliance with the predicted value of 72.80 mg $L^{-1}d^{-1}$. Addition of optimized EDTA (0.002 gL^{-1}) in the optimized combined metal stress medium (OCMS) resulted in further improvement in the lipid productivity of *A. obliquus.* The lipid productivity was improved from 73.23 $mgL^{-1}d^{-1}$ to 80.23 $mgL^{-1}d^{-1}$ with combination of optimized combined metal stress and EDTA concentration under N limited condition (OCMS+OE).

Table 4.6: Analysis of variance (ANOVA) for response surface quadratic model results of combined effect of metal stress under N limited condition in *A. obliquus*

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Model	9	2795.31	2795.31	310.59	19.84	0.002
Iron (A)	1	414.15	414.15	414.15	26.45	0.004
Magnesium (B)	1	173.48	173.48	173.48	11.08	0.021
Calcium (C)	1	13.23	13.23	13.23	0.84	0.400
Linear	3	600.86	600.86	200.29	12.79	0.009
Square	3	2173.19	2173.19	724.40	46.27	<0.001
Interaction	3	21.27	21.27	7.09	0.45	0.727
Residual Error	5	78.28	78.28	15.66		
Lack-of-Fit	3	77.63	77.63	25.88	80.16	0.012
Pure Error	2	0.65	0.65	0.32		
Total	14	2873.59				

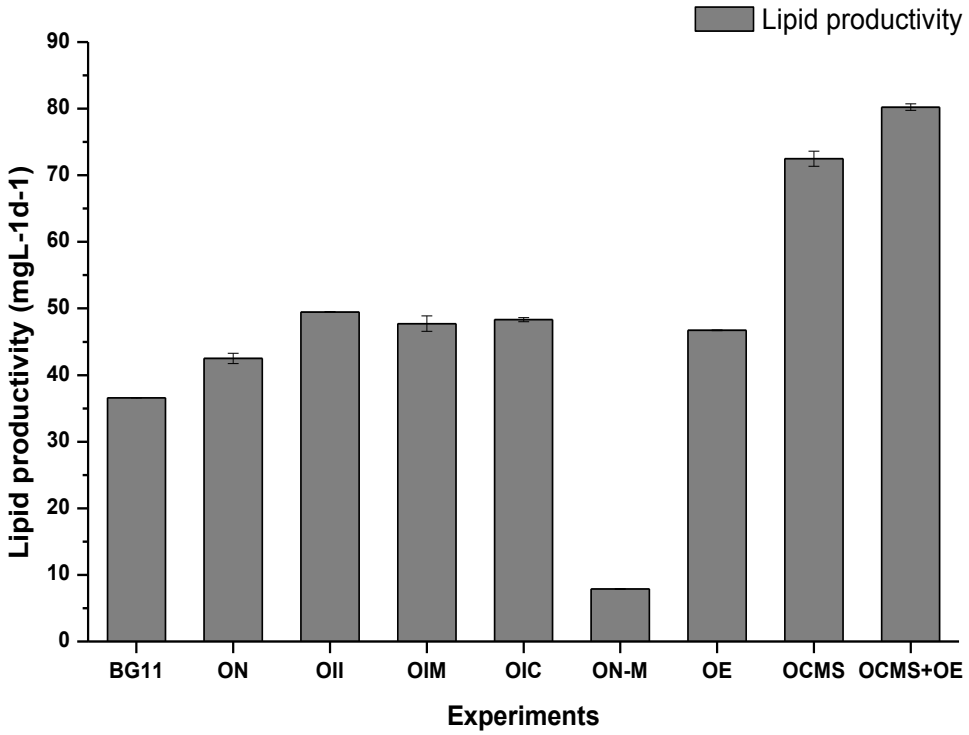


Figure 4.7: Effect of various experimental conditions on lipid productivity of *A. obliquus*

BG11- standard BG11 medium, ON-optimum N concentration, OII- optimum individual iron concentration OIM-Optimum individual magnesium concentration, ONP-M- optimum N concentration and optimum P without selected metals OCMS –optimum combined metal stress, OCMS+OE - optimum combined metal stress + optimum EDTA concentration

4.3.3.2 Effect of combined metal stress on biomass and lipid productivity of *C. sorokiniana* under N and P limited conditions using RSM

With *C. sorokiniana* for all the experimental runs (Exp. no. 1-15), lipid content and productivity along with biomass productivity were calculated at the late log phase (Day 14, Table 4.7). Lipid productivity was taken as the response to analyse the combined effect of the chosen metals on *C. sorokiniana*. The highest lipid productivity of 70.98 $mgL^{-1}d^{-1}$ was

obtained with a combination of 15 mgL^{-1} of Fe, 125 mgL^{-1} of Mg and 18 mgL^{-1} of Ca in Exp. 14 (OCMS). It was noted that the lipid productivity was increased by 2.12 folds with the combined metal stress (OCMS) as compared to control (ONP) (Figure 4.8). Moreover, the lipid content of *C. sorokiniana* under optimized combined metal stress was increased to 45.35%, while that with ONP condition was 34.25% and with normal BG11 medium was 18.5% (Table 4.7). The biomass productivity of *C. sorokiniana* during the combined metal stress was higher (156.43 $mgL^{-1}d^{-1}$) than ONP condition (99.29 $mgL^{-1}d^{-1}$) and was comparable to normal BG11 medium (154.86 $mgL^{-1}d^{-1}$) (Table 4.7). From these results, it was evident that the optimum combined metal stress under N and P limited conditions not only compensates for the decrease in biomass productivity caused by limitation of N and P but also improves the lipid content.

With the results obtained from Box-Behnken design and data analysis, a quadratic polynomial equation was derived based on multiple regression analysis. The equation with actual variables was:

$$R1 = 54.85 + 7.33\ Fe\ (A) + 345\ Mg\ (B) - 298\ Ca\ (C) - 0.5\ Fe^2\ (A^2) - 1.54\ Mg^2\ (B^2) + 1.14\ Ca^2\ (C^2) + 1.89\ Fe \times Mg\ (AB) - 2.74\ Fe \times Ca\ (AC) + 2.27\ Mg \times Ca\ (BC) \quad \text{(Eq. 4.6)}$$

Where, R1 is the observed response i.e., lipid productivity of *C. sorokiniana* grown with chosen metal stress conditions under optimized N and P concentrations in BG11 growth medium. A, B and C are the coded values of independent factors Fe, Mg and Ca concentrations respectively.

Table 4.7: Box-Behnken model results of combined effect of metal stress under N and P limited condition in *C. sorokiniana*

Ex. No.	Iron (A)	Magnesium (B)	Calcium (C)	Lipid productivity ($mgL^{-1}d^{-1}$)	Biomass productivity ($mgL^{-1}d^{-1}$)	Lipid content (%)
1	9	150	27	46.3	124.29	37.25
2	12	125	27	53.7	135	39.78
3	15	125	36	53.89	130.71	41.23
4	15	150	27	65.5	132.86	49.3
5	12	150	36	60.39	130	46.45
6	9	125	18	51.61	118.57	43.53
7	12	100	18	53.05	115.71	45.85
8	12	125	27	54.61	131.43	41.55
9	12	125	27	56.23	139.29	40.37
10	12	100	36	48.22	122.86	39.25
11	12	150	18	56.13	133.5	42.05
12	9	100	27	43.9	121.43	36.15
13	9	125	36	45.46	122.86	37
14	15	125	18	70.98	156.43	45.35
15	15	100	27	55.55	115	48.3
BG 11	6	75	36	28.87	154.86	18.5
ONP	6	75	36	33.44	99.29	34.25
ONP-M	0	0	0	8.49	20.36	41.75

*ONP-optimum N and phosphorous concentration, ONP-M- optimum N and phosphorous concentration without selected metals

The statistical significance of the designs was determined by the F-test for ANOVA (Table 4.8). The statistical relationship between the response and selected factors at 95% confidence level showed that the regression analysis is statistically significant since the p value for the model was 0.039 (< 0.05) (Table 4.8). The value of the determination coefficient R^2 = 90.66% indicates that only 9.34% of the total variations in the model cannot be explained, thus the model fits quite well (Table 4.8). In this experimental design, the factors Fe (A) and Mg (B) were found to be significant (Table 4.8). From the table 4.8 it can

be concluded that the metal with most notable impact on lipid productivity was Fe ($p = 0.003$) followed by Mg ($p = 0.048$) and then Ca ($p = 0.074$).

For the validation of RSM experimental design, triplicate experiments were performed at optimal combined metal stress condition (15 mgL^{-1} of Fe, 125 mgL^{-1} of Mg, and 18 mgL^{-1} of Ca). A lipid productivity of 70.94±0.92 $mgL^{-1}d^{-1}$ was obtained which is in compliance with the predicted value of 68.53 $mgL^{-1}d^{-1}$. Most notably the addition of EDTA (3 mgL^{-1}) in the optimized combined metal stress medium (OCMS) has further improved the biomass productivity (158.27 $mgL^{-1}d^{-1}$) and lipid content (48.67%) of *C. sorokiniana*. The lipid productivity was improved from 70.98 $mgL^{-1}d^{-1}$ to 77.03 $mgL^{-1}d^{-1}$ with combination of optimized combined metal stress and EDTA concentration under N and P limited condition (OCMS+OE) (Figure 4.8).

Table 4.8: Analysis of variance (ANOVA) for response surface quadratic model results of combined effect of metal stress under N and P limited condition in *C. sorokiniana*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Model	9	676.547	676.547	75.172	5.39	0.039
Iron (A)	1	430.15	430.15	430.15	30.86	0.003
Magnesium (B)	1	95.121	95.121	95.121	6.82	0.048
Calcium (C)	1	70.954	70.954	70.954	5.09	0.074
Linear	3	596.21	596.21	198.737	14.26	0.007
Square	3	15.439	15.439	5.146	0.37	0.779
Interaction	3	64.898	64.898	21.633	1.55	0.311
Residual Error	5	69.7	69.7	13.94		
Lack-of-Fit	3	66.422	66.422	22.141	13.51	0.07
Pure Error	2	3.278	3.278	1.639		
Total	14	746.246				

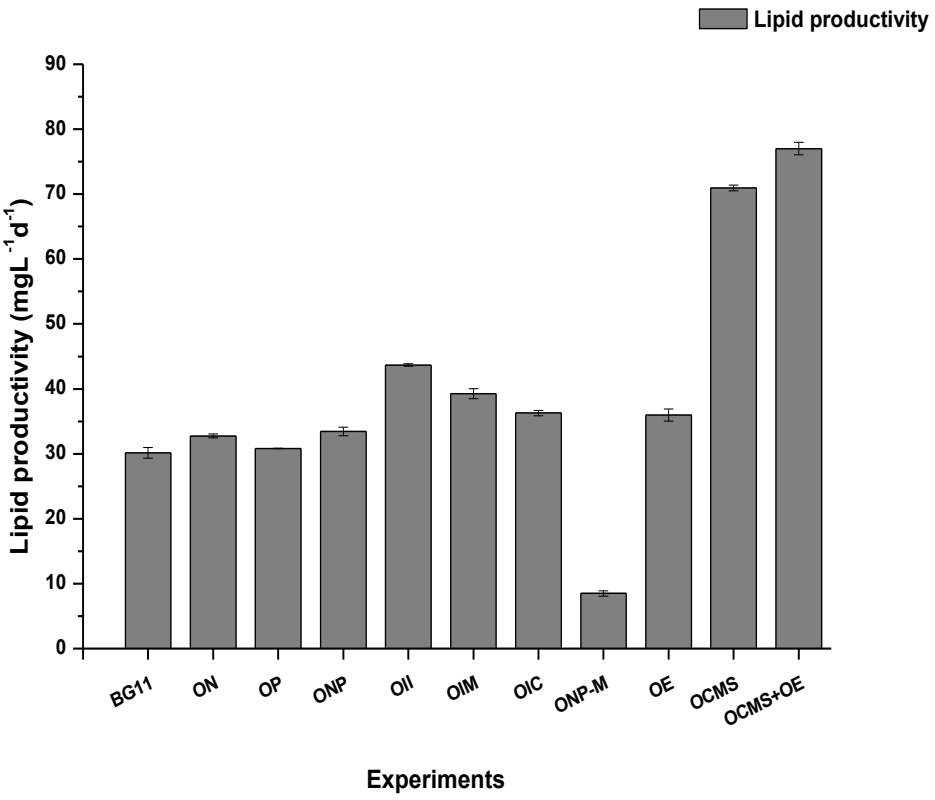


Figure 4.8: Effect of various experimental conditions on lipid productivity of *C.* sorokiniana

BG11- standard BG11 medium, ON-optimum N concentration, OP-optimum P concentration, ONP-optimum N and P concentration, OII- optimum individual iron concentration OIM-Optimum individual magnesium concentration, ONP-M- optimum N concentration and optimum P without selected metals OCMS –optimum combined metal stress, OCMS+OE - optimum combined metal stress + optimum EDTA concentration

The combined metal stress showed better lipid productivity in both strains as compared to the individual metal stresses and nutrient limitation alone. The results obtained in this study, indicated that the optimized combination of metals impacts positively on lipid productivity through both, enhanced lipid accumulation as well as biomass generation. RSM results also indicated Fe and Mg as the most influential metals for lipid productivity in *A. obliquus* and *C. sorokiniana*. From RSM results of both strains Ca was not found to

significantly influence the lipid productivity in this study. Iron assists in N assimilation by cells, therefore despite the lower concentration of N available in the media; presence of higher concentration of Fe enhances the assimilation of available N by microalgal cells (Cao et al., 2014, Wei et al., 2013, Wan et al., 2014). The Mg is an important constituent of chlorophyll molecule and therefore can positively influence microalgal biomass production (Gorain et al., 2013). Gorain et al. (2013) observed an improvement in biomass yield of up to 1.5 gL^{-1} (36% rise) in *S. obliquus* with Mg concentration of 150 mgL^{-1}.

The selected metals have also shown positive impact on the lipid accumulation of *A. obliquus*. Under OCMS conditions, higher lipid content of *A. obliquus* and *C. sorokiniana* was observed as compared to ON, ONP and normal BG11 (Table 4.5 and 4.7). Iron, which is a vital constituent of several enzymes and coenzymes in the lipid biosynthesis pathway, was found to be the most influential metal in this study to enhance the lipid productivity (Abd El Baky et al., 2012). An increase in lipid content of microalgae with Fe supplementation suggests that higher Fe concentration modulates the flux through certain metabolic pathways related to lipid accumulation.

Magnesium was observed to be the second most influential metal for enhancing lipid productivity in this study. Magnesium is known to promote the activity of acetyl-CoA carboxylase, the enzyme that regulates the first committing step of microalgal lipid biosynthesis (Ren et al., 2014, Lv et al., 2010). Therefore, improvement in the enzymatic activity of acetyl-CoA carboxylase could have resulted in the high lipid accumulation in *A. obliquus* and *C. sorokiniana* with Mg supplementation. Gorain et al. (2013) observed an improvement in lipid content of *S. obliquus* up to 27.6% (1.44 fold increase) with Mg supplementation (100 mg L^{-1}) in the media.

4.3.4 Effect of combined metals stress on photosynthetic performance and chlorophyll content of *A. obliquus* and *C. sorokiniana*

Pulse amplitude modulation (PAM) fluorometry is now being widely used as an *in situ* tool for assessment of the physiological conditions of microalgae under different stress conditions (Napoléon et al., 2013). By this technique, fluorescence emission of pigments which are directly associated with energy conversion reactions during the photosynthesis of photosystem II can be measured (Jiang et al., 2012). These fluorescence quotients provide information on the light absorption and utilization rate by the photosystem II complex (PSII) and characterize the physiological response of microalgal cells to changes in environmental conditions, *viz*., nutrient starvation or photo inhibition (Ramanna et al., 2014). The combined metal stress enhanced the biomass productivity of *A. obliquus* under N limited conditions, which is evident from the improvement in chlorophyll content and photosynthetic performance (Table 4.6). The relative electron transport rate (rETR) is a determination of the rate of linear electron transport through PS II which indicates the photosynthetic performance of microalgae (Ramanna et al., 2014). When *A. obliquus* was grown in standard BG11 medium, the rETR value was 39.7±0.44. However, under ON condition (750 mg L^{-1}), even though the lipid productivity of *A. obliquus* improved, its photosynthetic activity was hampered which is evident from the lower rETR value of 29.83±0.45 at the late log phase (Figure 4.9a). The OCMS condition (Fe 9 mg L^{-1}, Mg 100 mg L^{-1} and Ca 27 mg L^{-1}) on the other hand showed an improvement in photosynthetic performance with the rETR value of 49.17±0.25 (Figure 4.9a).

Maximum quantum efficiency of photosystem II (Fv/Fm) also indicated the positive influence of metals on photosynthetic performance. At the late log phase in *A. obliquus*, the

Fv/Fm value with OCMS was 0.616±0.002, while, that of ON was 0.385±0.01 respectively (Table 4.9a). Addition of optimized EDTA with optimized combined metal stress (OCMS+OE) showed further improvement in photosynthetic physiology with a rETR value of 50.91±0.37 (Figure 4.9a) and Fv/Fm value of 0.624±0.005 (Table 4.9a). Higher chlorophyll content was also observed for *A. obliquus* under OCMS condition as compared to ON. At the late log phase, the chlorophyll content for OCMS+OE was much higher (45.44 mgL^{-1}) than the experiment with ON (16.42 mgL^{-1}) (Table 4.9a).

Similarly, the combined metal and nutrient stress enhanced the biomass productivity of *C. sorokiniana* which was also evident from the improvements in photosynthetic performance (Figure 4.9b, Table 4.9b). At late log phase when *C. sorokiniana* was grown in standard BG11 medium alone, the rETR value was 32.88±0.65. Under ONP condition, even though the lipid productivity of *C. sorokiniana* was improved, its photosynthetic activity was hampered which was apparent from the lower rETR value of 19.77±0.63 at the late log phase (Figure 4.9b). Under the optimum combined metal stress condition (OCMS), an improvement in photosynthetic performance with the high rETR value of 38.05±0.38 was observed (Figure 4). At the late log phase, a higher value of the F_v/F_m (0.601±0.036) was observed under OCMS condition as compared to the ONP condition (0.437±0.007) (Table 4.9b). Addition of EDTA with optimized combined metal stress condition (OCMS+OE) showed a further improvement in photosynthetic activity with a rETR value of 39.715±0.86 (Figure 4.9b) and F_v/F_m value of 0.626±0.001 (Figure 4.9b, Table 4.9b). An increase in chlorophyll content was also observed with the increase in metal concentrations in *C. sorokiniana* (Table 4.9b). Highest chlorophyll content of 47.75 mgL^{-1} was observed under OCMS+OE condition (Table 4.9b).

Both photosynthetic performance and chlorophyll content results indicated the positive influence of selected metals on growth physiology of *A. obliquus* and *C. sorokiniana*. Fe and Mg plays significant role in photosynthesis, where Mg is core molecule of chlorophyll and Fe is an important metal of photosystem II (Cao et al., 2014, Wan et al., 2014). In *C. sorokiniana* at late log phase, Fv/Fm value under OII condition was 0.504±0.025 and under OIM condition was 0.511±0.015 while under ONP condition it was 0.437±0.007. It is clearly evident from the results that the supplementation of media with higher concentrations of these metals, improved the photosynthetic performance which eventually resulted in an increased biomass production of *A. obliquus* and *C. sorokiniana*. Results of photosynthetic behaviour (rETR and Fv/Fm) and chlorophyll content of *A. obliquus* and *C. sorokiniana* in this study confirmed the contribution of metals to biomass generation and supported the RSM result, which highlights the significant influence of Fe and Mg on overall lipid productivity under N and P limited condition.

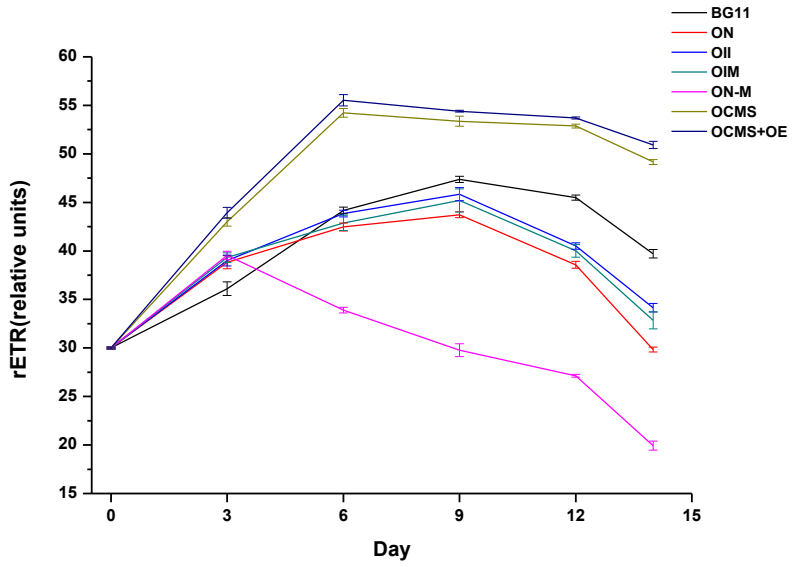


Figure 4.9a: Relative electron transport rate under various experimental conditions *A. obliquus*

BG11- standard BG11 medium, ON-optimum N concentration, OII- optimum individual iron concentration OIM-Optimum individual magnesium concentration, ON-M- optimum N concentration selected metals OCMS –optimum combined metal stress, OCMS+OE - optimum combined metal stress + optimum EDTA concentration

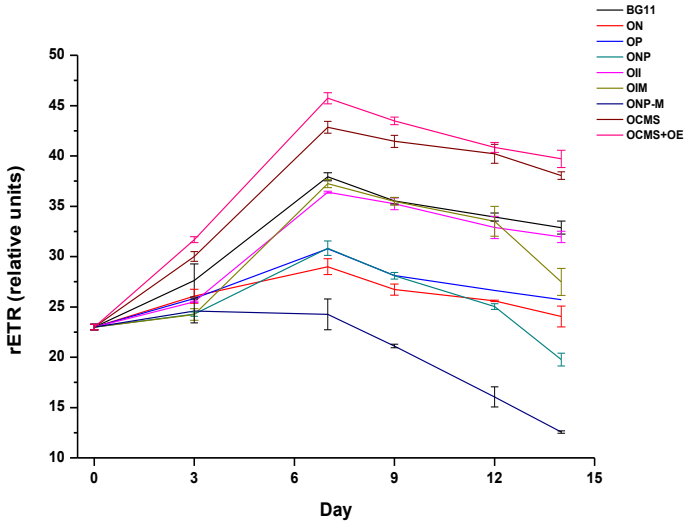


Figure 4.9b: Relative electron transport rate under various experimental conditions *C. sorokiniana*

BG11- standard BG11 medium, ON-optimum N concentration, OII- optimum individual iron concentration OIM-Optimum individual magnesium concentration, ONP-M- optimum N concentration and optimum P without selected metals OCMS –optimum combined metal stress, OCMS+OE - optimum combined metal stress + optimum EDTA concentration

Table 4.9a: Effect of various stress conditions on physiological parameters of *A. obliquus*

Experiment	F_v/F_m mid log phase	Fv/Fm late log phase	Chlorophyll content mid log phase (mgL^{-1})	Chlorophyll content late log phase (mgL^{-1})	Biomass productivity ($mgL^{-1}d^{-1}$)	Lipid content (%)
BG11	0.669±0.04	0.429±0.0	14.32±0.48	35.80±0.87	141.07±1.03	25.95±0.25
ON	0.637±0.05	0.385±0.02	12.20±0.44	16.42±0.1	109.29±0.47	38.89±0.81
OII	0.64±0.003	0.496±0.01	14.34±0.56	20.22±0.65	119.9±0.06	41.25±0.03
OIM	0.626±0.007	0.465±0.04	13.12±0.12	23.65±0.72	129.08±0.91	47.73±1.2
ON-M	0.612±0.05	0.290±0.11	5.66±0.25	8.16±0.07	18.64±0.06	42.25±1.14
OCMS	0.684±0.012	0.616±0.01	15.20±1.11	44.44±0.57	146.43±1.8	50.01±0.4
OCMS+OE	0.672±0.06	0.624±0.05	14.10±0.34	45.44±0.78	149±0.08	54.04±0.15

BG11- standard BG11 medium, ON-optimum N concentration, OII- optimum individual iron concentration OIM-Optimum individual magnesium concentration, ONP-M- optimum N concentration and optimum P without selected metals OCMS –optimum combined metal stress, OCMS+OE - optimum combined metal stress + optimum EDTA concentration

Table 4.9b: Effect of various stress conditions on physiological parameters of *C. sorokiniana*

Experiment	F_v/F_m mid log phase	F_v/F_m late log phase	Chlorophyll content mid log phase (mgL^{-1})	Chlorophyll content late log phase (mgL^{-1})	Biomass productivity ($mgL^{-1}d^{-1}$)	Lipid content (%)
BG11	0.618±0.023	0.500±0.018	16.44±1.07	38.54±1.72	154.86±1.2	18.5±0.14
ON	0.440±0.073	0.436±0.023	13.82±1.64	17.42±0.38	105±2.02	31.75±1.06
OP	0.437±0.035	0.453±0.032	15.23±0.76	19.16±0.87	108.5±2.1	28.4±0.57
ONP	0.471±0.039	0.437±0.007	11.20±0.91	16.44±0.49	99.29±3	33±0.71
OII	0.602±0.02	0.504±0.025	14.10±1.35	20.34±1.4	116.07±0.5	37.63±0.32
OIM	0.584±0.012	0.494±0.015	14.65±0.49	22.48±0.28	106.54±2.5	36.05±0.12
ONP-M	0.392±0.011	0.281±0.037	7.66±0.96	9.16±0.55	20.36±1.5	41.75±1.0
OCMS	0.666±0.01	0.601±0.04	16.88±0.62	46.82±0.73	156.43±1	45.35±1.4
OCMS+OE	0.667±0.014	0.626±0.018	17.10±0.28	47.75±0.142	158.27±0.4	48.67±0.46

BG11- standard BG11 medium, ON-optimum N concentration, OP-optimum P concentration, ONP-optimum N and P concentration, OII- optimum individual iron concentration OIM-Optimum individual magnesium concentration, ONP-M- optimum N concentration and optimum P without selected metals OCMS –optimum combined metal stress, OCMS+OE - optimum combined metal stress + optimum EDTA concentration

4.4 Conclusion

The combined strategy of supplementation of Fe, Mg and EDTA with Ca depletion under N and P limitation resulted in higher biomass and lipid accumulation compared to individual N and P stress conditions. Developed combined strategy alleviated the constraint of low biomass production under N and P limitation and improved the overall lipid productivity. For *A. obliquus*, the highest lipid productivity of 80.23 $mgL^{-1}d^{-1}$ was achieved with the developed strategy under limited N (750 mg L^{-1}) which was 2.18 fold higher than BG11 medium and 1.89 fold higher than the N limited condition alone. Similarly, for *C. sorokiniana*, highest lipid productivity of 77.03 $mgL^{-1}d^{-1}$ was achieved with the developed strategy under limited N (500 mgL^{-1}) and P (10 mgL^{-1}) which was 2.67 fold higher than BG11 medium and 2.35 fold higher than N and P limited condition. The RSM results indicate that Fe and Mg were the metals with significant influence on lipid productivity in both strains. Iron was the most influencing metal for enhancing lipid productivity followed by magnesium.

CHAPTER FIVE

GENE EXPRESSION ANALYSIS OF KEY GENES INVOLVED IN BIOSYNTHESIS OF FATTY ACID (ACCase) AND CARBON FIXATION (rbcL) UNDER SELECTED NUTRIENT AND METAL STRESSED CONDITIONS

SINGH, P., KUMARI, S., GULDHE, A., SINGH, G. & BUX, F. 2017. ACCase and rbcL gene expression as a function of nutrient and metal stress for enhancing lipid productivity in *Chlorella sorokiniana*. *Energy Conversion and Management,* 148**,** 809-819.

5.1 Introduction

Nutrient alteration can significantly improve lipid content in microalgae (Vonlanthen et al., 2015). Numerous studies have been conducted to evaluate the effects of major nutrients such as N and P on the lipid productivity in different strains of microalgae as discussed elsewhere (Griffiths et al., 2014, Sun et al., 2014, Chu et al., 2014). However, most of these studies arbitrated the effects based on lipid accumulation and biomass production, without understanding the effect on growth physiology, photosynthetic performance and expression of key genes at molecular level at different stages of microalgae cultivation. In microalgae, photosynthesis and lipid synthesis are two different pathways catalysed by different groups of enzymes and are controlled by different set of genes. Earlier studies have highlighted the

potential role of various genes such as *rbcL, accD, dgat, me, pepc*, etc. in regulating the growth and other metabolic processes of microalgae (Fan et al., 2014, Msanne et al., 2012).

The enzyme ribulose-bisphosphate carboxylase, catalyses the first step in carbon fixation where CO_2 combines with ribulose 1, 5-bisphosphate to yield two molecules of three-carbon compound, glycerate-3-phosphate. The simple carbon sugars produced by photosynthesis are then utilized in different metabolic processes to produce other organic compounds such as amino acids, lipids and compounds for cell structure. In microalgae, this enzyme consists of two types of protein subunits, the large subunit and small subunit (Uemura et al., 1997, Andersson and Backlund, 2008). All enzymatically active binding sites are reportedly located in the large subunit (*rbcL* gene) of the enzyme (Andersson and Backlund, 2008). This enzyme also coordinates the activity of several other co-enzymes of Calvin cycle and thus is identified as a key enzyme related to the growth physiology of the microalgae.

Lipid biosynthesis in microalgae starts in the chloroplast and is catalysed by a multifunctional enzyme complex. The enzyme, Acetyl-CoA carboxylase (ACCase) plays a crucial role in fatty acid biosynthesis, which catalyses acetyl-CoA conversion to malonyl-CoA, which subsequently enter the lipid biosynthesis pathway (Liang and Jiang, 2013). Many plants contain two forms of ACCase: heteromeric/ bacterial and homomeric/ eukaryotic ACCase. Heteromeric ACCase is the key enzyme for fatty acid synthesis in the chloroplast and has four subunits, but only the beta subunit (*accD*) is synthesized in the chloroplast (Salie and Thelen, 2016, Huerlimann and Heimann, 2013).

Both *accD* and *rbcL* are the two most exploited genes in both plant and microalgae genetic engineering studies (Salie and Thelen, 2016, Jusoh et al., 2015, Fan et al., 2014).

Both the genes are known to play critical roles in controlling the overall photosynthesis and lipid biosynthesis respectively. Therefore, understanding the expression level of these two genes under stress conditions would provide a better understanding of physiological response of microalgae under various stress conditions. In this study, the effect of macronutrients (N and P) and metals (Fe and Mg) individually and in combination (OCMS+OE) on expression of selected key genes (*rbcL* and *accD*) in *A. obliquus* and *C. sorokiniana* was investigated using reverse transcriptase quantitative PCR at different phases of its cultivation.

Transcriptome studies would provide an overall idea of the changes in different gene expression levels in response to different stress conditions (Liang and Jiang, 2013, Fan et al., 2016, El Amrani et al., 2015) and is an appropriate tool to provide a better understanding of various metabolic pathways governing growth and lipid accumulation in microalgae. In transcriptome analysis, for RNA sequencing, mRNA is converted from cDNA to an RNA-sequencing library. By sequencing the millions of DNA fragments in the library (known as 'reads') with next-generation sequencing, an accurate measure of the relative abundance of each transcript and variation can be obtained (Van Verk et al., 2013). The obtained sequence can be translated into information of gene expression levels by using bioinformatics tools. Therefore, for understanding the overall up and down regulation of different genes during lipid synthesis under optimized stress conditions, transcriptome analysis was performed in this study. Owing to its high lipid productivity *A. obliquus* was selected for transcriptome analysis; expression analysis was performed with samples from developed combined stress strategy and BG11 control conditions.

5.2 Materials and methods

5.2.1 Microalgae cultivation conditions for gene expression analysis

Microalgae, *A. obliquus* and *C. sorokiniana* were cultivated under optimized nutrients and metal stress conditions individually as well as in combination as described in chapter 4 section 2. The selected microalgae strains, *A. obliquus* was cultivated under, ON, OII, OIM, ON-M, OCMS+OE and control BG11 medium and *C. sorokiniana* was cultivated under ON, OP, ONP, OII, OIM, ON-M, OCMS+OE and in control BG11 medium. All the experiments were performed in triplicate. Similar cultivation conditions were maintained for all the experimental runs as mentioned in chapter 3 section 3.2.l.

5.2.2 Total RNA extraction and cDNA synthesis

Samples were drawn at mid (day 7) and late log phase (day 14) of the cultivation period of each experiment and the biomass was harvested by centrifugation. Immediately after harvesting, approximately 100 mg of wet biomass was ground in liquid nitrogen using pestle and mortar. Thereafter, the total RNA was extracted from the powdered sample using QIAGEN RNeasy Mini Kit following the manufacturer instructions. The quality and quantity of the extracted total RNA was analysed by electrophoresis using 1.2 % agarose gel and by spectrophotometric analysis (Nano-Drop 1000 spectrophotometer, Coleman Technologies Inc., USA) respectively. The first-strand cDNA was synthesized from the total extracted RNA using QuantiTect® Reverse Transcription Kit (Qiagen, USA) following the manufacturer instructions. The cDNA obtained was used as a template for the subsequent qPCR assays. Primers were selected based on previous literature to target the key functional

genes involved in photosynthesis (*rbcL*) and fatty acid biosynthesis (*accD*) pathway and used for expression-level quantitative detection (Table 5.1).

Table 5.1: Oligonucleotide sequences of primers used for relative gene expression studies

Genes	Function	Sequence (5^1-3^1)	Optimized annealing temp	Amplicon size (bp)	Reference
accD	conversion of acetyl Co-A to malonyl Co-A	(F) TTTGGTTTGTGCTTCTGGTG (R)CACCACCAGTTGTTGGAGAA	58.0	149	(Li et al., 2015)
rbcL	carbon fixation in Calvin cycle	(F) CTTTCCAAGGTCCTCCTCAC (R) TCTCTCCAACGCATAAATGG	56.3	208	(Fan et al., 2014)
β Actin	component of cell cytoskeleton	(F) GCTCAACTCCTCCACGCT (R) GTCCTTGCGGATGTCCAC	58	187	(Fan et al., 2014)
16S rRNA	component of small subunit of ribosome	(F) GTGSTGCAYGGYTGTCGTCA (R) ACGTCRTCCMCACCTTCCTC	60	147	(Maeda et al., 2003)

F- Forward; R- Reverse

5.2.3 Quantitative real-time PCR (qPCR)

For the qPCR assays, cDNA corresponding to 50 ng of total RNA was used per transcript to be quantified. The oligonucleotide sequences of primers used for gene expression study are described in table 5.1. Actin and 16S rRNA based two genes were tested to be used as reference gene for the RT qPCR assay normalization. Since the expression level of 16S rRNA gene was found to be unaffected by different experimental factors, 16S rRNA based gene was used as reference genes for normalization the gene expression data in this study.

The qPCR assay was performed using CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA). Briefly, the reaction mixture contained SsoFast™

EvaGreen® Supermix (Bio-Rad USA) (12.5 µL), forward and reverse primers (0.4 µM each) and template DNA (50 ng) in a final volume of 25 µL. A mixture of all PCR reagents containing 5 µL of sterile Milli-Q® (Millipore, Billerica, MA, USA) water instead of DNA template in each qPCR assay was taken as a negative control. RT-qPCR amplification protocol for the targeted genes consists of initial denaturation for 3 min at 95°C, followed by 40 cycles of three steps consisting of 15 s at 95°C, 20 s at appropriate annealing temperatures and 30 s at 72°C. Optimized annealing temperature for each primer was mentioned in table 5.1. The fluorescence signals were measured at the end of each extension step. The sample was considered negative if the fluorescent signal did not increase within 40 cycles. At the completion of qPCR amplification cycles, melting curve (Tm) analysis of the products was performed by reducing the temperature to 65°C then heating to 95°C at a rate of $0.5°Cs^{-1}$. Fluorescence was monitored continuously to confirm amplification specificity. The Tm peaks were calculated based on the initial fluorescence curve (F/T) by plotting the negative derivative of fluorescence versus temperature (dF/dT versus T).

The $2^{-\Delta\Delta CT}$ method was used to analyse the relative gene expression (Li et al., 2015, Schmittgen and Livak, 2008). The data obtained represented the fold change (increase or decrease) of the target gene in the treated samples of both strains (ON, OP, ONP, OII, OIM, ON-M, ONP-M, OCMS+OE) relative to the control sample (BG11) and was normalized to the expression of 16s rRNA reference gene.

5.2.4 Transcriptome analysis

5.2.4.1 Library construction and transcriptome analysis

RNA was extracted from *A. obliquus* cultures grown under BG11 and OCMS+OE and purified by using the QIAGEN RNeasy Mini Kit following manufacturer's instruction. The quality of the extracted total RNA was analysed as discussed in section 2.3. The Ribosomal RNAs (rRNAs) were removed using the Ribozero™ Magnet kit (plant leaf, Epicentre Biotechnologies, WI, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized QuantiTect® Reverse Transcription Kit (Qiagen) following manufacturer's instructions. Multiplexed paired-end libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA). Genome sequencing was carried out on an Illumina MiSeq platform. The resulting paired-end reads were quality trimmed and normalized. The resulting high quality reads of each library were quality controlled by using the program FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before assembly. De novo assembly of the transcriptome was performed by Trinity software (release 2014-07-17), according to the protocol. The abundance of each transcript was evaluated by RSEM (RNA-Seq by Expectation-Maximization) tool in every sample. R package RSEM was used to evaluate the expression levels of each transcript in every library by estimating the abundance of reads that aligned to the transcript. Differential expression of transcripts across samples was identified by using an R package called edgeR. The significance of differential expression was evaluated by the fold change (≥ 2) and p-value (<0.05).

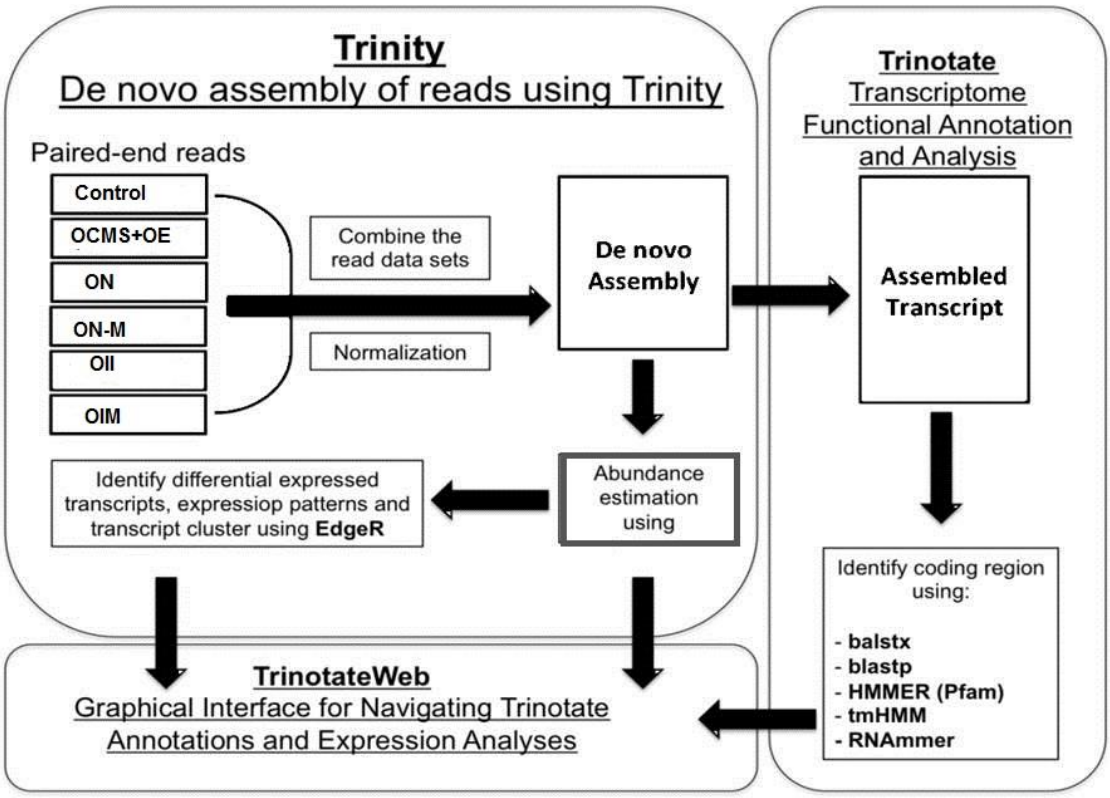


Figure 5.1 Schematic representation of RNA de novo assembly and analysis of transcripts

5.3 Results and discussion

5.3.1 RNA extraction, cDNA analysis and selection of housekeeping gene

RNA integrity and purity are considered to be the most crucial parameters for gene expression studies. Ribosomal RNA (rRNA) is the most abundant amongst all other subtypes of RNA. In this study, extracted RNA was analysed by gel electrophoresis. On the gel, there were two clear bands of 28S rRNA and 18S rRNA (Figure 5.2a) which indicated the integrity of isolated RNA.

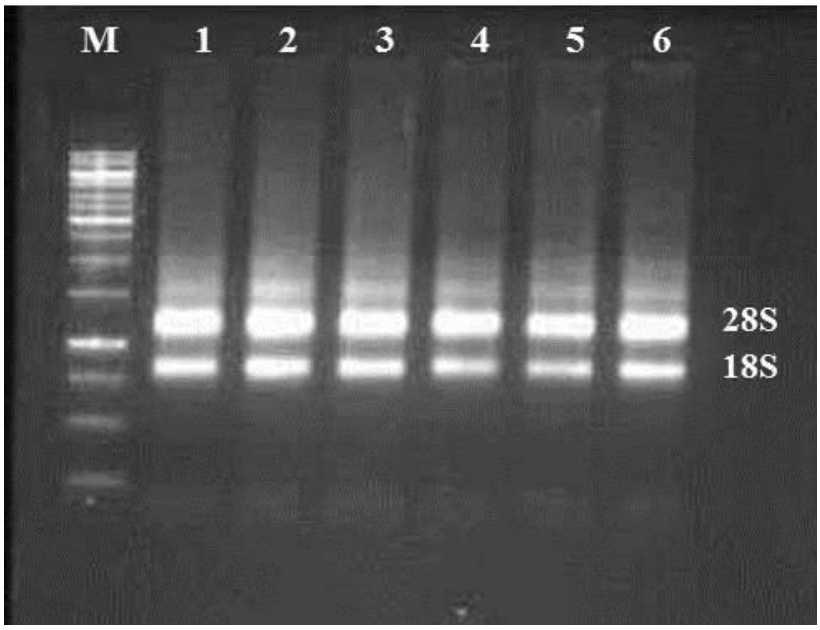


Figure 5.2a: Agarose gel electrophoresis of total RNA extracted from *A. obliquus* under different stress conditions. M = Marker, 1 = BG11, 2 = OCMS+OE, 3 = OII, 4 = OIM, 5 = ON, 6 = ON-M

The quality of the synthesised cDNA is also monitored to check its applicability for downstream applications. Figure 5.2b represents the gel electrophoresis image of the RT-qPCR product obtained from synthesized cDNA.

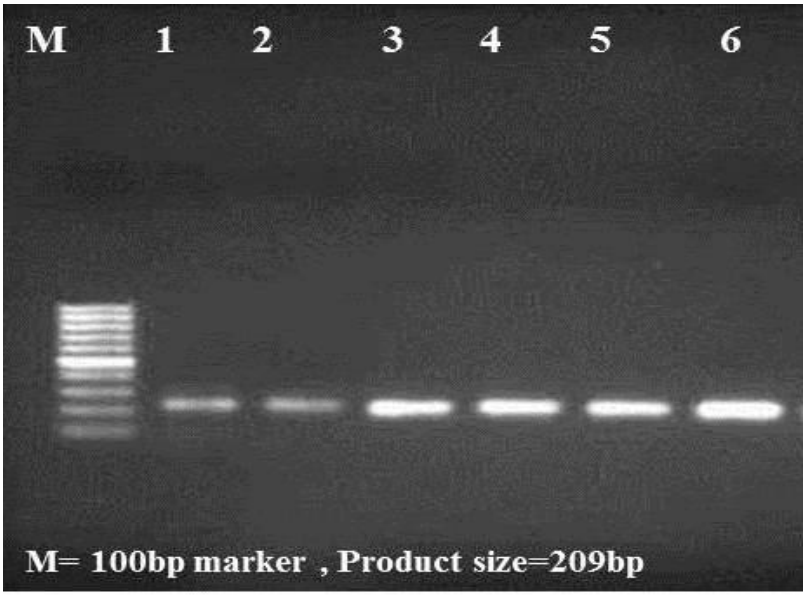


Figure 5.2b: Agarose gel electrophoresis of RT-qPCR product obtained from synthesized cDNA of *A. obliquus*. 1= ON-M, 2 = ON, 3 = OII, 4 = OIM, 5 = OCMS+OE, 6 = BG11

Data normalisation in real-time RT-qPCR is a further major step in gene quantification analysis. A stable endogenous control (housekeeping genes) should be included in any relative RT-qPCR study to improve its reliability and to correct sample to sample variations. The 16s rRNA is considered as the most conserved region during evolution and thus has been used for many phylogenetic studies (Maeda et al., 2003, Bowers et al., 2006). In this study, 16S rRNA based gene was selected as reference gene for relative gene expression study. The 16S rRNA gene has shown most stable expression in all the experimental conditions (control and stress). Figure 5.3 represents the amplification curve of 16S gene of *A. obliquus* under various stress conditions at different stages of cultivation. Melt curve analysis have done to assure whether the intercalating dye PCR/qPCR assays have produced single, specific products. In figure 5.3, the single melt peak indicates the pure, single amplicon.

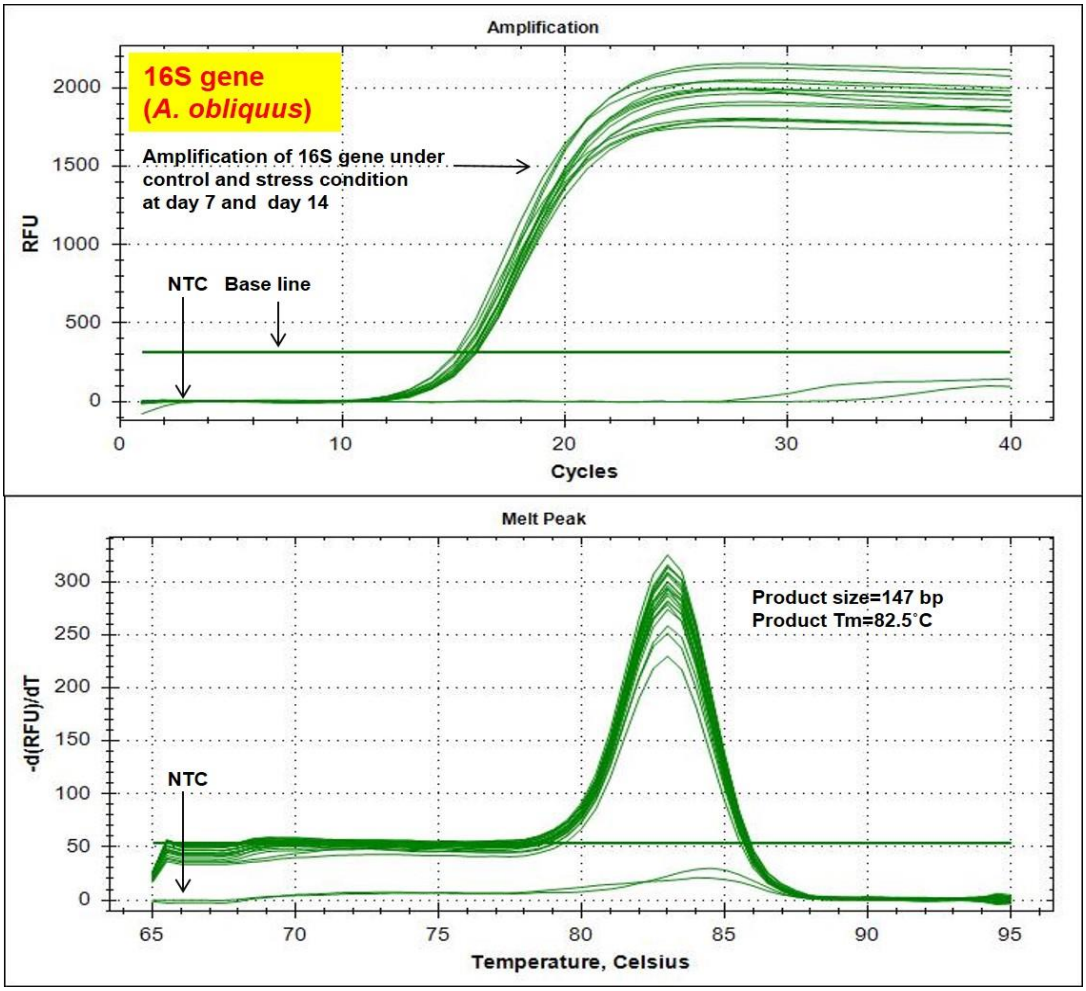


Figure 5.3: Effect of various stress condition on amplification curve of 16S rRNA gene of *A. obliquus*

5.3.2 Effect of individual and combined metals stress on the expression of *rbc*L gene of *A. obliquus* and *C. sorokiniana*

Impact of different experimental conditions at different stages of microalgal cultivation on *rbcL* gene expression is illustrated in figure 5.4 for *A. obliquus* and in figure 5.5 for *C. sorokiniana*. Microalgal cells grown under BG11 medium were taken as control for

relative fold change in the expression. In the mid log phase, N stress resulted in 70% decrease in *rbcL* gene expression in *A. obliquus* relative to the BG11 control (Figure 5.4 and 5.5). A combined N and P stress condition in *C. sorokiniana* have resulted in 89.2 % reduction in the expression of the *rbcL* gene relative to BG11 medium. Nitrogen deficiency can severely affect protein synthesis and reduce photosynthetic rates as evidenced in this study (Adams et al., 2013). Similarly, phosphorous plays a significant role in cellular metabolic processes related to energy transfer and signal transduction during photosynthesis. This down regulation of *rbcL* gene also translated in reduced photosynthetic performance (F_v/F_m and rETR) under N and P limited condition in both the strains (Table 4.9a and 4.9b; Figure 4.9a and 4.9b Chapter 4). Fan et al. (2014) also reported a similar decrease in expression of *rbcL* gene under N and P stress condition in *C. pyrenoidosa*. In their study expression of *rbcL* gene was decreased 2-5 times under nutrient (N and P) deficient condition as compared to nutrient sufficient condition.

Furthermore, at mid log phase, the individual experiments with optimum Fe (OII) and Mg (OIM) showed increase in expression of the *rbcL* gene as compared to individual N and P stress conditions. In *A. obliquus*, high Fe supplementation (OII) in N stress condition has shown a 36% increase in *rbcL* gene expression as compare to the control BG11 (Figure 5.4). Similarly, in *C. sorokiniana*, a 14.85% increase in *rbcL* gene expression was observed with high Fe (OII) concentration under N and P stress condition (Figure 5.5). Iron is an important metal in the regulation of photosystem I, photosystem II and electron transport during photosynthesis (Wan et al., 2014, Abd El Baky et al., 2012). Carbon fixation in microalgae requires high energy in the form of ATP, which is generated by electron transfer chain. High concentration of Fe could enhance the ATP production and thus accelerate the carbon

fixation (Petrou et al., 2014). Wan et al. (2014) have also studied the effect of Fe concentrations on the *rbcL* gene expression of *C. sorokiniana* and observed a 3.8×10^2 upregulation in the expression of *rbcL* gene under Fe sufficient condition as compared to Fe deficient condition.

In this study, an increase in Mg supplementation to 150 mgL^{-1} under N and P stress conditions (ON in *A. obliquus* and ONP in *C. sorokiniana*) have resulted in an increase of 33.55% and 38% in *rbcL* gene expression in *A. obliquus* and *C. sorokiniana* respectively at mid log phase as compared to control (Figure 5.4 and 5.5). Magnesium plays a crucial role in photosynthesis as it is a core molecule of chlorophyll and helps in light capture during photosynthesis (Wang et al., 2014). Magnesium also reported to regulate the activity of the RuBisCo enzyme by providing the optimal pH for activation of the enzyme (Andersson and Backlund, 2008). The pH of the stroma depends on Mg concentration; high Mg concentration provides optimal pH of 7-8 for RuBisCo which results in increase in the enzymatic activity by binding CO_2 and Mg ion at the active sites (Parry et al., 2008).

At late log phase, a drastic reduction in the *rbcL* gene expression was observed under all experimental conditions as compared to the mid log phase except in (OCMS+OE) experiment, where gene expression of rbcL was still high in both microalgal strains. At late log phase, combined stress (OCMS+OE) experiment have resulted in 2.5 fold increase in *rbcL* gene expression in *A. obliquus* and 2.75 fold increase in rbcL gene expression in *C. sorokiniana*. However, very low expression of *rbcL* gene was observed with the experiment where Fe and Mg was absent in to the medium. In *A. obliquus* expression of *rbcL* gene expression showed 95.84% reduction at mid log phase and 97.86% reduction at late log phase under ON-M condition compared to control. In *C. sorokiniana* expression of *rbcL* gene

expression showed 99.5% reduction at mid log phase and 99.8% reduction at late log phase under ONP-M condition compared to control. Thus, it is clearly evident that addition of metals has assisted the microalgae to accelerate the photosynthetic performance, which eventually improved the biomass yields. The individual effect of metals on *rbcL* expression also varied significantly both under mid and late log phase. At late-log phase, a slight increase in the *rbcL* expression of *A. obliquus* was observed under OII conditions (0.530 fold) as compared to OIM (0.11 fold) (Figure 5.4). The results from quantum efficiency of photosystem II (F_v/F_m) (Table 4.9a and 4.9b, Chapter 4) and relative electron transfer rate (Figure 4.9a and Figure 4.9b, Chapter 4) of this study further confirmed the above observations.

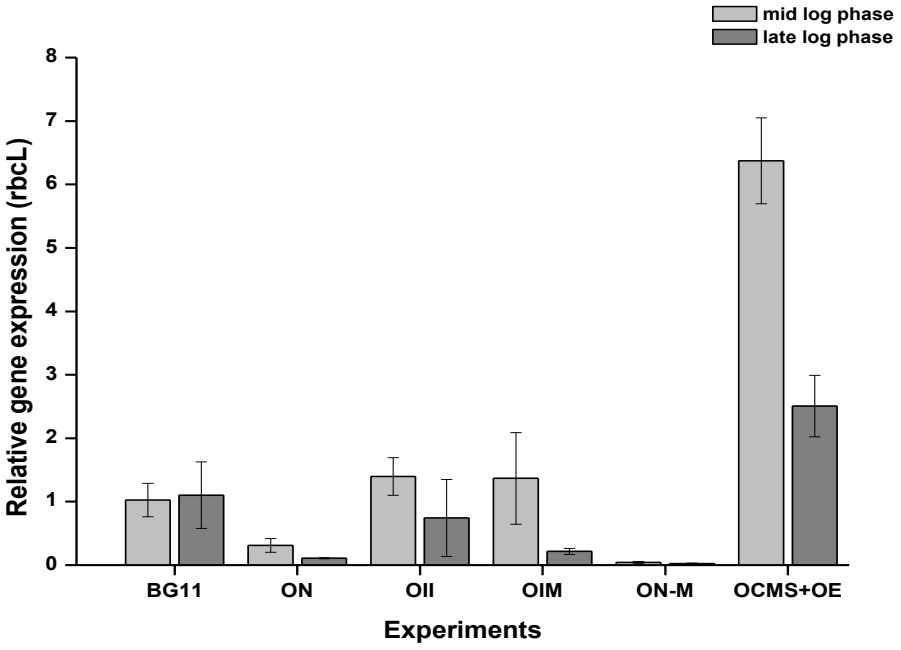


Figure 5.4: Effect of various experimental conditions on the relative expression of *rbcL* gene at mid log phase and at late log phase in *A. obliquus*

BG11- standard BG11 medium, ON-optimum N concentration, OII- optimum individual iron concentration OIM-Optimum individual magnesium concentration, ON-M- optimum N concentration without selected metals, OCMS –optimum combined metal stress, OCMS+OE - optimum combined metal stress + optimum EDTA concentration

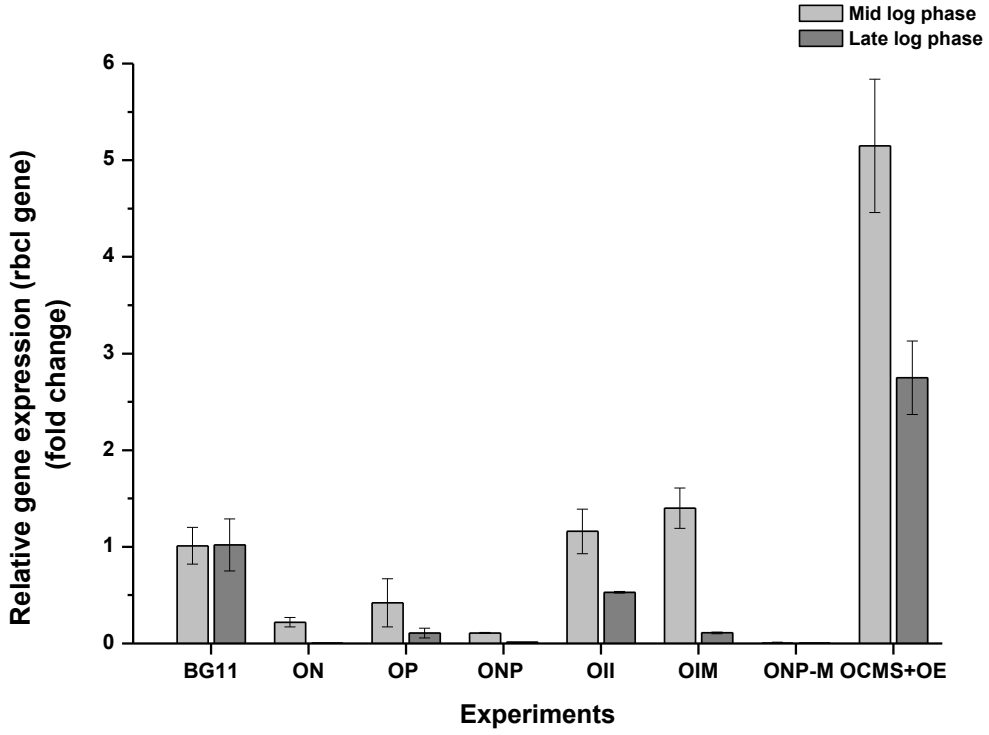


Figure 5.5: Effect of various experimental conditions on the relative expression of *rbcL* gene at mid log phase and at late log phase in *C. sorokiniana*

BG11- standard BG11 medium, ON-optimum N concentration, OP-optimum P concentration, OPN-optimum N and P concentration, OI- optimum individual iron concentration OMG-Optimum individual magnesium concentration, ONP-M- optimum N concentration and optimum P without selected metals, OCMS+ OE - optimum combined metal stress + optimum EDTA concentration

5.3.3 Effect of individual and combined metals stress on the expression of *accD* gene of *A. obliquus* and *C. sorokiniana*

Acetyl-CoA carboxylase (ACCase) catalyses the first rate-limiting step in the fatty-acid biosynthetic pathway through the formation of malonyl-CoA from acetyl-CoA. Effects of individual nutrients stress (N, P) individual metals stress (Fe, Mg) as well combination of nutrients and metals on expression of ACCase was investigated. The *accD* gene expression pattern of *A. obliquus and C. sorokiniana* genes at mid and late log phase are depicted in figure 5.6 and figure 5.7 respectively. An increase in expression level of *accD* was observed under all the experimental conditions. In *A. obliquus,* expression of *accD* gene was increased by 2 fold at mid log phase and 3.6 fold at late log phase under N limited conditions as compared to the control experiment (Figure 5.6). Similarly, in *C. sorokiniana*, compared to control (BG11), N limitation resulted in 2.83 fold increase in expression of *accD* gene at mid log phase and 3.24 fold increase at late log phase respectively (Figure 5.7). Fan et al. (2014) reported 39.8 fold increase in the *accD* level under N deprived condition in *Chlorella pyrenoidosa*. Phosphorus limitation also resulted in 1.92 fold increase in mid log and 2.93 fold increases in late log phase in *C. sorokiniana* (Figure 5.7). Fan et al. (2014) have also reported 4 fold increase in *accD* level under P deficient condition in *Chlorella pyrenoidosa*. Nitrogen and phosphorous deprivation conditions could lead to reduced cell division. Reduced cell division shifts the lipid biosynthetic pathways to synthesize more neutral lipids than synthesizing membrane lipids required for the cell wall formation (Zhang et al., 2013). Subsequent accumulation of NADH due to the lower photosynthetic rate inhibits enzyme citrate synthase and prevents acetyl-CoA from entering in to the TCA cycle (Shin et al., 2015, Salie and Thelen, 2016). Elevated concentrations of acetyl-CoA activate acetyl-CoA

carboxylase, thereby leading to enhanced lipid accumulation in microalgal cells (Li et al., 2015).

In this study, at late log phase, the expression of *accD* gene was found to be upregulated under individual as well as under combined metal stress conditions. This is probably due to fact that lipid accumulation occurs in the late log phase in microalgae when there is limited nutrients in the media (Khatoon et al., 2017). In *A. obliquus,* high Fe supplementation under N limited condition has resulted in 7.68 fold increase in *accD* gene expression as compared to control (Figure 5.6). In *C. sorokiniana* an upregulation of 8.46 fold was observed at late log phase when the culture was grown under high Fe concentration (OII), which was almost twice as compared to ONP condition (Figure 5.9). At late log phase, 6.05 fold and 6.89 fold increases in *accD* gene expression was observed under OIM condition in *A. obliquus* and *C. sorokiniana* respectively (Figure 5.6 and 5.7). Iron-sulphur complex is necessary to maintain stability of chloroplast and stroma as it is required for photophosphorylation in ATP production (Lv et al., 2010, Huang et al., 2014, Concas et al., 2014). Wan et al. (2014) reported high expression of *accD* gene with increase in Fe concentrations in *C. sorokiniana*. Iron and magnesium are reported to play a crucial roles in activity of ACCase (Liu et al., 2008). ACCase activity is associated with the pH of the chloroplast, Mg ion concentration and ATP production. An increase in the Mg ion is reported to improve ACCase activity in plants (Sasaki and Nagano, 2004). In this study, a higher expression of *accD* was visible under high Mg concentration at mid log phase in both the strains compared to other conditions (Figure 5.6 and 5.7). Expression of *accD* gene was 4.94 fold in *A. obliquus* and 5.05 fold in *C. sorokiniana* at mid log phase as compared to control

BG11 condition (Figure 5.6 and 5.7). This indicates the significant influence of magnesium on the activity of *accD*.

Combination of high Fe, high Mg along with EDTA supplementation under N and P limitation (OCMS+OE) have resulted in upregulation of 10.25 fold and 9.79 fold in the expression of *accD* gene in *A. obliquus* and *C. sorokiniana* respectively at late log phase. Results from the lipid quantification have also shown similar pattern. Lipid content of *A. obliquus* and *C. sorokiniana* were 25.95% and 18.5% in BG11 medium respectively. Under OCMS+OE conditions lipid contents were increased to 54.04% in *A. obliquus* and 48.67% in *C. sorokiniana* respectively (Table 4.9a and 4.9b, Chapter 4). Both Fe and Mg thus found to positively influence the lipid biosynthesis under limited N and P conditions in *A. obliquus* and *C. sorokiniana*.

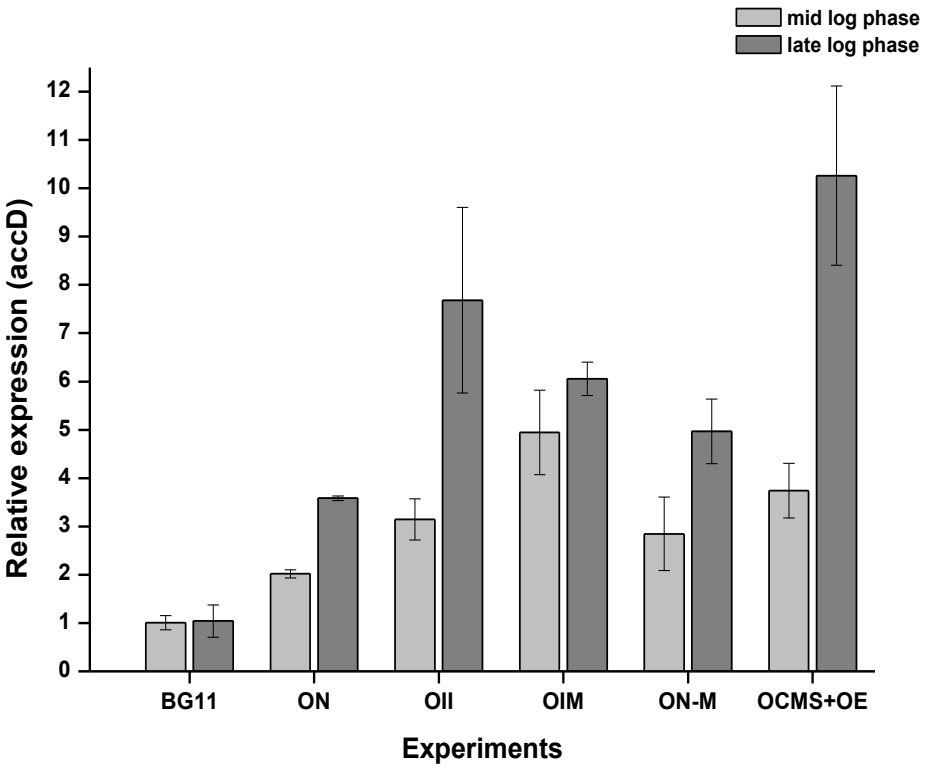


Figure 5.6 Effect of various experimental conditions on the relative expression of *accD* gene at mid log phase and at late log phase in *A. obliquus*

BG11- standard BG11 medium, ON-optimum N concentration, OII- optimum individual iron concentration OIM-Optimum individual magnesium concentration, ON-M- optimum N concentration without selected metals OCMS –optimum combined metal stress, OCMS+OE - optimum combined metal stress + optimum EDTA concentration

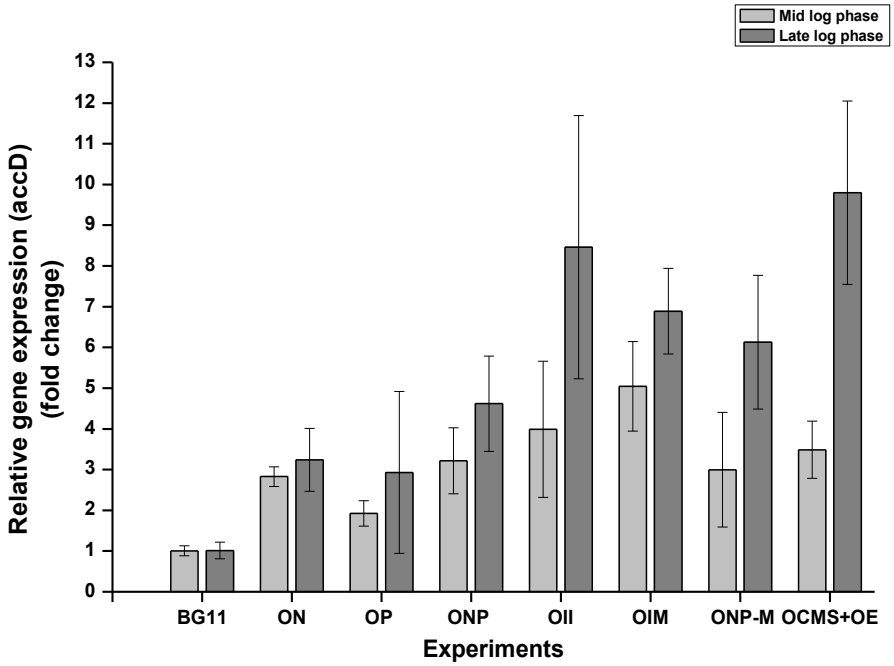


Figure 5.7: Effect of various experimental conditions on the relative expression of *accD* gene at mid log phase and at late log phase in *C. sorokiniana*

BG11- standard BG11 medium, ON-optimum N concentration, OP-optimum P concentration, ONP-optimum N and P concentration, OI- optimum individual iron concentration OMG-Optimum individual magnesium concentration, ONP-M- optimum N concentration and optimum P without selected metals, OCMS+ OE - optimum combined metal stress + optimum EDTA concentration

5.3.4 Transcriptome analysis

Microalga, *A. obliquus* had shown highest lipid productivity under optimized conditions and thus were used for further transcriptome analysis to understand the regulation of different genes involved in lipid biosynthesis. *A. obliquus* was grown under different stress conditions and the expression pattern was compared to the control BG11 sample. Among all the stress conditions, maximum variation in the transcriptome level gene expression was observed under OCMS+OE condition. Table 5.2 and Table 5.3 depict the variation in the transcriptome level gene expression under optimized combined stress conditions as compared to the control BG11 medium. Transcriptome analysis results have shown positive influence of combined stress strategy on the genes related to key cellular components and photosynthetic pathway.

Genes related to the ribosomal proteins have been upregulated under OCMS+OE condition. The expression level of 30S ribosomal proteins S7 was increased by 3 folds, S3 by 3-6 folds, S11 by 4 folds and S14 by 4 fold under OCMS+OE condition (Table 5.2). These proteins are involved in the binding and assembly of 16S rRNA to the 30S ribosomal unit (Carter et al., 2000). Expression of genes related to the cellular metabolism such 50S ribosomal protein L20 and L12 was also found to be upregulated under OCMS+OE condition as compared to the control. 50S ribosomal protein L20 are involved in assembly of 50S ribosomal RNA and it also binds with 23S rRNA (Wower et al., 1998). The 50S ribosomal protein L12 is essential for correct translation of genes in to protein (Ogle and Ramakrishnan, 2005, Wahl et al., 2000). Ribosomes are considered as cellular translational machinery, as they are responsible for the protein synthesis from mRNA (Zhou et al., 2015). Translation of the protein from mRNA is essential for, cell division, cell growth and development of other

cellular components of the microalgal cells (Zhou et al., 2015). Therefore, the above results indicate the increased transcription and translation process under the optimized conditions which in turn resulted in increased production of specific enzymes required for the cellular and metabolic activity. In total, expression of 12 genes involved in the process of transcription and translation was found to be increased under OCMS+OE condition as compared to control BG11 medium (Table 5.2). These transcriptome analysis results have validated the biomass productivity result from chapter 4, where a high biomass productivity of 149 $mgL^{-1}d^{-1}$ was observed under OCMS+OE condition despite of N limitation as compare to the biomass productivity of 141 $mgL^{-1}d^{-1}$ under BG11 medium (Table 4.9a; Chapter 4).

Table 5.2: Effect of combined metal and EDTA stress condition on the expression of genes related to key cellular components

Genes	Function	Fold increase
30S ribosomal protein S7	Primary rRNA binding proteins, it binds directly to 16S rRNA	3
30S ribosomal protein S3	Part of the 30S ribosomal subunit	6
30S ribosomal protein S3	Part of the 30S ribosomal subunit	5
30S ribosomal protein S3	Part of the 30S ribosomal subunit	3
30S ribosomal protein S14	Binds 16S rRNA, required for the assembly of 30S	4
30S ribosomal protein S11	Binds 16S rRNA, required for the assembly of 30S	4
DNA-directed RNA polymerase subunit beta	Largest and catalytic component of RNA polymerase IV which mediates 24-nt short-interfering RNAs (siRNA) accumulation.	3
DNA-directed RNA polymerase subunit beta N-terminal	DNA-dependent RNA polymerase catalyses the transcription of DNA into RNA using the four ribonucleotide triphosphates as substrates.	3
DNA-directed RNA polymerase subunit alpha	Largest and catalytic component of RNA polymerase II which synthesizes mRNA precursors and many functional non-coding RNAs.	4
50S ribosomal protein L20, chloroplastic	Binds directly to 23S ribosomal RNA and is necessary for the in vitro assembly process of the 50S ribosomal subunit	1
50S ribosomal protein L12, chloroplastic	Forms part of the ribosomal stalk which helps the ribosome interacts with GTP-bound translation factors. Is thus essential for accurate translation	10
Uncharacterized membrane protein ycf78	Essential for cell growth. May be involved in binding chloroplast DNA to either the chloroplast envelope or the thylakoid membrane.	6

Transcriptome analysis results have also shown an upregulation in the expression of genes involved in the photosynthesis under these conditions (Table 5.3). A 9 fold increase in the photosystem II protein D1 and 3 fold increases in the photosystem II protein D2 were observed in the combined stress condition (OCMS+OE) (Table 5.3). Photosystem II protein D1 provides electron to PSII and other electron accepters in microalgae and D2 is essential for stability of PSII. Upregulation of Photosystem II protein D1 and D2 genes could be as a result of supplementation of high Fe and Mg concentrations to the medium under N and P limited conditions. Similarly, a 2 fold increase in the expression of Cytochrome f gene was observed under optimized conditions. Cytochrome f gene is essential for the electron transfer between PSI to PSII during photosynthesis and for transfer of protons to the thylakoid to balance energy gradient by FE-S which provides energy in form of ATP for all enzymatic activities in photosynthesis (Hope, 1993, Wicke et al., 2011). High Fe concentrations in the combined stress condition probably contributed in the increase in the gene expression of cytochrome f due to the increased photosynthesis rate under this condition. This confirms the results of photosynthetic performance of the *A. obliquus* under OCMS+OE conditions, where high rETR and high Fv/Fm was observed under combined stress conditions (Figure 4.9a and Table 4.9 a). Lefebvre-Legendre et al. (2015) studied the effect of Fe availability in the expression of TAA1 gene, which is responsible for translation of psA subunit of PSI. They observed a decrease in the expression of TAA1 gene under Fe limited condition. In their study, the expression of TAA1 gene was increased with the re-supplementation of Fe. Similarly, Shin et al. (2015), studied the effect of N deprivation on the growth of *Dunaliella tertiolecta* by integrating transcriptome analysis. They observed reduction in the expression

of photosynthetic genes as well as genes related to photorespiration MTHFR, GGH and metE. However, in the present study genes related to photosynthesis were upregulated under combined stress conditions (OCMS+OE) even though the N was limited. Transcriptome analysis of non-model organisms is still difficult due to lack of full genome sequences. In this study, the expression analysis of *A. obliquus* was based on the other sequenced microalgal strains belonging to Chlorophyta division.

Table 5.3: Effect of combined metal and EDTA stress condition on the expression of gene related to photosynthetic pathway

Genes	**Function**	**Fold increase (OCMS+OE)**
Photosystem II D2 protein	The D1/D2 (PsbA/PsbA) reaction center heterodimer binds P680, the primary electron donor of PSII as well as several subsequent electron acceptors. D2 is needed for assembly of a stable PSII complex	3
Photosystem II protein D1		9
Cytochrome f	Component of the cytochrome b6-f complex, which mediates electron transfer between photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions	2
Light-dependent protochlorophyllide reductase subunit N	The light-independent (dark) form of protochlorophyllide reductase plays a key role in the ability of algae, and to form chlorophyll in the dark	3
Light-independent protochlorophyllide reductase subunit N	Component of the dark-operative protochlorophyllide reductase (DPOR) that uses Mg-ATP and reduced ferredoxin to reduce ring D of protochlorophyllide (Pchlide) to form chlorophyllide a (Chlide) (ChlN-ChlB) is the catalytic component of the complete This reaction is light-independent.	5
Chloroplast envelope membrane protein	GTPase involved in protein precursor import into chloroplasts. Seems to recognize chloroplast-destined precursor proteins and regulate their presentation to the translocation channel through GTP hydrolysis. Binds GTP, GDP, XTP, but not ATP.	1

5.4 Conclusion

Gene expression of *rbcL* and *accD* gene clearly demonstrated the roles of Fe and Mg in enhancing growth as well as lipid accumulation in *A. obliquus* and *C. sorokiniana* under N and P limited conditions. Both the genes (*rbcL* and *accD*) showed higher expression in optimized Fe experiment in late log phase which shows significant role of Fe in improving both growth physiology as well as lipid biosynthesis. An upregulation in expression of *accD* under optimized magnesium experiment was observed in mid as well as late log phases, which clearly indicated the impact of Mg on lipid biosynthesis. Transcriptome analysis has also confirmed the effect of combination of metals on genes related to key cellular components and photosynthesis. Results of gene expression and transcriptome analysis correlated well with photosynthetic performance and lipid content results.

CHAPTER SIX

BIOCHEMICAL COMPOSITION ANALYSIS AND EVALUATION OF SCALABILITY OF DEVELOPED STRATEGY

6.1 Introduction

Microalgae are considered to be one of the most promising feedstocks for many industrial applications due to its unique lipid composition, pigments, high protein content, carbohydrate content, and bio-active molecules (Benemann, 2013, Mata et al., 2010). Lipids are mainly used for biodiesel production, while protein component makes algae a promising feedstock for food, feed and health industry. The carbohydrates can be utilized for bioethanol and other chemicals (Singh et al., 2015). Microalgae cultivation conditions, not only affects the growth of the microalgae, but it also influences the biochemical composition of the microalgal biomass. In chapter 4 lipid productivity of the selected microalgal strains have been enhanced by optimization of nutrient and metal concentrations. Thus, it is necessary to assess the effect of developed strategy on biomass composition for other microalgal applications in the biorefinery approach.

Biodiesel fuel properties are determined by the fatty acid composition of feedstock lipids. The chain length of the fatty acid, percentage composition of saturated and unsaturated fatty acids present in the feedstock lipids influences the fuel properties of the biodiesel (Guldhe et al., 2015). Previous studies have shown the suitability of fatty acid composition of

microalgal lipid for biodiesel production (Abomohra et al., 2016, Abd El Baky et al., 2012). The presence of highly unsaturated fatty acid could render biodiesel properties off the specification. The European standard for biodiesel EN 14214 specifies 12% as a maximum limit for linolenic acid methyl esters (Nascimento et al., 2012). Polyunsaturated fatty acids are highly susceptible for oxidative cleavage due to a number of factors such as temperature, irradiation and nutrient stress (Guldhe et al., 2016).

Moreover, the success of lipid enhancement strategies depends upon the ease of its applicability and scalability. Several lipid enhancement strategies are developed by researchers, however very few have been tested in large scale cultivation systems. The open cultivation system is considered good for microalgal biomass generation for low value commodity products such as biodiesel. At the open cultivation systems environmental factors such as such as temperature, photoperiod, seasonal variation and CO_2 supply play crucial role and affect the microalgal growth and productivities. However, it is difficult to control these environmental conditions at open cultivation systems and altering these conditions incurs extra production cost. The variations in environmental factors of open systems could hamper the efficiency of the developed lipid enhancement strategy.

Thus, it is important to access the developed strategy at outdoor scaled up microalgal cultivation system. This chapter describes the effect of the individual and combinations of selected macronutrients (N, P), selected metals (Fe, Mg, Ca) and EDTA on the biochemical composition of selected microalgal strains (*A. obliquus* and *C. sorokiniana*). Biodiesel conversion and effect of nutrients (N, P) and metals on fatty acid profiles of *A. obliquus* and *C. sorokiniana* have been investigated. Furthermore, assessment of the developed lipid enhancement strategy for large scale open cultivation systems has also performed with *A.*

obliquus owing to its high lipid productivity to confirm the applicability of the developed strategy. Reliability of developed lipid enhancement strategy for *A. obliquus* was also studied by applying it to other microalgal isolates from screening studies of Chapter 3.

6.2 Materials and methods

6.2.1 Microalgal lab scale cultivation

Microalgae, *A. obliquus* were grown under ON, OII, OIM, ON-M and OCMS+OE experimental condition and *C. sorokiniana* were grown under ON , OP, ONP, OII, OIM ,ONP-M and OCMS+OE experimental condition as discussed elsewhere. For assessing the reliability of the lipid enhancement strategy developed in Chapter 4 for *A. obliquus*, all 7 microalgal isolates from screening study of Chapter 3 were cultivated with optimized combined metal stress and EDTA concentration under N limited conditions. Standard BG11 medium was used as the control. Cultivation parameters have been maintained as described in chapter 3, section 3.2.1. Biomass of the microalgal strain has been determined as described in chapter 3, section 3.2.2. Biomass was harvested at late log phase by centrifugation and freeze dried biomass is used for biochemical composition analysis.

6.2.2 Large scale cultivation in open circular pond (3000 Litre)

To determine the scalability of developed strategy, outdoor large scale trail was conducted. *A. obliquus* was cultivated under optimized as well as under BG11 medium (control) in an open circular pond (3000 L). The water temperature was 20-24 ºC and light intensity was 90-120 µmol m^{-2} s^{-1} during cultivation**.** Mixing and aeration of the microalgal

culture was accomplished by submersible pumps having a flow rate of 110 L min^{-1}. Samples were collected at initial, mid and late log phase for biomass, chlorophyll and photosynthetic efficiency analysis. Biomass was harvested at late log phase by centrifugation and freeze dried biomass was used for lipid quantification.

6.2.3 Biochemical composition analysis

6.2.3.1 Lipids and fatty acid analysis

Extraction of lipid from microalgal biomass was done as described in chapter 3 section 3.2.3. The extracted lipids were subjected to the simultaneous esterification and transesterification processes employing sulphuric acid as a catalyst and methanol as an acyl acceptor. The reaction conditions were 30:1 methanol to oil molar ratio; temperature: 60 °C; catalyst concentration: 10% w/w of oil; time: 4 h; hexane: 1ml (Guldhe et al., 2014). The stirring rate was kept constant at 200 rpm in orbital shaker incubator (Model TU-454, mrc Ltd., Israel). The fatty acid methyl esters (FAME) were then analysed by gas chromatography (Shimadzu GC-2014, Japan) with a flame ionization detector and a capillary column (SP2380, Supelco Analytical, and USA). The oven temperature was programmed to start at 60 °C and kept at hold for 2 min, thereafter increased the temperature to 160 °C at a ramp rate of 10 °C.min^{-1} and then to 240 °C at a ramp rate of 7 °C.min^{-1} and again kept at hold for 1 min at 240 °C. The injector and detector temperature was 250 °C and nitrogen was used as carrier gas (Guldhe et al., 2014). A 37 component FAME standard was used to identify peaks and methyl heptadeconoate (C17:0) was used as internal standard to calculate percentage FAME conversion.

6.2.3.2 Determination of total carbohydrate

Microalgal biomass was hydrolysed by mixing 100 mg of biomass in 10 mL of 2% H_2SO_4 and autoclaved at 121 °C for 20 min. After hydrolysis the sample was diluted up to 100 mL with distilled water and centrifuged at 2683 *g* for 10 min. Supernatant was used to determine sugar content by using Anthrone method and glucose was used as the standard (Pancha et al., 2014). Anthrone reagent was prepared by dissolving 100 mg of Anthrone (9, 10-dihydro-9- oxoanthacene) in 50 mL of ice cold 95% sulphuric acid (H_2SO_4). 4 mL of Anthrone reagent was added against 1 mL of glucose standard solutions and sample. The solution was kept in a boiling water bath for 10 min. Green colour of the resultant solution was observed by taking optical density at 630 nm (Guldhe et al., 2016). Carbohydrate content was determined by the calibration curve prepared using glucose standards.

6.2.3.3 Determination of protein content

Protein content was determined by Bradford method (Msanne et al., 2012). A 100 µL of 1 M NaOH was added to 10 mg of dried microalgae powder and incubated in water bath at 80 °C for 10 min. 900 µL of H_2O was added to the hydrolysed sample to bring volume up to 1 mL then the mixture was centrifuged at 12,000 *g* for 10 min. The supernatant was used to determine the protein concentration by the Bradford method. Different concentrations of bovine serum albumin were used to prepare the calibration curve (Guldhe et al., 2016).

6.2.4 Statistical analysis

Data analysis was done using the Minitab Statistical Software. Significance of results and differences among various treatments were evaluated for duplicate set of data by using

one-way analysis of variance (ANOVA). Posthoc Turkey's test (p = 0.05) was used for comparisons among the different means. Data was represented as Mean ± SD (Standard deviation) in tables, while in graphs; SD values were represented as error bars.

6.3 Results and discussion

6.3.1 Effect of nutrient and metal stress on the biochemical composition of *A. obliquus* and *C. sorokiniana*

Lipids, carbohydrates and proteins are the main constituents of microalgal biomass. Alterations in nutrient medium can affect the biochemical composition of microalgae (Griffiths et al., 2014, Juneja et al., 2013). Microalgae are reported to accumulate energy dense molecules such as lipids and carbohydrates under different stress conditions (Huang et al., 2014, Adams et al., 2013). In this study, the effect of nutrients and selected metals individually and as well as in combination on the biochemical composition of both *A. obliquus and C. sorokiniana* were investigated (Table 6.1 and 6.2).

Although microalgae are promising feedstocks for biodiesel production, cultivation of microalgae only for biodiesel production is a cost intensive process. Thus, an integrated biorefinery approach is required to improve the economics of microalgal biodiesel production where various other products can also be produced. Thus, in this study, the effect of various stress conditions on carbohydrate and protein content and productivities have also been studied. Similar to lipid content, an enhancement in the carbohydrate content of *A. obliquus* and *C. sorokiniana* was observed, when the microalgae was grown under different stress conditions (Table 6.1 and 6.2). Lipids and carbohydrates are the preferred storage products

under various stress conditions because, they are hydrophobic in nature, have highly reduced states, are efficiently packed in small compartment of cells and can also be used during adverse conditions for cell survival and proliferation (Pancha et al., 2014). Highest carbohydrate content was obtained under the ON-M (26.34 %) and ONP-M (26.7%) condition for *A. obliquus* and *C. sorokiniana* respectively (Table 6.1 and 6.2). In microalgae, during nutrient limitation conditions, photosynthetic carbon flow changes into different ways to channel metabolic energy into various energy rich compounds like carbohydrates and lipids (Shin et al., 2015). Highest carbohydrate productivities of 35.6 $mgL^{-1}d^{-1}$ and 33.84 $mgL^{-1}d^{-1}$ were observed under OCMS+OE condition for *A. obliquus* and *C. sorokiniana* respectively which were significantly higher than all other conditions. The high productivity observed under combined stress condition was due to the high carbohydrate content along with high biomass productivity under this condition.

In contrast to lipid and carbohydrate, protein content was decreased under both N and P limitation (Table 6.1 and 6.2). Nitrogen limitation resulted in a decrease in protein content from 45.7% to 18.61% in *A. obliquus.* Protein content of *C. sorokiniana* was also decreased under N and P limitation with an overall reduction of up to 42% under ONP condition as compared to BG11 medium. In this study, lowest protein content of 13.045% was observed under ON-M for *A. obliquus* and 11.4% under for *C. sorokiniana* under ONP-M condition for *C. sorokiniana* (Table 6.1 and 6.2). Pancha et al. (2014), studied the effects of N stress on the biochemical composition of *Scenedesmus* sp. and observed a 60% decrease in protein content under N deficient condition. A probable elucidation towards decrease in protein content under nutrient limited cultivation condition is that the during stress microalgal cells might

have degraded the nitrogenous compounds to maintain intracellular N quota for their normal metabolic function (Griffiths et al., 2014, Pancha et al., 2014).

However, under OCMS+OE condition the protein content was increased as compare to the N and P limitation in both the strains. For *A. obliquus*, 21.75% of protein content was observed under OCMS+OE condition, which was higher than all the protein content of all individual stress conditions. Similarly, for *C. sorokiniana* 23.26% of the protein content was observed under OCMS+OE (Table 6.1 and 6.2). Increase in protein content also indicates the improvement in the growth physiology under combined nutrient and metal stress condition (OCMS+OE) (Table 6.1 and 6.2). In *A. obliquus*, the highest protein productivity was observed with the BG11 medium (Table 6.1). The second highest protein productivity was observed under OCMS+OE condition which was significantly higher as compared to the other stress conditions. A 1.59 fold increase in the protein productivity of *A. obliquus* was observed under OCMS+OE conditions as compared to ON condition. Similarly, protein productivity of *C. sorokiniana* under OCMS+OE condition was significantly higher than other stress conditions (Table 6.2). A 1.8 fold increase in the protein productivity of *C. sorokiniana* was observed under OCMS+OE condition as compared to the ONP condition. A slight increase in the protein productivity was observed under individual Fe and Mg optimized conditions in both strains. For *A. obliquus*, lowest protein productivity of 2.43 $mgL^{-1}d^{-1}$ was observed under ON-M condition, this is due to low protein content and low biomass productivity at this condition. Similarly, for *C. sorokiniana* the lowest protein productivity of 2.23 $mgL^{-1}d^{-1}$ was observed under ONP-M condition.

The developed strategy not only resulted in high lipid productivity but it also showed substantial carbohydrate and protein productivities. Thus, this strategy can be useful for the

biorefinery approach of microalgal biodiesel production, where lipid extracted biomass (LEA) could be used for other applications such as feed and other biofuels.

Table 6.1: Biochemical composition of *A. obliquus* under various stress conditions

Experiment	Lipid content (%)	Lipid Productivity ($mgL^{-1}d^{-1}$)	Protein Content (%)	Protein productivity ($mgL^{-1}d^{-1}$)	Carbohydrate content (%)	Carbohydrate productivity ($mgL^{-1}d^{-1}$)
BG11	25.95±0.04	36.6±0.02	45.7±0.62	64.47±1.33	16.05±1.83	22.642±2.42
ON	38.89±0.81	42.5±0.79	18.61±0.86	20.34±1.03	22.83±0.42	24.95±0.57
OII	41.25±0.03	49.48±0.02	20.5±0.34	24.59±0.42	23.32±0.43	27.97±0.53
OIM	36.98±0.80	47.73±1.2	20.62±0.29	26.61±0.10	22.98±0.14	29.66±0.03
ON-M	42.25±0.56	7.88±0.03	13.05±1.18	2.43±0.25	26.34±1.70	4.91±0.38
OCMS+OE	54.04±0.15	80.23±0.49	21.75±0.07	32.41±0.12	23.9±0.25	35.6±0.35

BG11- standard BG11 medium, ON-optimum N concentration, OII- optimum individual iron concentration OIM-Optimum individual magnesium concentration, ON-M- optimum N concentration without selected metals, OCMS –optimum combined metal stress, OCMS+OE - optimum combined metal stress + optimum EDTA concentration

Table 6.2: Biochemical composition of *C. sorokiniana* under various stress conditions

Experiment	Lipid content (%)	Lipid productivity ($mgL^{-1}d^{-1}$)	Protein Content (%)	Protein productivity ($mgL^{-1}d^{-1}$)	Carbohydrate content (%)	Carbohydrate productivity ($mgL^{-1}d^{-1}$)
BG11	18.5±0.14	30.15±0.8	35.56±0.98	55.07±1.15	12.89±0.64	19.96±1.94
ON	31.75±1.06	32.75±0.3	18.23±0.18	19.14±0.1	20.42±0.34	21.44±0.67
OP	28.4±0.57	30.81±0.04	20.98±0.45	24.93±0,89	16.44±0.97	17.84±1.4
ONP	33±0.71	33.44±0.64	20.56±0.58	20.41±1.09	22.88±0.62	22.72±1.32
OII	37.63±0.32	43.69±0.18	19.12±0.62	23.07±0.83	21.22±0.95	24.63±0.95
OIM	36.05±0.12	37.78±0.76	20.45±0.49	22.28±0.01	18.34±0.59	19.98±1.1
ONP-M	41.75±1.0	8.50±0.42	11.4±1.19	2.32±0.06	26.7±0.98	5.44±0.6
OCMS+OE	48.67±0.46	77.03±0.98	23.26±0.09	36.82±0.03	21.38±0.48	33.84±0.88

BG11- standard BG11 medium, ON-optimum N concentration, OII- optimum individual iron concentration OIM-Optimum individual magnesium concentration, ONP-M- optimum N concentration and optimum P without selected metals, OCMS –optimum combined metal stress, OCMS+OE - optimum combined metal stress + optimum EDTA concentration

6.3.2 Fatty acid analysis and biodiesel conversion

The fatty acid compositions of *A. obliquus* and *C. sorokiniana* grown using normal BG11 medium and under optimum combined metal stress with optimized EDTA (OCMS+OE) concentration are depicted in figure 6.1 and 6.2. The main constituents of the fatty acid composition of *A. obliquus* and *C. sorokiniana* in both the cases were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3). However, there was sizeable difference in the percentage composition of individual fatty acids; saturated and unsaturated fatty acids. The percentage composition of saturated fatty acids such as palmitic acid and stearic acid has increased considerably in both strains under combined stress condition compared to standard BG11 condition (Figure 6.1 and 6.2). However, the percentage composition of the unsaturated fatty acids such as oleic acid, linoleic acid and linolenic acid were decreased under the stress conditions (Figure 6.1 and 6.2). Previous reports have shown that unsaturated fatty acids undergo oxidative damage as they are susceptible to changes in cultivation condition (light, temperature, CO_2) and nutrient stress (L. Tao et al., 2013).

In *A. obliquus*, total percentage composition of saturated fatty acid under BG11 was 48.09±0.18% which was increased to 61.01±1.72% under optimized metal stress condition (OCMS+OE) whereas; the total percentage composition of unsaturated fatty acid was decreased from 50.87±0.19% in BG11 to 37.59±1.92% under OCMS+OE conditions. Similar fatty acid composition was *observed* for *C. sorokiniana*, where saturated fatty acid under BG11 was 48.48±1.6%, while it was found to increase up to 60.57±0.58% under OCMS+OE condition. The unsaturated fatty acid composition in *C. sorokiniana*, under BG11 condition

was 51.52±1.6%, while it was 39.43±0.56% under OCMS+OE condition. Fuel properties of biodiesel depend on lipid characteristics of microalgae. The composition of fatty acids, chain length, degree of unsaturation etc., dictates the fuel properties such as cetane number, viscosity, cold flow properties, calorific value and oxidative stability (Singh et al., 2014, Guldhe et al., 2015). High percentages of saturated fatty acids are considered to be apt for the oxidation stability and high cetane number, while polyunsaturated fatty acids are considered suitable for cold flow properties. Even though polyunsaturated fatty acids are responsible for good cold flow properties of biodiesel, higher percentage of polyunsaturated fatty acids may hamper the oxidative stability which leads to rancidity of biodiesel (Singh et al., 2014). Thus, European standards (EN14214) have set the specified limit of 12% for linolenic acid methyl ester (C18:3) in biodiesel (Singh et al., 2014). In this study, linolenic acid methyl ester content of *A. obliquus* (7.86±0.08%) and *C. sorokiniana* (7.14±0.3%) under combined stress condition were well within the specified limits. The linolenic acid percentage composition of *A. obliquus* under BG11 was 11.27±0.35% which was within the specified limits and further reduced to 7.86±0.08% under optimized stress condition (OCMS+OE) giving better oxidative stability to the biodiesel. In *C. sorokiniana* the linolenic acid percentage composition under BG11 was 16.3±0.93% which was above the specified limits of 12% according to EN14214. Under optimized stress condition (OCMS+OE) in *C. sorokiniana* the linolenic acid percentage composition reduced to 7.14±0.3% giving better oxidative stability to the biodiesel. The high FAME conversion of 94.76±0.53% for *A. obliquus* and 93.89±0.47% for *C. sorokiniana* was observed in this study using acid catalyst, which was slightly below the EN14214 specified limit of 96.5% (Singh et al., 2014), and thus require further investigation to optimize conversion strategies.

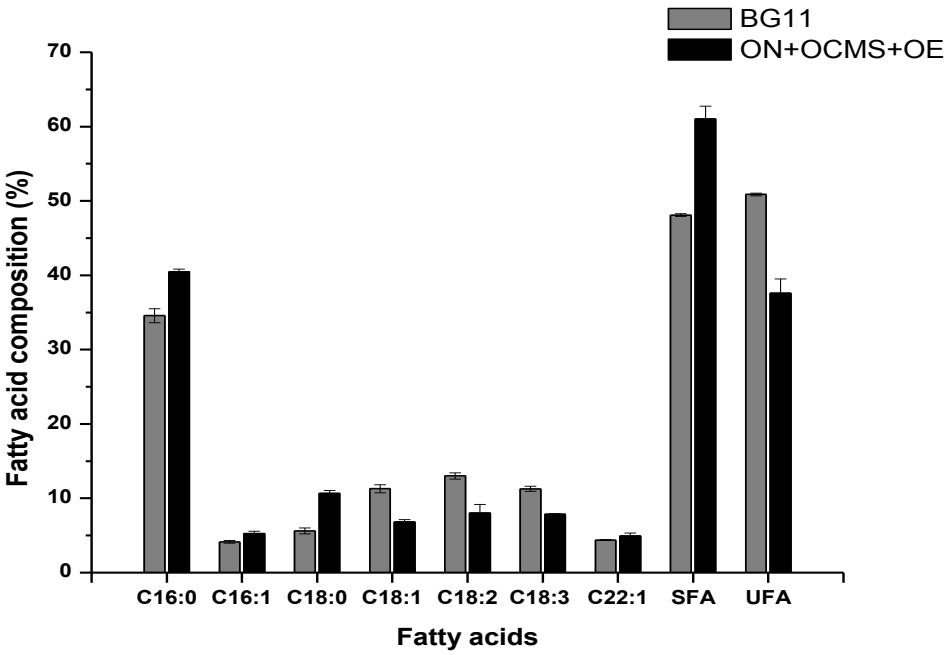


Figure 6.1: Fatty acid profile of *A. obliquus* grown under combined stress condition and normal BG11 medium

BG11– Standard BG11 medium, ON + OCMS + OE – Optimum N + optimum combined metal stress + optimum EDTA concentration

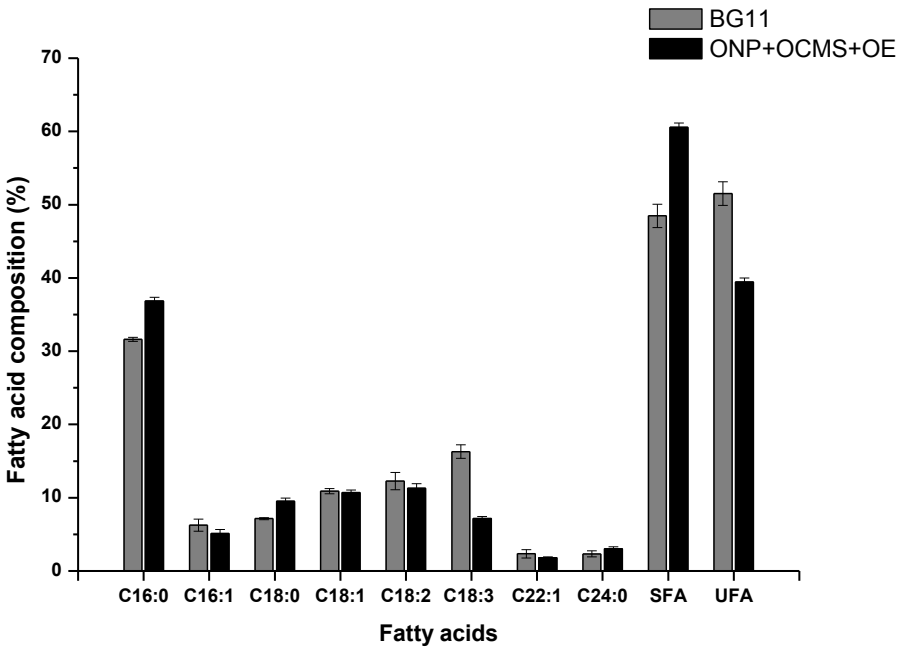


Figure 6.2: Fatty acid profile of *C. sorokiniana* grown under combined stress condition and normal BG11 medium.

BG11– Standard BG11 medium, ONP +OCMS + OE – Optimum N and P + optimum combined metal stress + optimum EDTA concentration

6.3.3 Assessment of developed strategy from lab to open circular pond (3000 L)

Biodiesel production from microalgae involves several crucial steps starting from isolation of microalgae from the environment, screening and selection of potential microalgal strains, development of a lipid enhancement strategy and application of the developed strategy to large scale. In this study, *A. obliquus* was grown in open circular ponds (3000 L) under OCMS+OE, condition, and BG11 as control. The selection of *A. obliquus* was not only based on its highest lipid productivity among all selected strains but also because of its known robustness such as high tolerance under variability in pH, potential to flourish in

wastewater and in open ponds, and its resistance against bio contaminants (Kim et al., 2016, Gruber-Brunhumer et al., 2015). Lipid productivity observed under OCMS+OE condition was 20.20 mgL^1d^{-1} while it was 9.69 $mgL^{-1}d^{-1}$ in the control pond. The lipid productivity was increased by 2.08 folds in open pond by using the developed strategy in this study. The biomass and photosynthetic efficiency results were also shown similar trends as the lab scale results (Table 6.3). However at large scale, lipid and biomass productivities were lower as compared to lab scale study in both the conditions (OCMS+OE and BG11). This is probably because of the temperature and light variations at open cultivation system which could have directly influenced the biomass and lipid productivities in comparison to lab-scale controlled conditions.

Table 6.3: Physiological parameters of *A. obliquus* grown under developed strategy at large scale cultivation (open circular pond 3000L)

Experiment open circular pond (3000L)	**Fv/F_m Mid log phase**	**Fv/Fm late log phase**	**Chlorophyll content mid log phase (mgL^{-1})**	**Chlorophyll content late log phase (mgL^{-1})**	**Biomass productivity ($mgL^{-1}d^{-1}$)**	**Lipid content (%)**
BG11	0.654±0.005	0.661±0.04	11.32±1.2	25.43±0.32	75.17±0.71	12.9±0.42
OCMS+OE	0.665±0.2	0.688±0.01	10.89±0.54	35.3±0.66	105.75±0.59	19.5±0.7

BG11- standard BG11 medium, OCMS + OE - optimum combined metal stress + optimum EDTA concentration

6.3.4 Applicability of the developed strategy

Applicability of the lipid enhancement strategy of combined metals and EDTA stress (OCMS+OE) was confirmed by testing it with all the seven microalgal isolates taking normal

BG11 medium as a control. Among these, *A. obliquus*, *Chlorococcum* sp. KZN05 and *Chlorella* sp. KZN07 has shown approximately 2 fold increase in lipid productivity, while *C. sorokiniana* and *Gloeocystis* sp. KZN03 showed approximately 1.75 fold increase in lipid productivity. The other two isolates *viz*. *Nannochloropsis* sp. KZN02 and *Chlorella* sp. KZN06 showed around 1.5 fold increase (Figure 6.3). The developed strategy has shown variable influence on the lipid productivity of different microalgal strains. The developed strategy has shown increase in lipid productivity of all the strains and thus can be applied for cultivation of other microalgal strain.

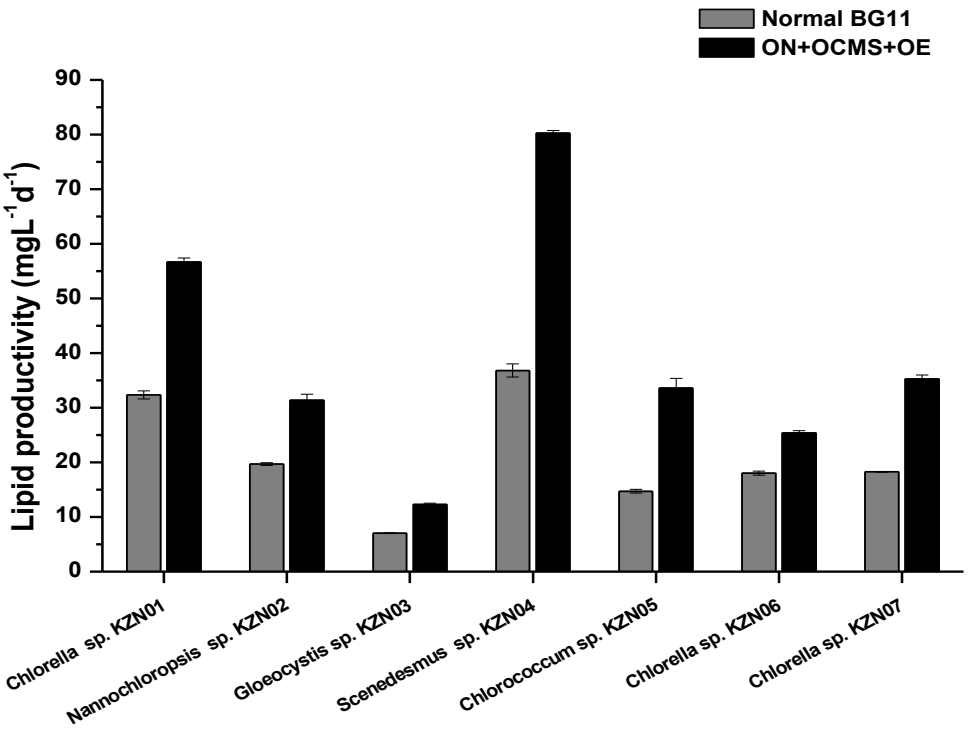


Figure 6.3: Applicability of developed strategy with other isolated microalgal strains

BG11– Standard BG11 medium, ON + OCMS + OE – Optimum N + optimum combined metal stress + optimum EDTA concentration

6.4 Conclusion

The developed lipid enhancement strategy not only improved the lipid accumulation but also enhances carbohydrate productivity with adequate protein yields, which improves the prospects of utilizing microalgal biomass for other applications. Fatty acid profile of both strains proved its suitability for biodiesel production. Easy applicability of this strategy at large scale open cultivation systems and efficiency in various microalgal strains makes it an effective tool for sustainable and economical microalgal biodiesel production process.

CHAPTER SEVEN

GENERAL CONCLUSIONS

In recent years, microalgae have emerged as a potential biodiesel feedstock due to its traits such as fast growth rate, high lipid accumulation, suitable fatty acid composition and adaptability towards a wide range of climatic and environmental conditions. Despite these advantages, the large scale microalgal biodiesel production is still far from fruition due to the unfavourable economics. One possible solution for improving the economics of microalgal cultivation is to select a suitable strain and investigate its response to different cultivation conditions for enhancement of the overall lipid productivity. Thus, the main focus of this study was to develop an easily applicable and scalable lipid enhancement strategy based on nutrient, metal and EDTA stress for indigenous microalgal strains by investigating growth physiology, photosynthetic performance, lipid accumulation and expression of ACCase and *rbcL* genes at molecular level.

Significant aspects of the research findings are summarized as follows:

Isolation, screening and selection of microalgae

A total of 7 green indigenous fresh water microalgal isolates were screened to select potential biodiesel feedstock. Results from this study showed that, the microalgal growth pattern, growth rate, cultivation period, biomass production and lipid content varies from species to species. Among all seven green microalgal strains, *A. obliquus* and *C. sorokiniana* have shown high lipid productivity of 36.82 and 32.34 $mgL^{-1}d^{-1}$ respectively. The highest

lipid content of 26.03% was observed *A. obliquus* whereas *C. sorokiniana* was identified as the highest biomass producing microalgae with biomass productivity of 165 $mgL^{-1}d^{-1}$. Thus *A. obliquus* and *C. sorokiniana* have selected for further lipid enhancement study involving different cultivation strategies. The selected strains were also characterized using phylogenetic analysis for identification and confirmation of the species at molecular level. The finding of screening study highlights the importance of this initial step for successful microalgal biodiesel production.

Development of a lipid enhancement strategy

The study resulted in developing an integrated lipid enhancement strategy by altering the trace metal composition in the BG11 medium. This includes supplementation of Fe, Mg and EDTA while depleting Ca concentration in the original BG11 medium. This approach allowed for alleviating the low biomass constraint of N and P limitation and improving the overall lipid productivity to a significant level in all the strains tested in this study. For *A. obliquus*, the highest lipid productivity of 80.23 $mgL^{-1}d^{-1}$ was achieved with developed strategy under limited N (750 mg L^{-1}) which was 2.18 fold higher than BG11 medium and 1.89 fold higher than the N limited condition alone. Similarly, for *C. sorokiniana*, highest lipid productivity of 77.03 $mgL^{-1}d^{-1}$ was achieved with the developed strategy under limited N (500 mgL^{-1}) and P (10 mgL^{-1}) which was 2.67 fold higher than BG11 medium and 2.35 fold higher than N and P limited condition alone. Photosynthetic performance and RSM results of both strains denote the role of Fe and Mg in improving both biomass yield and lipid accumulation. Iron was found to be the most influencing metal for enhancing lipid productivity followed by magnesium. The findings highlight the importance of metals and

EDTA, which can be used to alter the microalgal physiology based on cultivation strategy and target application.

Gene expression analysis

The gene expression studies based on the key genes involved in photosynthesis (*rbcL)* and lipid biosynthesis (*accD*) further demonstrated the role of Fe and Mg in enhancing growth as well as lipid accumulation in *A. obliquus* and *C. sorokiniana* under limited N and P condition. Both the genes (*rbcL* and *accD*) showed higher expression in optimized Fe experiment in late log phase which shows significant role of Fe in improving both growth physiology as well as lipid biosynthesis. An upregulation in expression of *accD* during the optimized Mg experiment was observed in mid as well as late log phases, which indicated the clear impact of Mg on lipid biosynthesis. Moreover, the transcriptome analysis based on next generation sequencing of whole transcripts of RNA extracted from optimized and control experiments has assisted in further confirmation of the increased gene expression of differential genes related to key cellular components and photosynthesis. Results from this study have assisted in elucidating the role of combined metal and EDTA stress on growth and lipid biosynthesis at the molecular level. The results from this study will form the basis for further studies targeting genetic modifications to develop hyper lipid producing microalgal strains.

Biochemical composition analysis

The developed lipid enhancement strategy not only improved the lipid accumulation in *A. obliquus* and *C. sorokiniana* but also enhanced the carbohydrate productivity with adequate protein yields, which improves the prospects of utilizing microalgal biomass for

other applications through a biorefinery approach. Fatty acid composition of the *A. obliquus* and *C. sorokiniana* showed suitable lipid profile for biodiesel production. Percentage compositions of linolenic acid methyl ester in *A. obliquus* (7.86±0.08%) and *C. sorokiniana* (7.14±0.3%) under combined stress condition were well within the specified limits of European standards (EN14214). The high FAME conversion of 94.76±0.53% for *A. obliquus* and 93.89±0.47% for *C. sorokiniana* was observed in this study using acid catalyst, which was slightly below the EN14214 specified limit of 96.5%.

Scalability and universal applicability of developed lipid enhancement strategy

The open pond trial at 3000L showed an increase of 2.08 fold in lipid productivity of *A. obliquus* which highlights easy scalability of this technique. The developed strategy showed substantial improvement in lipid productivity, when applied for other isolated microalgal strains. Easy applicability of this strategy to large scale open cultivation system and efficiency in various microalgal strains makes it an effective tool for sustainable and economical biodiesel production using indigenous microalgal strains.

RECOMMENDATIONS

Based on the findings from this work, the recommendations are as follows:

- Microalgae from the stressed environment could have higher potential of lipid accumulation. Extensive isolation and screening studies are needed to identify potential microalgal strains for biodiesel production.
- Cultivation of microalgae in wastewater is gaining interest due to economic and environmental benefits. Results from this study have proved the role of metals and EDTA on improving biomass yield and lipid accumulation. This strategy can also be implemented with low cost growth mediums such as wastewater which needs thorough lab scale investigation and large scale trials.
- Other metals apart from Fe, Mg and Ca investigated in this study could also have impact on lipid and other metabolite production in microalgae. Thus, other micronutrients need to be extensively studied for their role on microalgal physiology and metabolites production.
- Whole genome sequencing of the potential microalgal strains is recommended
- Many studies are focused on lab scale experiments. Developed cultivation strategies needs to be evaluated at large scale for commercial application.
- Life cycle analysis of biodiesel production using developed strategy is recommended at demonstration scale production facility for its commercial implementation.

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APPENDICES

Appendix one

BG11 nutrient medium (Chapter 3)

Component	Quantity
$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (disodium salt)	0.001 g
$NaCO_3$	0.02 g
Trace metal mix A5	1.0 ml
Agar (if needed)	10.0 g
Distilled water	1.0 L

Trace metal mix

Component	Quantity
H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$NaMoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg
Distilled water	1.0 L

Appendix two

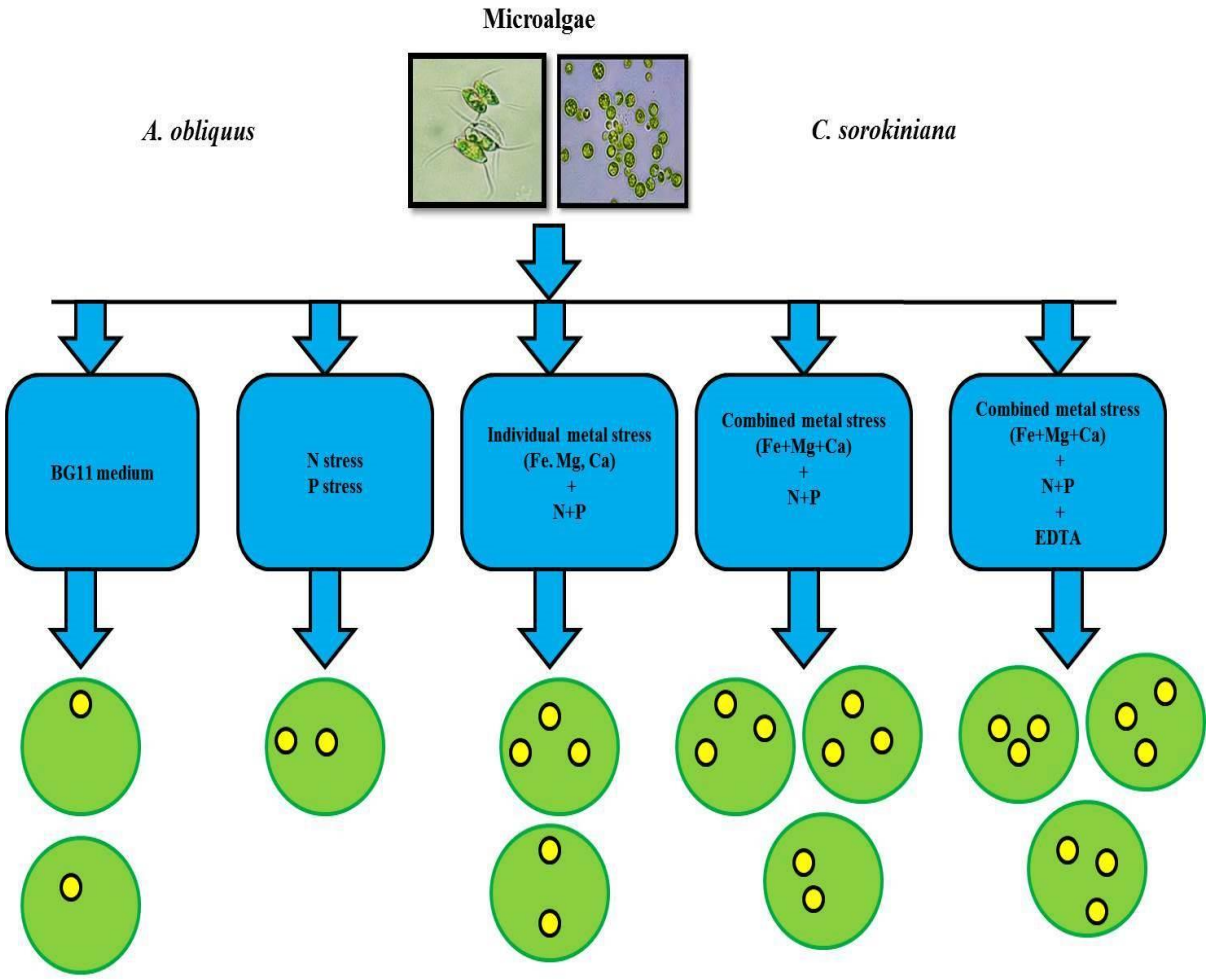
Molecular identification of *A. obliquus* and *C. sorokiniana* (Chapter 3)

Gel electrophoresis image of genomic DNA with high range ladder

Gel electrophoresis image of PCR product with DIR primer (750bp product size)

Appendix three

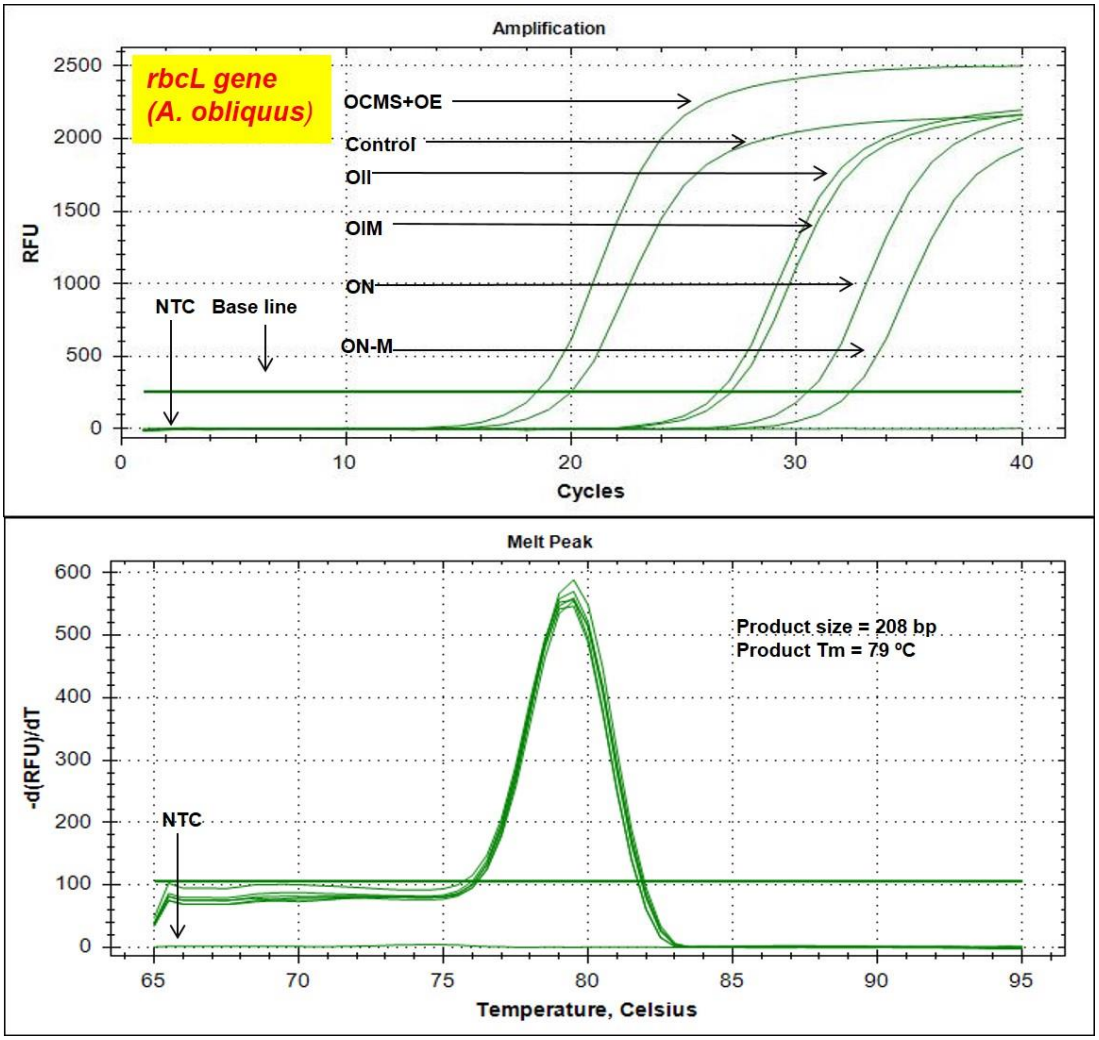
Graphical representaion of lipid enhancement strategy



Effect of different stress conditions on lipid accumulation and biomass productivity

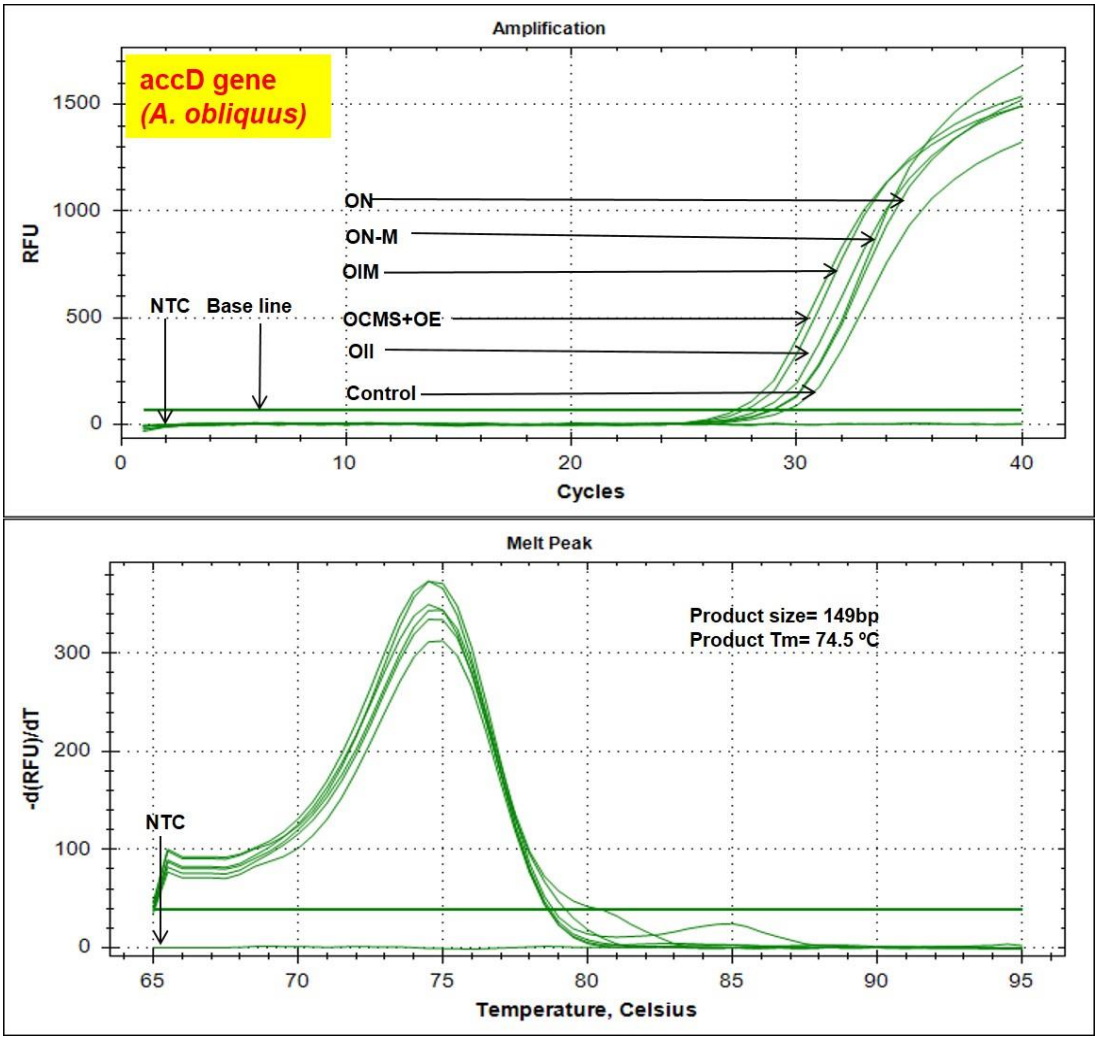
Appendix four

Effect of various stress condition on the amplification of *rbcL* gene



Appendix five

Effect of various conditions on the amplification of *accD* gene



Appendix six

Transcriptome analysis: variation in the gene expression of *A. obliquus* under different stress condition (Chapter 5)

Transcription ID	OCMS+OE	OII	OIM	ON	ON-M	Uniport ID	Protein
TRINITY_DN6520_c0_g1_i1	6	0	0	0	0	YCF78_ACUOB	Uncharacterized membrane protein ycf78
TRINITY_DN32851_c0_g1_i1	2	0	0	0	0	YCF78_ACUOB	Uncharacterized membrane protein ycf78
TRINITY_DN17721_c0_g1_i1	3	0	0	0	0	RR7_ACUOB	30S ribosomal protein S7, chloroplastic
TRINITY_DN6149_c0_g1_i1	6	0	0	0	0	RR3_ACUOB	30S ribosomal protein S3, chloroplastic
TRINITY_DN20620_c0_g1_i1	5	0	0	0	0	RR3_ACUOB	30S ribosomal protein S3, chloroplastic
TRINITY_DN6149_c0_g2_i1	3	0	0	0	0	RR3_ACUOB	30S ribosomal protein S3, chloroplastic
TRINITY_DN9850_c0_g1_i1	0	2	0	0	0	RR2B_ACUOB	30S ribosomal protein S2, chloroplastic 2
TRINITY_DN6983_c0_g1_i2	0	1	0	0	0	RR2B_ACUOB	30S ribosomal protein S2, chloroplastic 2
TRINITY_DN4944_c0_g2_i1	4	0	0	0	0	RR14_ACUOB	30S ribosomal protein S14, chloroplastic
TRINITY_DN28968_c0_g1_i1	3	0	0	0	0	RPOC2_ACUOB	DNA-directed RNA polymerase subunit beta'
TRINITY_DN840_c0_g1_i1	5	0	0	0	0	RPOC1_ACUOB	DNA-directed RNA polymerase subunit beta
TRINITY_DN2797_c0_g1_i1	2	0	0	0	0	RPOC1_ACUOB	DNA-directed RNA polymerase subunit beta
TRINITY_DN3739_c0_g2_i1	0	0	0	0	1	RPOB1_ACUOB	DNA-directed RNA polymerase subunit beta N-terminal section
TRINITY_DN7352_c0_g4_i1	3	0	0	0	0	RPOB1_ACUOB	DNA-directed RNA polymerase subunit beta N-terminal section
TRINITY_DN7352_c0_g5_i1	1	0	0	0	0	RPOB1_ACUOB	DNA-directed RNA polymerase subunit beta N-terminal section
TRINITY_DN5966_c0_g1_i1	4	0	0	0	0	RPOA_ACUOB	DNA-directed RNA polymerase subunit alpha
TRINITY_DN214_c0_g2_i1	1	0	0	0	0	RK20_ACUOB	50S ribosomal protein L20, chloroplastic
TRINITY_DN7254_c0_g1_i1	10	0	0	0	0	RK12_ACUOB	50S ribosomal protein L12, chloroplastic
TRINITY_DN6781_c0_g1_i1	0	0	0	1	0	RDPO_ACUOB	Probable reverse transcriptase
TRINITY_DN5833_c0_g1_i1	3	0	0	0	0	PSBD_ACUOB	Photosystem II D2 protein
TRINITY_DN4889_c0_g1_i1	9	0	0	0	0	PSBA_ACUOB	Photosystem II protein D1
TRINITY_DN863_c0_g1_i1	0	1	0	0	0	PSAA_ACUOB	Photosystem I P700 chlorophyll a apoprotein A1
TRINITY_DN5462_c0_g1_i1	2	0	0	0	0	CYF_ACUOB	Cytochrome f
TRINITY_DN2405_c0_g2_i1	0	1	0	0	0	CYB6_ACUOB	Cytochrome b6
TRINITY_DN6629_c0_g2_i1	3	2	0	0	0	CHLN_ACUOB	Light-independent protochlorophyllide reductase
TRINITY_DN23646_c0_g1_i1	5	0	0	0	0	CHLN_ACUOB	Light-independent protochlorophyllide reductase:
TRINITY_DN7313_c0_g4_i1	0	2	3	1	1	CEMA_ACUOB	Chloroplast envelope membrane protein
TRINITY_DN7429_c3_g2_i1	1	0	6	0	0	CEMA_ACUOB	Chloroplast envelope membrane protein

Appendix seven

Table 1: Grouping information using Tukey method for *A. obliquus* carbohydrate productivity under various conditions (chapter 6)

Conditions	Mean	Grouping				
OCMS+OE	35.603	A				
OIM	29.658		B			
OII	27.967		B	C		
ON	24.949			C	D	
BG11	22.625				D	
ON-M	4.911					E

Means that do not share a letter are significantly different. A is highest level and E is lowest level.
R-Sq = 99,39% R-Sq(adj) = 98,88%

Table 2: Grouping information using Tukey method for *A. obliquus* protein productivity under various conditions

Conditions	Mean	Grouping				
BG11	64.479	A				
OCMS+OE	32.41		B			
OIM	26.61			C		
OII	24.585			C		
ON	20.335				D	
ON-M	2.433					E

Means that do not share a letter are significantly different. A is highest level and E is lowest level.
R-Sq = 99,93% R-Sq(adj) = 99,86%

Table 3: Grouping Information using Tukey method for *C. sorokiniana* carbohydrate productivity under various conditions (chapter 6)

Conditions	Mean	Grouping				
OCMS+OE	34.650	A				
OII	24.361		B			
ONP	22.746		B	C		
BG11	21.495		B	C	D	
ON	21.180		B	C	D	
OIM	20.012			C	D	
OP	17.528				D	
ONP-M	5.322					E

Means that do not share a letter are significantly different. A is highest level and E is lowest level.
S = 1,005 R-Sq = 99,13% R-Sq(adj) = 98,37%

Table 4: Grouping information using Tukey method for *C. sorokiniana* protein productivity under various conditions

Conditions	Mean	Grouping					
BG11	55.113	A					
OCMS+OE	37.204		B				
OII	24.237			C			
OIM	22.624			C			
OP	22.083			C	D		
ON	19.305				D	E	
ONP	16.917					E	
ONP-M	2.377						F

Means that do not share a letter are significantly different. A is highest level and F is lowest level.
R-Sq = 99,88% R-Sq(adj) = 99,78%

Appendix eight

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Trends and novel strategies for enhancing lipid accumulation and quality in microalgae

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ABSTRACT

In order to realize the potential of microalgal biodiesel there is a need for substantial impetus involving interventions to radically improve lipid yields upstream. Nutrient stress and alteration to cultivation conditions are commonly used lipid enhancement strategies in microalgae. The main bottleneck of applying conventional strategies is their scalability as some of these strategies incur additional cost and energy. Novel lipid enhancement strategies have emerged to research forefront to overcome these challenges. In this review, the latest trends in microalgal lipid enhancement strategies, possible solutions and future directions are critically discussed. Advanced strategies such as combined nutrient and cultivation condition stress, microalgae–bacteria interactions, use of phytohormones EDTA and chemical additives, improving light conditions using LED, dyes and paints, and gene expression analysis are described. Molecular approaches such as metabolic and genetic engineering are emerging as the potential lipid enhancing strategies. Recent advancements in gene expression studies, genetic and metabolic engineering have shown promising results in enhancing lipid productivity in microalgae; however environmental risk and long term viability are still major challenges.

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Combined metals and EDTA control: An integrated and scalable lipid enhancement strategy to alleviate biomass constraints in microalgae under nitrogen limited conditions

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ABSTRACT

The commercial realization of microalgal biodiesel production necessitates substantial impulsion towards development of strategies to improve lipid yields upstream. Nitrogen stress is the most widely used lipid enhancement strategy; yet, it is associated with compromised biomass productivity. In this novel approach, combined effect of metals and EDTA on lipid productivity of *Acutodesmus obliquus* was investigated under nitrogen limited conditions. The effect of metal concentrations, individually and in combination, on microalgal lipids and biomass production is a scarcely exploited area. Combined metal stress alleviates the constraint of low biomass production under nitrogen limitation and improved the overall lipid productivity. Highest lipid productivity of 73.23 mg L^{-1} d^{-1} was achieved with a combination of iron 9 mg L^{-1}, magnesium 100 mg L^{-1} and calcium 27 mg L^{-1} at limited nitrogen (750 mg L^{-1}). This was 1.72 fold higher than nitrogen stress alone and 1.99 fold higher than BG11 medium. Iron was found to be most significantly influencing metal followed by magnesium in response surface methodology data analysis. The enhanced photosynthetic performance and chlorophyll content further confirmed the significant impact of iron and magnesium on the microalgal biomass. The addition of EDTA to the optimised metal combination further improved the lipid productivity to 80.23 mg L^{-1} d^{-1} (2.18 fold). At 3000 L open cultivation pond this strategy has resulted in an increase of 2.08 fold in lipid productivity. Higher biodiesel conversion rates were also observed with this easy, universally applicable and scalable lipid enhancement strategy.

1. Introduction

Biodiesel production from microalgae is potentially feasible as these tiny photosynthetic microbes present substantial biomass and lipid productivity compared to other edible and non-edible plant based feedstocks [1–3]. Furthermore, microalgal lipids are majorly constituted of suitable fatty acid composition for the biodiesel production [4–6]. Though there are several remarkable advantages of microalgae over other feedstocks, biomass production is still an expensive process due to a number of factors associated with microalgal cultivation [7,8]. One possible solution for improving the economics of microalgal cultivation is to select a suitable strain and investigate its response to different cultivation conditions for enhancement of the overall lipid productivity [9–11]. It is well known that a number of factors could influence lipid accumulation in microalgae, such as nitrogen starvation or limitation [12,13], phosphate limitation [14] high salinity, carbon source concentration, light intensity, and temperature [15–17]. Under the stress conditions, microalgae tend to accumulate energy in dense forms such as lipids [2,10,18]. Recent reports have shown the potential of combining different nutrients and abiotic stress factors to improve microalgal lipid productivity. To obtain maximum yields, it is imperative to have knowledge of the synergistic effects of factors as well as significance of each factor with regards to lipid accumulation [15,19–22]. Breuer et al. [15] investigated the effects of light, pH, and temperature on TAG accumulation under nitrogen deficient conditions and found pH and temperature to be major influencing factors for TAG accumulation. The highest TAG content (40%) was obtained at pH 7 and 27.6 °C which was independent of light variation. Ji et al. [23] studied the effects of variation in temperature, light intensity and photoperiod on *Desmodesmus* sp. and observed high biomass production at a combination of temperature: 30 °C, light intensity: 98 μmol m^{-2} s^{-1} and photoperiod: 14:10 (L:D Light:Dark photoperiod). The effects of environmental factors such as light intensity and temperature have

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ACCase and rbcL gene expression as a function of nutrient and metal stress for enhancing lipid productivity in *Chlorella sorokiniana*

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ABSTRACT

Development of novel and scalable strategies for improving lipid and biomass yield is a prerequisite for sustainable biodiesel production from microalgae. Currently, nutrient stress is the widely employed lipid enhancement strategy in microalgae, yet, it is associated with compromised biomass productivity. In this study, an alternative approach for lipid enhancement in *Chlorella sorokiniana* is proposed to alleviate the constraint of low biomass production under nutrient stress. This is achieved through combining nutrient stress along with selected metals and EDTA stress. A substantial increase in lipid yield (77.03 mg L^{-1} d^{-1}) was achieved when the nutrient (nitrogen and phosphorus) stress BG11 media was supplemented with a combination of iron (15 mg L^{-1}), magnesium (125 mg L^{-1}), calcium (18 mg L^{-1}) and EDTA (3 mg L^{-1}). This was further validated by quantifying the expression levels of two key genes involved in photosynthesis (*rbcL*) and lipid biosynthesis (*accD*) pathways. Under this developed strategy a 5.15-fold increase in *rbcL* and 9.79-fold increases in *accD* gene expression were noted in comparison to the culture grown in BG11 medium. A significant correlation could also be drawn between the expression of *rbcL* and *accD* genes to biomass yields, photosynthetic performance and lipid productivity. The proposed strategy could be easily applied at various commercial scale microalgal cultivation systems for enhancing lipid content without compromising the biomass yield.

1. Introduction

Microalgal traits such as fast growth rates, high lipid accumulation, suitable fatty acid composition and adaptability towards wide range of climatic and environmental conditions makes it an apt feedstock for biodiesel production [1]. Despite of several advantages over other feedstocks, the microalgal biodiesel is still far from the commercial scale production due to unfavourable economic feasibility [2–4]. Ensuring high lipid accumulation in biomass could improve the economics of the microalgal biodiesel production. Lipid accumulation in microalgae is determined by several factors such as nutrient limitations, high salinity, carbon source concentration, light intensity, and temperature [5–7]. These conditions can be altered to achieve high lipid productivities in microalgal biomass. However, apart from nutrient, alterations in most of these conditions to improve lipid yields in microalgae are either energy intensive or strenuous to be applied at large scale [8–10]. Alteration of nutrient concentration could be the best possible solution, as it is easily applicable and scalable.

Nitrogen (N) and phosphorous (P) stress are the most effective strategies to enhance lipid accumulation in microalgae but these are associated with low biomass production that reduces overall lipid productivity [11]. Thus, there is need for alternate strategies that can be coupled with N and P stress to improve overall lipid productivity. Metals such as iron (Fe), calcium (Ca), magnesium (Mg), copper (Cu), and zinc (Zn) are known to play major role in microalgal physiology. Some of the metals such Fe, Ca and Mg reportedly improved biomass and lipid yields, when added to the microalgae growth media [12,13]. However, most of the previous studies focused on lipid accumulation and biomass production, without elucidating its effect on growth physiology, photosyn-

Abbreviations: ACCase, acetyl-CoA carboxylase; BG11, standard BG11 medium; Ca, Calcium; Fe, Iron; g L^{-1}, gram per litre; g, gram; OCMS, optimum combined metal stress; OCMS, optimum combined metal stress; OCMS+OE, optimum combined metal stress + optimum EDTA concentration; OII, optimum individual iron concentration; OIM, optimum individual magnesium concentration; ON, optimum nitrogen concentration; ONP, optimum nitrogen and phosphorus concentration; ONP-M, optimum nitrogen concentration and optimum phosphorus without selected metals; OP, optimum phosphorus concentration; rbcL, ribulose-1,5-bisphosphate carboxylase/oxygenase; rETR, relative electron transport rate; rpm, rotation per minute; mg, milligram; mL, millilitre; mg L^{-1}, milligram per litre; Mg, magnesium.

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Regular Article

Investigation of combined effect of nitrogen, phosphorus and iron on lipid productivity of microalgae *Ankistrodesmus falcatus* KJ671624 using response surface methodology

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ABSTRACT

Enhancement of lipid accumulation is essential to improve the commercial feasibility of microalgal biodiesel production. An oleaginous microalgal strain, *Ankistrodesmus falcatus* KJ671624 was evaluated for its potential as a biodiesel feedstock in this study. The collective effect of nutrient (nitrogen, phosphorous and iron) stresses on the lipid productivity of the selected strain was studied by response surface methodology. The highest lipid content of 59.6% and lipid productivity of 74.07 mg L^{-1} d^{-1} was obtained under nutrient stress with nitrogen 750 mg L^{-1}, phosphorus 0 mg L^{-1} and iron 9 mg L^{-1}. The photosynthetic behaviour validates the high lipid productivity under combined nutrient stress condition. Saturated fatty acid composition was increased by 38.49% under selected nutrient stress condition compared to BG11 medium. The enhanced lipid accumulation with suitable lipid profile (C16:0, C18:1, C18:2, C18:3) and biodiesel conversion of 91.54 ± 1.43% achieved in *A. falcatus* KJ671624 further confirm its potential as a promising feedstock for biodiesel production.

1. Introduction

Crude oil is currently the world's primary source of liquid fuel. The increasing demand for fuel has led to a continuous increase in the price of fossil fuels and emission of green house gases (GHGs). Microalgae have the potential to generate higher quantities of biomass and more suitable lipids for biodiesel production than crop plants whilst they require less cultivation area and no arable land [1]. Despite of these advantages, high production cost is still a bottleneck to successful commercialization [2]. Enormous amounts of microalgal lipids are required to cater to the biodiesel demand. Most of oleaginous microalgal strains accumulate up to 20–50% lipids based on the dry cell weight [3]. Exploiting novel strains with high lipid accumulation capacity could meet the enormous lipid requirement. Moreover lipid accumulation capacity in microalgae can be improved by applying various biochemical strategies [4–6].

Exploitation of microalgal strains with high biomass and cellular lipid productivity is essential for sustainable biodiesel production. Several strains have been screened for biodiesel production by researchers worldwide, but very few have undergone further research. *Ankistrodesmus falcatus* is a fast growing (2.5 doublings a day), fresh water [7] microalgae which is well studied for sewage purification [8]. Several studies have highlighted the potential of *Ankistrodesmus* sp. for biodiesel production with high lipid productivity and suitable fatty acid profile [7,9,10]. Jayanta et al. [11] studied growth and lipid accumulation in *A. falcatus* under salinity stress. They found highest growth rate to be 0.313 day^{-1} and lipid content of 55.3% at salinity of 160 mM. Nascimento et al. [12] screened algal strains for biodiesel production based on their lipid productivity and fuel quality. In their study *A. falcatus* showed lipid productivity of 56.07 mg L^{-1} d^{-1}. Nascimento et al. [12] also estimated fuel properties of *A. falcatus* based on fatty acid composition and found higher cetane number (50.52) than the ASTM specified limits (min 47). Talebi et al. [5] reported *Ankistrodesmus* sp. as suitable algal strain for biodiesel synthesis; however, slow growth rate was their concern. They also suggested further investigation in cultivation conditions for improving the prospects of this strain as a biodiesel feedstock. Research on this promising strain for enhancing lipid accumulation capacity could therefore improve its viability as sustainable biodiesel feedstock.

Factors such as nutrients stress (nitrogen, phosphorus, iron and salt); temperature, light intensity, photoperiod and pH directly influence biomass and lipid content of microalgae [13–17]. Nitrogen is a constituent of protein synthesis, essential for cell division and growth of microalgae [18]. At adequate nitrogen

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CHAPTER

4

Microalgae Isolation and Basic Culturing Techniques

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1. INTRODUCTION

Microalgae are ubiquitous organisms that are the primary producers for life on the earth. Rapid growth rates and the simple structure of microalgae make them interesting organisms for biotechnological applications. Cellular components of microalgae such as carbohydrates and proteins can be utilized for various purposes including biofuels and products of nutritional and pharmaceutical value (Chisti, 2007; Harun et al., 2010; Singh et al., 2014). Researchers, industries, and governments are now focusing on the development of green processes. This has extended to the exploitation of microalgae, especially for biofuels and pharmaceutical products.

Microalgae are a diverse group of organisms and found in various natural habitats. They exhibit variation in their nutritional requirements as well as metabolite production (Rawat et al., 2013). Isolation and culture development of microalgal strains of interest are essential primary steps toward laboratory investigations and successful commercial application. Bioprospecting of microalgae for a particular product or application is a multistep process comprised of sampling of environments, purification and isolation of monocultures, determining nutrient requirements and cultivation parameters, screening of microalgal species, measuring growth, and developing suitable cultivation techniques (Mutanda et al., 2011).

This chapter describes the commonly adopted techniques in isolation, screening, growth measurement, determining cultivation conditions and nutrient requirements, and cultivation techniques. Choice of suitable technique depends upon the microalgal strain and its application.

2. MICROALGAL ISOLATION AND PURIFICATION TECHNIQUES

Sampling and isolation of microalgae from natural habitats is a well-established procedure. Depending on their different habitats, algal strains vary in their ease of cultivation under laboratory conditions. A key step in the isolation of microalgae is to provide culture conditions that mimic their natural habitats. Microalgae are

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Full Length Article

Biodiesel synthesis from microalgal lipids using tungstated zirconia as a heterogeneous acid catalyst and its comparison with homogeneous acid and enzyme catalysts

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Heterotrophic cultivation of microalgae using aquaculture wastewater: A biorefinery concept for biomass production and nutrient remediation

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Assessment of municipal wastewaters at various stages of treatment process as potential growth media for *Chlorella sorokiniana* under different modes of cultivation

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Evaluating the potential of cytokinins for biomass and lipid enhancement in microalga *Acutodesmus obliquus* under nitrogen stress

Nirmal Renuka, Abhishek Guldhe, Poonam Singh, Faiz Ahmad Ansari, Ismail Rawat, Faizal Bux*

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Abhishek Guldhe [a], Carla V.R. Moura [b], Poonam Singh [a], Ismail Rawat [a], Edmilson M. Moura [b], Yogesh Sharma [c], Faizal Bux [a, *]

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Microalgal cultivation using aquaculture wastewater: Integrated biomass generation and nutrient remediation

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Biodiesel synthesis from microalgae using immobilized *Aspergillus niger* whole cell lipase biocatalyst

Abhishek Guldhe [a], Poonam Singh [a], Sheena Kumari [a], Ismail Rawat [a], Kugen Permaul [b], Faizal Bux [a, *]

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An innovative electrochemical process to alleviate the challenges for harvesting of small size microalgae by using non-sacrificial carbon electrodes

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Evaluation of operating conditions for sustainable harvesting of microalgal biomass applying electrochemical method using non sacrificial electrodes

Rohit Misra, Abhishek Guldhe, Poonam Singh, Ismail Rawat, Thor Axel Stenström, Faizal Bux *

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Electrochemical harvesting process for microalgae by using nonsacrificial carbon electrode: A sustainable approach for biodiesel production

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