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Co-production of Biodiesel and Alpha Linolenic Acid from *Desmodesmus* sp. MCC34

THESIS

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Under the Supervision of
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CERTIFICATE

This is to certify that the thesis entitled "**Co-production of biodiesel and alpha linolenic acid from *Desmodesmus* sp. MCC34**" submitted by **A. Senthil Nagappan**, ID No. **2009PHXF407P** for the award of Ph. D Degree of the Institute, embodies original work done by him under my supervision.

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Abstract

In the present scenario of increasing global temperature which is linked to greenhouse gas emission and continued depletion of non-renewable fossil fuels, there is urgent need for alternate renewable fuels. Microalgae, which can fix carbon dioxide and have advantages such as higher photosynthetic efficiency than plants and high lipid content, can be an ideal source of biofuel. In this context, twenty one algal strains comprising three locally isolated strains were scrutinized for lipid production. The *Desmodesmus* sp. MCC34, which emerged as the promising species due to higher lipid productivity, was further optimized for its growth by varying growth parameters (light intensity, photoperiod, pH, salinity) and concentrations of substrate (nitrate, phosphate and sulphate). The mass cultivation of *Desmodesmus* sp. MCC34 in raceway pond resulted in areal biomass productivity of 13946.23 kg ha^{-1} $year^{-1}$ and volumetric biomass productivity of 56.94 mg L^{-1} day^{-1} during Mar 2013 which decreased to 6262.28 kg ha^{-1} $year^{-1}$ and 25.57 mg $L^{-1}day^{-1}$ during Nov. A unique growth model, which predicts biomass productivity of *Desmodesmus* sp. MCC34 in outdoor cultivation in raceway pond was developed and validated. Successful harvesting of *Desmodesmus* sp. MCC34 was demonstrated with the help of flocculation aids, ferric chloride (150 mg L^{-1}) and chitosan (90 mg L^{-1}). The nitrogen deprivation was found to be a suitable strategy to increase lipid content not only in *Desmodesmus* sp. MCC34, but also in three other strains which also showed high lipid productivity. The nitrogen deprivation decreased chlorophyll, carotenoids and total protein content and increased the carbohydrate content in all four strains. The biodiesel of *Desmodesmus* sp. MCC34 had cetane number of 41 and iodine value of 140 g Iodine per gram biodiesel. The iodine value was not complying with the biodiesel standard which was due to significant amount of unsaturated fatty acid. The major fatty acids found in *Desmodesmus* sp. MCC34 were palmitic acid (C16), stearic acid (C18), oleic acid (C18:1), linoleic acid (C18:2) and alpha linolenic acid (C18:3;n-3). Among which, alpha linolenic acid, an omega 3 fatty acid and pharmaceutically important compound, was found in higher concentration in *Desmodesmus* sp. MCC34 (24% of the total fatty acids and 4.2 mg g^{-1} biomass). A method was developed for simultaneous production of pure alpha linolenic acid as well as high quality biodiesel from the same lipid present in *Desmodesmus* sp. MCC34 using silver ion chromatography. By this method, alpha linolenic acid methyl ester (ALAME) of 90% purity could be obtained. The residual lipid (after removing ALAME) showed improved biodiesel property such as cetane number of 54 and

iodine value of 94 g I_2 per 100 g. Among the solvents screened for lipid extraction, the Folch solvent mix showed highest FAME yield (49 mg g^{-1}) from dry biomass while the Bligh and Dyer solvent mix showed highest yield (46 mg g^{-1}) from the wet biomass. A cost effective procedure of extracting lipid from biomass by simple diffusion process called static extraction was developed. By this method, we could extract the maximum attainable lipid in biomass in shorter time under favorable solvent-biomass ratio and surface area factor, without the need of energy consuming steps such as cell lysis and biomass-solvent stirring. In study involving optimization of transesterification process, the base catalyst (KOH) was found to be more efficient than acid catalyst (HCl) in terms of temperature requirement, reaction time and catalyst amount. The optimal temperature, reaction time, solvent-oil ratio (ml/g) and catalyst amount for alkali catalyzed transesterification were 60°C, 1 minute, 300 and 0.2% (w/v) KOH respectively. This study suggests that along with good quality biodiesel production, considerable amount of other products such as alpha linolenic acid, carotenoids and carbohydrate can also be obtained from mass production of *Desmodesmus* sp. MCC34 matching with suggested biorefinery approach.

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List of Abbreviations

Abbreviations	Denotations	Expanded Form
ALAME	_	Alpha linolenic acid methyl ester
GLAME	_	Gamma linolenic acid methyl ester
FAME	_	Fatty acid methyl ester
ALA	_	Alpha linolenic acid
SFA	_	Saturated fatty acid
MUFA	_	Monounsaturated fatty acid
PUFA	_	Polyunsaturated fatty acid
SBR	_	Solvent-biomass ratio
SAF	_	Surface area factor
GC-MS	_	Gas chromatography-Mass spectrometry
NMR	_	Nuclear magnetic resonance spectroscopy
RPTLC	_	Reverse phase Thin layer chromatography
ITS	_	Internal transcribed spacer
CBC	_	Compensatory base change
CCUBGA	_	Centre for conservation and utilization of blue green algae
IARI	_	Indian agricultural research institute
W_L	g	Weight of the total lipid
W_b	g	Weight of the dry biomass
X	$g\ L^{-1}$	Biomass concentration at time t
X2	$g\ L^{-1}$	Biomass concentration at end of exponential phase
X1	$g\ L^{-1}$	Biomass concentration at start of exponential phase

t	day	Time between start and end of exponential phase.
S_p	mM	Dipotassium hydrogen phosphate concentration
S_n	mM	Sodium nitrate concentration
S_s	mM	Sulphate concentration
K_{Sp}	mM	Michaelis Menten constant for phosphate
K_{Sn}	mM	Michaelis Menten constant for nitrate
K_{Ss}	mM	Michaelis Menten constant for sulphate
I_Z	μ mol photon m^{-2} s^{-1}	Light intensity at given depth
I_s	μ mol photon m^{-2} s^{-1}	Optimum light intensity for maximum growth
T_{max}	°C	Maximum survival temperature of algae
T_{min}	°C	Minimum survival temperature of algae
T_{opt}	°C	Optimal temperature of algae
T	°C	Temperature at time t
N	%	Percentage of each fatty acid
M	Dalton	Molecular weight of each fatty acid
D	_	Number of double bond of each fatty acid
k	min^{-1}	Mass transfer coefficient of lipid extraction process
k_1	min^{-1}	Mass transfer coefficient of washing step
k_2	min^{-1}	Mass transfer coefficient of diffusion step
L	(% of the dry biomass	Lipid concentration at given time
M	(% of the dry biomass	Maximum amount of extractable lipid
M1	(% of the dry biomass	Maximum amount of lipid obtained from washing step
M2	(% of the dry biomass	Maximum amount of lipid obtained through diffusion step

Chapter 1: Introduction

1.1 General introduction

Energy is the driving factor of world economy and demand for it had risen exponentially since the start of 20th century. The global energy consumption was 13 billion ton oil equivalent in 2015 and predicted to increase further by 50% in year 2030 (Nejat *et al.,* 2015). The energy is required for domestic, industrial and transportation purposes. While domestic and industrial plants derive the energy through electricity, nuclear power, etc., the transport sector requires energy in the form of potable and combustible nature. Initially, coal was used as mobile fuel in trains and ships, later it was replaced by more combustible petroleum. Even though the use of petroleum for domestic purposes is in existence for more than 2000 years, the modern history of petroleum started with the establishment of oil extraction plant in Pennsylvania, USA by Edwin Drake in the year 1859. In 1909, the first oil well in Middle East was drilled in Iran. Now, more than 40 million metric ton of petroleum is extracted per day chiefly from the gulf countries, with little contribution from other parts of the world. The fossil fuels are non-renewable resources and are predicted to drain out by the year 2080 (Nejat *et al.,* 2015). In search of renewable resources, wind, solar and biomass were identified. Among biofuel from biomass, biodiesel and bioethanol are used in automobiles. In particular, biodiesel which is used as fuel in automobiles since 1920 has the advantage over bioethanol in terms of direct usage in engines without any blending. There are three generations of biodiesel. The first and second generations of biodiesel were obtained from plant sources (Zhang *et al.,* 2016).

The biodiesel from microalgae belongs to the third generation. Microalgae have been exploited as biodiesel source since 1970 with aquatic species program of USA being the pioneer in producing algal biodiesel (Sheehan *et al.,* 1998). The algal biodiesel, which is obtained through transesterification reaction of algal oil has the distinctive advantage of higher biomass productivity over first and second generation biofuel. The algal biomass productivity in outdoor condition is 80-200 tons ha^{-1} $year^{-1}$, which is 15-20 times higher than that of agricultural crops (Chisti 2016). The other advantages of algae are its ability to grow in waste water, sequestration of heavy metals (Sarwa & Verma 2013) and production of valuable by-products (Mohan *et al.,* 2016). Also, certain microalgae such as nitrogen fixing blue green algae can also grow without exogenous supply of combined nitrogen compounds, therefore minimizing cost involved in mass cultivation. Lot of unique characteristics exists among different classes of microalgae.

Microalgae are thallophytes i.e., primitive organisms unlike higher plants, lacking roots, stems and leaves (Borowitzka *et al.,* 2016). Due to simpler structure, they can adapt to the extreme environments. These photosynthetic organisms have chlorophyll a as primary pigment. The chlorophyll b, which serves as the accessory pigment to chlorophyll a in green algae, is absent in cyanobacteria. Although primarily autotrophic in nature, they can be heterotrophic and mixotrophic also, when a suitable carbon source such as starch, glucose, etc. is available in their habitat. The habitat of microalgae can be either fresh water or marine.

1.2 Biodiesel production from microalgae

The biodiesel production from microalgae can be achieved either by dry or wet route. The dry route involves drying and subsequent pulverization of the algae, which are conducted prior to lipid extraction while the wet route skips these steps. The energy consuming process in dry route is the drying whereas in wet route, it is the extraction of lipid process. One more variation in biodiesel production from microalgae is the in-situ transesterification in which lipid extraction and transesterification is carried out in a single step (Go *et al.,* 2016).

1.2.1 Mass cultivation of microalgae

The microalgae can be mass cultivated by adopting either open pond or closed photobioreactor. Both of these methods have pros and cons. The main advantage of open pond cultivation is that it requires minimal capital cost for installation (Hasheminejad *et al.,* 2011) but suffers from the problem of contamination, lesser biomass productivity compared to photobioreactor and constant loss of water through evaporation. Though, the photobioreactors are free from contamination and shows higher biomass productivity (Singh *et al.,* 2011), the drawback lies with its higher installation cost. The type of product to be obtained from microalgae, dictates the choice of cultivation. For example, low valued products such as biofuel can utilize open pond cultivation whereas food and pharmaceutical products can be obtained through photobioreactors.

The open pond cultivation is of two types; raceway pond and static ponds. A raceway pond consists of two runways separated by wall and connected in end in which the water moves in opposite direction. The mixing is usually provided by a paddle wheel. The advantage of raceway pond is

uniform distribution of nutrients, biomass and gases, which is achieved through mixing by paddle wheel (Chen *et al.,* 2011). Growth inhibition due to oxygen is reduced in this pond. This kind of pond is operated in Taiwan and India for growing *Chlorella* and *Spirulina* respectively as protein sources (Spolaore *et al.,* 2006). In a static pond, the mixing is not provided mechanically. Construction of these ponds in windy area provides natural mixing as in case of Australian saline ponds meant for *Dunaliella* cultivation and Mexican Caracol ponds for *Spirulina* (Ben-Amotz 2004). Cultivation in both type of open ponds are suitable for species which can grow in extreme conditions, for example *Dunaliella* can grow in extreme saline conditions whereas *Spirulina* can grow in extreme alkaline conditions. This advantage of species is preferable since they can outperform the local contaminants in growth.

The photobioreactors are of various kinds; airlift, bubble column, vertical and tubular. Mixing in a photobioreactor is provided through CO_2 via inlet. The temperature of photobioreactor is maintained by either submersing the whole system in pool of water or spraying cold water over it (Hoh *et al.,* 2016). Light is provided either naturally by placing the system in outdoor condition or artificially in indoor condition through fluorescent tubes. The photobioreactors are used for producing high valued products such as omega 3 fatty acids (Singh *et al.,* 2011). The fermentors are also used for cultivating algae in heterotrophic mode. Algae can also grow in absence of sunlight provided a suitable carbon source is supplemented. Advantage of fermentor over photobioreactor is the higher biomass productivity but at higher feed cost. Solazyme, a leading company in commercialization of algal products, uses this kind of systems for production; Martek is another company which produces decosahexaenoic acid through fermentation from species such as *Cryptethecodinium* (Spolaore *et al.,* 2006).

1.2.2 Harvesting microalgae

The harvesting is next step in microalgal biodiesel production once sufficient algal biomass is produced through mass cultivation. The foremost problem that microalgae pose for harvesting is its size. These organisms are microscopic, ranging from 2-10 µm, therefore harvesting of microalgae through filtration requires filters having smaller pore size (Chatsungnoen & Chisti 2016), which can be achieved only through the process of ultrafiltration. The ultrafiltration is slow and expensive which leads to clogging of filter pores leading to substantial cake resistance (Wan

et al., 2015). The other way of overcoming this problem is to increase the particle size, which is done by supplementing flocculation aids. The common flocculation aids used for algal harvesting are ferric chloride, aluminum potassium sulphate, chitosan and cationic flocculants (Hasheminejad *et al.,* 2011). Ferric chloride is a cation which can form bonds with negatively charged algae to form flocs; these large sized flocs can be retained on filters much easily than single algae (Wan *et al.,* 2015). Aluminum potassium sulphate is a popular flocculating agent used in cleaning waste water and has been recently applied for harvesting algae also (Razack *et al.,* 2015). Use of chemical flocculants, although cheaper than organic flocculants, is detrimental to algae when added in higher concentration. It can also lead to problem in downstream processing (Wan *et al*., 2015). On other hand, organic flocculant such as chitosan, starch, etc. are environmental friendly and does not affect algal growth; therefore, reusability of media is possible (Milledge & Heaven 2013). Apart from flocculation, there are other method of harvesting such as electroflocculation, centrifugation and sedimentation (Milledge & Heaven 2013). Electroflocculation, an energy intensive process, involves immersing electrodes (typically made of iron or aluminum) in water containing algae, which results in discharge of ions, in this case iron or aluminum, thereby resulting in flocculation. Advantage of electroflocculation, over chemical flocculant is that it does not release chloride or sulphate into the water like chemical flocculants. Centrifugation is concentrating algae by centrifugal force; it is not suitable for low cost products, as it requires excessive energy to operate. Sedimentation is a slow process, suitable for certain algae which can sediment naturally due to their larger size. Although sedimentation is cost effective, it is poor in concentrating algae since it retains large water content. The choice of harvesting method depends on cost and type of product.

1.2.3 Lipid extraction

Once algae are harvested, the next step in biodiesel production from microalgae is lipid extraction. The lipid can be extracted from dry or wet algal biomass (Guldhe *et al.,* 2016). The lipid extraction by dry route requires extensive energy for drying the algae (Quinn *et al.,* 2014). Sun-drying can be a process of choice in semi-arid places such as Shekawathi region of Rajasthan, India. Solar drying is yet another mode for drying the algal biomass economically which requires the installation of solar dryer equipment. Both drying process are not suitable for thermo labile products. The drying becomes a time-limiting step during winters, during which the lipid

extraction can be performed directly on wet algae. A study had shown that wet route requires compulsory cell lysis in order to increase the rate of lipid extraction (Martin 2016). Cell lysis is again an energy intensive step causing cost burden to biodiesel production from microalgae. The cell lysis techniques include homogenization, ultrasonication, microwave radiation, electric pulse field, etc. The supercritical fluid extraction using carbon dioxide, ethanol, etc. is environmental friendly process (Hasheminejad *et al.,* 2011), which involves instantaneous lipid extraction from intact biomass without lysis of cell. But it incurs additional cost to algal biodiesel production.

There are varieties of solvents which are used in extracting lipid from microalgae. The two most commonly used solvents for lipid extraction are Bligh Dyer and Folch solvent mixtures (Bligh & Dyer 1959; Folch *et al.,* 1957). Both of them use chloroform and methanol in varied proportion in order to extract lipid. These systems are very efficient as they are polar: non polar mixtures in which polar solvent breaks hydrogen bonding between membrane lipid and protein thereby extracting polar lipids, whereas non polar solvent extracts the non-polar lipid (Halim *et al.,* 2012). Solvents such as ethanol, methanol, dichloromethane-methanol mixture and hexane-ethanol mixture are also reported for lipid extraction from microalgae. These solvents have advantage over the gold standard systems such as Bligh Dyer and Folch, as they do not use chloroform; chloroform is classified as B2 carcinogen therefore ruling out scaling up option (Sheng *et al.,* 2011). Finally, the choice of solvent in industrial scale depends up on the end product and environmental safety (Olmstead *et al.,* 2013b).

1.2.4 Transesterification

Transesterification is the process of converting lipid such as triacylglycerol, phospholipid and glycolipid into fatty acid methyl ester (Figure 1. 1). According to bureau of Indian standards, any mixture containing 96% fatty acid methyl ester is biodiesel (Tyagi *et al.,* 2010). The difference between biodiesel and lipid is its viscosity. Biodiesel is less viscous, with specific gravity of 0.8 g ml^{-1}, and unlike lipid does not clog the diesel engines when directly used. The transesterification process uses alcohol such as methanol, ethanol, isopropanol and butanol for producing methyl, ethyl, isopropyl and butyl esters (Canakci & Van Gerpen 2001), among which methanol is preferred transesterifying reagent. Higher the molecular weight of alcohol, faster is the reaction (Canakci & Van Gerpen 2001). This reaction is catalyzed either by alkali or acid or enzyme. The

transesterification reaction catalyzed by alkali such as KOH and NaOH is a rapid process while the acid catalyzed reaction involving HCl or H_2SO_4 is slower (Tyagi *et al.*, 2010). The enzymatic process by using immobilized lipase for transesterification has the advantage of reusability and the selective enrichment of fatty acid is reported through the use of specific lipases (Akoh *et al.*, 2007; López *et al.*, 2016).

The lipid extraction and transesterification steps can be combined in a single step called in-situ transesterification for obtaining biodiesel directly from biomass. The in-situ transesterification can be done for both wet and dry algae (Ehimen *et al.*, 2010). The wet algae in-situ transesterification can be catalyzed only by an acid catalyst since the use of alkali results in soap formation (Choi *et al.*, 2014) whereas, in-situ transesterification of dry algae can be carried out using acid and alkali catalyst (Xu & Mi 2011).

1.3 Biodiesel properties

The quality of biodiesel depends mainly on fatty acid profile. The two main properties that reflect the quality of biodiesel are cetane number and iodine value. Cetane number is indication of how quick a fuel can ignite and iodine value indicates oxidation stability of fuel (Monteiro *et al.*, 2008). Cetane number is determined by specially designed engine where the pressure is increased by tightening an axle until the time gap between fuel injection and ignition is 2.4 ms (Gautam & Martin 2000); the cetane number is calculated from the proportion of cetane (hexadecene) and isocetane to achieve the same time period of 2.4 ms. Cetane number can also be calculated theoretically based on fatty acid profile (Islam *et al.*, 2015). Theoretical calculation shows that higher the saturated fatty acid methyl ester content, higher is the cetane number. The iodine value is calculated by placing lipid in a dark chamber along with measured amount of a halogen, by which halogen and lipid reacts and unused halogen is titrated in order to determine the iodine value (Monteiro *et al.*, 2008). Theoretical calculation shows that higher the unsaturated fatty acid methyl ester content, higher is the iodine value (Islam *et al.*, 2015).

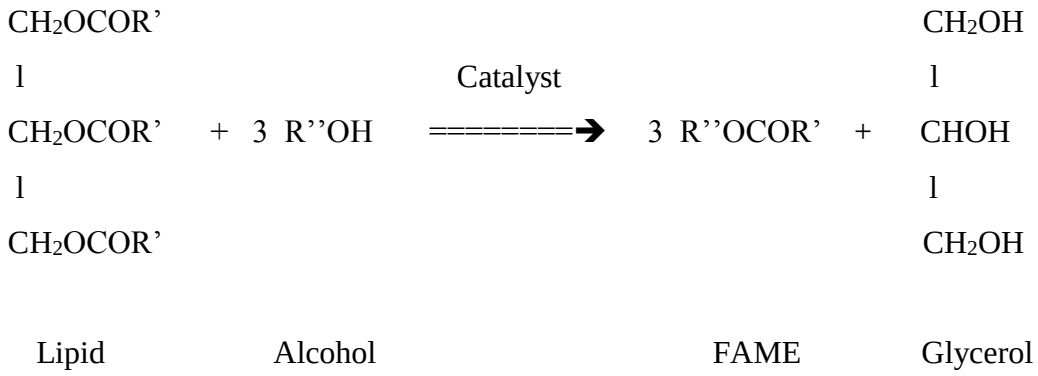


Figure 1.1 The transesterification process. R'-fatty acid and R''-alkyl or hydrogen

The lipid with higher iodine value is not desirable since they are easily oxidized and cannot be stored for long time. Biodiesel with high oleic acid content is preferred since they have good flow properties, cetane number and oxidation stability (Mendoza *et al.,* 1999).

1.4 Algal species for lipid production

Many algae are projected as suitable candidates for higher lipid production. *Botryococcus* is high in hydrocarbon content but is a slow growing organism (Moutel *et al.,* 2016). *Nannochloropsis* is a marine organism which is rapid grower at the same time has high lipid content (Caporgno *et al.,* 2016). *Dunaliella* species are halotolerant marine species which are able to tolerate not only high salinity but high temperature and are robust organisms, rich in by-products such as carotenoids and glycerol, which can be a value addition (Bonnefond *et al.,* 2016). *Chlorella* is a fresh water species rich in chlorophyll and proteins, fast growers and when subjected to stress conditions they can yield higher lipids (Illman *et al.,* 2000). Similarly *Scenedesmus* is a fresh water species and on specific conditions yield higher lipid (Breuer *et al.,* 2013). Cyanobacteria are of two types; nitrogen fixers and non-nitrogen fixers; nitrogen fixing cyanobacteria such as *Anabaena* and *Nostoc* does not contain high lipid content but due to prokaryotic in nature they adapt to harsh environmental conditions (Vargas *et al.,* 1998) while non-nitrogen fixers such as *Spirulina* and *Synechocystis* are well studied organism and having their genome sequenced, make them preferable to genetic manipulation. In general, organisms isolated from local environment have definite advantage over non-native organism for mass cultivation as they can withstand local environmental conditions (Singh *et al.,* 2011). The term lipid productivity is the combination of lipid content and growth rate and is considered as the key characteristic to look upon on algae for lipid related production such as biodiesel (Griffiths & Harrison 2009).

1.5 Biorefinery Approach

Although microalgae are projected as promising source for biodiesel, the cost of producing biodiesel from microalgae is higher than producing diesel from fossil fuel. The co-production of value added product is suggested to overcome this problem (Dong *et al.,* 2016). The by-products of biodiesel production from microalgae are pigments, carbohydrate, protein and omega 3 fatty acids. The carbohydrate can be used to make biogas and bioethanol, proteins can be used as animal and fish feed and the omega 3 fatty acids such as eicosapentaenoic acid (EPA), decosahexaenoic

acid (DHA) and alpha linolenic acid (ALA) have health benefits and are pharmaceutically important compounds (Cuellar-Bermudez *et al.,* 2015). Alpha linolenic acid (ALA) can lower the cholesterol, bad lipids in blood; it can act as anti-inflammatory agent, protects nervous system, also prevents secondary coronary infection; it also offers skin protection by reducing scaling and roughness (Kim *et al.,* 2014).

Omega 3 fatty acids are fatty acids in which the first double bond from the methyl side is present on 3rd carbon (Cuellar-Bermudez *et al.,* 2014). These fatty acids are commercially obtained from plant and fish sources. The cod fish, source of cod liver oil, are rapidly dwindling in numbers in oceans; therefore other sources have been identified. The marine diatoms such as *Crypthecodinium* and *Phaedactylum* are the other sources which are commercially exploited in recent times (Spolaore *et al.,* 2006). Martek Corporation of USA uses fermentation technology to produce DHA from *Crypthecodinium* (Spolaore *et al.,* 2006). Similarly Nestle produces child infant formula fortified by DHA obtained from a marine diatom and DHA from microalgae unlike fish are not smelly and cheaper to produce; therefore, suitable for vegetarians; EPA on other hand is costlier to purify from microalgae (Spolaore *et al.,* 2006). ALA is other omega 3 fatty acid which is chiefly obtained from linseed oil (Kim *et al.,* 2014) and commercial production of ALA is not reported from microalgae.

1.6 Lipid analysis

Measurement of lipid is done by staining algae with lipophilic stains such as nile red (9-diethylamino-5H-benzo[a]phenoxazine-5-one) (Morschett *et al.,* 2016). According to the amount of lipid, fluorescence of nile red increases. Although this method is rapid, it poses problem with lipid estimation in algae due to presence of thick cell wall; solvents such as DMSO are used to mobilize the dye into the cell (Mutanda *et al.,* 2011). The infra-red spectroscopy is also used to quantify lipid based on C=O peak height which corresponds to fatty acid methyl ester. Its non-destructive method and latest FTIR requires no mixing with potassium bromide (Mutanda *et al.,* 2011). The most popular method to quantify lipid is by gravimetric method which utilizes specific solvent to extract lipid which is usually Bligh Dyer solvent mizture (Bligh & Dyer 1959). This method requires higher quantity of biomass in order to avoid experimental errors and depends on solvent selectivity. The disadvantage of this method is the chance of extracting other components

along with lipids which could lead to error. Notwithstanding, gravimetric method is most popular lipid quantifying method.

The transesterified lipid is identified for constituent fatty acid composition by gas chromatography (Tyagi *et al.,* 2010). The other method for detecting fatty acid is HPLC which uses reverse phase column; the detector used is refractive index detector (Delmonte *et al.,* 2005). Separation of unsaturated fatty acid is versatile through reverse phase column (Delmonte *et al.,* 2005). Thin layer chromatography (TLC) is used for preliminary screening in which a non-polar mobile phase such as hexane: diethyl ether: glacial acetic acid – 8:2:0.2 can separate hydrocarbons, triacylglycerol and fatty acids in normal TLC plate. Visualization can be carried out by charring or iodine vapor. Preparative TLC plate can be used for quantitative purpose (Amenta 1964). Quantification of individual component is by scrapping component containing portion of silica gel and performing potassium dichromate reduction test. This is a cumbersome process but the cost and time involved are very less compared to the sophisticated gas chromatography or HPLC which are very expensive.

The purification of omega 3 fatty acids such as alpha linolenic acid can be achieved through crystallization, winterization, HPLC, urea addition and silver ion chromatography (Sajilata *et al.,* 2008). Compared to other techniques, urea crystallization and silver ion chromatography are cheaper and easier to perform. Urea in methanol, on crystallization, forms bonds with saturated fatty acid leaving unsaturated fatty acid in mother liquor (Gunstone *et al.,* 1976). Unsaturated fatty acid can be easily separated by washing the crystals with hexane whereas saturated fatty acid can be separated easily by dissolving in water followed by washing with hexane. The downside of this technique is the lower yields. The other cheap technique is silver ion chromatography (Morris 1966). The silver ions are able to form reversible bond with unsaturated fatty acid. More the unsaturated a fatty acid is, more the bonding, this leads to greater retention in column. Then column is eluted stepwise by pure hexane and hexane-acetone mixture. Preliminary screening for unsaturated fatty acid can be done using either reverse phase TLC or silver ion TLC (Cruz-Hernandez *et al.,* 2004). Reverse phase TLC utilizes silica gel bonded to C18 in which acetonitrile was used as mobile phase. Visualization is achieved through iodine vapor. Other technique is silver ion TLC, in which ordinary TLC plate is impregnated with silver by silver nitrate solution. The

silver ion chromatography gives high yield in comparison to urea crystallization at the same time cheaper than HPLC (Sajilatha *et al.,* 2008).

1.7 Lipid synthesis at cellular level

Denovo fatty acid synthesis occurs in chloroplast. First committing step in fatty acid synthesis is acetyl-CoA carboxylase (ACC) activity which condenses two acetyl CoA to form malonyl CoA. Malonyl-CoA Acyl ACP transacylase (MAT) joins this malonyl CoA to ACP protein. Then 4 carbon molecule is elongated to 16 carbon molecule by fatty acid synthase (type 2 FAS). It has discrete subunits such as ketoacyl ACP synthase (KAS), ketoacyl ACP reductase (KAR), hydroxyacyl ACP Dehydrase (HAD) and enoyl CoA ACP reductase (EAR). In addition to this *Nannochloropsis* possesses type 1 FAS. ACP is either cleaved from acyl moeity by thioesterase/ hydrolase to form fatty acid or directly assimilated into kennedy pathway by glycerol phosphate acyl transferase (GPAT) and lyso phosphatidic acid acyl transferase (LPAT). Fatty acid gets elongated(C>20) in endoplasmic reticulum by cytosolic accase, FAS, desaturase and elongases. Double bonds are added to fatty acid by desaturase enzymes.TAG formation occurs by Kennedy pathway. Generally enzymes of this pathway are considered to be located in endoplasmic reticulum. Recent studies suggest localization of these enzymes in chloroplast also (Fan *et al.*, 2011). GPAT adds first acyl moiety to glycerol backbone. LPAT adds second acyl moiety; LPAT is present in plastid (Li *et al.,* 2012). Phosphatidic acid phosphatase removes phosphate in diacylglycerol leaving diacylglycerol acyltransferase (DGAT) to add 3rd acyl moiety. DGAT is present in endoplasmic reticulum (Li *et al.,* 2012). These acyl moieties are obtained either through denovo fatty acid synthesis or through phosphodiacylglycerol acyltransferase. The latter is called acyl-CoA independent mechanism (Dahlqvist *et al.,* 2000). Certain lipiases and DAG: DAG transacylase, PL/GL: DAG acyltransferase also takes part in this reaction (Li *et al.,* 2012).

1.8 Lipid accumulation

In comparison to sulphate, phosphate and iron stress, the nitrogen deprivation results in higher lipid accumulation (Zienkiewicz *et al.,* 2016). Amount of lipid accumulation is species specific (Gong *et al.,* 2013). Under nitrogen deprivation, the lipid content increases either by increasing the size of triacylglycerol droplet or the number of droplets that are found dispersed in chloroplast

and cytoplasm (Guarnieri *et al.*, 2013). Also nitrogen deprived biomass contains lipids whose fatty acid composition is suitable for good quality biodiesel (Olmstead et al., 2013).

The physiological changes along with lipid accumulation associated with nitrogen deprivation are of importance on the aspect of biorefinery approach and also it throws light on the stress tolerant mechanism of different algae. Decrease in chlorophyll content due to nitrogen deprivation is observed in various microalgal species (Ma *et al.,* 2016; Liu *et al.,* 2012b; Recht *et al.,* 2012; Valenzuela *et al.,* 2013) possibly to reduce reactive oxygen species formation (Mus *et al.,* 2013). The mechanism proposed for triacylglycerol/lipid accumulation behavior during nitrogen deprived condition is the alternative route of NADPH. It is directed to triacylglycerol rather than regular anabolic sink. If not directed to triacylglycerol, reactive oxygen species accumulates which in turn damage the cellular membrane (Liu *et al.,* 2012b) leading to triacylglycerol/lipid accumulation. Also, triacylglycerol production makes available of NADP+. The protein content decreases for nitrogen recycling (Ördög *et al.,* 2016; Bertozzini *et al.,* 2013; Rismani-Yazdi *et al.,* 2012; Kraft *et al.,* 2008). Compared to chlorophyll, protein is 40% of biomass of algae and constitutes 15% of total cellular nitrogen; therefore the degradation of proteins can provide cells to make new enzymes for altered metabolism (Darley 1977). Protein with high nitrogen content like rubisco and ribosomal protein decreases rapidly compared to protein with low nitrogen content (Schmollinger *et al.,* 2014). Carbohydrate is reported to increase in this condition as shown in diatom *Skeltonema*, *Chlamydomonas*, *Neochloris oleoabundans* (Bertozzini *et al.,* 2013; Cakmak *et al.,* 2012). Algal carbohydrates are made of starch and cellulose which can be easily converted to sugar and then to bioethanol/biogas. Carotenoids act as antioxidative agent by screening off excess light and thus preventing unsaturated fatty acid lipid peroxidation (Zhu *et al.,* 2014). An increase in zeaxanthin and decrease in violoxanthin and carotenoids has been reported in *Nannochloropsis oceania*, *Chlamydomonas* and *Dunaliella tertiolecta* (Dong *et al.,* 2013; Cakmak *et al.,* 2012; Mendoza *et al.,* 1999). FAME profile changes in favor of biodiesel quality when subjected to nitrogen deprivation. The reasons behind these changes have to be understood. General trend following nitrogen deprivation is decrease in polyunsaturated fatty acid and increase in monounsaturated fatty acid whereas, saturated fatty acid generally shows no change (Breuer *et al.,* 2012; Olmstead *et al.,* 2013a).

The type of lipid affects the quality of biodiesel. Unlike plants, majority of algal lipids are membrane lipids (Nakamura & Li-Beisson 2016). These membrane lipids are polar in nature consisting of phospholipids and glycolipids. The lesser content of phospholipid and glycolipid and higher content of triacylglycerol make biodiesel production easier since it can skip degumming step in downstream refining process (Olmstead *et al.,* 2013a). Proportion of triacylglycerol and membrane lipid varies from species to species. While freshwater *Chlorella* is reported to have 25% triacylglycerol in total lipids, the *Nannochlorpsis* constitute 40% triacylglycerol (Olmstead *et al.,* 2013a). Phospholipid and glycolipid significantly gets reduced during nitrogen deprivation in *Chlorella* and *Nannochloropsis* (Olmstead *et al.,* 2013a; Xiao *et al.,* 2013).

Gaps in existing research

- The cost of commercial biodiesel production from microalgae is too high.
- There is no proper growth model for microalgae grown in raceway pond.
- Harvesting of microalgae is not optimized.
- The bio-refinery aspect is not explored.
- Improper selection of algal strain.

Objective of the proposed research are as follows

- Isolation, identification and characterisation of native algal strains.
- Optimization of growth parameters for higher lipid production and accumulation.
- Large scale cultivation of microalgae for biodiesel and co-products formation.
- Development of mathematical model for mass cultivation of microalgae.
- Identification of suitable harvesting method, lipid extraction, transesterification and purification of co-product from selected strain.

Chapter 2: Materials and Methods

2.1 Screening and characterization of microalgal strains for growth and lipid production

2.1.1 Isolation and purification of microalgal strains from their natural habitat

The samples were collected from the water bodies located in Shekhawati regions of Rajasthan, India (Geographic co-ordinates: 28°22'N 75°36'E) in the year 2010 and were purified according to the methods of Andersen (2005). Natural samples were taken to the laboratory and 1 ml of the samples were incubated into 100 ml sterilized growth media (composition is given in section 2.1.1.1) in a 250 ml culture flask. The flasks were incubated at temperature 25±1˚C, 16 light/8 dark h cycle and illumination 3,000 lux, until microalgal growth was apparent. From the fully grown culture, 200 µl of sample was taken and streaked on agar plates for strain isolation. The agar plates were prepared by dissolving 1.5 g of agar (Hi-media) in 100ml of BG11 media by heating and then pouring the agar solution in petri dish before solidification. The petri dishes were kept under standard growth conditions, till the formation of colonies on agar surface. The individual colonies observed, were aseptically transferred to the test tubes containing 10 ml of BG11 media.

2.1.1.1 Culture media

BG11 media was used for the isolation and cultivation of microalgal strains and its composition is given in Table 2. 1 and 2. 2. For nitrogen fixing cyanobacterial strains, media without a nitrogen source (sodium nitrate) was used, whereas for the halotolerant strains (*Dunaliella genera*), BG11 media was supplemented with salt (sodium chloride) of 1%.

2.1.1.2 Culture conditions

The cultures were grown in 250 ml conical flask containing 100 ml media under laboratory conditions of 16:8 hours light/dark photoperiod, light intensity of 3000 lux (41 µmol photon m^{-2} s^{-1}) provided by cool white fluorescent tubes and temperature of 25±1˚C. Cultures were manually shaken twice a day. The light intensity was measured using a digital lux meter (HTC, India).

Table 2.1 The composition of BG11 media

Salt	**g L^{-1}**
Sodium nitrate	1.5
Magnesium sulphate	0.075
Di-potassium hydrogen phosphate	0.04
Calcium chloride	0.036
Sodium carbonate	0.02
Citric acid	0.006
Ferric ammonium citrate	0.006
Trace metal solution	1 ml

Table 2.2 The composition of trace metal solution used in BG 11 media

Salt	**g L^{-1}**
Boric acid	2.86
Manganese chloride	1.81
Ethylenediaminetetraacetic Acid	1.00
Zinc sulphate	0.22
Copper sulphate	0.08
Cobalt nitrate	0.05
Sodium molybdate	0.04

2.1.2 Microalgal strain identification

2.1.2.1 Morphological characterization

A light microscope (Olympus,USA) was used to study the morphology of the microalgal cells under 40X and 100X magnification. The cell size was measured using Image J software (NIH, USA). An attachable digital camera (Olympus, USA) was used for capturing the image. The preliminary identification based on morphology was carried out with the help of standard monograph (Bellinger & Sigee 2010).

2.1.2.2 Strain identification through rDNA sequencing and phylogenetic tree construction

Further characterization of the microalgal strain was done with the help of rDNA sequencing, in which the sequence of a portion of internal transcribed spacer (ITS2) region of ribosomal RNA (rRNA) is used to identify a species. The steps involved in this technique are described below.

2.1.2.2.1 Genomic DNA isolation from microalgae

The DNA from microalgae was extracted and purified according to the protocol of Doyle (1989) with minor modification. One ml of the microalgal cells was centrifuged at 6000 rpm for 10 minutes; supernatant was discarded and the pellet was re-suspended in a buffer containing 5% CTAB (cetyltrimethyl ammonium bromide) detergent. Glass beads of 2-5 mm in size were added in the pellet and vortexed for 15 to 30 seconds to rupture the cells. Proteins were removed with the help of phenol-chloroform mixture and DNA was finally precipitated from the aqueous solution with ethanol.

2.1.2.2.2 PCR amplification and sequencing

For the PCR amplification of the genomic DNA, specific primers targeting ITS2 region and partial rRNA gene were selected. The selection was made based on the literature reports, where these primers have been used for algal identification (White *et al.,* 1990). The sequences of the primers are as follows:

Forward: 5’-TCCGTAGGTGAACCTGCGG-3’and
Reverse: 5’-TCCTCCGCTTATTGATATGC-3’

The 25 µl PCR reaction mixture contained the following:

- 25 nanogram of DNA,
- 2 µl of 10X PCR buffer containing 15mM $MgCl_2$,
- 2 µl of 0.25 mM dNTPs,
- 0.5 unit TAQ polymerase
- 1µM of forward and reverse primers each.

Temperature condition was:

- Initial denaturation: 2 minutes at 94 °C
- 30 cycles of following conditions
    - 4 min at 95 °C for denaturation
    - 1 min at 60 °C for annealing
    - 1 min at 72 °C for elongation
- Final elongation: 7 min at 72 °C.

The amplified DNA was visualized by gel electrophoresis technique. Briefly 0.8 g agarose (SD Fine, India) was dissolved in 100 ml TAE buffer by heating and then poured in a gel tray for setting. The gel was placed in electrophoresis tank (Biorad, USA) containing TAE buffer and the apparatus was run at 120 V (voltage) and 100 A (current) for 15 minutes. The amplified DNA, thus obtained by PCR was sent for sequencing by Sanger dideoxy method (Xcelris lab, Ahmedabad, India) for further analysis.

2.1.2.2.3 Sequence search and phylogenetic tree construction

The sequences of the partial rRNA region, obtained for different microalgal strains were analyzed by the NCBI's Basic Local Alignment Search Tool (BLAST). The highly matching sequences to the partial rRNA region having lower expect value (e-value) were retrieved from NCBI database in FASTA format. Then the matching sequences were aligned with our sequence using multiple alignment program of CLUSTALX followed by the construction of phylogenetic tree by neighbor joining alignment method using the same program. The reliability of constructed tree was tested with the method of bootstrap sampling, again available in the same program, with sample size of 1000. The Tree view program was used for visualizing the final tested phylogenetic tree.

2.1.2.3 Compensatory base change analysis

2.1.1.3.1 Annotation of ITS2 region

The sequence of rDNA contains various ITS regions such as ITS1, ITS2, etc. The exact location of ITS2 region has to be identified within the given sequence with correct starting and ending nucleotides. This was done using ITS2 region prediction software available in the following link http://its2.bioapps.biozen trum.uni-wuerzburg.de.

2.1.1.3.2 ITS2 structure prediction

The annotated ITS2 regions of each species were used for predicting their structure using RNA structure software. The structure predicted with lowest free energy was selected for further analysis.

2.1.1.3.3 Structure alignment and prediction of compensatory base change

In order to predict compensatory base changes (CBC) between two ITS2 regions, firstly their structures had to be aligned which was achieved using RNA structure aligning program, '4SALE'. After alignment, the program finds the CBC between all the species and presents the results in the form of matrix.

2.1.3 Preliminary screening of microalgae for lipid productivity

2.1.3.1 Microalgal strains

The organisms used in the present study comprises of already existing algal strains in our laboratory and also strains which were isolated from their natural habitat specifically for the present work. Table 2. 3 provides the description about the microalgal strains selected for the experiment, the nature of their habitat and the media used to culture them under laboratory conditions

2.1.3.2 Specific growth rate and biomass productivity

The specific growth rate and biomass productivity were measured from the growth of the experimental cultures. The growth of algal strains was monitored by measuring their biomass

production over exponential phase of growth. Biomass concentration was derived from the chlorophyll readings by following regression equation

Biomass concentration ($g\ L^{-1}$) = S * O.D (1)

where,

S is slope of line through the points (x1,y1), (x2,y2) and (x3,y3), where x1,x2 and x3 are chlorophyll absorbances and y1,y2 and y3 are known biomass concentrations,

O.D is absorbance of chlorophyll from biomass of unknown concentration.

The resulting regression equations for different species are shown in Table 2. 4.

2.1.3.2.1 Chlorophyll and carotenoids estimation

Chlorophyll and carotenoids were extracted with methanol under dark condition to prevent degradation of the pigment. On completion of extraction (4^{th} hour), algal pellet was removed by centrifugation at 9,000 rpm for 10 minutes and absorbances were recorded at 470, 665 and 650 nm using JascoV-630 spectrophotometer. From which, the chlorophyll a, b and carotenoids were calculated according Lichtenthaler and Wellburn 1983 by following equation

.

Chlorophyll a (Ca, µg/ml) = $(15.65*A_{665})-(7.34*A_{650})$ (2)

Chlorophyll b (Cb, µg/ml) = $(27.05*A_{650})-(11.21*A_{665})$ (3)

Carotenoids (µg/ml) = $[(1000*A_{470})-(2.86*Ca)-(129.2*Cb)]/245$ (4)

2.1.3.2.2 Dry weight measurement

For the dry weight measurement, 100 ml microalgal cells were collected by centrifugation at 11,000 rpm for 2 min at room temperature. The pellets obtained were washed twice with distilled water and dried at 80°C for 48 hrs and weighed on a digital weighing balance.

2.1.3.2.3 Measurement of specific growth rate and biomass productivity

The specific growth rate (µ day^{-1}) of the microalgal strains were calculated by the following equation:

$$\mu = (\ln X2 - \ln X1) / t \quad (5)$$

where,

X2 (g L^{-1}) is the final concentration of the biomass, during the exponential phase,

X1 (g L^{-1}) is the initial concentration of the biomass during the exponential phase,

t (day) is the time duration of the exponential phase.

Biomass productivity (BP mg L^{-1} day^{-1}) represents the amount of biomass produced in a given time. It was calculated as per following formula,

$$BP = (X2 - X1)/t \quad (6)$$

2.1.3.3 Lipid content and lipid productivity

In order to measure lipid content and lipid productivity, the lipid was extracted according to method of Bligh and Dyer (Bligh & Dyer 1959). Equal amount of each algal biomass was harvested by centrifugation at 6000 rpm for 10 minutes; the supernatant was discarded and to the pellet, the Bligh Dyer solvent (Chloroform: methanol: water – 1:2:0.8) was added. To the mixture 1.8 ml water and 1 ml chloroform was added for the phase separation and the organic phase containing the lipid was selectively removed into a glass vial. The solvent was evaporated and the amount of lipid left was measured by weighing. The lipid yield parameters were calculated as follows.

$$L = (W_L/W_b)*100 \quad (7)$$

$$\text{Lipid productivity (mg } L^{-1} \text{ day}^{-1}) = (BP * L)/100 \quad (8)$$

where,

L is the lipid yield in %

W_L is the total lipid weight and W_b is the dry weight of biomass

BP is biomass productivity in mg L^{-1} day^{-}

Table 2.3 The organisms selected for the study

S.No.	Species	Natural habitat	Media
1	*Anabaena doliolum*	Fresh water	BG 11 without nitrate
2	*Westiellopsis*	Fresh water	BG 11 without nitrate
3	*Nostoc calcicola*	Fresh water	BG 11 without nitrate
4	*Nostoc* sp. 1	Fresh water	BG 11 without nitrate
5	*Anabaena cycadeae*	Fresh water	BG 11 without nitrate
6	*Anabaena cylindrica*	Fresh water	BG 11 without nitrate
7	*Tolypothrix*	Fresh water	BG 11 without nitrate
8	*Nostoc muscorum*	Fresh water	BG 11 without nitrate
9	*Anabaena fertilissima*	Fresh water	BG 11 without nitrate
10	*Synechocystis*	Fresh water	BG 11
11	*Nostoc* sp. (g15)	Fresh water	BG 11 without nitrate
12	*Nostoc* sp. (g17)	Fresh water	BG 11 without nitrate
13	*Anabaena* sp. (g14)	Fresh water	BG 11 without nitrate
14	*Anabaena* sp. (g24)	Fresh water	BG 11 without nitrate
15	*Anabaena* sp. (g19)	Fresh water	BG 11 without nitrate
16	*Chlorella minutissima*	Fresh water	BG 11
17	*Dunaliella salina*	Marine	BG 11 with 1 % NaCl
18	*Dunaliella tertiolecta*	Marine	BG 11 with 1 % NaCl
19	*Desmodesmus* sp. MCC34 (natural isolate)	Fresh water	BG 11
20.	*Scenedesmus* sp. MCC26 (natural isolate)	Fresh water	BG 11
21.	*Chlorella* sp. MCC32 (natural isolate)	Fresh water	BG 11

Table 2.4 The regression equation of different species

S.No.	Species	Regression equation of biomass concentration (g L^{-1})
1	*Anabaena doliolum*	O.D *1.51
2	*Westiellopsis*	O.D *1.12
3	*Nostoc calcicola*	O.D * 0.85
4	*Nostoc* sp. (g15)	O.D * 1.3
5	*Nostoc* sp. (g17)	O.D * 0.87
6	*Nostoc* sp. 1	O.D * 0.73
7	*Anabaena cycadeae*	O.D * 0.6
8	*Anabaena* sp. (g14)	O.D * 1.22
9	*Anabaena cylindrica*	O.D * 0.85
10	*Anabaena* sp. (g24)	O.D * 0.47
11	*Tolypothrix*	O.D * 1.46
12	*Anabaena* sp. (g19)	O.D * 0.61
13	*Nostoc muscorum*	O.D * 0.65
14	*Anabaena fertilissima*	O.D * 0.86
15	*Synechocystis*	O.D * 1.05
16	*Scenedesmus* sp. MCC32	O.D * 0.23
17	*Chlorella* sp. MCC26	O.D * 0.67
18	*Dunaliella salina*	O.D * 0.71
19	*Dunaliella tertiolecta*	O.D * 0.61
20	*Chlorella minutissima*	O.D * 0.61
21	*Desmodesmus* sp. MCC34	O.D * 0.26

2.2 Factors affecting lipid productivity

2.2.1 Effect of light intensity

For the study of light intensity on lipid productivity, culture was grown under five light intensities: 14, 27, 41, 54 and 84 μ mol photon m^{-2} s^{-1} (1000, 2000, 3000, 4000 and 6000 lux respectively). A 100 ml media with manual shaking twice a day was implemented and constant temperature of 24±1°C was maintained. The significant difference between the values was analyzed by One way ANOVA followed by Tukey's post hoc honestly significant difference test.

2.2.2 Effect of photoperiod

The algae were exposed to three photoperiods: 24 h Light/0 h Dark (continuous illumination), 9L/15D and 12L/12D h cycle. A 100 ml media with manual shaking twice a day was implemented and constant temperature of 24±1°C was maintained.

2.2.3 Effect of pH and salinity

In-order to check the effects of salinity on lipid productivity, sodium chloride was added to BG11 media in concentration ranging from 0.5 to 1.5% (w/v). The experiments involving the effect of pH on lipid productivity, the pH were set in the range of 6 to 9 using 0.1 N hydrochloric acid and 0.1 N sodium hydroxide. A continuous light intensity of 41 μ mol photon m^{-2} s^{-1} and temperature of 24±1°C were maintained. A 100 ml media with manual shaking twice a day was implemented.

2.2.4 Effect of trophic conditions

The trophic conditions correspond to maintaining a mixotrophic and heterorophic conditions. The media was supplemented with glucose and glycerol as alternate or additional carbon source for algae; glucose and glycerol was tested under two concentrations: 0.5 and 1% (w/v). Photoautotrophically grown algae without addition of glucose or glycerol, served as control. The same cultural conditions as described in the above sections were followed.

2.2.5 Effect of Nutrients

The effects of four different nitrogen sources, $NaNO_3$, $NaNO_2$, NH_4Cl and urea (nitrogen concentrations of 2.9 mM, 0.15 mM, 1.45 mM and 14.6 mM) on lipid productivity were studied, in which nitrogen free BG 11 media served as control. To determine the optimal phosphorus concentration in medium for maximum lipid productivity, four concentrations of di-potassium hydrogen phosphate were tested: 0.002, 0.02, 0.04 and 0.2 g L^{-1}, in which K_2HPO_4 free media served as control. In order to make up the loss of potassium during the variation of K_2HPO_4 in the experiment, an equimolar concentration of KCl was used. To find out the optimal concentration of sulfur, the media was supplemented with 0.015, 0.03, 0.075 and 0.2 g L^{-1} $MgSO_4$, in which $MgSO_4$ free media served as control. In order to make up the loss of magnesium during the variation of $MgSO_4$ in the experiment, an equimolar concentration of $MgCl_2$ was used. Other components such as calcium chloride, ferric ammonium citrate and sodium carbonated were also varied to find the optimum lipid productivity. A continuous light intensity of 41 μ mol photon m^{-2} s^{-1} and temperature of 24±1°C were maintained. A 100 ml media with manual shaking twice a day was implemented.

2.2.6 Lipid accumulation studies through nutrient deprivation

2.2.6.1 Nutrient deprivation

For the study of accumulation of lipid in *Desmodesmus* sp. MCC34, the media was deprived of essential nutrients such as nitrate, phosphate, sulphate and iron. For nitrogen deprived media, the BG 11 was used in which sodium nitrate was replaced with equimolar concentration of sodium sulphate; likewise for the phosphate deprivation, di-potassium hydrogen phosphate was replaced with equimolar concentration of potassium chloride, for the sulphate deprivation, magnesium sulphate was replaced with equimolar concentration of magnesium chloride and for the iron deficiency, ferric ammonium citrate was replaced with equimolar concentration of citric acid and ammonium chloride.. A continuous light intensity of 41 μ mol photon m^{-2} s^{-1} and temperature of 24±1°C were maintained. A 100 ml media with manual shaking twice a day was implemented.

2.2.6.2 Biochemical changes associated with nitrogen deprivation

Four species were selected for this study and they were *Desmodesmus* sp. MCC34, *C. minutissima, D. salina and D. tertiolecta*

2.2.6.2.1 Biomass production

The change in biomass concentration after 10 and 20 days of nitrogen deprivation in four species were determined according to section 2.1.3.2. All the four species were inoculated at a uniform cell density of 0.1 g L^{-1} in the BG 11 medium containing either 2.9 mM nitrogen (control) or 0 mM nitrogen in nitrogen deficient conditions.

2.2.6.2.2 Lipid content

The lipid content was measured as given in previous section 2.1.3.3

2.2.6.2.3 Chlorophyll and carotenoid estimation

The estimation of chlorophyll and carotenoids were carried out as given in section 2.1.3.2.1.

2.2.2.6.4 Protein estimation

Protein was estimated using Lowry method (Lowry *et al.,* 1951). 0.5 M sodium hydroxide and 0.8 M sodium carbonate were mixed in 1 liter of de-ionized water to make reagent A. 10 mM sodium potassium tartrate and 40 mM copper sulphate were mixed in de-ionized water to make reagent B. 50 ml of Reagent A was added to 1 ml of Reagent B to make reagent C. Then 2 ml of reagent C was added to the algal cells, followed by 5 ml of 1 N Folin's reagent to obtain a blue coloration. The microplate reader (Stat fax 2100, Awareness technology Inc., USA) was used for measuring absorbance at 630 nm.

2.2.2.6.5 Carbohydrate measurement

The carbohydrate was measured using phenol sulphuric acid method (Masuko *et al.,* 2005). The microalgal exopolysaccharide secretion was removed by centrifugation (5,000 rpm for 10 minutes) and the algal pellet was washed three times with de-ionized water. A 50 µl of 80% phenol was added to the sample, followed by, addition of 2 ml concentrated sulphuric acid to develop a yellow

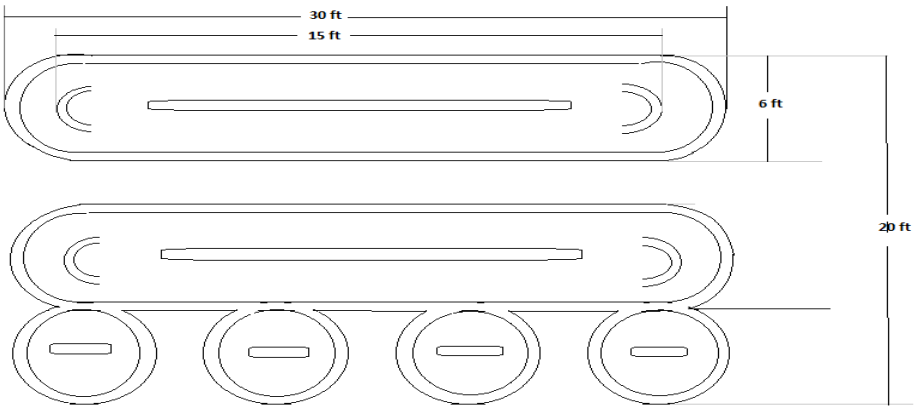
colour. The microplate reader (Stat fax 2100, Awareness technology inc, USA) was used for measuring absorbance at 492 nm.

2.2.7 Mass cultivation of *Desmodesmus* sp. MCC34

The plastic tubs with a capacity of 10 L and baby pools with a capacity of 100 L were used for pilot scale culturing as well as for preparing inoculums for raceway pond in green house, under natural light and temperature conditions (Figure 2.1).

The mass cultivation was carried out in 1000 L capacity raceway pond. The structure of the pond is shown in Figure 2.2, where a green net was used for covering the raceway pond in order to prevent photo-inhibition effects of the sunlight. Also shown in figure is the polyhouse, inside which the raceway pond is present. The light and temperature prevailing in the region of Pilani, Rajasthan, India for period of 12 months is given in Table 2.5. The media (BG11) was inoculated with 10% inoculum prepared in green house as well as in raceway adjacent circular ponds. The mixing was provided manually twice a day, as shown in Figure 2.2 (C). A constant water level was maintained throughout the cultivation period.

Figure 2.1 The pilot scale cultivation in green house. (A) 10 liter plastic tubs (B) 100 liter baby pools (C) The green house.



A

Figure 2.2 Mass cultivation by raceway pond (A) Blueprint of raceway pond (B) Poly house (C) *Desmodesmus* sp. MCC34, being manually mixed in raceway pond.

Table 2.5 The average temperature and radiation for different months during the year 2013 in Pilani

	January	February	March	April	May	June	July	August	September	October	November	December
Max Temp. (°C)	30.0	34.1	40.6	45.6	47.2	46.7	45.0	42	40.6	39.4	36.1	29.3
Min Temp. (°C)	−0.6	1.6	4.4	10.7	15.2	18.9	20.3	20.7	17.3	9.4	3.9	1.1
Avg. Temp. (°C)	14	17	23	28	32	34	31	30	29	26	20	16
Average solar radiation (Mj m^{-2} day^{-1})	20	24	29	34	36	35	29	28	27	24	20	18

2.2.8 Growth model for the raceway pond

Various factors affect the growth of algae in a raceway pond, namely; intensity and duration of light, shading, humidity, temperature, substrate concentration, etc. (Gharagozloo *et al.,* 2014; Huesemann *et al.,* 2016). Therefore a model was generated, to determine the effect of various factors on the growth of microalgal strains in a raceway pond.

The biomass concentration obtained in the raceway pond at any given time is explained by the formula,

$$X = X_0 * \exp(\mu net * t) \quad (9)$$

where X_0 and X are the initial and final concentration of the biomass at time zero and time "t" & the net specific growth rate is denoted as $\mu_{net.}$

The net specific growth rate, μ_{net} is explained by

$$\mu_{net} = \mu_{max} * f(L) * f(S) * f(t) \quad (10)$$

where μ_{max} is maximum specific growth rate and f(t), f(s) and f(L) are the temperature, substrate and light intensity functions respectively.

Function f(S) is given by the Monod equation and described as

f(S) is equal to 1 if S greater than 2 times of K_S

or

$f(S) = S/(K_S + S)$ if S is lesser than 2 times of K_S (11)

where 'S' is the concentration of the given substrate and K_S is the Michaelis Menten constant. The Michaelis Menten constant is the concentration of a given substrate when the reaction rate is at the half maximal.

The Steeles equation explains the light function f(L) in better fashion than a Monod model since former incorporates the Beer lamberts law to explain the changes in light intensity along the water column. According to the equation,

f(L) is equal to 1 if intensity of light is greater than I_s

or

$f(L) = I_Z/I_s * e^{1-(I_Z/I_s)}$ if intensity of light is lesser than I_s (12)

where the intensity of light at depth Z is given by I_Z and the optimum intensity of light for growth is given by I_s.

The temperature f(t) is explained by

f(t) is equal to zero if given temperature is less than minimum temperature survived by the species i.e., $T<T_{min}$

or

$$f(t) = \frac{(T-T_{max})(T-T_{min})^2}{((T_{opt}-T_{min})[(T-T_{opt})(T_{opt}-T_{min})-(T_{opt}+T_{min}-2T)(T_{opt}-T_{max}))} \quad (13)$$

or

f(t) is equal to zero if given temperature is higher than the maximum temperature that species can survive.

where T_{min}, T_{max} and T_{opt} are minimum, maximum and optimal temperature for the tested microalgae

Based on above equations, the following equation can be formed in order to calculate the concentration of biomass on any given day based on temperature and light conditions.

$$X = X_0*\exp(\mu_{max}*Sn/(K_{Sn}+Sn)*(Sp/(K_{Sp}+Sp)*Ss/(K_{Ss}+Ss))*(I_Z/I_s*\exp(1-(I_Z/I_s))*(((T-T_{max})(T-T_{min})^2)/((T_{opt}-T_{min})*[(T-T_{opt})*(T_{opt}-T_{min}))-((T_{opt}+T_{min}-2T)(T_{opt}-T_{max}))])) \quad (14)$$

2.3. Characterization of lipid extract for biodiesel and alpha linolenic acid methyl ester production

2.3.1 Fatty acid methyl ester separation and the column performance

2.3.1.1 Fatty acid methyl ester (FAME) preparation

The gas chromatographic technique requires the conversion of fatty acids as fatty acid methyl ester (FAME) for their analysis; therefore a prior sample preparation was done to generate fatty acid methyl esters. For production of fatty acid methyl esters (FAME), 3% methanolic HCl was added to the extracted lipid and the mixture was incubated at 70°C for 2 hours followed by the addition of equal volume of hexane and water. Mixture was vortexed vigorously for few seconds. The top hexane layer containing FAME was carefully transferred into separate vial.

2.3.1.2 FAME analysis

Gas Chromatography (GC-2010, Shimadzu) was used for the analysis of FAME and two types of columns were selected to standardize the separation process.

Capillary column

A highly polar biscyanopropyl capillary column (SP-2560, 0.2 um x 100m x 0.25mm ID, Agilent, USA) was used. The temperature of the column was maintained between 140 and 200°C, at a rise of 1°C per minute and the temperature of injector was set at 260°C. The flame ionization detector was maintained at a temperature of 270°C. The carrier gas used was nitrogen with a flow rate of 1.21 ml min^{-1}. A mixture containing 36 components of fatty acid methyl ester (Supelco, USA) was used as standard for analysis of FAME.

Packed Column

DEGS Column (3 m long and 3 mm internal diameter, Nucon Engineers, India) coated with 10% diethylene glycol succinate was used. The carrier gas was nitrogen, along with air and hydrogen acting as fuel, the flow rates of both the gases was maintained around 30 to 40 ml min^{-1}. The temperatures of oven, detector and injector were set as 190, 230 and 240°C respectively. The same standards used in capillary column were also used here for the detection of FAME.

2.3.2. Mass spectra analysis of important fatty acid methyl esters

The identification of fatty acid methyl esters were done with mass spectrometer (Shimadzu; GC-MS-QP-2010 plus, Japan), having following settings; the temperature of source and quadruple were 230 and 155°C, operating in the electrospray ionization mode at 70 ev and m/z generated for the ions in the range 50 - 500. The spectral data of standard compounds available from NIST (National Institute of standards and technology) library and AOCS (American Oil Chemists society) website, were used to interpret the mass as well as the fragmentation pattern of the FAME.

2.3.4 Biodiesel property of the selected microalgae

Biodiesel property such as cetane number and iodine value was calculated from percentage of fatty acid methyl esters by empirical equation (Sinha *et al.,* 2016).

$$\text{Iodine value } (IV) = \Sigma\ (254*D*N)/M \quad (15)$$

$$\text{Cetane number } (CN) = 46.3+ (5458/ (\Sigma\ (560*N)\ /M)) - 0.225*IV \quad (16)$$

where N is percentage of each fatty acid, M is molecular weight of each fatty acid and D is number of double bond of each fatty acid.

2.3.5 Alpha linolenic acid methyl ester separation and characterization

2.3.5.1 Alpha linolenic acid methyl ester (ALAME) separation and identification through mass spectra

ALAME was separated from its closed related isomer gamma linolenic acid methyl ester (GLAME) and other FAME by biscyanopropyl capillary column of gas chromatography as detailed in above section 2.3.1.2. The identification of ALAME compound through mass spectroscopy was carried out under same operating condition as detailed in section 2.3.2.

2.3.5.2 Identification through NMR

The ^{1}H NMR spectra of purified ALAME was obtained by Bruker advance 400 MHZ spectrometer. The tetramethylsilane was used as internal standard. The chemical shifts were recorded in parts per million. The solvent in which sample was dissolved was deutreated chloroform.

2.3.6 ALAME production

ALAME content in 100g of dry biomass was calculated according to following equation

ALAME in 100g biomass (g) = % ALAME in FAME

* FAME content in 100 g biomass (17)

2.3.7 Purification of alpha linolenic acid methyl ester

2.3.7.1 ALAME preparation

The ALAME was prepared by adding 1 ml of 3N methanolic HCl to 0.1 g of lipid extract and incubating at 80°C for 3 h. After which, 1 ml hexane and 1 ml water were added to the mixture and vortexed vigorously for few seconds. The top hexane phase, containing ALAME was transferred into a separate vial.

2.3.7.2 Reverse phase thin layer chromatography

The readymade reverse phase thin layer chromatographic plates, bonded with c-18 silica gel (Merck, India) was also used to separate the ALAME from unsaturated fatty acids and their ester derivatives, using acetonitrile as the mobile phase. The visualization was done by exposing the plate to iodine vapors.

2.3.7.3 Silver ion thin layer chromatography

The silver impregnated thin layer chromatography plates were prepared by dipping ready-made TLC plate (Merck, India) in 4% methanolic silver nitrate solution (methanol/water-9/1,v/v) with hexane/diethyl ether (9/1,v/v) as mobile phase which was used for visualization of purified ALAME as well as other unsaturated fatty acids.

2.3.7.4 Purification of ALAME by silver ion chromatography

For ALAME and FAME purification, *Desmodesmus* sp. MCC34 biomass was used and performed using argentated (silver ion) column chromatography as described earlier with minor modification (Sajilata *et al.,* 2008). The solvent used for elution was petroleum ether (Boiling point 40 to 60°C) with combination of acetone. A 100 mg of crude FAME was loaded which is less than 5% of packed column weight. The column used was 1cm in diameter and 7 cm in height. The silver impregnated silica gel column was sequentially eluted twice with 5 ml of 100% petroleum ether (Eluent A and B), followed by two times elution with 5 ml solvent mixture (99% petroleum ether and 1% acetone; Eluent C and D) and finally three times elution with 5 ml solvent mixture (98% petroleum ether and 2% acetone; Eluent E, F and G). Table 2. 6 shows the composition of eluent used in silver impregnated silica gel column. The silver impregnated thin layer chromatography plates were prepared by dipping ready-made TLC plate (Merck, India) in 4% methanolic silver nitrate solution (methanol/water-9/1,v/v) with hexane/diethyl ether (9/1,v/v) as mobile phase which was used for visualization of purified ALAME as well as other unsaturated fatty acids.

Table 2.6 Composition of eluent used in silver impregnated silica gel column

Solvent Name	**Composition**	**Volume (ml)**
A	petroleum ether	5
B	petroleum ether	5
C	99% petroleum ether and 1% acetone	5
D	99% petroleum ether and 1% acetone	5
E	98% petroleum ether and 2% acetone	5
F	98% petroleum ether and 2% acetone	5
G	98% petroleum ether and 2% acetone	5

2.4 Optimization of downstream processes for biodiesel and alpha linolenic acid production

2.4.2 Harvesting of algal biomass

Two methods were experimented for the efficient harvesting of the microalgal strains.

2.4.2.1 Harvesting by filtration using flocculation aid

Ferric chloride and chitosan were used as the flocculating agent to flocculate algae in the present study. Several concentrations of $FeCl_3$ and chitosan were added to 5 ml of culture, hand shaken for few seconds and left still for 30 minutes. An aliquot was taken at a height of two-thirds from the bottom. The optical density (OD) of the aliquot was measured at 685 nm to evaluate the flocculation activity (Lv *et al.*, 2016). The flocculating activity was calculated using the following equation:

Flocculating activity = (1-A/B) * 100 (18)

where, A is the optical density at 685 nm of the sample taken at $2/3^{rd}$ height after flocculation and B is the optical density at 685 nm of the algal culture before the flocculation.

2.4.2.2 Harvesting by filtration without flocculation aid

In order to look at the possibility of harvesting without any flocculation aid, a simple filtration experiment was carried out on direct algal cultures using muslin cloth of average pore size 2 mm. Filtration efficiency or the amount of biomass filtered was calculated by following equation,

Filtration efficiency /Biomass filtered % = (1-A/B) * 100 (19)

where, A is the optical density of filtrate at 685 nm and B is the optical density of the algal culture before the filtration measured at 685 nm.

2.4.3 Optimization of lipid extraction protocol

2.4.3.1 Screening of solvents for lipid extraction

For screening of solvents for lipid extraction, *Desmodesmus* sp. MCC34 was used. The solvents used for screening are given in Table 2. 7. For lipid extraction by wet biomass, 25 ml of the culture volume was centrifuged. Supernatant was discarded and to the pellet, 5ml of respective solvent was added. This was left for 24 hour. Next day solvent containing lipid was transferred to a fresh vial and solvent was evaporated. For lipid extraction from dry biomass, 50 mg of algae was taken in culture tube. To this 5ml of solvent was added. This was left for 24 hour. Next day solvent was decanted to a fresh vial and solvent was evaporated. In case of ternary solvent system such as Bligh-Dyer mix (Chloroform: Methanol: Water-1:2:0.8) and binary solvent system such as Folch (Chloroform: Methanol-2:1), usual phase separation was not carried out. The dried lipid extract was subjected to transesterification and the FAME obtained by this reaction was subsequently quantified as given in previous sections.

2.4.3.2 Effect of cell lysis on lipid extraction

A concentrated algal (*Desmodesmus* sp. MCC34) solution of 10 g biomass of per liter was used for the present study. Cell lysis was carried out using different methods. Sonication was done using probe sonicator. The frequency was maintained at 15 KHz for the time span of 4 minutes. Autoclaving of cells were carried out at 121 C for 15 minutes at a pressure of 1.5 MPa. Automated microwave oven set at frequency of 2000 MHz, temperature of 200°C, time span of 4 minutes were used for applying microwave radiation. Water was added once in 30 seconds to avoid complete drying out of algae during this procedure as higher temperature results in constant evaporation.

2.4.3.3 Factors affecting lipid extraction

2.4.3.3.1 Solvent biomass ratio

To study the effect of SBR, ml g^{-1}, on lipid extraction of *Desmodesmus* sp. MCC34, a 3, 7, 13 and 28 ml of Folch solvent (Chloroform: methanol – 2:1 v/v) were added to 1 g biomass placed in flat

bottomed test tubes of 1.7 cm internal diameter. Then, the lipid was measured at varying point of time.

2.4.3.3.2 Surface Area factor

The surface area of biomass expose to bulk solvent was found to affect lipid extraction. Therefore a term is introduced to quantify the surface area effects. The term is the surface area factor (SAF, cm^2 ml g^{-1}) which is calculated as,

SAF = (surface area of total biomass exposed *
total volume of solvent) / (weight of biomass) (20)

The desired surface areas of total biomass were achieved by loading 1 g of biomass in different sizes of flat bottomed test tubes and beaker. For example, 1 g of dry biomass loaded in a test tube of 1 cm internal diameter has a lower surface area exposure of 0.79 cm^2 to the bulk solvent whereas the same biomass loaded in a beaker of 4 cm internal diameter has a higher exposure of 12.6 cm^2 (Figure 2. 3). Throughout this experiment, a constant solvent biomass ratio (SBR) was maintained by adding 5 ml of Folch solvent. The lipid was measured at varying point of time.

2.4.3.4 The static vs mixing extraction

Static extraction method involves adding solvent to the biomass and incubating it at room temperature without stirring until lipid extraction is attained to the maximum. For the extraction, 1 g of dried biomass of *Desmodesmus* sp. MCC34 was added to 5 ml of Folch solvent in a closed beaker having internal diameter of 4 cm and incubated at room temperature without shaking. For comparison purpose, lipid extraction was carried by stirring the biomass with solvent at 200 rpm at room temperature. Lipid was measured at varying point of time. Experiment was done in triplicates.

Table 2.7 List of solvents screened for lipid extraction from *Desmodesmus* sp. MCC34

S.No	Solvent	Reference
1	Chloroform:Methanol-2:1	Folch *et al.*, 1957
2	Petroleum ether:Toluene-1:2	Xu & Mi 2011
3	Dichloromethane:Methanol-2:1	Xu & Mi 2011
4	Dichloromethane:Methanol-1:1	Xu & Mi 2011
5	Methanol: Methyl tertiary butyl ether-1.5:5	Sheng *et al.*, 2011
6	Ethyl acetate:ethanol-1:1	Sheng *et al.*, 2011
7	Ethyl acetate:ethanol-2:1	Sheng *et al.*, 2011
8	Ethyl acetate:hexane-2:3	Ryckebosch *et al.*, 2013
9	Hexane:Ethanol-1:2.5	Sheng *et al.*, 2011
10	Hexane:Ethanol-1:0.9	Sheng *et al.*, 2011
11	Toluene:Methanol-3:1	Xu & Mi 2011
12	Dichloromethane:ethanol-1:1	Ryckebosch *et al.*, 2013
13	Hexane:Isopropanol-2:3	Sheng *et al.*, 2011
14	Toluene:Methanol-1:1	Xu & Mi 2011
15	Hexane:Isopropanol-3:2	Sheng *et al.*, 2011
16	Chloroform:Methanol:Water-1:2:0.8	Bligh & Dyer 1959
17	Petroleum ether:Toluene-3:1	Xu & Mi 2011
18	Methyl tertiary butyl ether	
19	Petroleum ether	
20	Ethanol	
21	Chloroform	
22	Isopropanol	
23	Ethyl acetate	
24	Toluene	
25	Hexane	
26	Methanol	
27	Acetone	
28	Butanol	
29	Dichloromethane	

2.4.3.5 Analysis of lipids by Thin Layer Chromatography (TLC)

Thin layer chromatography was used for the qualitative as well as quantitative analysis of the lipids. The readymade TLC plates (Merck, India) were used for separating the compounds, with the help of hexane: diethyl-ether: glacial acetic acid (8:2:0.2) as mobile phase. Identification of the spots on the TLC plates was achieved by staining them with iodine vapor.

The lipid samples were quantified with the help of preparative TLC plates using dichromate reduction method. For making the preparative plates, 10 g of silica gel (Himedia, India) was mixed with 30 ml of de-ionized water to make a free flowing slurry. This slurry was quickly poured on a glass plate (12cm x 9cm) to form an even surface, it was air dried and finally incubated in an oven at 100^{o}C for one hour to activate the plates. In order to quantify the lipids, following steps were followed as described by Amenta, 1964: the standards and samples were spotted on the TLC plate; plate was run using the mobile phase (hexane: diethyl-ether: glacial acetic acid in the ratio of 8:2:0.2), till the solvent front reached 3/4th of the plate height; plates were dried in-order to evaporate the mobile phase; one part of the plate containing the sample was covered with aluminum foil, followed by exposure of the half covered plate to the iodine vapor (Figure 2. 4). The final step was to scrap carefully the sample portion of silica gel corresponding to standard spot as identified by iodine vapor. This scrapped silica gel was transferred to a test tube and 2 ml of 2% potassium dichromate in concentrated sulphuric acid was added to it. The solution was heated at 80°C in oven with intermittent mixing for 1 hour. The spectrophotometric absorbance was taken at 405 nm using microplate reader (Awareness Inc., USA), to quantify the lipids.

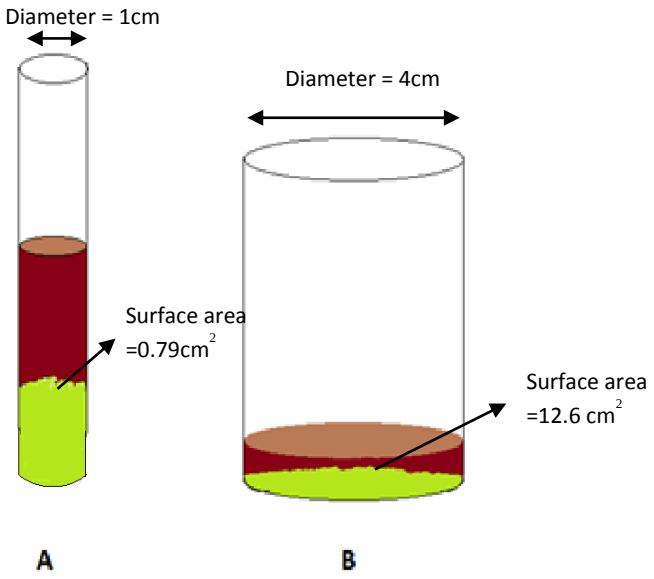


Figure 2.3 Surface area of total biomass exposed to bulk solvent in two different setups. A represents 1 g of biomass in a test tube of internal diameter 1 cm giving 0.79 cm^2 and B is same 1 g of biomass in a beaker of 4cm internal diameter giving 12.6 cm^2 exposures.

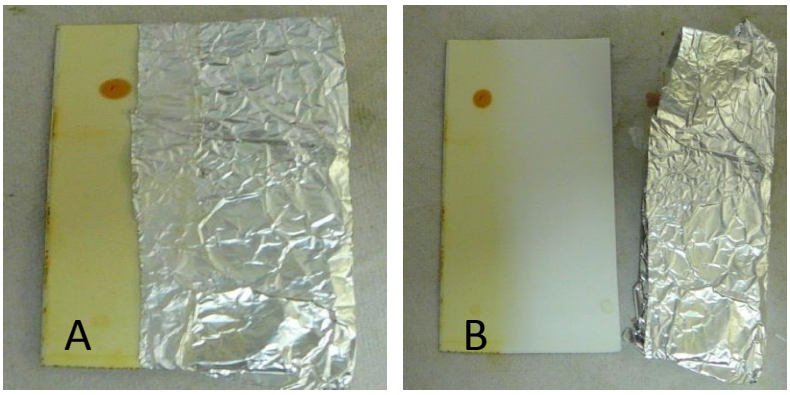


Figure 2.4 Quantitative analysis of lipids by preparative TLC plates (A) The covering of half the plate with aluminium foil and (B) iodine vapor staining of half the portion.

2.4.3.5 Kinetic Study

The lipid extraction from any given algae follows first order rate kinetics as described by Ficks law. The equation is as follows

$$K \frac{dL}{dt} = (M-L) \quad (21)$$

where K is mass transfer coefficient (min^{-1}), t is time (min), L is lipid concentration at given time (% of the dry biomass), M is maximum amount of lipid extractable from biomass (% of the dry biomass).

The above differential equation was solved to derive the value of K. The Patricelli model also describes lipid extraction but includes two steps instead of diffusion alone (McConnell & Farag 2013). The first step is the washing step which involves instant lipid extraction from outside cell surface. The second step is the diffusion process which depends on concentration gradient of solute across the cell wall. The equation is as follows

$$\frac{dL}{dt} = M1\,(1-\exp(-k_1 t)) + M2(1-\exp(-k_2 t)) \quad (22)$$

where M1 is maximum attainable lipid from the washing process, M2 is maximum attainable lipid from the diffusion step, k_1 is the washing step's mass transfer coefficient (min^{-1}) and k_2 is the diffusion process's mass transfer coefficient (min^{-1}). The kinetic parameters were obtained by solving the above differential equation.

2.4.4 Transesterification reaction

The factors affecting transesterification reaction such as temperature, catalyst amount, solvent-oil ratio and time were optimized. For the entire study, the lipid extracted from wet biomass of *Desmodesmus* sp. MCC34 by Folch's solvent mixture, yielding 40±2 mg lipid g^{-1} biomass was used. Experiments were conducted in triplicates and during the study of each individual factor, other factors were kept constant. For acidic transesterification, HCl was used as catalyst and the common conditions were solvent to oil ratio (ml/g) of 5, catalyst of 2% of solvent volume, time of 15 minutes and temperature of 50°C. The alkaline transesterification was carried out using KOH as catalyst and the common conditions were solvent to oil ratio (ml/g) of 500, catalyst of 2% of solvent volume, time of 15 minutes and temperature of 40°C.

Chapter 3: Results and Discussion

3.1 Screening and characterization of microalgal strains for growth and lipid production

3.1.1 Isolation and purification of microalgal strains from their natural habitat

Three pure microalgal strains were isolated from the natural samples through streak plate technique. They were subjected to microscopic observation and strains were identified and characterized based on their morphological characters, followed by sequencing. After which, strains were deposited in Microbial Culture Collection in IARI, New Delhi and obtained accession number as MCC34, MCC32 and MCC26.

3.1.2 Microalgal strain identification

3.1.2.1 Morphological characterization

Desmodesmus sp. MCC34

The organism formed two cell colony under normal conditions, while four cell colonies were seen when organism was subjected to nitrogen deprived conditions. The coenebia were arranged linearly and the cells were not covered with any mucilaginous sheath (Figure 3. 1 A). Cells were oval shaped and had spines at four corners of the unit. On an average, the individual cell's size was found to be 4.6 µm which had typical green color pigmentation. These characters suggest the organism belong to the class chlorophyta, family *Scenedesmacae* and genera *Desmodesmus*.

Scenedesmus sp. MCC32

Desmodesmus and *Scenedesmus* are closely related species that can been distinguished based on the presence and absence of spine (Fawley *et al.*, 2011). *Desmodesmus* had spines whereas *Scenedesmus* lack spines (Figure 3. 1 B). Other morphological features used for identification were presence of two cell colony structure instead of four cells as seen in *Desmodesmus*. The cells were oval shaped but not as elongated as *Desmodesmus*. The individual cells on an average were 15 µm in size which is approximately three times larger than *Desmodesmus* sp. MCC34. The coenebia, arranged in linear manner was not surrounded by mucilaginous sheath and had green pigmentation. These characteristics, suggests that it belongs to genera *Scenedesmus* of family *Scenedesmacae*.

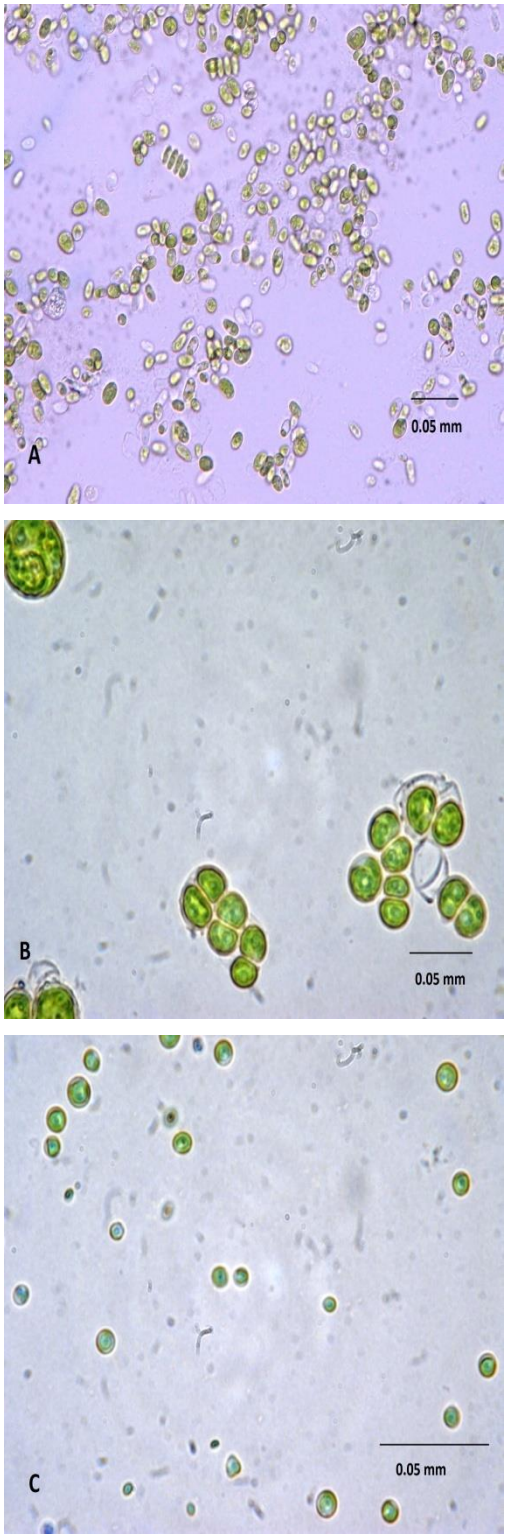


Figure 3.1 Light microscopy observations of (A) *Desmodesmus* sp. MCC34, (B) *Scenedesmus* sp. MCC32 and (C) *Chlorella* sp. MCC26.

***Chlorella* sp. MCC26**

Cells were round in shape with no colony forming tendencies (Figure 3.1 C). On an average, the individual cells were of 2.5 um in size. It had typical green pigmentation with no mucilaneous sheath around them. These characters suggest that it belongs to genera *Chlorella* of family *Chlorellaceae*.

3.1.2.2 Strain identification through rDNA sequencing and phylogenetic tree construction

Further characterization of the algal strain was done through rDNA sequencing as mentioned in section 2.1.1.2. Following which the sequences were submitted to the nucleotide repository, Genbank of NCBI. The details of the annotation of these three sequences are as follows

- ✓ *Scenedesmus sp*. SSKV3/MCC32: internal transcribed spacer (ITS) 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence; 680 bp linear DNA; Accession: KF731761.1 GI: 568218861
- ✓ *Desmodesmus sp*. SSKV2/MCC34: ITS 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and ITS 2, partial sequence; 560 bp linear DNA; Accession: KF731760.1 GI: 568218860
- ✓ *Chlorella sp*. SSKV1/MCC26: ITS 1, partial sequence; 5.8S ribosomal RNA gene and ITS 2, complete sequence; and 28S ribosomal RNA gene, partial sequence; 735 bp linear DNA; Accession: KF731759.1 GI: 568218859

When these sequences were compared with sequences present in the database, it was found that the *Scenedesmus sp*. SSKV3/MCC32 had 99% similarity with *Scenedesmus rubescens* and *Scenedesmus reticulata*. It also shared a close relationship with other sequences of *Scenedesmus* genera, as shown in phylogenetic tree (Figure 3.2). The *Desmodesmus sp*. SSKV2/MCC34 sequence had 98% similarity with *Desmodesmus* sp. IB-01. The phylogenetic tree data suggests that this sequence also shared a close relationship with other sequences of *Desmodesmus* genera (Figure 3.3). The *Chlorella sp*. SSKV1//MCC26 sequence had only 87 % similarity with *Chlorella sorokiniana*. The construction of phylogenetic tree suggests that this sequence shared a close relationship with other sequences of *Chlorella* genera (Figure 3.4). After confirming their identity through microscopic observations and rDNA sequencing, all species were deposited in CCUBGA, IARI microbial culture collection, New Delhi, India.

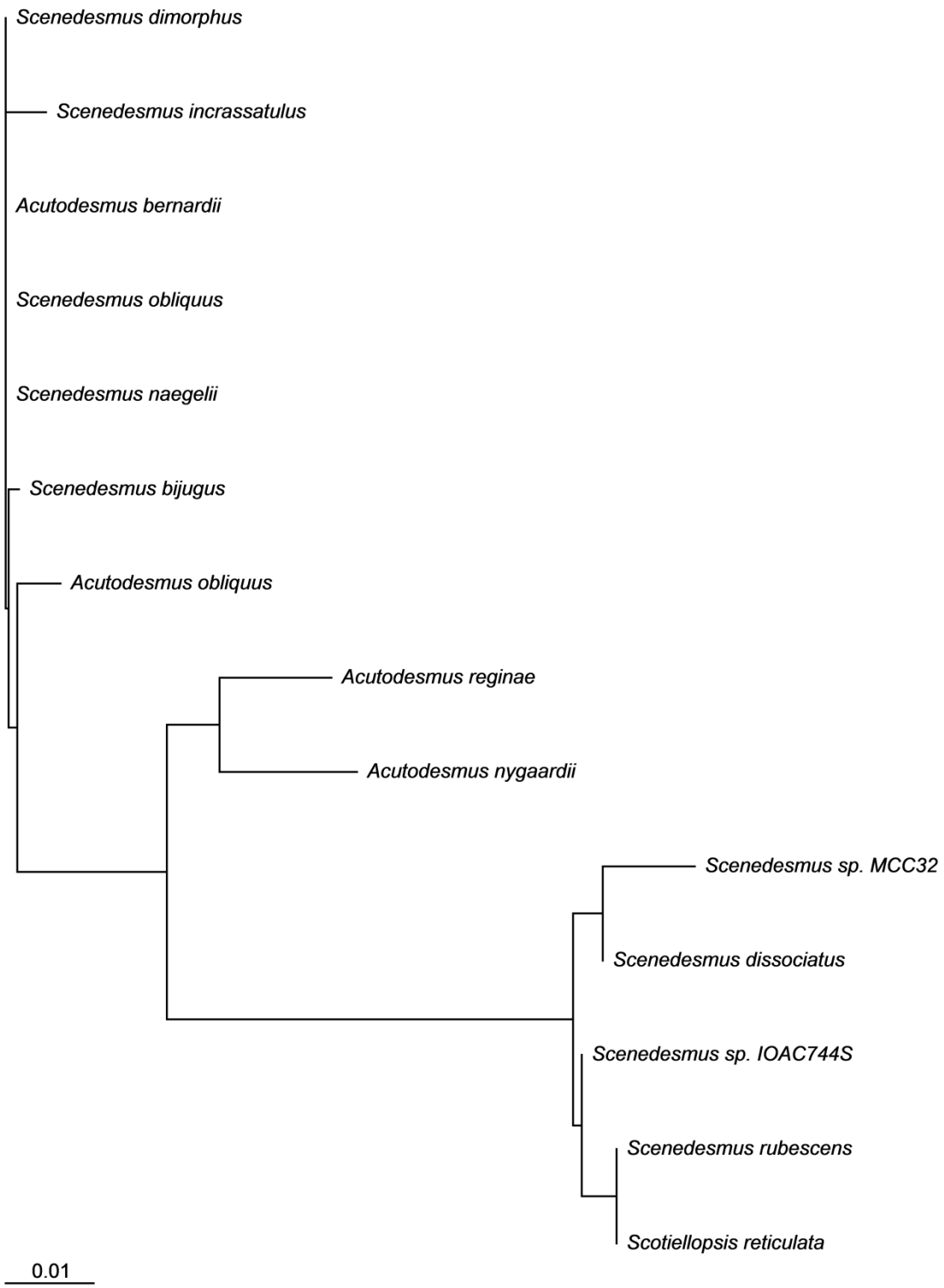


Figure 3.2 Phylogenetic tree for *Scenedesmus* sp. MCC32, constructed by neighbor joining alignment method of CLUSTALX program.

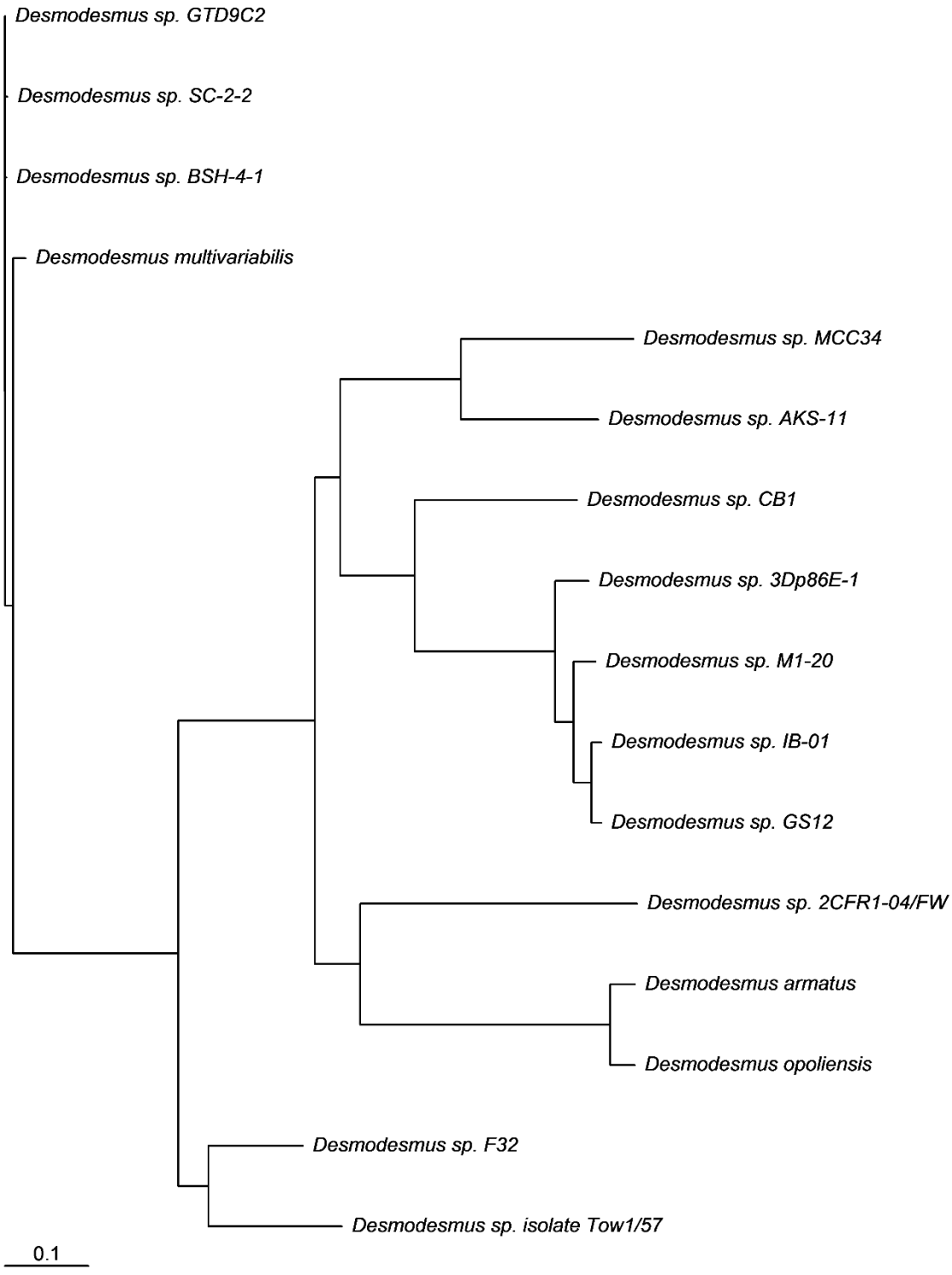


Figure 3.3 Phylogenetic tree for *Desmodesmus* sp. MCC34, constructed by neighbor joining alignment method of CLUSTALX program.

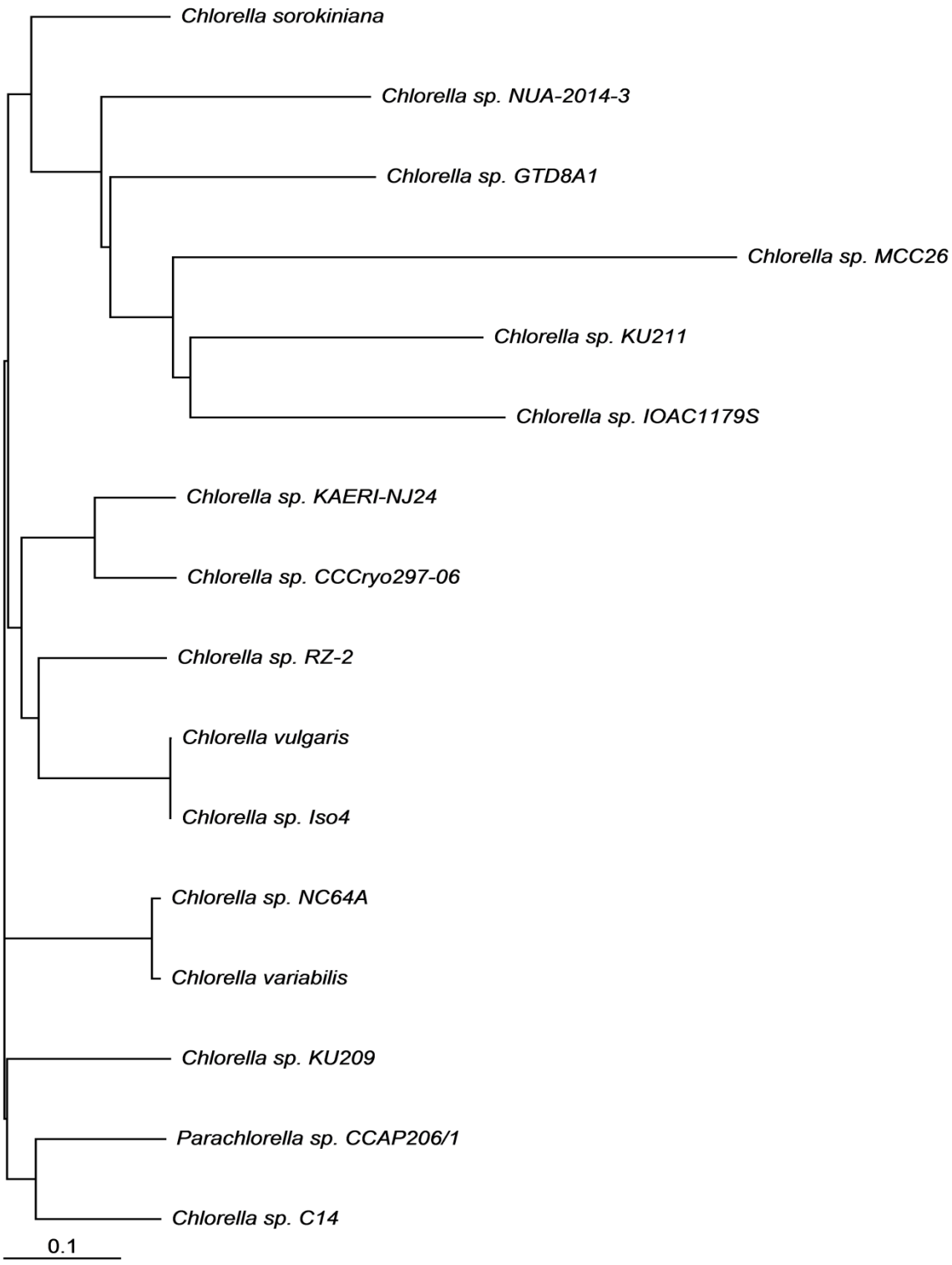


Figure 3.4 Phylogenetic tree for *Chlorella* sp. MCC26, constructed by neighbor joining alignment method of CLUSTALX program.

3.1.2.3 Compensatory base change analysis

Chlorella sp. SSKV1/MCC26 sequence showed only 87% similarity to *Chlorella sorokiniana*, therefore, to confirm the novelty, it was subjected to compensatory base change (CBC) analysis. Two species are different if it has at least one compensatory base changes in ITS2 region (Müller *et al.,* 2007). In-order to identify compensatory base changes, the sequence was first analyzed by BLAST followed by annotation of ITS2 region in rRNA. A series of ITS2, rRNA structures were predicted for the annotated ITS2 region using RNA structure software and the free energy values were calculated (Table 3.1). The structure predicted with a low free energy was selected for further analysis.

Structure alignment followed by the prediction of compensatory base change using "4SALE" program led to the identification of at least one compensatory base change for *Chlorella* sp. MCC26 among all closely related *Chlorella* species (Table 3.2). Figure 3.5 shows the ITS2 structure and location of compensatory base changes (CBCs). The nucleotide pairing of guanine to cytosine in helix II of ITS2 structure as shown by pointed arrows in Figure 3.5, is a unique base pairing found only in *Chlorella* sp. SSKV1 / MCC26 whereas other *Chlorella* species has adenine to uracil base pairing. This unique base pairing gave rise to CBC and indicates that *Chlorella* sp. SSKV1 / MCC26 species is indeed a novel species.

3.1.3 Preliminary screening of microalgae for lipid productivity

Lipid productivity is a key parameter to look in algae for biodiesel and alpha linolenic acid production. Lipid productivity combines the specific growth rate, biomass productivity and lipid content; therefore all these parameters were measured as a preliminary step towards screening for lipid productivity.

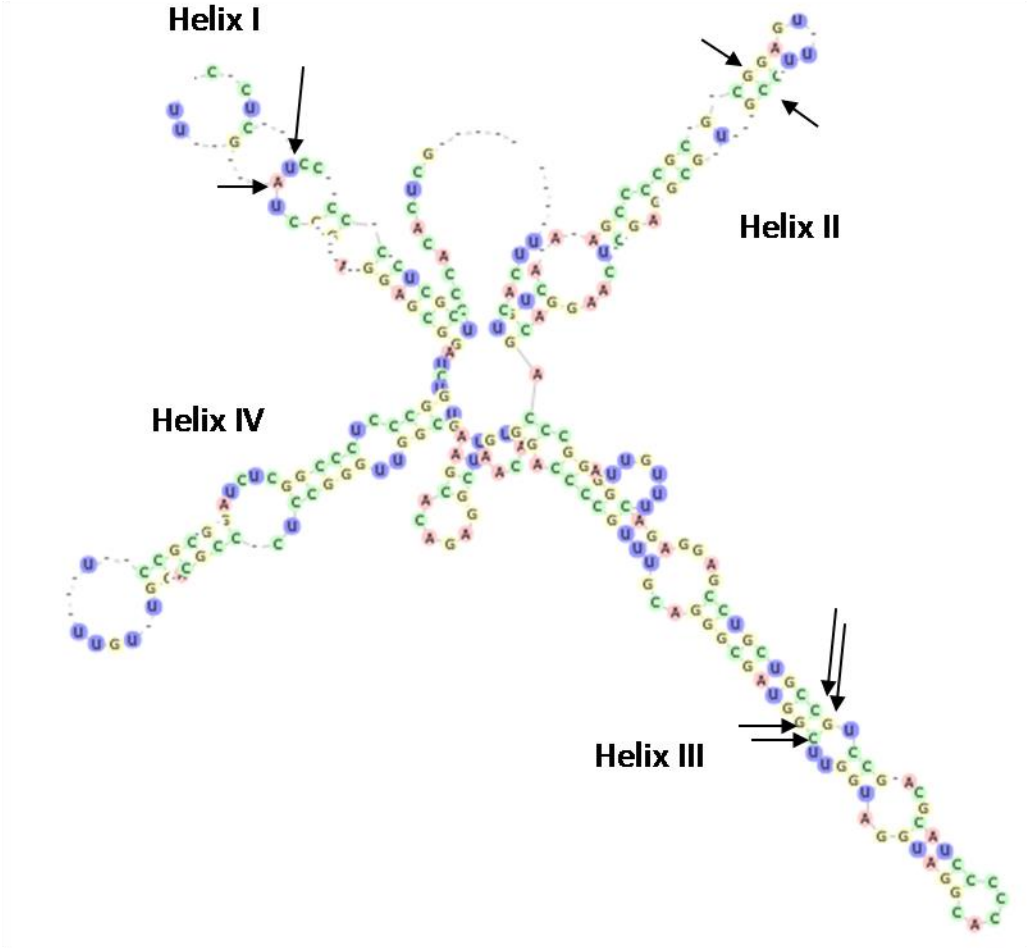


Figure 3.5 ITS2 predicted structure of *Chlorella sp.* MCC26. Four compensatory base changes with other species are shown in arrows.

Table 3.1 RNA structure of ITS2 region of *Chlorella* sp. MCC26 and its free energy as predicted by RNA structure software.

ITS structure	Energy
1	-107.5
2	-98.2
3	-87.1
4	-81.9
5	-76.1
6	-60.1
7	-60

Table 3.2 CBC tree matrix using 4 sale program of different *Chlorella* species as retrieved from NCBI database.

species	*Chlorella sorokiniana*	*Hindakia_tetrachotoma*	*Chlorella_sp._SAG222*	*Chlorella_sp._CCAP 222*	*Dictyosphaerium_sp*	*Chlorella sp. MCC26*	*Didymogenes_anomala*	*Chlorella_sp_IFRPD 1018*	*Chlorella_sp_CCCryo297-06*	*Chlorella_variabilis*	*Chlorella vulgaris*	*Chlorella_spMRBG1*
Chlorella sorokiniana	0	0	0	1	0	1	0	3	0	1	0	0
Hindakia_tetrachotoma	0	0	0	0	0	2	1	1	0	0	0	0
Chlorella_sp._SAG222	0	0	0	1	0	1	0	3	0	1	0	0
Chlorella_sp._CCAP 222	1	0	1	0	1	1	1	3	1	1	0	0
Dictyosphaerium_sp	0	0	0	1	0	1	0	3	0	1	0	0
Chlorella sp. MCC26	1	2	1	1	1	0	1	3	1	1	1	1
Didymogenes_anomala	0	1	0	1	0	1	0	4	0	2	0	0
Chlorella_sp_IFRPD 1018	3	1	3	3	3	3	4	0	3	1	3	3
Chlorella_sp_CCCryo297-06	0	0	0	1	0	1	0	3	0	1	0	0
Chlorella_variabilis	1	0	1	1	1	1	2	1	1	0	1	1
Chlorella vulgaris	0	0	0	0	0	1	0	3	0	1	0	0
Chlorella_spMRBG1	0	0	0	0	0	1	0	3	0	1	0	0

3.1.3.1 Specific growth rate and biomass productivity

In order to select suitable strain, twenty one algal strains were screened for their specific growth rate and biomass productivity. As shown in Table 3.3, the green algae, in general, had higher specific growth rates as compared to other microalgal strains. These findings are in agreement with earlier reports of higher growth rates of green algal species over other microalgal strains (Nascimento *et al.*, 2013). The cyanobacterial strains *Anabaena doliolum* and *Nostoc muscorum* along with *Dunaliella salina, Chlorella minutissima, Dunaliella tertiolecta*, and *Desmodesmus* sp. MCC34 showed higher growth rate among the algal strain. Growth, when expressed in terms of biomass productivity also showed the similar pattern among these six strains. The growth curves of these six species are shown in Figure 3. 6.

3.1.3.2 Lipid content and lipid productivity

Lipid content for the six microalgal strains are shown in Table 3. 4. The lipid content was found to be highest in *C. minutissima* (23%; $p<0.05$) followed by *Desmodesmus* sp. MCC34 (20%), *Dunaliella salina* (19%), as well as *Anabaena doliolum* (19%) *Nostoc muscorum* (17%), and *Dunaliella tertiolecta* (12%). These results are almost in tune to earlier report showing 20% lipid content in *Chlorella sp*, 14–20% in *D. salina* (Griffiths & Harrison 2009; Lim *et al.*, 2012). Lipid content in *Anabaena doliolum* (19%) and *Nostoc muscorum* (17%) were found to be higher than the reported values of 8–13% possibly due to difference in extraction techniques and solvent used (Vargas *et al.*, 1998).

Lipid productivity is the expression of lipid content along with growth of algae, is a superior parameter to compare the potential of lipid production. Lipid productivity calculated for six microalgal strains is also represented in Table 3.4. *Desmodesmus* sp. MCC34 showed significantly higher lipid productivity (13.4 mg L^{-1} day^{-1}, $p < 0.01$) than rest of the species followed by *D. salina* (9.4 mg L^{-1} day^{-1}), *C. minutissima* (6.5 mg L^{-1} day^{-1}), *D. tertiolecta* (3.8 mg L^{-1} day^{-1}), *N. muscorum* (2.9 mg L^{-1} day^{-1}), *and A. doliolum* (2.6 mg L^{-1} day^{-1}). Based on above observations, *Desmodesmus* sp.MCC34 was used for further investigations.

Table 3.3 The specific growth rate and biomass productivity of different species. Data are mean ±standard deviation (n=3).

S.No.	Species	Specific growth rate (day^{-1})	Biomass productivity ($mg\ L^{-1}\ day^{-1}$)
1	*Anabaena doliolum*	0.12±0.01	13.67±0.01
2	*Westiellopsis*	0.08±0.002	7.53±0.04
3	*Nostoc calcicola*	0.06±0.002	2.6±0.02
4	*Nostoc* sp. (g15)	0.1±0.002	6.9±0.01
5	*Nostoc* sp. (g17)	0.08±0.001	5.72±0.03
6	*Nostoc* sp. 1	0.09±0.002	10.38±0.08
7	*Anabaena cycadeae*	0.07±0.003	3.79±0.01
8	*Anabaena* sp. (g14)	0.06±0.003	9.45±0.03
9	*Anabaena cylindrica*	0.07±0.005	8.63±0.04
10	*Anabaena* sp. (g24)	0.05±0.002	13.3±0.06
11	*Tolypothrix*	0.09±0.006	6.07±0.09
12	*Anabaena* sp. (g19)	0.06±0.001	4.15±0.02
13	*Nostoc muscorum*	0.15±0.01	17.58±0.01
14	*Anabaena fertilissima*	0.09±0.002	1.32±0.01
15	*Synechocystis*	0.08±0.002	13.02±0.08
16	*Scenedesmus* sp. MCC32	0.09±0.001	12.58±0.17
17	*Chlorella* sp. MCC26	0.05±0.002	9.9±0.06
18	*Dunaliella salina*	0.23±0.01	49.34±0.15
19	*Dunaliella tertiolecta*	0.17±0.01	31.1±0.05
20	*Chlorella minutissima*	0.16±0.01	27.79±0.02
21	*Desmodesmus* sp. MCC34	0.26±0.01	65.9±0.1

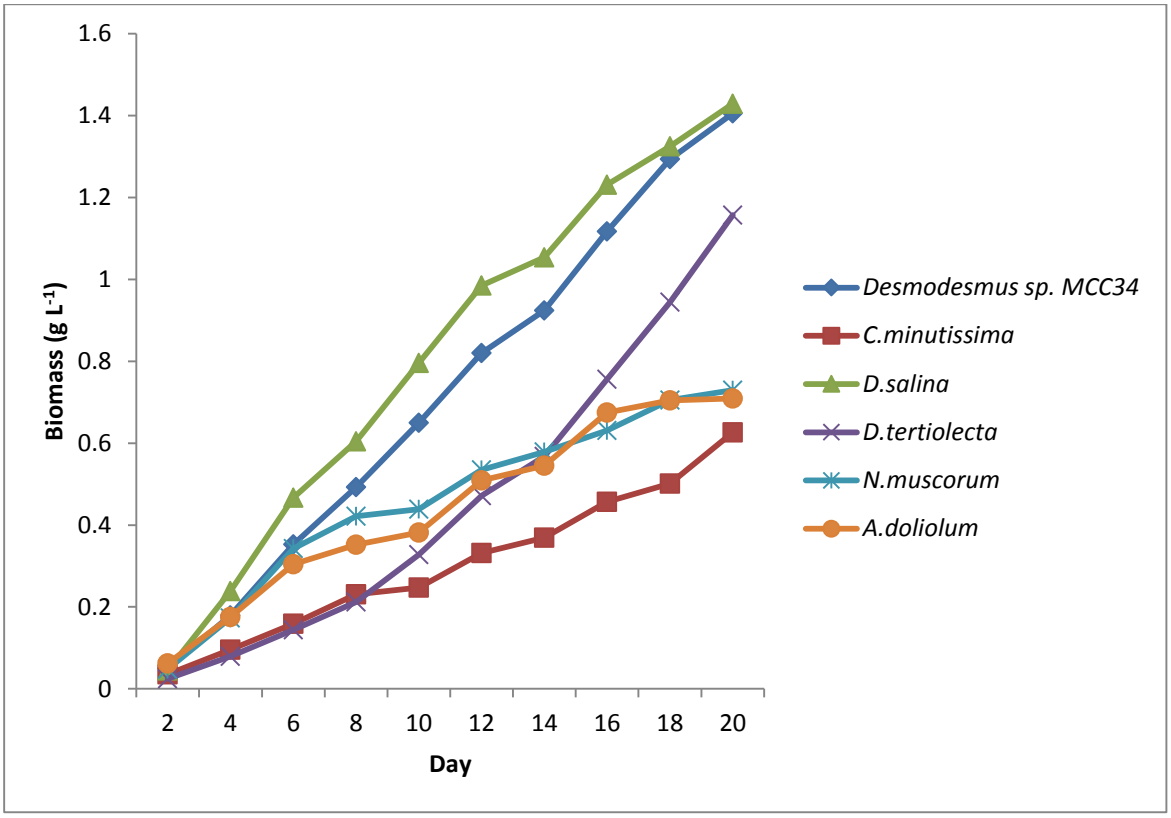


Figure 3.6 Growth curves of six algal species: *A. doliolum*, *N. muscorum*, *D. salina*, *C. minutissima*, *D. tertiolecta*, and *Desmodesmus* sp. MCC34

Table 3.4 Lipid production characteristics of different species. Data are mean ±standard deviation (n=3).

S.No.	Species	Lipid content (%)	Lipid productivity (mg L^{-1} day^{-1})
1	*Anabaena doliolum*	19.02±0.52	2.6±0.01
2	*Westiellopsis*	9.3±0.66	0.7±0.04
3	*Nostoc calcicola*	6.55±0.12	0.17±0.01
4	*Nostoc* sp. (g15)	9.85±0.41	0.68±0.01
5	*Nostoc* sp. (g17)	9.62±0.52	0.55±0.03
6	*Nostoc* sp. 1	10.69±2.33	1.11±0.09
7	*Anabaena cycadeae*	9.75±0.25	0.37±0.01
8	*Anabaena* sp. (g14)	3.28±0.18	0.31±0.01
9	*Anabaena cylindrica*	6.95±0.2	0.6±0.03
10	*Anabaena* sp. (g24)	10.15±0.47	1.35±0.06
11	*Tolypothrix*	7.74±1.99	0.47±0.07
12	*Anabaena* sp. (g19)	9.88±0.58	0.41±0.02
13	*Nostoc muscorum*	17.01±1.21	2.99±0.01
14	*Anabaena fertilissima*	7.6±3.16	0.1±0.01
15	*Synechocystis*	3.61±0.48	0.47±0.03
16	*Scenedesmus* sp. MCC32	11.45±2.5	1.44±0.2
17	*Chlorella* sp. MCC26	11.62±0.77	1.15±0.07
18	*Dunaliella salina*	18.97±0.66	9.36±0.29
19	*Dunaliella tertiolecta*	12.25±0.04	3.81±0.06
20	*Chlorella minutissima*	23.46±0.27	6.52±0.05
21	*Desmodesmus* sp. MCC34	20.35±0.5	13.41±0.2

3.2 Factors affecting lipid productivity

Based on the lipid productivity discussed in earlier section, *Desmodesmus* sp. MCC34 was chosen to determine the effect of various chemical and physiological factors on lipid productivity.

3.2.1 Effect of light intensity

It is an established fact that algal growth is influenced by light intensity and the algal growth leads to lipid production. The lipid productivity at different light intensities was tested and the results obtained are shown in Figure 3. 7. It is observed that out of five intensities tested, the lipid productivity was higher at 54 and 41 μ mol photon m^{-2} s^{-1}($p<0.05$), giving rise to approximately 15 mg L^{-1} day^{-1} of biomass. Beyond these light intensities, no further increase in lipid productivity was observed. Liu et al., showed that the growth of *Scenedesmus species* was saturated at a light intensity of 84 μ mol photon m^{-2} s^{-1} (Liu *et al.*, 2012a). However, in the present study, we observed that a much lower light intensity (41 μ mol photon m^{-2} s^{-1}) could lead to growth saturation. Such a low light saturation value is favorable for mass cultivation as they can grow well during winter season when light intensities are generally low.

3.2.2 Effect of photoperiod

Previous studies have suggested a photoperiod of 16:8 and 18:6 light: dark hours as an optimum growth conditions for *Chlorella* and *Nannochloropsis*; with longer light periods resulting in decrease in the growth yields (Khoeyi *et al.*, 2012; Wahidin *et al.*, 2013). However, the results of *Desmodesmus* sp. MCC34 (Figure 3. 8) indicates that a continuous illumination of culture (24:0 light and dark period) showed higher lipid productivity (15.1 mg L^{-1} day^{-1}; $p<0.05$). Therefore, this species can provide higher lipid yield under photobioreactor setups where artificial illumination is provided continuously.

3.2.3 Effect of pH and salinity

To test the effect of pH on microalgal growth and lipid productivity, *Desmodesmus* sp. MCC34 was grown at different pH (Figure 3. 9) and it was observed that the optimum lipid productivity

was obtained at pH 8 ($p<0.05$). Earlier studies have shown that a neutral pH is conducive for normal microalgal growth whereas higher pH increases the lipid production (Gardner *et al.*, 2011). However, in the present study the lipid productivity did not increase in the medium having pH beyond 8.

Existing reports indicates that salt stress inhibits growth but increases lipid content in certain microalgal species (Takagi *et al.*, 2006). However, even a slight increase of 0 to 0.5% salinity had cut down the lipid productivity by more than 50% in *Desmodesmus* sp. MCC34 (Figure 3. 10), indicating that salinity stress does not induce lipid production at least in this algae. Such a decline in lipid content may be attributed to fresh water habitat of *Desmodesmus* sp. MCC34 where increase in salinity severely inhibited growth, hence the lipid productivity as shown in the case of mixed microalgae cultures isolated from an inland water body (Mohan & Devi 2014).

3.2.4 Effect of trophic conditions

The light penetration into the denser culture is more restricted than the diluted culture. This phenomenon termed as self-shading limits the microalgal growth especially at higher cell density (Hewes 2016). To overcome this effect and enhance the biomass production as well as lipid productivity, mixotrophic cultivation of target algae was adopted, in which organic carbon serves as energy source in addition to light. In the present study, glucose and glycerol were tested as the organic carbon sources on the growth of *Desmodesmus* sp. MCC34 in the presence of light and presented in Figure 3.11.

Among the two concentrations tested, 0.5% (w/v) of glucose yielded a maximum lipid productivity of 160 mg L^{-1} day^{-1}(Figure 3. 11). Earlier report involving batch cultivation of *Chlorella zofingiensis,* showed maximal lipid productivity of 351 mg L^{-1} day^{-1} with 5% (w/v) glucose (Liu *et al.*, 2010). Although this is twofold the productivity of *Desmodesmus* sp. MCC34, the yield of lipid per gram of glucose for *Desmodesmus* sp. MCC34 is 320 mg whereas *C. zofingiensis*'s yield is only 70 mg lipid per gram of glucose. This suggests the economical utilization of substrate by *Desmodesmus* sp. MCC34. The cost of glucose is 490$ per tonne or 0.49$ per kg (Fei *et al.*, 2015).

The cost of production of per kg lipid from *C. zofingiensis* using glucose is 6.9 $ whereas it costs only 1.5$ for *Desmodesmus* sp. MCC34 based on the cost assessment by Fei *et al.*, 2015.

Addition of 0.5% (w/v) glycerol to the media resulted in lipid productivity of 20 mg L^{-1} day^{-1} whereas 1% (w/v) led to decreased productivity of 16 mg L^{-1} day^{-1}. In spite of glycerol supplementation, the lipid productivity was not substantially enhanced from that of control which is phototrophic cultivation.

The glucose and glycerol were unable to stimulate growth in dark indicating *Desmodesmus* sp. MCC34 does not adapt to heterotrophic conditions.

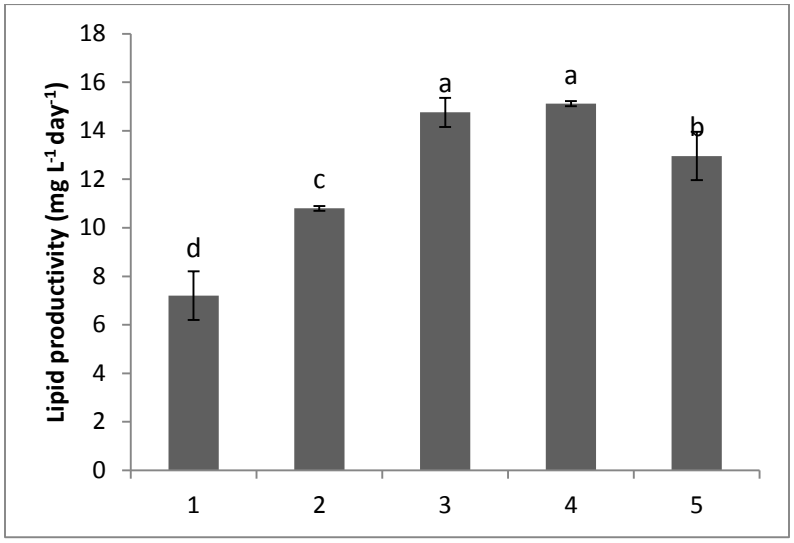


Figure 3.7 Lipid productivity of *Desmodesmus* sp. MCC34 at various light intensities (1) 14, (2) 27, (3) 41, (4) 54 and (5) 84 μ mol photon m^{-2} s^{-1}. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

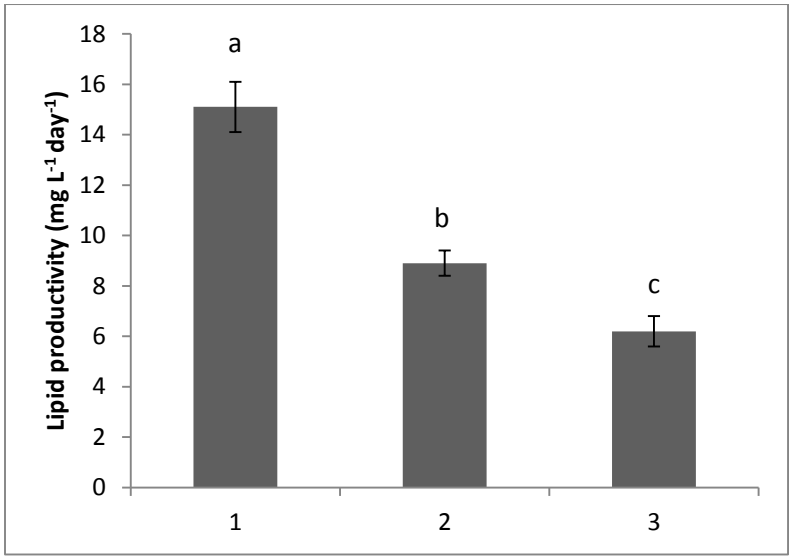


Figure 3.8 Effect of photoperiod on lipid productivity of *Desmodesmus* sp. MCC34 (1) 24L/0D, (2)12L/12D, (3) 9L/15D hours. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

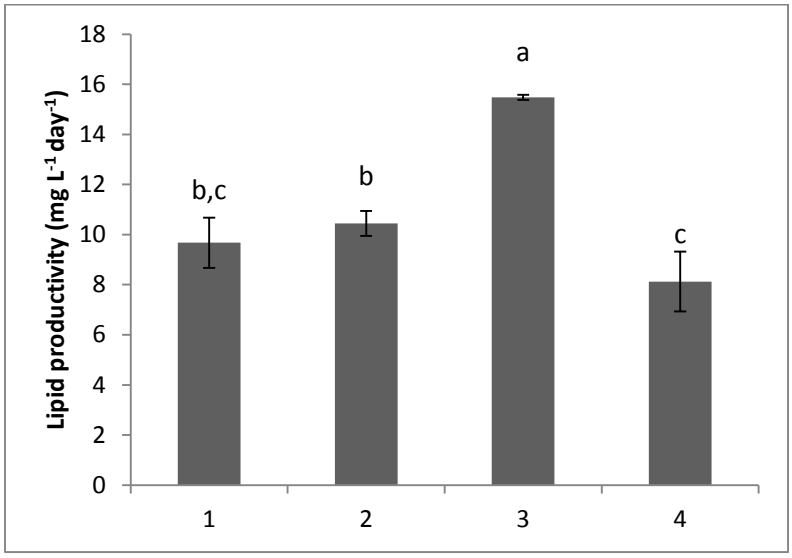


Figure 3.9 Lipid productivity of *Desmodesmus* sp. MCC34 under different pH (1) 6, (2) 7, (3) 8 and (4) 9 pH. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

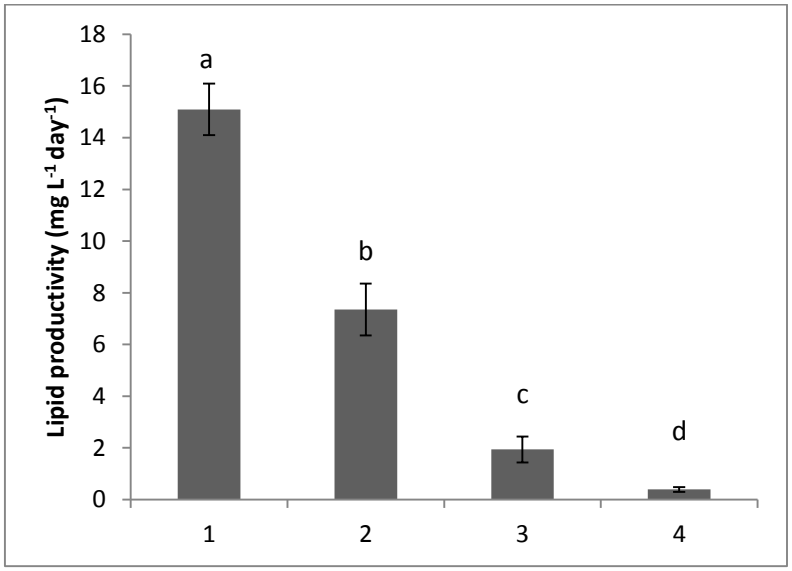


Figure 3.10 Lipid productivity of *Desmodesmus* sp. MCC34 under different concentration of NaCl (1) 0 % (control), (2) 0.5 %, (3) 1 % and (4) 2 % NaCl. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

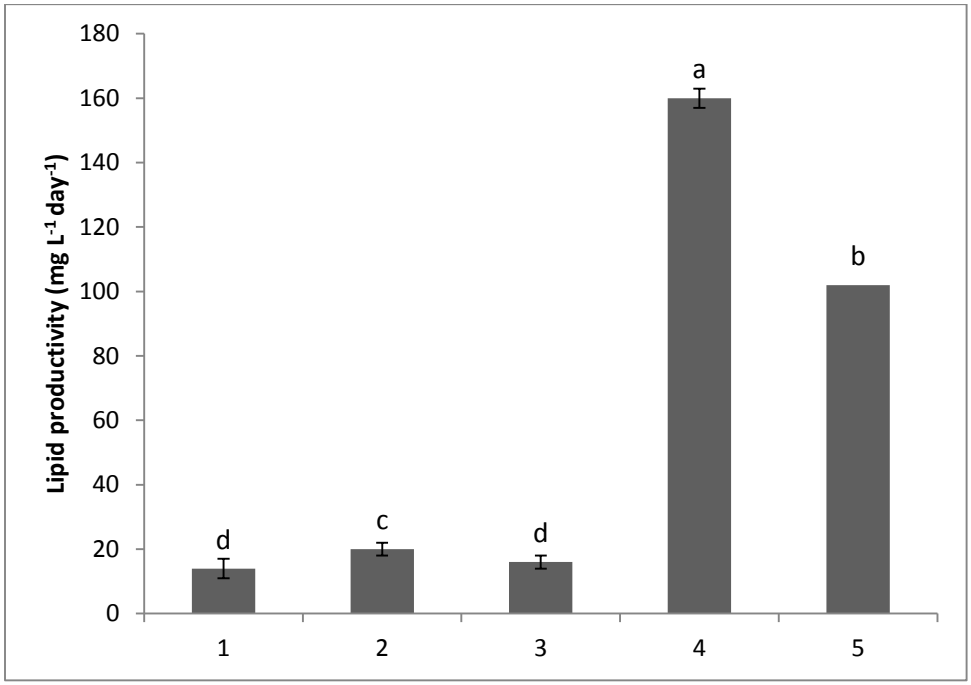


Figure 3.11 Lipid production of *Desmodesmus* sp. MCC34 under different mixotrophic condition, (1) Control (Photoautotrophically grown algae without addition of glucose or glycerol was used as control), (2) 0.5 % glycerol, (3) 1 % glycerol, (4) 0.5 % glucose and (5) 1 % glucose. All are w/v. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

3.2.5 Effect of Nutrients

3.2.5.1 Nitrogen source

The recommended media for the cultivation of photoautotrophic algae contain inorganic nitrogen compounds such as nitrate, nitrite, ammonium and urea (Hsieh & Wu 2009). However, microalgae are also shown to utilize different sources of organic nitrogen such as yeast extract, peptone, malt extract, etc. (Erdoğan *et al.*, 2016). In comparison to organic nitrogen sources, inorganic nitrogen sources are preferred because of lesser growth of contaminating organisms and their low cost. Since these nitrogen compounds follow different pathways for the biosynthesis of protein, it is expected that, lipid productivity will also be altered when we supply different nitrogen sources for the cultivation of algae. With this aim, *Desmodesmus* sp. MCC34 was cultivated in the presence of different inorganic nitrogen sources, in which nitrogen-free media served as control (Figure 3.12). Overall trend showed that lipid productivity increased with increasing concentration of nitrogen sources up to 2.9 mM, irrespective of nitrogen source used. Beyond the concentration of 2.9 mM, either there was no change in lipid productivity, as in case of sodium nitrite or there was decrease in lipid productivity, as in case of sodium nitrate and urea. A decrease in lipid productivity at higher sodium nitrate concentration as seen earlier by Rodriguez et al., 1992 is attributed to substrate inhibition effect. In terms of yield, highest lipid productivity of 15.2 mg L^{-1} day^{-1}($p<0.05$) was achieved with 2.9 mM nitrogen concentration of sodium nitrate which is 10-fold higher than the reported value of 1.4 mg L^{-1} day^{-1} for *Scenedesmus obtusus* (Chandra *et al.*, 2016). As far as growth is concerned, either nitrate or nitrite shows almost similar growth rate, at least in *Synechococcus* (Deng et al., 2011). The lipid productivity of 2.8 mg L^{-1} day^{-1} demonstrated by nitrogen free control culture can be attributed to the nitrogen stress induced lipid accumulation as reported earlier (Breuer *et al.*, 2012; Gong *et al.*, 2013; Praveenkumar *et al.*, 2012). The low lipid productivity by culture grown with ammonium salt as compared to other sources may have resulted from production of toxic ammonia from ammonium ions under alkaline pH (Eustance *et al.*, 2013). The mass cultivation with urea as nitrogen source is a promising option, since it is cheaper than other inorganic nitrogen sources (Hsieh & Wu 2009). Thus, in order to obtain higher lipid productivity, 2.9 mM nitrogen concentration of sodium nitrate or sodium nitrite can be used for mass cultivation of *Desmodesmus* sp. MCC34.

3.2.5.2 Phosphorus

The phosphorus is a major macronutrient required for the growth of microalgae and acts as growth limiting source in mass cultivation (Solovchenko *et al.,* 2016). It is an essential component of cell membrane and also necessary for anabolism of DNA and RNA. The synthesis of lipid as well as maintenance of good growth requires optimal supply of phosphorus is required in growth medium. Since the production of lipid is dependent on growth, the lipid productivity of *Desmodesmus* sp. MCC34 under different concentrations of di-potassium hydrogen phosphate was investigated. The culture medium lacking phosphorus served as control. It was found that the lipid productivity increased with the increasing concentration of phosphorus till 0.2 mM phosphorus with a highest lipid productivity of 14.2 mg L^{-1} day^{-1} (Figure 3. 13; $p<0.05$). A decrease in lipid productivity at higher phosphorus concentration as evident in this study and also reported earlier by Martinez et al (1999) can be attributed to substrate inhibition effect. In an earlier study involving *Scenedesmus*, a maximum lipid productivity of 6.9 mg L^{-1} day^{-1} was obtained at phosphorus concentration of 6.6 mM phosphorus (Yin-Hu *et al.,* 2014). In comparison, the lipid productivity seen in *Desmodesmus* sp. MCC34 is not only two times higher that of the *Scenedesmus* but also at 13 times less concentration of phosphorus. The control, which lacked any exogenous addition of phosphorus, displayed a lipid productivity value of 3.7 mg L^{-1} day^{-1} possibly due to scavenging of stored phosphate (Powell *et al.,* 2009).

3.2.5.3 Sulfur

In comparison to nitrogen and phosphorus, sulfur is a less investigated compound in assessing the microalgal growth and lipid production. But still, it is an essential macronutrient, serving as key component of the amino acids such as cysteine and methionine and also a constituent of sulpholipids, which is part of membrane lipids. The form of sulfur used by microalgae for growth is sulphate (Mera *et al.,* 2016). The aim of the present study was to see the effects of different concentrations of the sulfur on lipid productivity of *Desmodesmus* sp. MCC34 using the salt of $MgSO_4.7H_2O$. The culture medium lacking sulfur served as control. The lipid productivity increased with the increasing concentration of sulfur until it reached 0.08 mM sulfur (Figure 3. 14). The higher concentration of sulfur (0.13 and 0.21 mM) generated no different response from that of 0.08 mM sulfur, in terms of lipid productivity ($p>0.05$). In terms of yield, maximal lipid

productivity of 15.2 mg L^{-1} day^{-1} was achieved with a concentration of 0.08 mM sulfur. This concentration of sulfur is nearly three times lower than the reported concentration of 0.22 mM sulfur required for achieving maximal lipid productivity in *Botryococcus braunii* (Tran *et al.*, 2010). As far as growth and substrate inhibition are concerned, Mera et al., 2016 reported the optimal sulfur concentration for achieving maximum growth in *Chlamydomonas moewusii* as 0.02 mM and observed growth inhibition at a concentration of 0.66 mM.

The screening of media components such as calcium chloride, ferric ammonium citrate and sodium carbonate for their effects on lipid productivity proved unfruitful, as there was no significant difference in lipid productivity between the control (media, which lacked these components) and media which had these components in them ($p>0.05$; Figure 3. 15 - 17). These results are similar with an earlier study, where these media components have not been found to affect the lipid productivity of microalgae, *Botryococcus braunii* (Tran *et al.*, 2010).

3.2.6 Lipid accumulation studies through nutrient deprivation

The microalgal growth depends on constant supply of nutrients. When these nutrients become depleted, their growth is inhibited and they are no longer able to utilize the photosynthetic product in growth (Du & Benning 2016). Hence the microalgae tend to store the carbon, obtained by carbon dioxide fixation, in the form of storage molecules such as lipid due to unavailability of nucleic acid and protein building blocks (Schmollinger *et al.*, 2014). The inhibition of growth along with continuing synthesis of lipid leads to lipid accumulation. For the assessment of lipid accumulation, the parameter of lipid content was chosen instead of lipid productivity throughout this study.

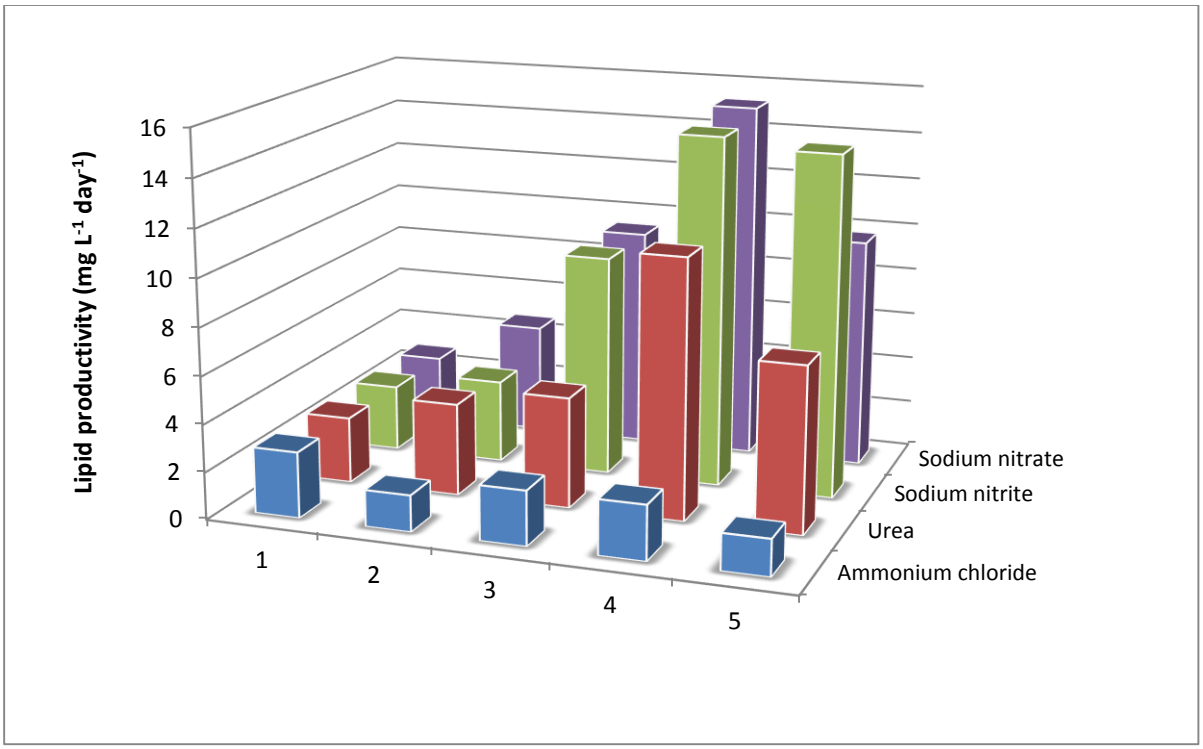


Figure 3.12 Effect of various nitrogen sources on lipid productivity by after 10 days of growth of *Desmodesmus* sp. MCC34 in a medium containing (1) 0 mM (control), (2) 0.15 mM, (3) 1.45 mM, (4) 2.9 mM and (5) 14.6 mM nitrogen in sodium nitrate, sodium nitrite, urea and ammonium chloride. Data are the mean of triplicates.

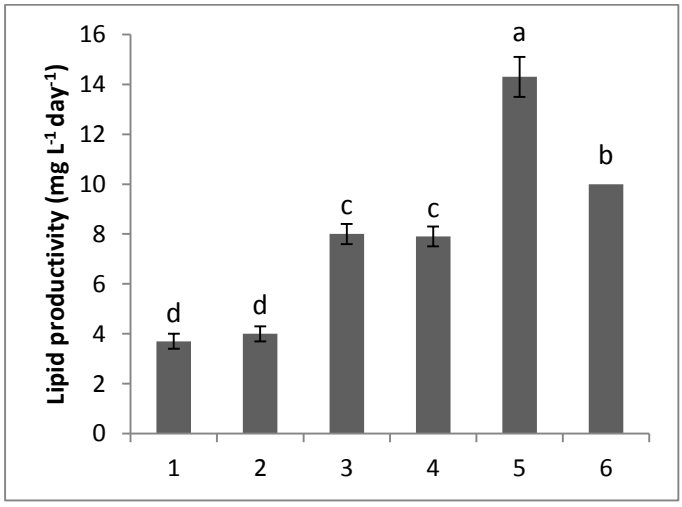


Figure 3.13 Lipid productivity after 10 days of growth of *Desmodesmus* sp. MCC34 in medium supplemented with different concentration of phosphorus, (1) 0mM (control), (1) 0.005 mM, (2) 0.02 mM, (3) 0.04 mM, (4) 0.2 mM and (5) 2 mM phosphorus in K_2HPO_4. Bars with different letters are significantly different at $P<0.05$.

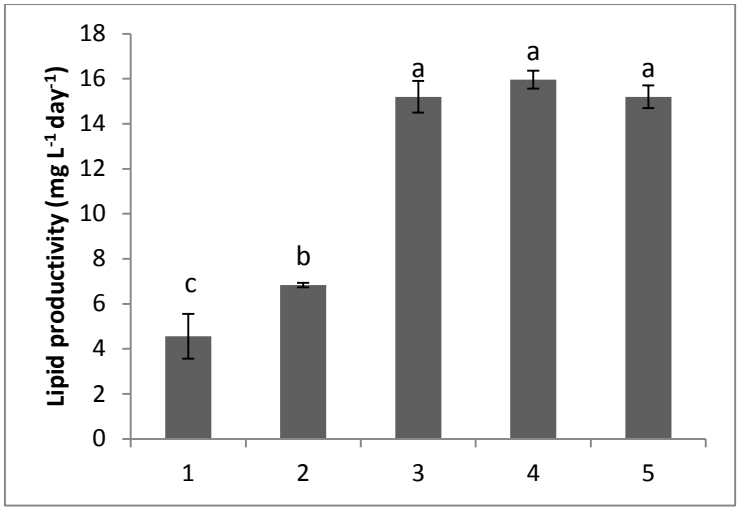


Figure 3.14 Lipid productivity after 10 days of growth of *Desmodesmus* sp. MCC34 in medium supplemented with different concentration of sulfur, (1) 0 mM (control), (2) 0.04 mM, (3) 0.08 mM, (4) 0.13 mM and (5) 0.21 mM sulfur in $MgSO_4.7H_2O$. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

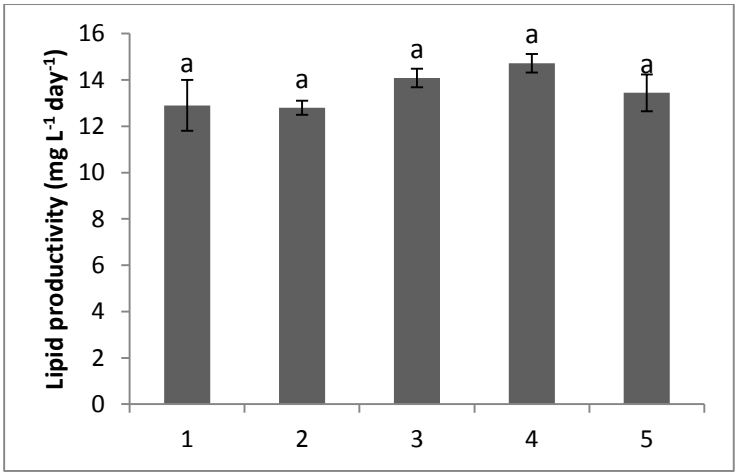


Figure 3.15 Lipid productivity after 10 days of growth of *Desmodesmus* sp. MCC34 in medium supplemented with different concentration of Na_2CO_3, (1) 0 g L^{-1} (control), (2) 0.005 g L^{-1}, (3) 0.01 g L^{-1}, (4) 0.2 g $L^{-1}$and (5) 1 g L^{-1} Na_2CO_3. Data are the mean of triplicates.

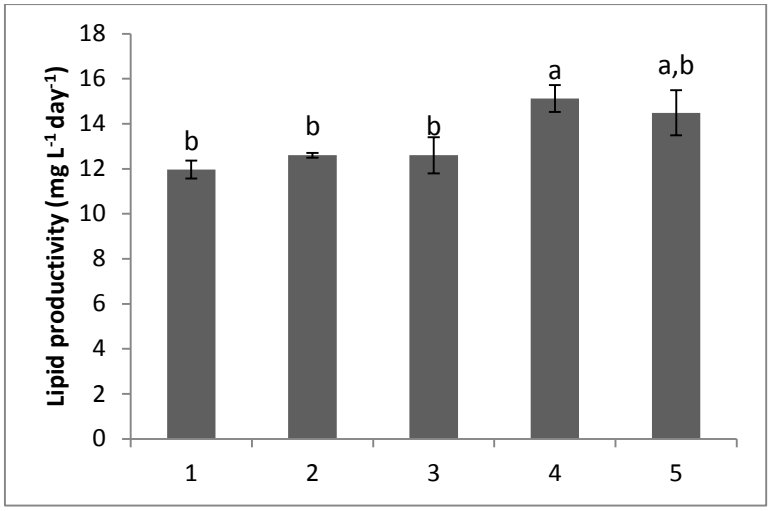


Figure 3.16 Lipid productivity after 10 days of growth of *Desmodesmus* sp. MCC34 in medium supplemented with different concentration of $CaCl_2$, (1) 0 g L^{-1} (control), (2) 0.01 g L^{-1}, (3) 0.036 g L^{-1} ,(4) 0.2 g L^{-1} and (5) 0.5 g L^{-1} $CaCl_2.2H_2O$. Data are the mean of triplicates.

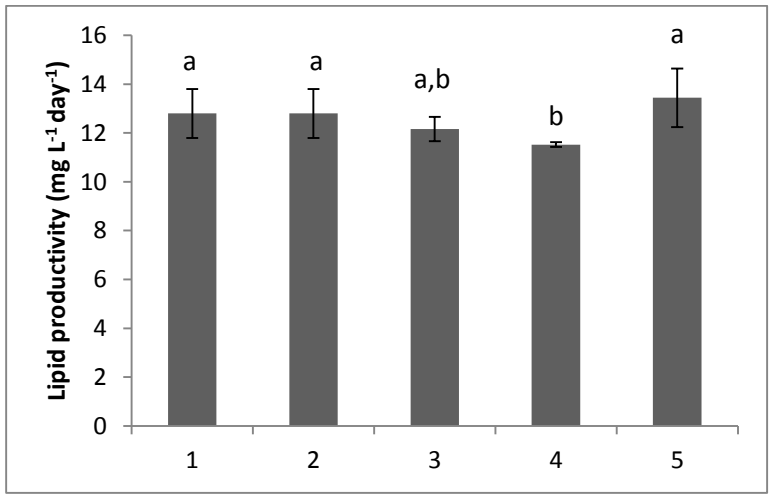


Figure 3.17 Lipid productivity after 10 days of growth of *Desmodesmus* sp. MCC34 in medium supplemented with different concentration of ferric ammonium citrate, (1) 0 g L^{-1} (control), (2) 0.05 mg L^{-1}, (3) 0.1 mg L^{-1}, (4) 0.6 mg L^{-1} and (5) 5 mg L^{-1} ferric ammonium citrate. Data are the mean of triplicates.

3.2.6.1 Nutrient deprivation

The lipid accumulation in algae can be induced by deprivation of nutrients such as nitrogen (Praveenkumar *et al.,* 2012), phosphate (Esakkimuthu *et al.,* 2016), sulphate (Ota *et al.,* 2016) and iron (Praveenkumar *et al.,* 2012). In this study, the *Desmodesmus* sp. MCC34 was subjected to such nutrient deficiencies and tested for their effect on lipid content, in which cultures grown in normal BG 11 media served as control. On day 10 of nutrient deficiency, a maximum increase of 30% over control under nitrogen deprivation, followed by 27% increase under phosphate deprivation were observed ($p<0.05$), whereas sulphate and iron did not show any change over control (Figure 3.18). The similar findings were reported on higher increase of lipid content under nitrogen deprivation than under phosphate deprivation in *Phaeodactylum* (Gong *et al.,* 2013). Similar trend was observed after 20 days of nutrient deficiency showing maximum increase in lipid content under nitrogen deprivation, with an exception under sulfur deprivation showing slight increase in lipid content over control (Gong *et al.,* 2013). A similar trend of higher lipid content in nitrogen deprivation over phosphate, sulphate and iron deprivation was also observed for *Chlorella* species (Praveenkumar *et al.,* 2012). The reason for higher lipid accumulation observed under nitrogen deprivation could be attributed to the arrest of biosynthesis of amino acid. The NADPH which is used for anabolism of amino acid is left unutilized causing its accumulation. Such excess NADPH inhibits citrate synthase, a TCA cycle enzyme, which leads to build up of cellular acetyl CoA leading to de-novo fatty acid synthesis (Guerra *et al.,* 2013). Thus, nitrogen deprivation can be adopted as an efficient strategy for large scale lipid production by *Desmodesmus* sp. MCC34.

3.2.6.2 Biochemical changes associated with nitrogen deprivation

The nitrogen deprivation, which resulted in higher lipid content in *Desmodesmus* sp. MCC34 than any other stress was chosen for studying biochemical changes associated with it. Along with *Desmodesmus* sp. MCC34, three more species (*C. minussitima*, *D. salina* and *D. tertiolecta*) which are known for higher lipid content were also selected on the aspect of bio-refinery approach, which is to see their potential for co-producing other value added products such as carbohydrate, protein, chlorophyll and carotenoids.

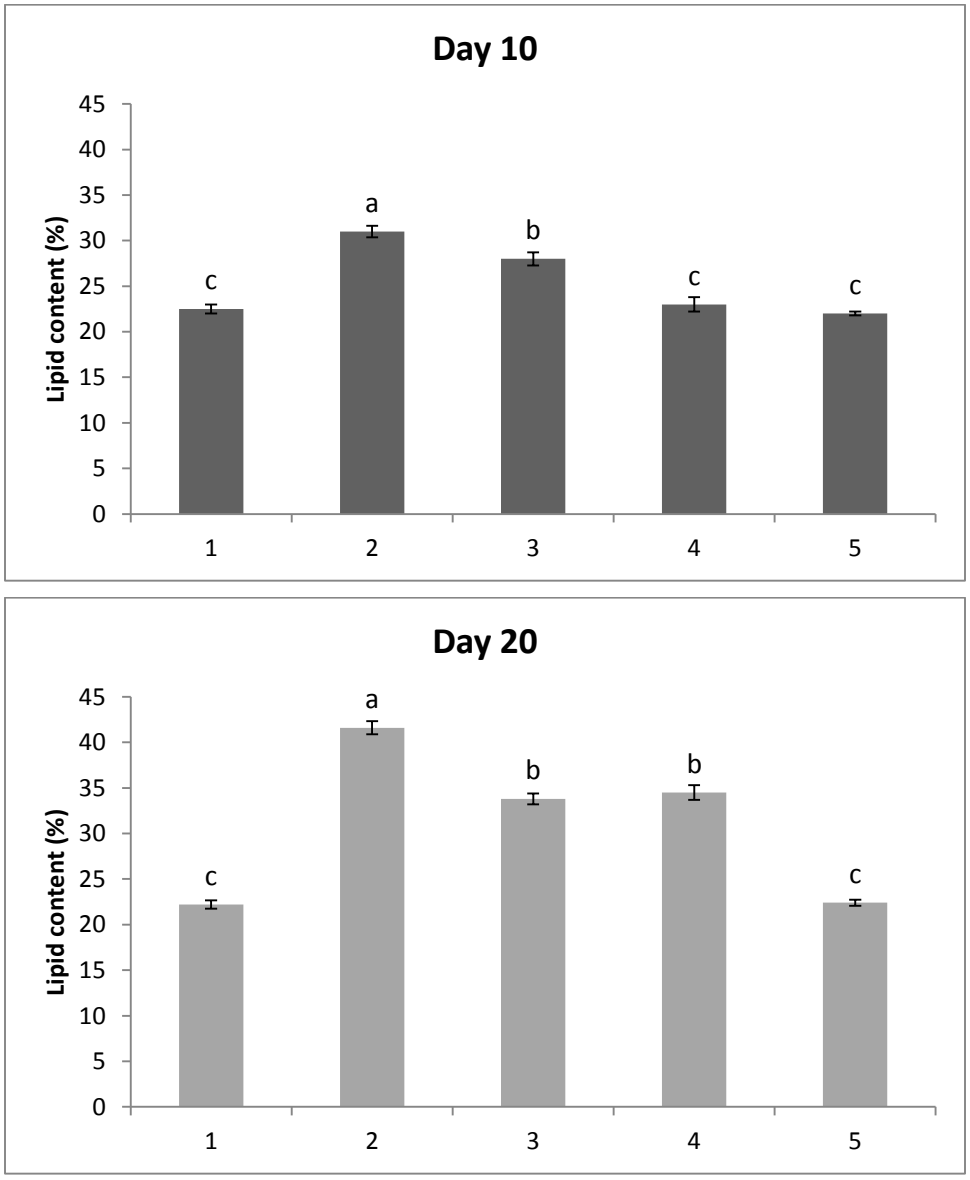


Figure 3.18 The effect of deprivation of various nutrients on lipid content on day 10 and 20, (1) Control, no deficiency, (2) Nitrogen deficiency, (3) Phosphate deficiency, (4) Sulphate deficiency, and (5) Iron deficiency. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

3.2.6.2.1 Biomass production

The nitrogen deprivation is reported to progressively affect the growth rate of algae before completely inhibiting the biomass production altogether (Breuer *et al.,* 2012; Ördög *et al.,* 2013). The extent of biomass production under nitrogen deprivation was found to be species specific (Ördög *et al.,* 2013). The present study aims at determining the change in biomass concentration after 10 and 20 days of nitrogen deprivation in four species. All the four species were inoculated at a uniform cell density of 0.1 g L^{-1} in the medium containing either 2.9 mM nitrogen (control) or 0 mM nitrogen in nitrogen deficient conditions. On day 10, the highest biomass production of 0.58 g L^{-1} was achieved with *D. salina*, followed by *Desmodesmus* sp. MCC34 (0.52 g L^{-1}) and *D. tertiolecta* (0.49 g L^{-1}; Figure 3.19). The least biomass production of 0.4 g L^{-1} was observed in *C. minutissima*. A similar trend of biomass production was observed in four species after 20 days of nitrogen deficiency. *D. salina* is shown to produce higher biomass than other algae because *D. salina* is reported to consume lesser nitrogen during biomass production, as compared to other algae such as *Chlorella* (Kim *et al.,* 2012). In an earlier study involving 30 different species of *Chlorella* and *Scenedesmus* subjected to nitrogen stress, *Scenedesmus* species in general was reported to produce higher biomass than *Chlorella* (Ördög *et al.,* 2013). At cellular level, the decrease in biomass production during nitrogen deprivation can be associated with downregulation of cell cycle proteins such as CUT4 and RIO kinase since this phenomenon was observed in nitrogen deprived, lipid accumulating *Chlorella* species (Guarnieri *et al.,* 2013).

3.2.6.2.2 Lipid content

A number of algal species are reported to show increase in lipid content when subjected to nitrogen deficiency and the degree of increase varies from species to species (Boyle *et al.,* 2012; Liang *et al.,* 2013; Xiao *et al.,* 2013; Liu *et al.,* 2016). But not all algal species produce lipid under nitrogen deprivation condition as shown by Breuer et al., 2012 and Gong et al., 2012. In the present study, four species, screened for change in amount of lipid showed a significant increase in lipid content after 20 days of nitrogen deprivation ($p<0.05$; Figure 3. 20 A) as compared to nitrogen sufficient medium. Among all the tested strain and highest increase was observed in *Desmodesmus* sp. MCC34 (doubled), followed by *C. minussitima* (1.5 times), *D. salina* (1.3 times) and *D. tertiolecta* (1.3 times). The literatures also suggest that the extent of lipid accumulation is species specific (Liang et al., 2013; Boyle et al.,2012; Xiao et al., 2013; Breuer et al., 2012; Gong et al.,2012).

Breuer *et al.,* 2012 compared nine microalgal species grown under nitrogen deprived condition for accumulation of lipid and reported that within 6 days of nitrogen deprivation, the lipid content of *Chlorella*, *Scenedesmus* and *Neochloris* increased four-fold whereas *Phaeodactylum*, *Nannochloropsis*, *Porphyridium* and *Isochrysis* doubled and there was no accumulation in case of *Dunaliella*. In another study involving four species, only *Nannochloropsis oculata* showed lipid accumulation, whereas *Chaetoceros*, *Phaeodactylum tricornatum* and *Pavlova virdis* failed to accumulate lipid under nitrogen deprivation (Gong *et al.,* 2013). The higher lipid production under nitrogen deprivation can be attributed to both acyl-CoA dependent and acyl-CoA independent pathways (Xiao *et al.,* 2013). In acyl-CoA dependent pathway, enzymes involved in de-novo fatty acid synthesis such as acetyl-CoA carboxylase and enzymes such as fatty acid elongase and long-chain-fatty-acid-CoA ligase are found to be over expressed in nitrogen deprived lipid accumulating microalgae such as *Nannochloropsis* (Liang *et al.,* 2013). On other hand, in acyl-CoA independent pathway, membrane lipids are converted to triacylglycerol through over expression of enzymes such as glycolipases and phospholipases, phosphodicaylglycerol acyl transferase and diacylglycerol acyltransferase, as reported in case of nitrogen deprived *Chlamydomonas* (Boyle *et al.,* 2012).

3.2.6.2.3 Chlorophyll

The formation of chlorophyll content in photosynthetic organism is the reflection of growth and in turn, the biomass production. The reduction in chlorophyll content is a mechanism adopted by cell in order to counteract the formation of reactive oxygen species during the stress of nitrogen limitation (Liu *et al.,* 2012b; Mus *et al.,* 2013). The present study observes change in the levels of chlorophyll a and chlorophyll b in four species after 20 days of nitrogen deprivation. As shown in Figure 3. 20 B & C, both types of chlorophyll, a and b decreased under nitrogen deprivation. The decrement of chlorophyll during nitrogen deprivation is also reported for number of microalgal species (Liang *et al.,* 2013; Liu *et al.,* 2012b; Recht *et al.,* 2012; Rismani-Yazdi *et al.,* 2012; Valenzuela *et al.,* 2013). In general, chlorophyll b was found to be more sensitive to nitrogen deprivation than chlorophyll a, which as a result increased the ratio of chlorophyll a to b in all the four species. The increase in the ratio of Chlorophyll a to chlorophyll b can be associated with decrease in activity of Photosystem II (PS II) and increase in the activity of Photosystem I, as shown in *Chlorella* species (Dong *et al.,* 2013). This difference in the activities of photosystems

results in a shift from non-cyclic to cyclic photophosphorylation, leading to inhibition of NADPH but the production of ATP is maintained, which is essential for lipid synthesis.

3.2.6.2.4 Carotenoids

Carotenoids are class of photosynthetic pigments, present in membrane proteins of thylakoids (Paliwal *et al.,* 2016), having two main functions; one is to help in light harvesting process and other is prevention of cell from photo-damage. They are high valued products which are commercially used in food industry as dye, in aquaculture and poultry as food additive and in cosmetic industry for its antioxidant properties (Paliwal *et al.,* 2016).

Carotenoids are synthesized from a common precursor geranylgeranyl pyrophosphate (GGPP), which is derived from the glycolysis products, pyruvate and glyseraldehyde 3 phospahte, through series of enzymatic activity belonging to deoxyxylulose 5-phosphate pathway (Cordero *et al.,* 2011). The GGPP is not only the precursor of carotenoids but terepenoid molecules such as phytol and tocopherol are also derived from it. Evidence suggests that the first committing step in carotenoid biosynthesis is the condensation of two GGPP molecules, catalyzed by phytoene synthase, which directs the carbon flux towards carotenoid biosynthesis pathway (Cordero *et al.,* 2011).

In this study, the changes in total carotenoids in four species were analyzed at the end of 20th day of nitrogen deprivation. A drastic reduction in carotenoid content in nitrogen deprived condition was observed over nitrogen sufficient control. While a 50% reduction in carotenoids was found in *C. minutissima, D. salina* showed 70% decrease followed by *D. tertiolecta* (70% decreases). But the carotenoid content remained unchanged in *Desmodesmus* sp. MCC34 (Figure 3. 20 D). It had been reported in *D. salina* that under nitrogen deprivation, although phytoene synthase activity remained unchanged, the β-carotene was found to accumulate (Lamers *et al.,* 2012; Sánchez-Estudillo *et al.,* 2006). Studies involving *Nannochloropsis* and *Dunaliella* had shown that during nitrogen deprivation, certain carotenoids such γ-carotene, zeaxanthin, etc. are found to decrease while lutein, α-carotene, β-carotene, etc. were found to increase (Dong *et al.,* 2013; Kim *et al.,* 2015). An earlier report of Dong et al., 2013, observed reduction of carotenoid content by 88% in *Nannochloropsis* during nitrogen limitation. The ratio of carotenoids to total chlorophyll increased

in all the tested species under nitrogen deprivation. This again is an indicator of loss of PSII activity (Dong *et al.,* 2013). Fig 3.20 B, C and D shows that the rate of degradation of carotenoids is slower in comparison to that of total chlorophylls in nitrogen deprived conditions of four algae. The reason for slower rate of carotenoids degradation could be that the antioxidant property of carotenoids can play an important role in minimizing the effects of reactive oxygen species which is produced as a result of oxidative stress under nitrogen deprivation (Zhang *et al*., 2013).

3.2.6.2.5 Protein

The protein in microalgae constitutes 40% of the total biomass and 15% of total cellular nitrogen (Darley 1977). During nitrogen deprivation, the nitrogen available in cell's protein can be mobilized to make essential enzymes needed for cell maintenance as well as lipid accumulation through protein degradation. It is reported that the degradation of protein by cysteine protease and amino acid oxidase during nitrogen deprivation leads to over accumulation of TCA cycle intermediates which are directed towards acetyl CoA production leading to de-novo fatty acid synthesis (Dong *et al.,* 2013). In one case, 18-fold increments in alpha ketoglutarate were observed in nitrogen deprived *Chlorella Vulgaris* (Guarnieri *et al.,* 2011). The regulatory control for lipid accumulation during nitrogen deprivation lies with the NADPH, which is accumulated in cells due to lack of amino acid biosynthesis; the excess NADPH inhibits the activity of citrate synthase, which in turn lead to over accumulation of acetyl CoA, a precursor of fatty acid (Guerra *et al.,* 2013).

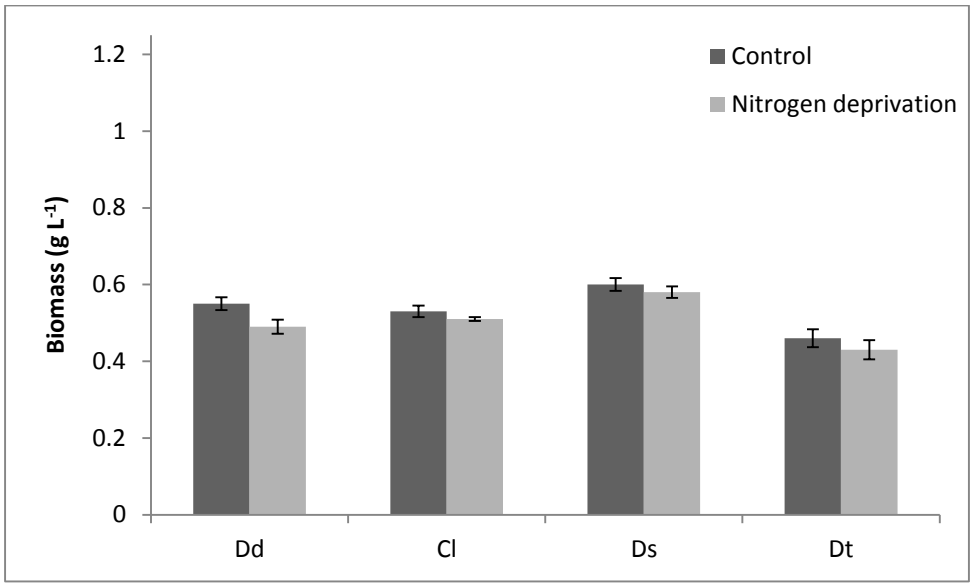
In this study, four species were observed for change in protein content after 20 days of nitrogen deprivation. Protein content decreased in all the tested species (Figure 3. 20 E) with maximal decrease (10%) was observed in *D. salina*. Such decrease in protein content during nitrogen deprivation had been reported in several microalgae (Bertozzini *et al.,* 2013; Ördög *et al.,* 2016; Cakmak *et al.,* 2012; Liu *et al.,* 2012b; Msanne *et al.,* 2012; Rismani-Yazdi *et al.,* 2012; Wan *et al.,* 2013). However, no change in the protein content was also reported for *Nannochloropsis* in nitrogen deprived condition (Xiao *et al*., 2012). On the aspect of biorefinery approach, *C.minutissima* showed higher protein content (25%) in 20 days of nitrogen deprivation, which was followed by *Desmodesmus* sp. MCC34 (22%).

3.2.6.2.5 Carbohydrate

The main carbohydrates of algae are cellulose and starch which are unlike carbohydrate, hemicelluloses and lignin found in higher plants (Ho *et al.,* 2012). Since cellulose and starch can be easily converted to simple sugar, glucose, the algal biostocks containing these carbohydrates are considered to be an ideal source for biogas and bioethanol production (Ho *et al.,* 2012). The carbohydrate is one of the two storage molecules produced by algae under stress condition, other being the lipid. The starch synthesis pathway include ADP-glucose pyrophosphorylase, which converts glucose 1 phosphate (derived from fructose 6 phosphate, a product of Calvin cycle) to ADP glucose, starch synthase converting ADP glucose to amylose and α-1,4-glucan branching enzyme converting amylose to starch (Rismani-Yazdi *et al.,* 2012). The lipid is synthesized through denovo fatty acid synthesis pathway. The pathways leading to these two compounds are known to be competitive in nature. In a comparative study involving a starchless mutant and a wild type *Chlamydomonas*, higher lipid content was observed in starchless mutant than starch producing wild type, implying the competitive nature of the two pathways (Li *et al.,* 2010).

In the present study, four algal species were tested for change in carbohydrate at 20th day of nitrogen deprivation. Carbohydrate was shown to increase in all the tested species under nitrogen deprivation (Figure 3. 20 F). The highest increase was observed for *D. tertiolecta* (30%), followed by *D. salina* (27%), *Desmodesmus* sp. MCC34 (18%) and *C. minutissima* (12%). There are mixed reports on the status of carbohydrate during nitrogen deprivation. In studies involving *Skeltonema*, *Chlamydomonas*, *Neochloris oleoabundans*, *Synechocystis* and *Synechococcus*, the carbohydrate is shown to increase during nitrogen starvation (Bertozzini *et al.,* 2013; Bajhaiya *et al.,* 2016;Cakmak *et al.,* 2012; Görl *et al.,* 1998; Osanai *et al.,* 2013; Rismani-Yazdi *et al.,* 2012), whereas in *Nannochloropsis* and *Phaeodactylum*, the carbohydrate remained unchanged (Recht *et al.,* 2012; Valenzuela *et al.,* 2012; Wan *et al.,* 2013; Xiao *et al.,* 2013). Increased carbohydrate levels in microalgae during nitrogen deprivation as found in this study, is an added advantage for biodiesel production from microalgae since carbohydrate can be used for co-producing biogas or bioethanol.

A



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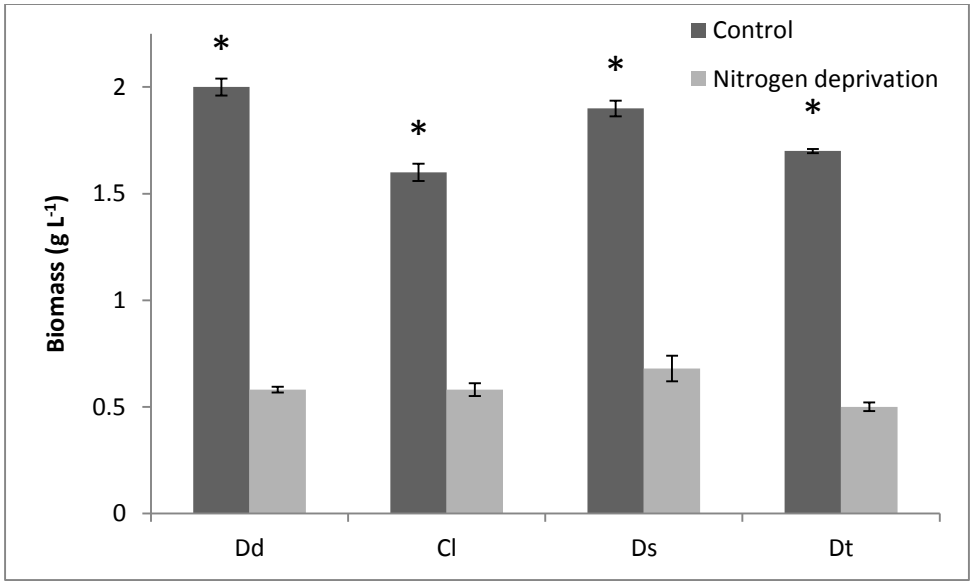

Figure 3.19 Change in biomass concentration. (A) Day 10 of nitrogen deprivation. (B) Day 20 of nitrogen deprivation. Dd - *Desmodesmus* sp. MCC34, Cl - *C. minutissima*, Ds- *D. salina* and Dt - *D. tertiolecta.* Data are the mean of triplicates. Asterisks indicate significant differences ($P < 0.05$).

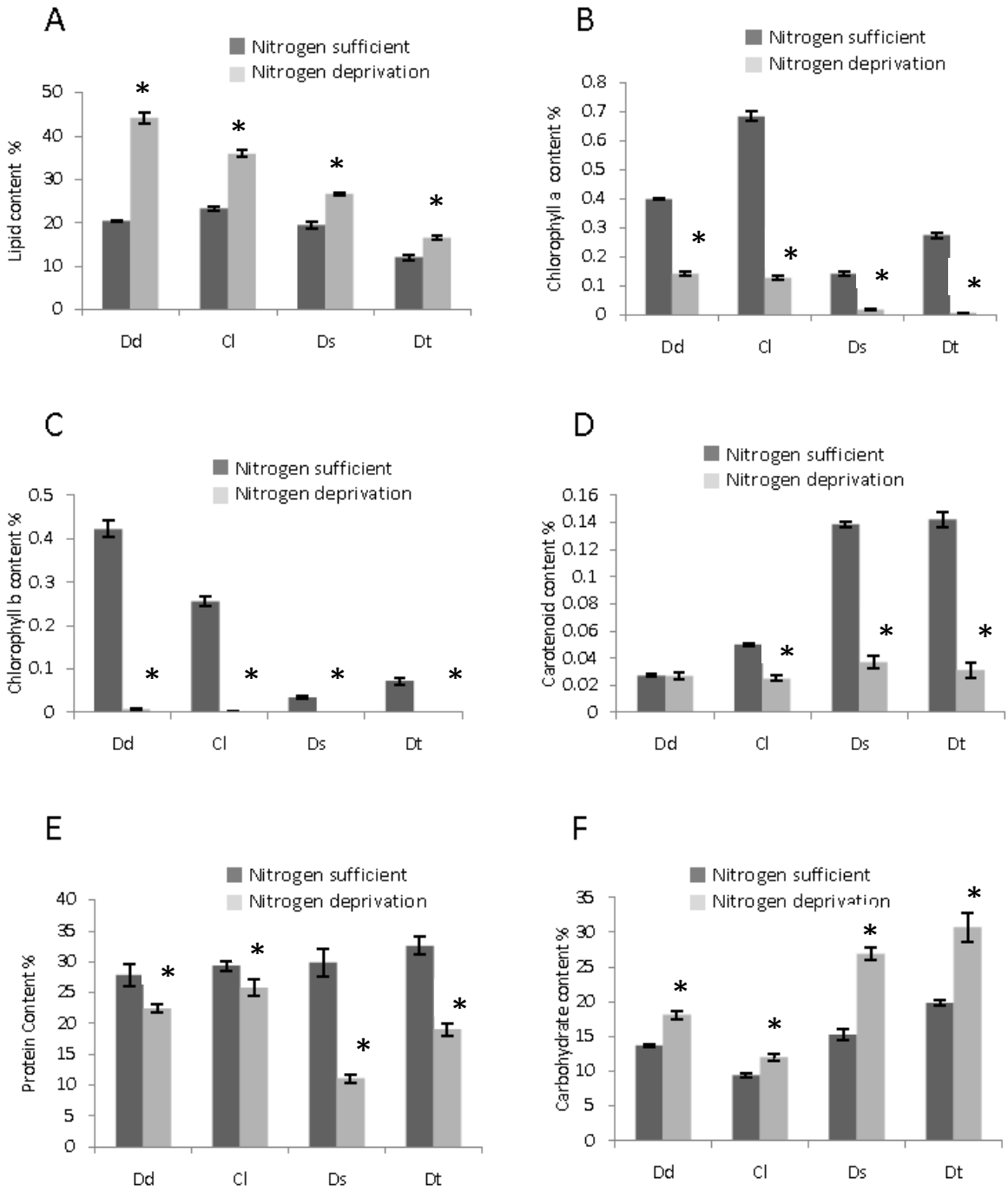

Figure 3.20 Changes in biochemical properties following 20 days of nitrogen deprivation, (A) Lipid content, (B) chlorophyll a, (C) chlorophyll b, (D) carotenoids, (E) Protein and (F) Carbohydrate. Dd - *Desmodesmus* sp. MCC34, Cl - *C.minutissima*, Ds - *D.salina* and Dt - *D.tertiolecta.* Data are the mean of triplicates. Asterisks indicate values that are significantly different from the control (nitrogen sufficient; $p<0.05$)

3.2.7 Mass cultivation of *Desmodesmus* sp. **MCC34**

Mass cultivation of algae is essential step for obtaining any industrial product. Several methods are suggested in literature for mass production of biomass; raceway pond and tubular bioreactors are the major ones that are widely used for this purpose. The growth conditions under raceway pond or tubular reactors are different from laboratory scale cultivation of algae.

Desmodesmus sp. MCC34 showing higher lipid productivity was optimized for different parameters needed for mass cultivation in a raceway pond. Areal biomass productivity and volumetric biomass productivity were calculated from growth data and expressed as kg dry biomass produced per hectare per year and mg dry biomass produced per liter per day, respectively (Figure 3.21).

Desmodesmus sp. MCC34 showed volumetric biomass productivity of 65.9 mg L^{-1} day^{-1} in laboratory condition. It was subjected to outdoor cultivation during the month of November and March 2013. The average temperature and light intensity for the month of Mar were 33°C and 735 µmol m^{-2} s^{-1} respectively, whereas, these values were 22 °C and 208 µmol m^{-2} s^{-1} respectively for Nov. The choice of these two months was based on availability of optimal light intensity and temperature prevailing in this area. Outdoor cultivation yielded areal biomass productivity of 13946.23 kg ha^{-1} $year^{-1}$ and volumetric biomass productivity of 56.94 mg L^{-1} day^{-1} during Mar which decreased to 6262.28 kg ha^{-1} $year^{-1}$ and 25.57 mg $L^{-1}day^{-1}$ during Nov. The difference in light intensity and temperature between these two months could be the reason for differences in both areal and biomass productivity. Maximum biomass concentration was reached at day 7 during Mar. In the month of Nov, biomass productivity did not match the production obtained during the month of Mar even after 13 days of incubation. These results are comparable with the earlier report on mass cultivation of *Scenedesmus*, a close relative of *Desmodesmus* species (Chinnasamy *et al.*, 2010; Griffiths and Harrison 2009; Lin and Lin 2011; Xia *et al.*, 2013).

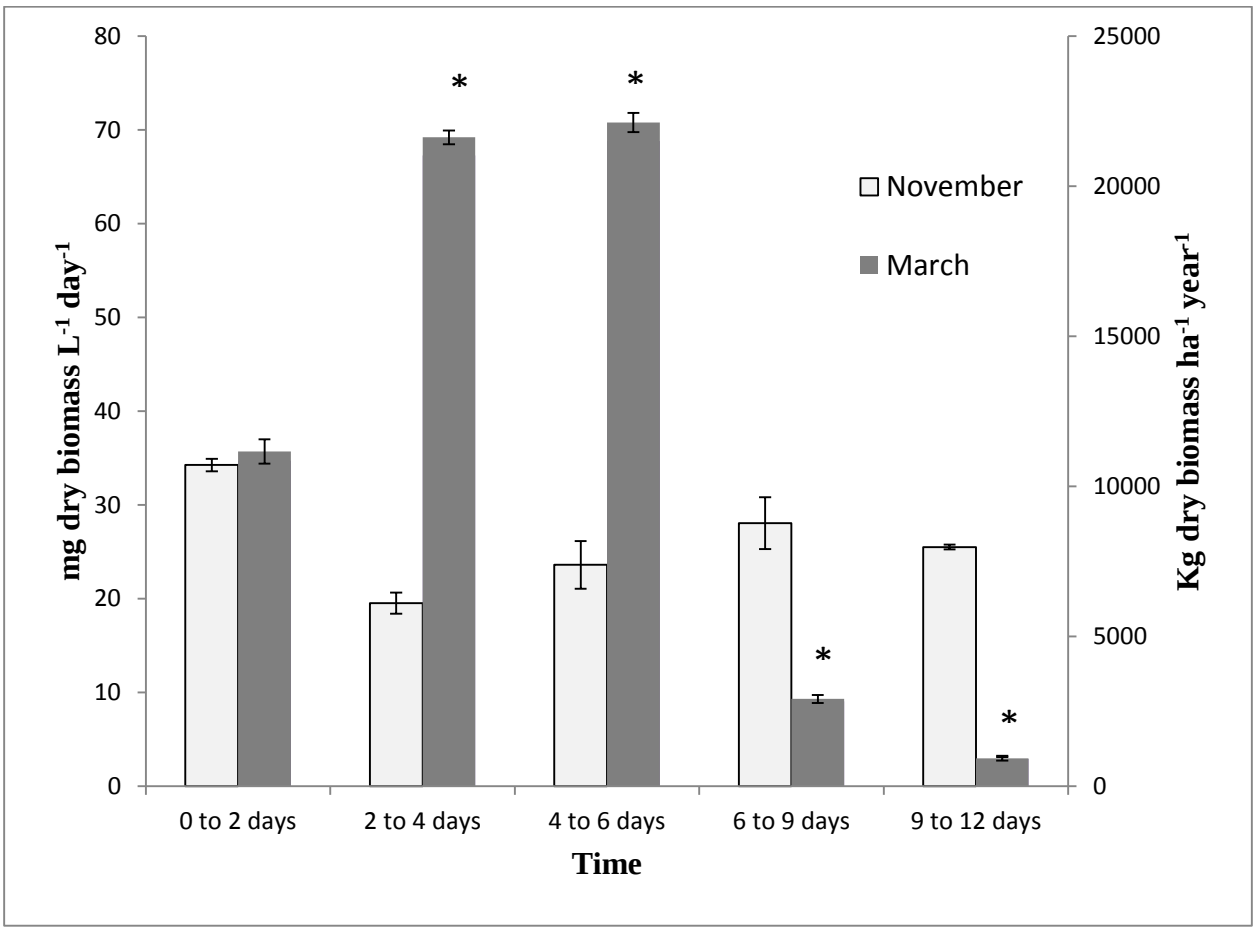

Figure 3.21 Volumetric and areal biomass productivity of *Desmodesmus* sp. MCC34, cultivated during the month of March and November, 2013 in raceway pond. Data are the mean of triplicates. Asterisks indicate the March's biomass productivity values that are significantly different from the November values ($p<0.05$).

3.2.8 Growth model for the raceway pond

Mass cultivation under raceway pond is subjected to diurnal and seasonal variation of temperature and light. These environmental factors along with intrinsic properties of algae such as molar extinction coefficient, optimal temperature, etc determine the final biomass productivity in outdoor cultivation. A growth model takes into account of these factors and their effect on growth, by which it predicts the biomass productivity (Nagappan & Verma 2016).

By knowing saturating light intensity, molar extinction coefficient, temperature growth profile, Michaelis-Menten constant of various substrates of any given algae, its outdoor mass cultivation productivity can be optimized using the equation as developed and explained in section 2.2.8. The model has been validated with the experimental results obtained under mass cultivation of *Desmodesmus* sp. MCC34 in raceway pond. Uniqueness of model is that it is developed for non-mixing condition.

A plot between specific growth rate and substrate concentration (Figure 3. 22), suggests that Michaelis Menten constant (K_S) for key macronutrients - nitrate, phosphate and sulphate to be 0.1, 0.0821 and 0.0068 mM respectively. Nitrate and sulphate were provided well above the minimum substrate concentration required to sustain maximum growth rate i.e., $S>>K_S$, therefore $Sn/(K_{Sn} +Sn)* Ss/(K_{Ss} +Ss)$ in equation 14 is equal to 1. Beyond day 4 during Mar and day 6 during Nov, phosphate concentration started to fall below the minimum substrate concentration required to sustain maximum growth rate (Table 3. 5), which led to decrease in value of f(s) or $Sp/(K_{Sp} +Sp)$ in model affecting the final biomass concentration. Light intensity decreases along the pond depth due to scattering and the light absorption by algae. These characteristics are explained by Steeles equation which incorporates saturating light intensity for growth (Equation 12). Light intensity at depth z is calculated using Beer Lamberts law. The molar extinction coefficient of *Desmodesmus* sp. MCC34 was found to be 0.22 $m^2 g^{-1}$ which is comparable with the value 0.2 $m^2 g^{-1}$ reported for *Nannochloropsis salina* (Huesemann *et al.*, 2013). Our result shows 75 μmol photon $m^{-2} s^{-1}$ as saturating light intensity for *Desmodesmus* sp. MCC34 (Figure 3. 23), whereas, 450 μmol photon

m^{-2} s^{-1} was reported as saturating light intensity for *Desmodesmus* in an earlier study (Xie *et al.*, 2013). Optimum temperature for the growth of *Desmodesmus sp*. MCC34 was found to be 35 °C corresponding to its natural habitat of mid arid region (Figure 3. 24). Thus, this local isolate has advantage of tolerating high temperature while sustaining normal growth as compared to other *Desmodesmus* strain having its growth rate affected at 24°C and above (Sánchez *et al.*, 2008). The average temperature term as calculated in $(((T-T_{max})(T-T_{min})^2)/ ((T_{opt}-T_{min})[(T_{opt}-T_{min})(T-T_{opt})-(T_{opt}-T_{max})(T_{opt}+T_{min}-2T)))$ in equation 14 or f(t) in equation 13 was found to be 0.97 for the month of March and 0.67 for November (Table 3. 6). Experimental values and the values predicted by our model for biomass concentration during month of Nov and Mar are shown in Figure 3.25. The closeness of two lines in the figure validates the proposed model for the given species and two conditions tested here. Thus, this model can be useful for developing strategy for mass cultivation of *Desmodesmus* sp. MCC34 for industrial application.

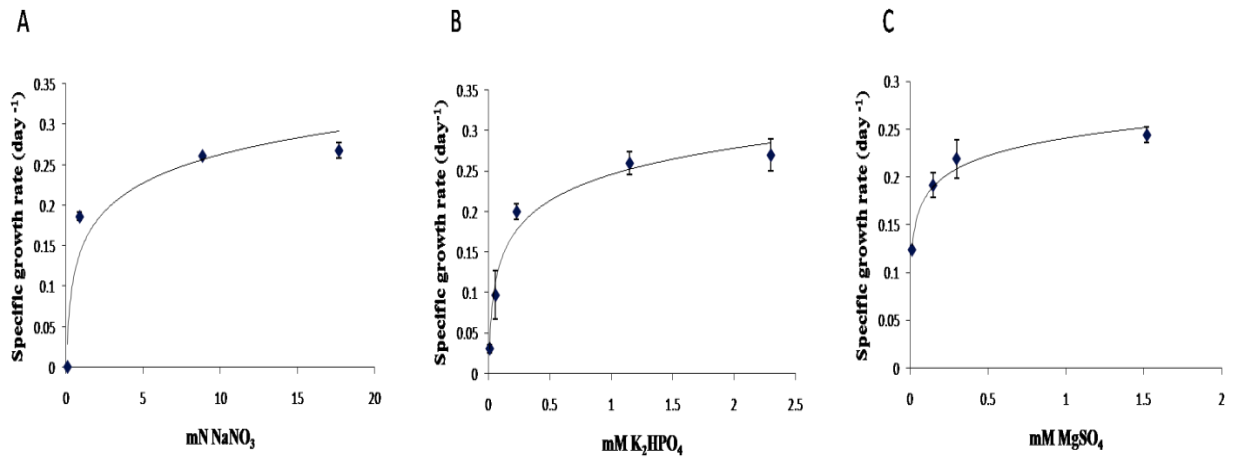

Figure 3.22 Relationship between various substrate concentration and specific growth rate. (A) $NaNO_3$, (B) K_2HPO_4 and (C) $MgSO_4$. The dots represent experimental values and line is generated by the model.

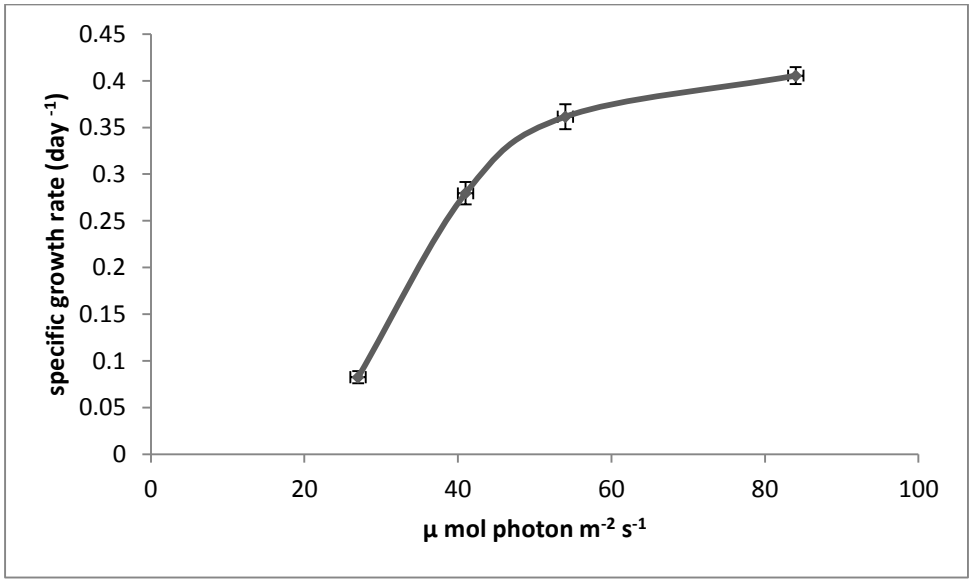

Figure 3.23 Specific growth rate at different light intensity for *Desmodesmus* sp. MCC34. Data are the mean of triplicates.

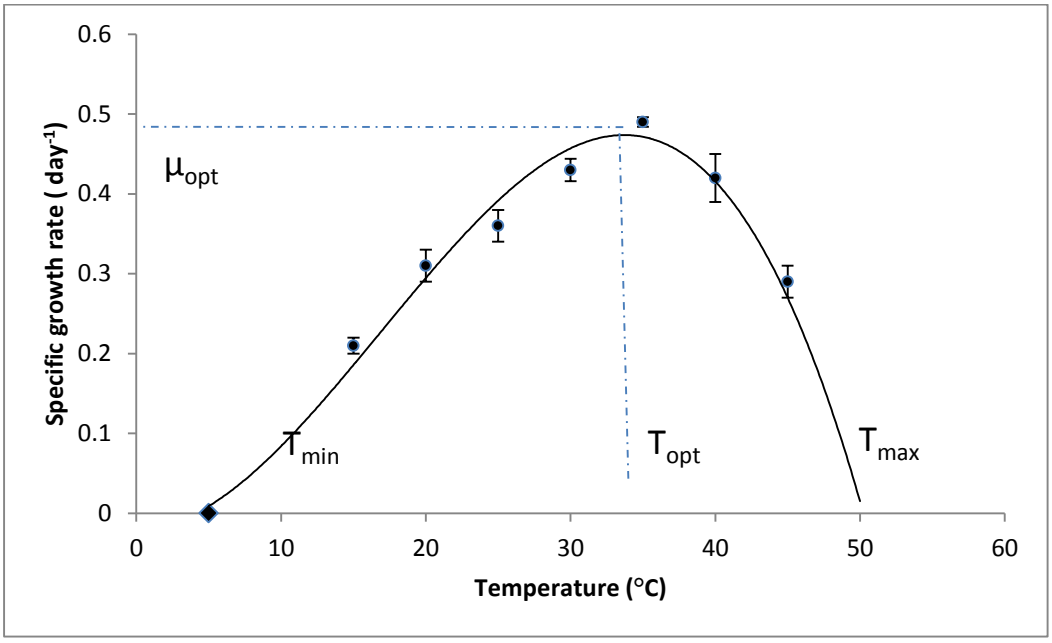

Figure 3.24 Relationship between temperature and specific growth rate. Data are the mean of triplicates.

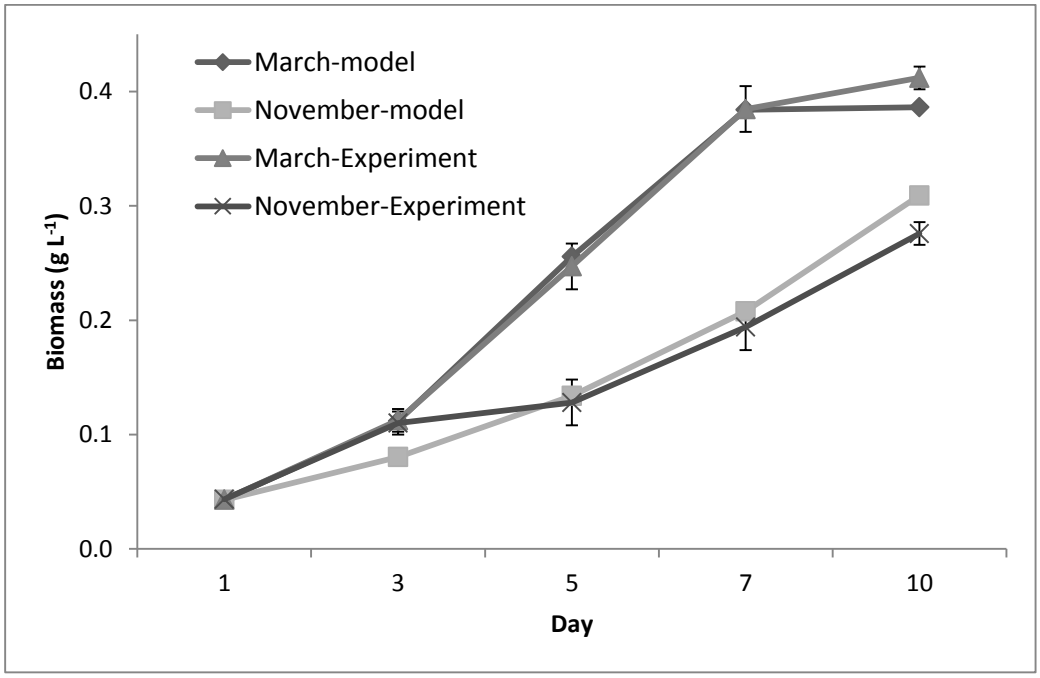

Figure 3.25 Comparison of growth values obtained by using model and experimental values obtained in raceway pond during November and March 2013. Data are the mean of triplicates.

Table 3.5 The substrate function values of phosphate during mass cultivation by raceway pond in the month of November (A) and March (B) during the year 2013.

A

Day	f(s)
2	1
4	1
6	0.95
9	0.9

B

Day	f(s)
2	1
4	0.94
6	0.85
9	0.67

Table 3.6 The temperature function values during mass cultivation by raceway pond cultivation in the month of November (A) and March (B) during the year 2013.

A

Day	f(t)
2	0.66
4	0.68
6	0.7
9	0.63
Average	0.67

B

Day	f(t)
2	0.99
4	1
6	0.95
9	0.94
Average	0.97

3.3 Characterization of lipid extract for biodiesel and alpha linolenic acid methyl ester production

Identification and quantification of the lipid extracts for the presence of fatty acid methyl esters (biodiesel) including alpha linolenic acid methyl ester (ALAME) was performed using the techniques of thin layer chromatography, gas chromatography and mass spectrometry.

3.3.1 FAME separation and the column performance

The fatty acid methyl ester (FAME), which is obtained from the lipid through transesterification, is a mixture of methylated fatty acids such as palmitic acid methyl ester, stearic acid methyl ester, etc. In order to determine the composition of FAME in microalgal lipid, the individual FAME has to be separated with gas chromatography, using suitable column. With this aim, two columns were selected; one was a packed column, and the other a capillary column. The advantage of packed column is that, it is cheaper and can handle large volume of sample, but is less efficient to resolve closely related fatty acid peaks such as gamma linolenic acid methyl ester and alpha linolenic acid methyl ester (Willard 1986). Figure 3.26 (A) shows the separation of fatty acids methyl ester of *Desmodesmus* sp. MCC34 lipid extract by 10% diethylglycol succinate packed column, where broader peaks of individual FAME molecules, indicating low resolution can be seen. In contrast, the capillary column with a narrow pore size and longer length (6 m) gave the advantage of increased resolution of fatty acids, showing sharper peaks (Figure 3.26 B). Despite the cost, capillary columns are preferred over packed columns for separation of FAME owing to increased resolution (Adewuyi & Oderinde 2012; Woo *et al.,* 2012). Therefore in the present study, capillary column was chosen over packed column for analysis of the compounds.

3.3.2. Mass spectra analysis of important fatty acid methyl esters

The important fatty acids as well as their isomers were identified from the selected microalgae with the help of mass spectrometry. The major FAMEs obtained from different species and their characteristic mass spectra are as follows.

3.3.2.1 Palmitic acid methyl ester

Figure 3.27 (A) shows mass spectra of palmitic acid methyl ester. The characteristic peak of palmitic acid methyl ester was observed as an ion with m/z=270 (Eglinton *et al.*, 1968), whereas the ion observed at m/z=239 represents a fragment of the parent ion, without methoxyl group; the methoxyl group could be seen as a molecular ion with m/z=31 in the mass spectra. The ion with m/z=76 is the resultant of Mclafferty rearrangement pattern characteristic of fatty acid methyl ester (Eglinton *et al.*, 1968), in which the hydrogen at 4^{th} position of aliphatic chain is transferred to carbo-methoxy group of fatty acid methyl ester through successive transition state, making this rearrangement sterically favourable. Some of the other fragment ions observed at m/z=87,101, 115, 128, 143, 157, were found to be the part of the fatty acid methyl esters of general formula, $CH_3COO(CH_2)_n$.

3.3.2.2 Oleic acid methyl ester

Figure 3.27 (B) shows mass spectra of oleic acid methyl ester, where we can see the characteristic peak of oleic acid methyl ester as ion with m/z=296 (Kleiman & Spencer 1973). The loss of methoxyl group from this compound gave rise to a fragment with m/z=264. The ion with m/z=222, represents a fragment without Mclafferty ion; this ion is resultant of a rearrangement pattern common to mass spectrometer in which a keto group undergoes β-cleavage and gains γ-hydrogen. The ions with m/z=152, 166, 180 represents, monoeic fatty acid methyl ester fragments of general formula C_nH_{2n-1}.

3.3.2.3 Linoleic acid methyl ester

Figure 3.27 (C) shows mass spectra of linoleic acid methyl ester, with the characteristic peak of parent ion at m/z=294 (Fasman & Sober 1977). The ion with m/z=220 represents the loss of

Mclaffarty fragment in linoleic acid methyl ester, while the ion with m/z=262 represents the linoleic acid methyl ester without methoxy group. The hydrocarbon fragments of general formula C_nH_{2n-3} having m/z= 67, 95, 81, 125, 109,135 are also seen.

3.3.2.4 Linolenic acid methyl ester

Figure 3.27 (D) & (E) shows mass spectra of two isomers of linolenic acid methyl ester. The characteristic peak of linolenic acid methyl ester of both isomers is the ion with m/z=292 (Fasman & Sober 1977). These isomers can be distinguished based on fragmentation pattern of omega ions. The omega ion of alpha linolenic acid methyl ester has m/z=108, which is due to the presence of first double bond in 3rd carbon from the end of aliphatic chain. While the omega ion of gamma linolenic acid methyl ester has m/z=150, due to the double bond present in 6th carbon from the end of aliphatic chain. The alpha ion fragment of alpha linolenic acid methyl ester has m/z=236 whereas the alpha ion fragment of gamma linolenic methyl ester has m/z= 194. The Mclaffarty ion is very small in abundance therefore difficult to observe. The hydrocarbon chain fragments of general molecular formula C_nH_{2n-5} predominates the spectra with ions beginning with m/z=79.

3.3.3 FAME profile of selected microalgae

FAME profile of six algal species which showed highest lipid productivity is given in Table 3.7. The major fatty acids found in these algae are palmitic acid (C16), stearic acid (c18), oleic acid (C18:1), linoleic acid (C18:2). While C16 and C18:3n-3 (ALAME) were present in *Desmodesmus* sp. MCC34, C16 and C18:2 were detected in *C. minussitima*, C16, C18:1 and C18:2 in *D. salina*, C14, C16, C18:1 and C18:2 in *D. tertiolecta*.

The major polyunsaturated fatty acids (PUFA) detected in algae in this study were linoleic acid (C18:2), linolenic acid (C18:3) and hexadecadienoic acid (C16:2). In case of fresh water algae (*C. minussitima* and *Desmodesmus* sp. MCC34), 50% of total fatty acid is PUFA which is known to provide fluidity and integrity to the membrane. These values falls in the similar range to fatty acid profile reported in literature for 30 different species of *Chlorella* and *Scenedesmus* (Ördög *et al.*, 2013). Palmitic acid (C16) is single abundant saturated fatty acid (SFA) found in all algae.

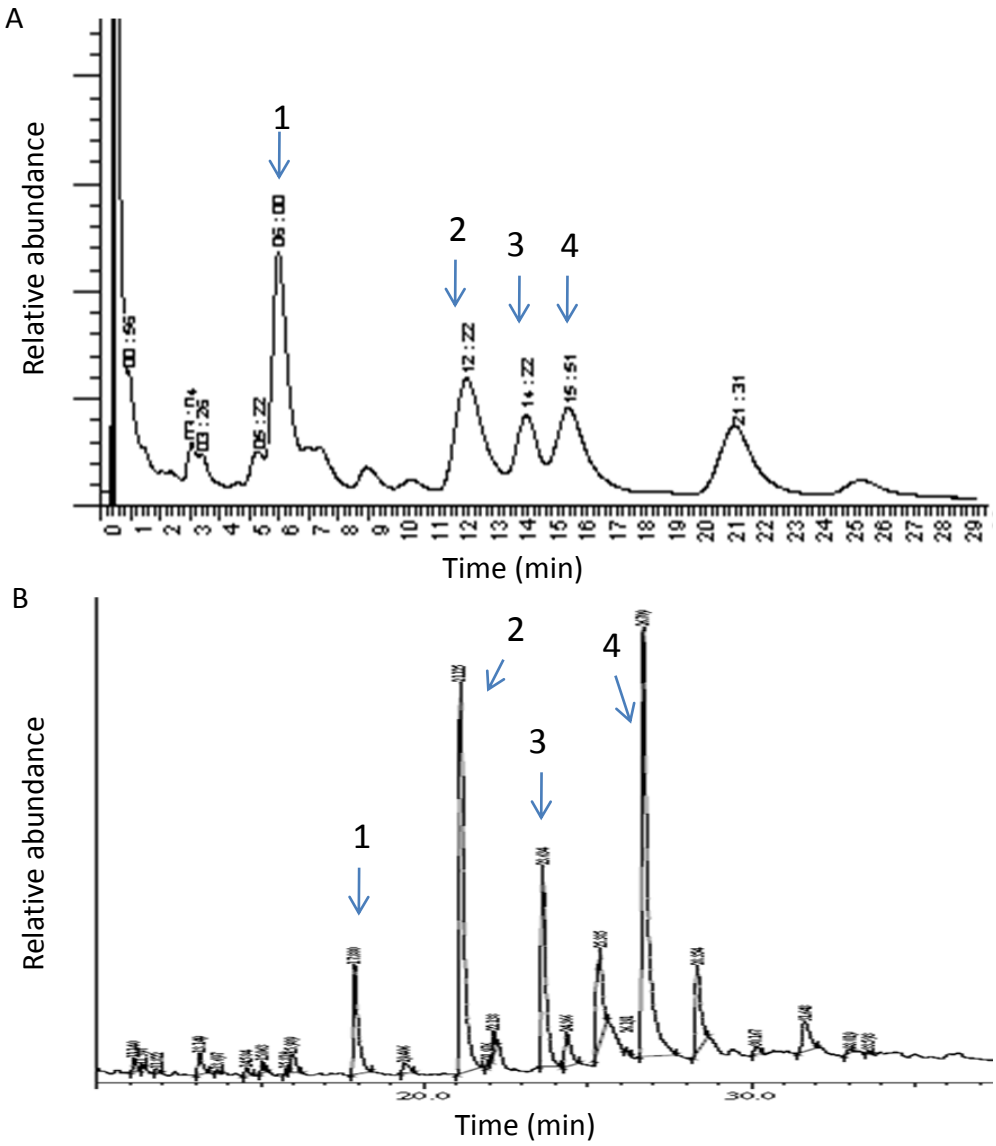

Figure 3.26 Difference in the peaks' structure obtained for fatty acid methyl ester using different columns (A) Resolution of fatty acids of *Desmodesmus* sp. MCC34 by packed column (B) Resolution of fatty acids of *Desmodesmus sp*. MCC34 by capillary column. 1 represents palmitic acid methyl ester, 2 is oleic acid methyl ester, 3 is linoleic acid methyl ester and 4 is alpha linolenic acid methyl ester.

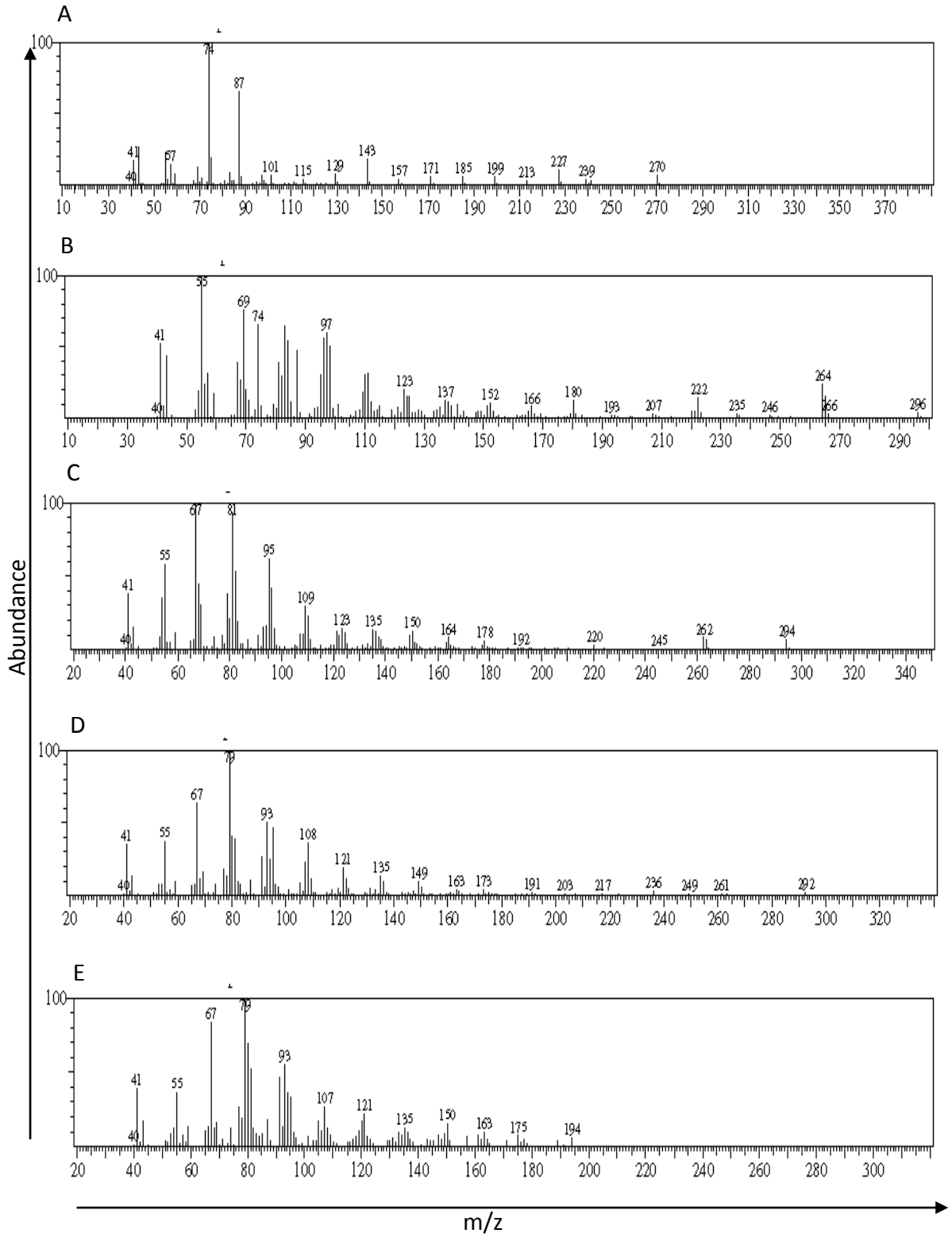

Figure 3.27 Mass spectra of (A) palmitic, (B) oleic, (C) linoleic acid, (D) alpha linolenic acid and (E) gamma linolenic acid methyl esters obtained from *Desmodesmus* sp. MCC34.

In *N. muscorum*, palmitic acid constitutes nearly 42% of total fatty acid as it is the final end product of de-novo fatty acid synthesis pathway. Oleic acid (C18:1) is the dominant mono unsaturated fatty acid (MUFA) found in all six algal species. Both species of halotolerant *Dunaliella* shows slightly higher proportions of this fatty acid compared to fresh water species and cyanobacteria. Such high concentration of oleic acid could be attributed to higher rate of carotenogenesis reported in *Dunaliella* species (Mendoza *et al.,* 1999). It is established fact that degree of unsaturation, isomerism and chain length of fatty acid determine the quality of biodiesel (Olmstead *et al.,* 2013a). SFA is reported to have good combustion and is free from the problem of oxidation but it suffers from poor cold flow properties (Jeong *et al.,* 2008). PUFA can provide required cold flow properties but is prone to oxidation (Olmstead *et al.,* 2013a). An alga with abundant MUFA is a better candidate as it provides good biodiesel qualities such as good combustion, oxidation stability and cold flow properties (Jeong *et al.,* 2008). The condition of nitrogen deprivation, had effects on fatty acid profile of algae with an increase in SFA and minimal decrease in PUFA (Table 3.8). MUFA especially oleic acid (C18:1) increased drastically in both *Dunaliella* species which is indicator of increased carotenogenesis during nitrogen deprivation (Mendoza *et al.,* 1999).

3.3.4 Biodiesel property of the selected microalgae

In order to assess quality of biodiesel, cetane number and iodine value were calculated and presented in Table 3.9. The cetane number is an indicator of speed of fuel combustion, whereas iodine value indicates oxidation of oil and its shelf life. The quality standard, set for cetane number, varies from country to country. The accepted cetane number is above 47 in Europe (EN 14214), above 40 in United States (ASTM 6751) and above 48 in India (BIS 15607:2005). Biodiesel from all species have shown cetane number complying with United States standard. According to Indian, European and United States standard, iodine value should not exceed 120 g I_2 per 100 g (Tyagi *et al.,* 2010). Iodine value of biodiesel from all species falls below 120 g I_2 per 100 g except for *Desmodesmus* sp. MCC34. This is due to higher amount of unsaturated fatty acid, predominately alpha linolenic acid, which is present in this species. Therefore, if we remove ALAME and PUFA from FAME, it may prove to be an ideal source of biodiesel. Nitrogen stress showed moderate decrease in iodine value and slight increase in cetane number of biodiesel obtained from algal species studied (Table 3.10).

Table 3.7 The FAME (%) profile of selected algae. Data are mean of triplicates, n = 3 ± SE.

FAME	*C. minutissima* (%)	*Desmodesmus sp* (%)	*D.salina* (%)	*D. tertiolecta* (%)	*N. muscorum* (%)	*A. doliolum* (%)
C14	7.5 ± 0.9	2.4 ± 0.1	2.3 ±0.1	18 ± 0.9	6.2 ± 0.5	7.9 ± 0.6
C16	26.5 ± 1.7	25.6 ± 2.1	20.4 ±1.4	23.7 ± 1.6	41.9 ± 3.5	25.9 ± 1.1
C16:1	1 ± 0.1	2.5 ± 0.2	7.2 ±0,3	3.4 ± 0.1	8.2 ± 1	15.5 ± 0.9
C16:2	12.7 ± 0.9	2.4 ± 0.2	11.2 ±0.6	3.6 ± 0.1	0	0.6
C18	2.8 ± 0.2	1.8 ± 0.1	3 ±0.1	8.7 ± 0.5	7.5 ± 0.6	1.3 ± 0.1
C18:1	8.5 ± 0.6	15 ± 1.6	26.2 ±1.8	18.3 ± 1.4	0.9	20.9 ± 1.6
C16:4	0.2	5.4 ± 0.3	0	0	0	0
C18:2	34.7 ± 2.5	15.8 ± 1.9	19.5 ±1.8	14.9 ± 1.1	22.1 ± 1.4	20.5 ± 1.5
C18:3(n-6)	0	4.3 ± 0.4	1.3	0	0	0
C18:3(n-3)	6.2 ± 0.4	24.8 ± 2.6	8 ±0.2	9.4 ± 0.8	13.3 ± 0.6	7.4 ± 0.8
C18:4	0	0	0.8	0	0	0
SFA[1]	37	30	26	50	56	35
MUFA[2]	9	18	33	22	9	36
PUFA[3]	54	53	41	28	35	28

[1]SFA-saturated fatty acid

[2]MUFA- monounsaturated fatty acid

[3]PUFA –polyunsaturated fatty acid

Table 3.8 The FAME (%) profile of selected algal species under nitrogen deprivation. Data are mean of triplicates, n = 3 ± SE.

FAME	*C. minutissima* (%)	*Desmodesmus* sp. (%)	*D. salina* (%)	*D. tertiolecta* (%)
C14	8.89 ±0.31	3.22 ±0.08	4.20 ±0.12	2.57 ±0.05
C16	25.51 ±0.67	31.39 ±1.01	23.43 ±0.68	23.76 ±0.77
C16:1	1.28 ±0.03	1.59 ±0.05	4.90 ±0.15	1.47 ±0.04
C16:2	8.81 ±0.17	0.42 ±0.02	7.42 ±0.18	4.23 ±0.19
C18	6.72 ±0.22	2.42 ±0.07	4.14 ±0.11	2.63 ±0.07
C18:1	6.34 ±0.18	12.37 ±0.32	30.33 ±0.99	34.27 ±1.10
C16:4	0.46 ±0.01	6.05 ±0.12	_	_
C18:2	33.92 ±0.98	15.99 ±0.37	14.58 ±0.54	16.07 ±0.61
C18:3(n-6)	_	2.86 ±0.08	0.57 ±0.08	_
C18:3(n-3)	8.07 ±0.28	23.70 ±0.77	9.59 ±0.21	15.01 ±0.49
C18:4	_	_	0.83 ±0.01	_
SFA[1]	41	37	32	29
MUFA[2]	8	14	35	36
PUFA[3]	51	49	33	35

[1]SFA-saturated fatty acid

[2]MUFA- monounsaturated fatty acid

[3]PUFA –polyunsaturated fatty acid

Table 3.9 Biodiesel properties of algae grown in normal BG11 media.

Biodiesel Property	*C. minutissima*	*Desmodesmus* sp. MCC34	*D. salina*	*D. tertiolecta*	*N. muscorum*	*A. doliolum*
Iodine value (g I_2 100 g^{-1})	109.3	143.9	111.7	76.0	81.5	88.5
Cetane number	48.9	41.5	48.7	56.1	55.0	53.5

Table 3.10 The biodiesel properties after 20 days of nitrogen deprivation

Biodiesel Property	*C. minutissima*	*Desmodesmus* sp. MCC34	*D. salina*	*D. tertiolecta*
Iodine value (g I_2 100 g^{-1})	104.9	133.3	99.5	105.9
Cetane number	49.9	43.7	51.4	50.2

3.3.5 Alpha linolenic acid methyl ester separation and characterization

3.3.5.1 ALAME separation and identification through mass spectra

Alpha linolenic acid or its methylated form (ALAME) is an omega 3 fatty acid and a pharmaceutically important compound found in selected lipids of selected species. In present study we have screened lipid for the presence of ALAME in six algal species by using mass spectra peak of fatty acid methyl ester (FAME).

The term ALAME and gamma linolenic acid methyl ester (GLAME) are interchangeably used in literature due to improper identification (Guil-Guerrero 2014). The separation of isomers of linolenic acid methyl ester was carried out successfully by the biscyanopropyl gas chromatography column and mass spectra clearly distinguished these two isomers (Figure 3. 28). The presence of characteristic peak of alpha linolenic acid (m/Z = 108 and m/z=236) indicates the presence of polyunsaturated fatty acid with n-3 terminal moiety and alpha ions fragment that has two double bonds (Figure 3.29).

3.2.5.2 Identification through NMR

ALAME was also confirmed by nuclear magnetic resonance (NMR) analysis. Figure 3.30 shows NMR spectrum of ALAME obtained from the purified lipid fraction of *Desmodesmus* sp.MCC34 and Table 3.11 shows various peaks obtained from proton NMR spectra. The signal of the peak and their integration values correspond to that of ALAME's, except a peak at 1.6 which should had an integration value of 2, instead had value of 24. This might be due to possible contamination which could have co–eluted with ALAME rich fraction during its purification.

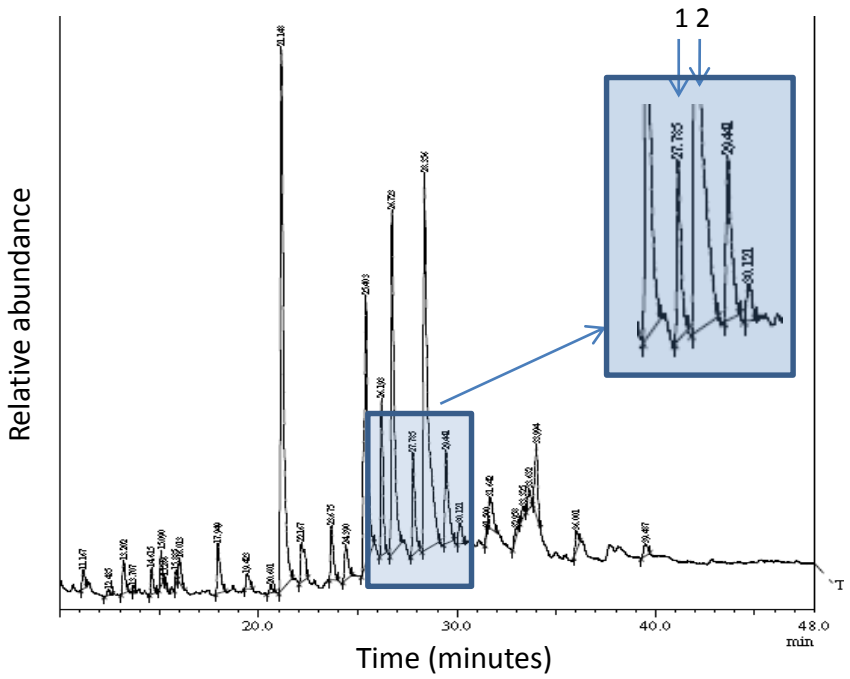

Figure 3.28 The separation of isomers (1) gamma linolenic acid methyl ester and (2) alpha linolenic acid methyl ester in *Desmodesmus* sp. MCC34 by biscyanopropanyl capillary column

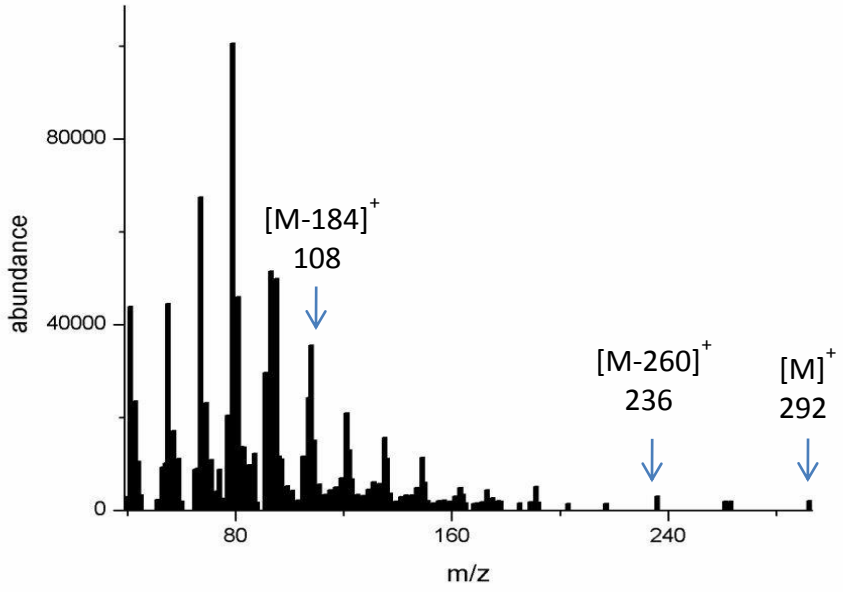

Figure 3.29 Mass spectra of alpha linolenic acid methyl esters. The source of ALAME is *Desmodesmus* sp. MCC34

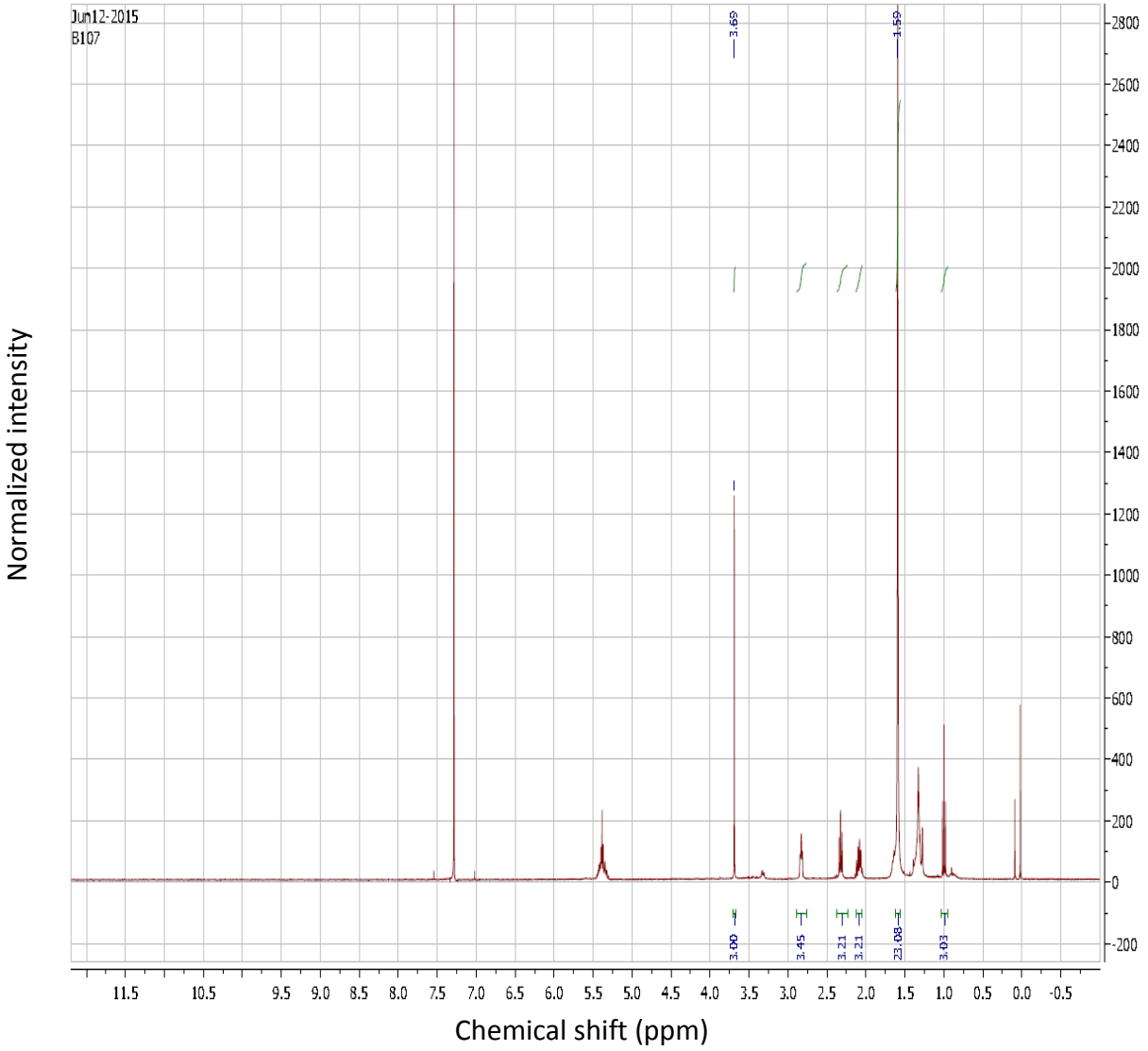

Figure 3.30 NMR spectra of ALAME of *Desmodesmus* sp. MCC34.

Table 3.11 NMR analysis of purified ALAME showing the signals and their interpretation

S.No	Signal (ppm)	Interpretation	Number of protons
1	7.6	$CDCl_3$	_
2	5.3	Olefenic proton	6
3	3.6	Methyl ester	3
4	2.8	Bis allylic proton	4
5	2.3	First methyl after ester	2
6	2.05	Allylic proton	
7	1.6	Second methyl after ester	2
8	1.3	Strong CH_2 peak	8
9	0.95	Last methyl group	3
10	0	TMS standard	_

3.3.6 ALAME production

ALAME content in 100g of dry biomass was calculated according to equation 20. As shown in Table 3.12, the ALAME content in 100g of dry biomass was found to be highest in *Desmodesmus* sp. MCC34 (4.37 g; $p<0.1$) followed by *D. salina* (1.25 g), *N. muscorum* (1.14 g), *C. minutissima* (1.11 g), *D. tertiolecta* (0.88 g) and *A. doliolum* (0.45 g). In order to assess composition of FAME, ALAME percentage within total FAME was calculated. Percent ALAME content in FAME was found to be highest in *Desmodesmus* sp. MCC34 (24.8%; $p<0.1$) followed by *N. muscorum* (13.3%), *D. tertiolecta* (9.4%), *D. salina* (8%), *A. doliolum (*7.4%) and *C. minutissima* (6.2%). The 24.8% of ALAME in FAME as shown by *Desmodesmus* sp. MCC34 is much higher than the reported value of 8% in *Scenedesmsus acutus* and 14 to 19% in *Chlorella* species (Batista *et al.*, 2013; Benvenuti *et al.*, 2014; Parsaeimehr *et al.*, 2015).

3.3.6.1 ALAME production under nitrogen stress

The fatty acid composition in algae is known to change with varying growth condition or by adding growth supplements in media. Earlier report involving *Chlorella* also suggests that a shifting from TAP acetate medium to acetylcholine supplemented medium increases the ALA content from 14.2% to 26.2% (Parsaeimehr *et al.*, 2015). *Desmodesmus* sp. MCC34 which showed 24.8% ALAME content in FAME was subjected to nitrogen deprivation and its effect on ALAME production along with growth and protein content was studied. Figure 3.31 compares ALAME production by algae grown separately for 20 days in normal condition and in nitrogen deprived media. ALAME content in the biomass showed significant increment under nitrogen deprived condition as compared to normal state ($p<0.05$). This can be attributed to increased accumulation of lipid during nitrogen deprivation as reported earlier for eleven algae (Breuer *et al.*, 2012; Ördög *et al.*, 2013).

The ALAME and lipid productivity depends on biomass production as shown in earlier section. Comparison of growth of *Desmodesmus* sp. MCC34 under normal media (control) and nitrogen deprived media suggested the first 20 days of nitrogen deprivation does not show much effect on biomass production followed by severe growth inhibition (Figure 3.32). Lipid content showed 0.7 fold increase in cells incubated under nitrogen deprived condition for 20 days, which was also reflected in ALAME content in the biomass. The protein is known to constitute 40% of algal

biomass and 15% of total cellular nitrogen (Darley 1977). It is also reported that the degradation of amino acid by cysteine protease and amino acid oxidase leads to over accumulation of TCA cycle intermediates which in turn are used for making alternate proteins that are essential for lipid metabolism (Dong *et al.,* 2013). In one case, 18-fold increments in alpha ketoglutarate were observed during nitrogen deprivation (Guarnieri *et al.,* 2011). As shown in Figure 3.33, the protein content in the cells grown in nitrogen deprived medium showed steady decline as compared to the cells grown in normal media. Thus the accumulation of ALAME in *Desmodesmus* sp. MCC34 during nitrogen deprivation can be associated with decrement in protein content

3.3.7 Purification of alpha linolenic acid methyl ester

A small scale purification of alpha linolenic acid was achieved with the help of RP-TLC and silver ion chromatographic technique. Method was standardized with the help of TLC and was implemented to purify ALAME through silver ion column separation

3.3.7.1 Reverse phase thin layer chromatography

The acetonitrile had been reported previously for the usage of mobile phase in RP-TLC for the separation of omega-3 fatty acid methyl esters (Jham *et al.,* 2005). Since ALAME is an omega 3 fatty acid, the same mobile phase was tested for its separation. The retention factor of separated ALAME along with other fatty acid methyl ester is shown in Table 3.13. ALAME had distinctive retention factor (RF) value indicating a clear separation from the rest of the fatty acid methyl ester.

3.3.7.2 Silver ion thin layer chromatography

Silver ion chromatography involves transfer of charge from silver ion to the double bond of unsaturated fatty acids. This phenomenon is utilized to separate different classes of fatty acid in which a non-polar mobile phase can elute saturated fatty acid faster in comparison to unsaturated fatty acid. The mobile phase generally usually used in this technique is hexane (Guil-Guerrero *et al.,* 2003; Sajilata *et al.,* 2008). The petroleum ether as mobile phase showed similar RF value in

Table 3.12 ALAME content in biomass and ALAME content in FAME. n = 3 ± SE. Different alphabetic superscripts denote significantly different means.

	C. minutissima	*Desmodesmus* sp. MCC34	*D. salina*	*D. tertiolecta*	*N. muscorum*	*A. doliolum*
ALAME content in biomass (g/100 g of dried biomass)	1.11±0.07 [a]	4.37 ±0.46 [b]	1.25 ±0.03 [a]	0.88 ±0.08 [d]	1.14 ±0.05 [a]	0.45±0.05 [e]
ALAME content in FAME (%)	6.18 ±0.18 [a]	24.82 ±0.61 [b]	8.02 ±0.21 [c]	9.36 ±0.41 [d]	13.33 ±0.51 [e]	7.36 ±0.21 [a]

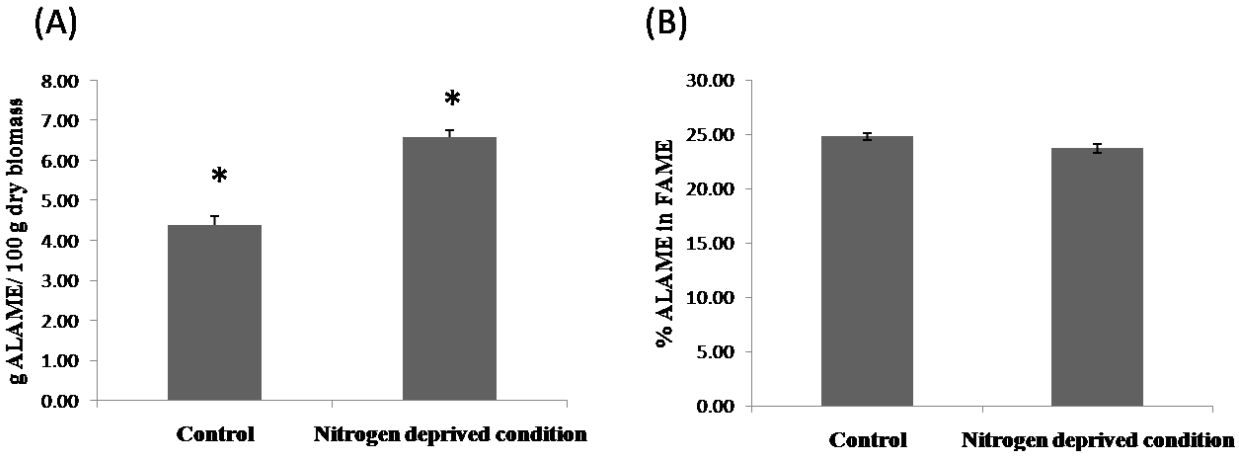

Figure 3.31 (A) The ALAME productivity (g 100 g^{-1} of dried biomass) and (B) the content of ALAME in FAME (%) in *Desmodesmus* sp. MCC34. Data is mean of triplicates. Asterisks indicates significant difference, $p<0.05$.

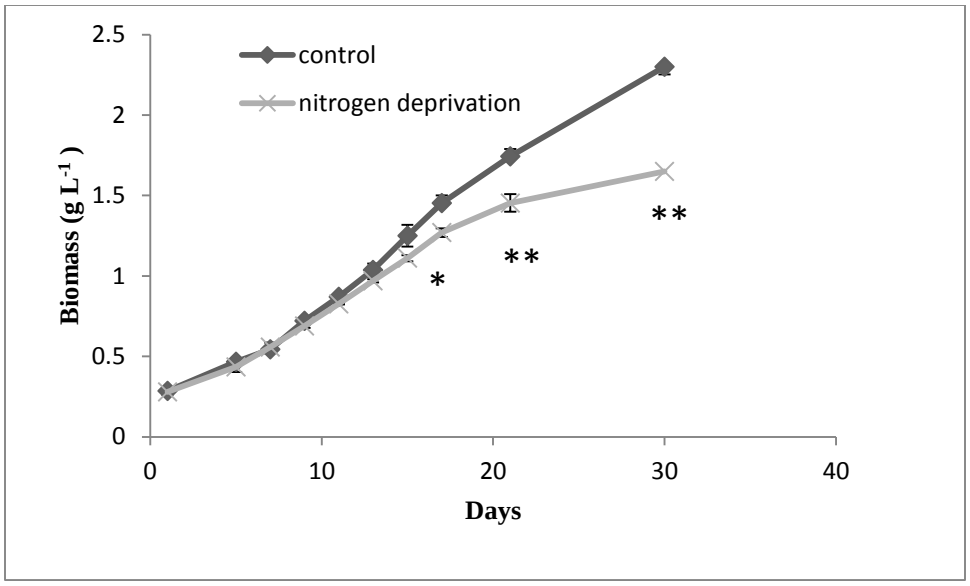

Figure 3.32 Comparison of growth rate between *Desmodesmus* sp. MCC34 grown in normal media (control) and in nitrogen deprived media for 20 days. Data is mean of biological triplicates. The symbol * indicates $p<0.05$ and ** indicates $p<0.01$.

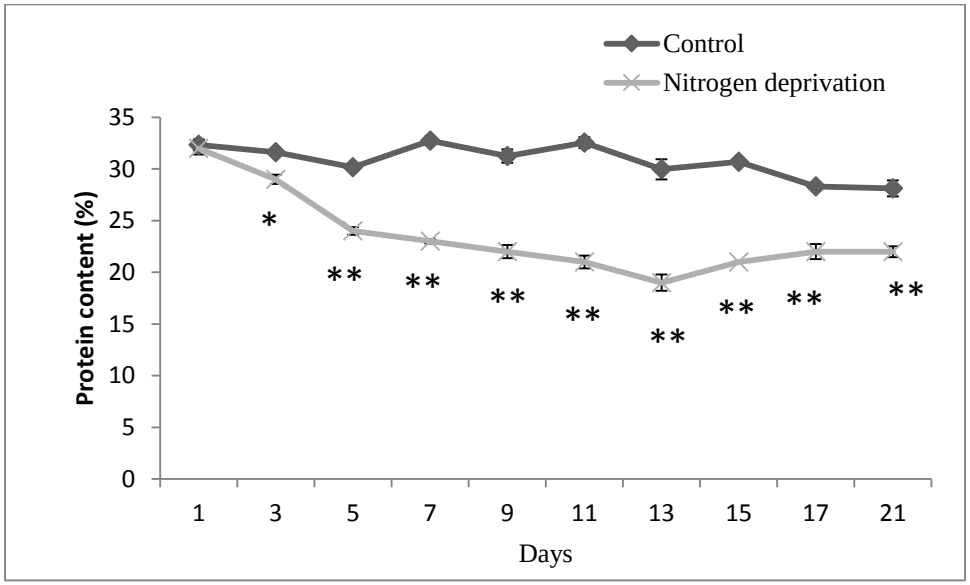

Figure 3.33 Protein content in *Desmodesmus* sp. MCC34 grown under normal condition (control) and in nitrogen deprived condition. Data is mean of biological triplicates. The symbol * indicates $p<0.05$ and ** indicates $p<0.01$.

obtaining ALAME by silver ion thin layer chromatography (Ag-TLC) as that of hexane (Table 3.13, Table 3.14 & Figure 3.34).

3.3.7.3 Purification of ALAME by silver ion chromatography

The silver impregnated silica gel column was sequentially eluted with eluent A, B, C, D, E, F and G. During the elution with eluent F and G, the ALAME could be separated in their respective fraction. After completion of the elution steps, the ALAME dominant fractions F and G were combined and rest of the fractions (A, B, C, D and E) were combined separately and analyzed through gas chromatography. The results of FAME analysis of these fractions are shown in Table 3.15, Table 3.16 and Figure 3.35. The fraction F and G yielded ALAME of 91% purity whereas gamma linolenic acid methyl ester (GLAME) dominated the impurity fraction. The similar method adopted earlier resulted in 100% purification of ALAME from linseed oil, as this oil does not contain GLAME, indicating that this method cannot separate the two species of linolenic acid (Guil-Guerrero *et al.*, 2003). In another study, the GLAME was purified from *Spirulina* using the same method by which a purity of 96% was obtained with recovery of 66% (Sajilata *et al.*, 2008).

3.3.7.4 Separation of ALAME from FAME for the improved biodiesel properties and value addition

The ALAME which was present dominantly in the *Desmodesmus* sp. MCC34 was separated from the lipid extract, as shown in the above section. The rest of the fractions if combined will result in FAME having good biodiesel properties.

Therefore, the combined fraction of A, B, C, D and E which had more of saturated, mono unsaturated fatty acid and lesser ALAME showed cetane number and iodine value (g I_2 per 100 g) of 51 and 99 respectively which are acceptable values for biodiesel (Table 3.17). Thus by using the proposed method, the same biomass can be used for biodiesel production as well as value added ALAME.

Table 3.13 The retention factor of fatty acid and their methyl ester derivatives using RP-TLC with acetonitrile as mobile phase

S.No	Compound Name	Retention factor
1	Oleic acid	0.4
2	Linoleic acid	0.52
3	Alpha Linolenic acid	0.61
4	Oleic acid methyl ester	0.33
5	Linoleic acid methyl ester	0.43
6	Alpha Linolenic acid methyl ester	0.54

Table 3.14 Retention factor of ALAME separated using mobile phases, petroleum ether and hexane.

S.No	Mobile phase	Retention factor
1	Petroleum ether:acetone(95:5,v/v)	0.3
2	Hexane:acetone(95:5,v/v)	0.337

Table 3.15 The FAME (%) profile as analyzed by RP-TLC of the individual fractions A, B, C, D, E, F and G for which the solvent composition is given in Table 2.5. Unit of FAME is mg.

	A	B	C	D	E	F	G
c18:1	2.745	3.9	8.1	0.3	0	0	0
c18:2	0	0	7.84	6.08	1.76	0	0
c18:3(n-3)	0	0	0.025	3.825	6.5	8	6.75

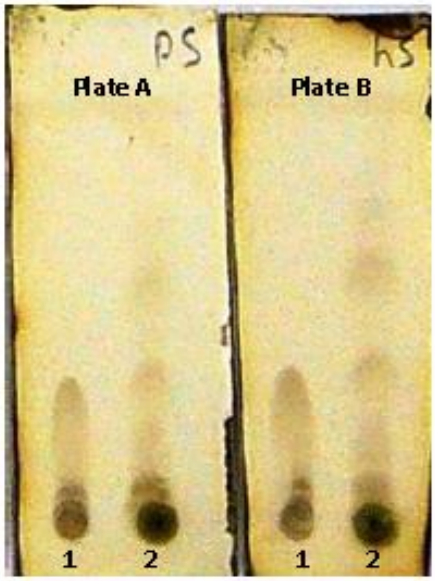

Figure 3.34 The FAME and ALAME were run on silver ion thin layer chromatography plates with petroleum ether (A) and hexane (B) as mobile phases for 5 minutes. The spot-1 represents standard ALAME and spot-2 is total FAME.

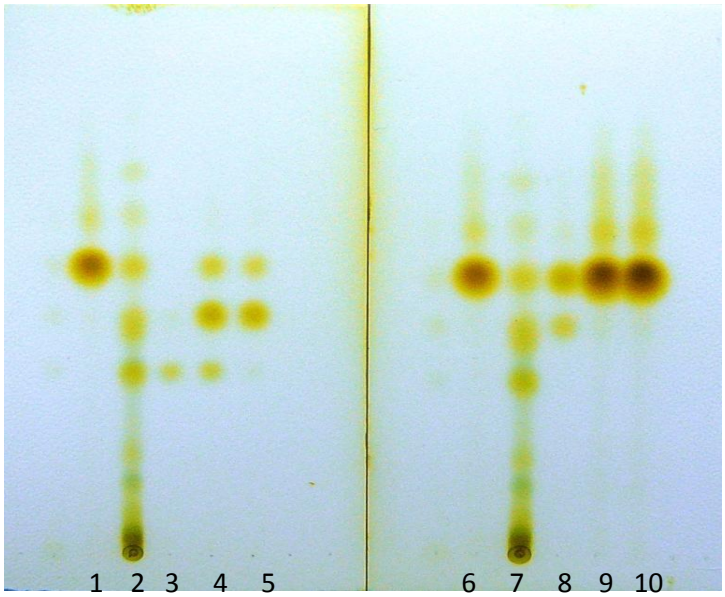

Figure 3.35 RP-TLC analysis of fractions eluted by argentated column chromatography. Lanes 1&6-ALAME ester standard, 2&7-crude biodiesel, 3-Eluent A, 4-Eluent B, 5-Eluent C, 8-Eluent D, 9-Eluent E and 10-Eluent F.

Table 3.16 The FAME (%) profile as analyzed by gas chromatography of the combined fractions. 1 represents the combined fraction of A, B, C, D and E; 2 represents the combined fraction of F and G. n = 3 ± SE.

Fatty acid methyl ester	**1 (%)**	**2 (%)**
C14	2.4±0.2	0
C16	33.4±1.3	0
C18	1.2±0.1	0
C16:1	3.1±0.2	0
C18:1	22.3±1.5	0
c16:2	2.2±0.3	0
C18:2	21.6±1.2	0
c18:3 (n-6)	1.1±0.2	7.7±1.4
C18:3 (n-3)	12.7±0.5	92.3±1.4
c16:4	0	0
c18:4	0	0

Table 3.17 The biodiesel properties of the crude FAME vs combined fractions. 1 represents the crude FAME and 2 is the combined fraction of A, B, C, D and E

Biodiesel Property	**1**	**2**
Iodine value(g I_2 100 g^{-1})	143.9	99.4
Cetane number	41.5	51.4

3.4 Optimization of downstream processes for biodiesel and alpha linolenic acid production

3.4.1 The downstream processes of biodiesel and alpha linolenic acid production

Harvesting, lipid extraction and transesterification are the downstream processes of biodiesel and alpha linolenic acid production from microalgae that often becomes the bottlenecks for its industrial production. Therefore, a systematic optimization of these steps is required in order to bring down the production cost.

3.4.2 Harvesting of algal biomass

3.4.2.1 Harvesting by filtration using flocculation aid

One of the hurdles for large scale biodiesel production from microalgae is harvesting of the algal biomass. The two commonly applied methods for harvesting microbial biomass are centrifugation and filtration; however there are some major drawbacks associated with these processes. The major disadvantage of centrifugation is the high energy cost involved in this process while filtration suffers from the problem of fouling (De Godos *et al.,* 2011). The flocculation followed by gravity sedimentation has also been tried as cost effective alternative (Wan *et al.,* 2015). With this aim, two flocculation aids, one representing chemical flocculent (ferric chloride) and other of bio-flocculent (chitosan) were chosen for testing the flocculation efficiency in four microalgae.

3.4.2.1.1 Flocculation with ferric chloride

The salts of multivalent cation like ferric chloride can bind to the negatively charged carboxyl groups on algal surface, thereby creating algal flocs which can be easily harvested (Wyatt *et al.,* 2012). In the present study, the optimum concentration of ferric chloride required for achieving highest flocculation efficiency in *Desmodesmus* sp. MCC34, *C. minutissima*, *D. salina* and *D. tertiolecta* were determined at pH 11. Table 3.18 shows that the highest flocculation efficiency of 98%, 99% and 98% were achieved for *Desmodesmus* sp. MCC34, *C. minutissima* and *D. salina* respectively under 150 mg L^{-1} $FeCl_3$ concentration while *D. tertiolecta* required a lesser concentration of 100 mg L^{-1} $FeCl_3$ for achieving highest flocculation efficiency of 99%. However,

at higher $FeCl_3$ concentration (150 mg L^{-1}), the flocculation became ineffective as shown in earlier report where the flocculation efficiency decreased significantly in *Chlorella zofingiensis* at $FeCl_3$ concentration of 125 mg L^{-1} Kim *et al.,* 2011 had reported efficient flocculation of a *Scenedesmus* species with combined treatment of 50 mg L^{-1} $FeCl_3$, and 1 g L^{-1} bio-flocculent.

3.4.2.1.2 Flocculation with chitosan

Chitosan is a bio-flocculent obtained from crustaceous sea species. The advantages of chitosan are the re-usability of flocculated media and unlike chemical flocculants, it does not contaminate the end products like biodiesel with metal ions (Oh *et al.,* 2001). In this study, the optimum concentration of chitosan required for achieving highest flocculation efficiency in four algal species were determined at pH 11. The highest flocculation efficiencies of 88, 85, 87 and 91% were achieved for *Desmodesmus* sp. MCC34, *C. minutissima*, *D. salina* and *D. tertiolecta* under chitosan concentration of 90 mg L^{-1} (Table 3.19). In an earlier study, chitosan at a concentration of 120 mg L^{-1} resulted in highest flocculation efficiency of 92% in *C. Vulgaris* (Rashid et al., 2013). In another study involving a *Chlorella* species, when chitosan of concentration 150 mg L^{-1} was used, a flocculation efficiency of 99% was obtained (Ahmad *et al.,* 2011). However, in the present study, the optimum concentration of chitosan for achieving highest flocculation efficiency in all the four algae was only 90 mg L^{-1} which is lesser than the reported concentrations in literature.

3.4.2.2 Harvesting by filtration without flocculation aid

The flocculation aid was found to be efficient in harvesting microalgal biomass, however it can still increase the production cost, and therefore harvesting by filtration without addition of any flocculation aid was experimented. The filtration of majority of microalgae is usually done using membrane filters which have pore size ranging from 0.1 to 0.4 µm (Nurra *et al.,* 2014). But membrane filtration is costly and is also associated with problems of high cake resistance (Nurra *et al.,* 2014). Therefore, a simple filtration experiment under atmospheric pressure was carried out using muslin cloth (average pore size 2 mm in diameter) to check the harvestable nature of microalgae. Four green algae (*Desmodesmus* sp. MCC34, *C. minutissima*, *D. salina* and *D. tertiolecta*) were tested for this purpose. Results are presented in Figure 3.36 and it shows that the

filtration efficiency of *Desmodesmus* sp. MCC34, *C. minutissima* and *D. salina* were as low as 23, 23.7 and 15.7 % respectively but a higher retention of 47 % biomass was observed for *D. tertiolecta* which is due to its large cell size of 10 µm. This study shows that microalgae such as *D. tertiolecta* can still be harvested by filtration without any flocculation aid and thereby reducing the cost borne by the harvesting process in biodiesel production. The microscopic observation of the algal cells retained during muslin cloth filtration is shown in Figure 3.37.

3.4.3 Optimization of lipid extraction protocol

3.4.3.1 Screening of solvents for lipid extraction

The effect of different solvents as well as combination of solvents on the extraction of lipids from wet and dry biomass of *Desmodesmus* sp. MCC34 were tested (Table 3.20 A and B). It was observed that the Folch solvent mix showed highest FAME yield (49 mg g^{-1}) from dry biomass, however it was less effective in extracting lipids from wet biomass (12.01 mg g^{-1}). Similarly the Bligh and Dyer solvent showed highest yield (46.83 mg g^{-1}) from the wet biomass but was unable to extract the lipids will same efficiency from dry biomass, yielding 13.34 mg g^{-1} FAME. Similar trends were observed for other solvent systems. These findings suggest that a different combination of solvent should be chosen for lipid extraction depending on the nature of biomass. It was found that the solvents which are predominantly non-polar in nature works well in case of dry biomass, whereas solvents that are polar shows better efficiency of FAME extraction from wet biomass. The better performance of Bligh & Dyer mix and Folch mix could be attributed to the fact that they are polar-non polar azeotrophic mixture suiting fresh water algae, which are generally made up of 50% polar lipid and 50% neutral lipid (Halim *et al.*, 2012). It is reported that the polar solvent makes strong hydrogen bond with polar lipid thereby releasing such lipid from associated protein, while neutral lipids makes weak vander waal bond with non-polar solvents (Halim *et al.*, 2012). Therefore the presence or absence of water content in the biomass acts as the deciding factor for solvent selection. Sheng et al (2011) has also reported that water content adversely affect the lipid extraction from *Synechocystis sp*. by Bligh and dyer solvent.

Table 3.18 Flocculation efficiency (%) of *Desmodesmus* sp. MCC34, *C. minutissima*, *D. salina* and *D. tertiolecta* using ferric chloride. n = 3 ± SE. Different alphabetic superscripts denote significantly different means.

Ferric chloride concentration (mg L^{-1})	*Desmodesmus* sp. MCC34	*C. minutissima*	*D. salina*	*D. tertiolecta*
0	61.31±1.17	52.29±2.36	57.82±1.73	66.99±0.94
50	89.21±0.19^{e}	93.31±3.58^{b,c,d}	82.52±3.15^{g}	86.97±0.19^{f}
100	95.34±0.6 ^{c,d}	97.26±1.46^{b}	96.05±0.19 ^{c}	99.56±0.51^{a}
150	98.41±1.16 ^{a}	99.13±0.38 ^{a}	98.54±0.75^{a}	94.55±0.13 ^{d}
200	56.1±1.7	54.77±7.23	62.75±4.26	65.91±2.63
250	51.27±0.25	47.97±4.33	45.9±3.62	59.65±1.13

Table 3.19 Flocculation efficiency (%) of *Desmodesmus* sp. MCC34, *C. minutissima*, *D. salina* and *D. tertiolecta* using chitosan. n = 3 ± SE. Different alphabetic superscripts denote significantly different means.

Chitosan (mg L^{-1})	*Desmodesmus* sp. MCC34	*C. minutissima*	*D. salina*	*D. tertiolecta*
0	30.4±2.11	23.2±1.45	24.2±2.19	35.2±1.37
30	39.5±3.12	28.7±3.19	30.9±1.18	46.7±2.81
60	83.6±1.76 ^{d}	79.2±2.55	83.2±1.92 ^{d}	90.2±2.19 ^{a,b}
90	88.9±2.66 ^{b,c}	85.6±2.12 ^{c}	87.1±2.16 ^{c,b}	91.1±2.1 ^{a}
120	88.6±1.74 ^{b,c}	86.1±1.17 ^{c}	86.2±1.39 ^{c}	93.4±2.67 ^{a}
150	78.4±2.17	72.6±3.35	72.2±0.59	83.5±1.71 ^{d}

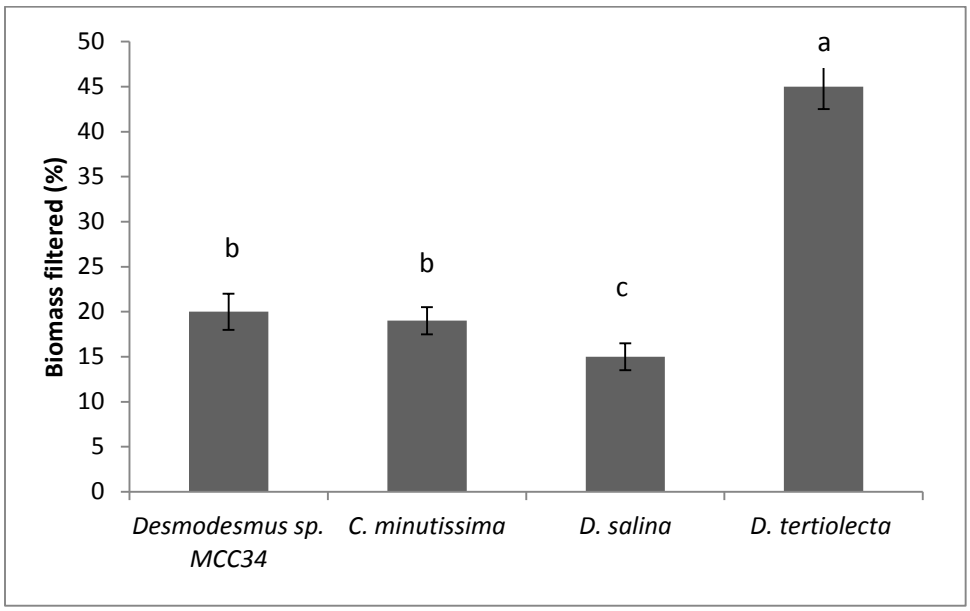

Figure 3.36 Filtration efficiency of *Desmodesmus* sp. MCC34, *C. minutissima*, *D. salina*, *D. tertiolecta* by direct filtration using muslin cloth. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

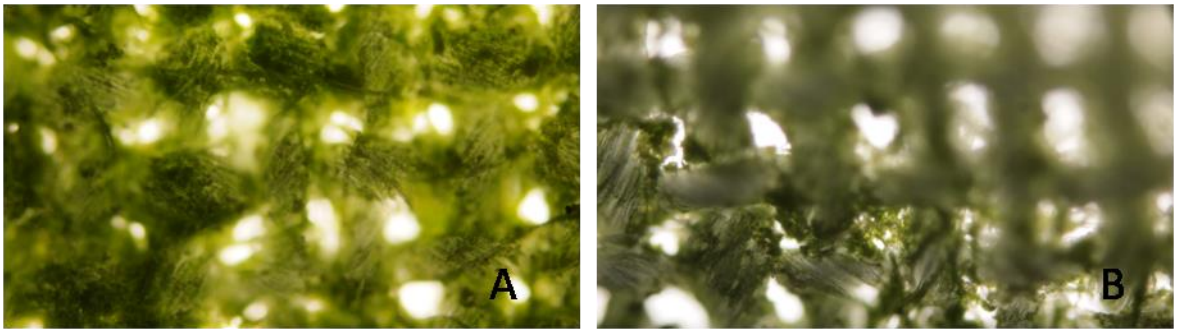

Figure 3. 37 Microscopic observations (10X) of muslin cloth filtration of (A) *Desmodesmus* and (B) *D. salina*

Even though Folch as well as Bligh & Dyer solvents perform better they are not used in industrial scale because of the presence of chloroform, which is carcinogenic in nature. Chloroform on decomposition also yields phosgene gas which can modify the target lipid (Matyash *et al.*, 2008). Dichloromethane: methanol (2:1) and butanol which are next best solvent systems are recommended for lipid extraction from dry and wet biomass due to their less toxic nature and ease in scalability.

3.4.3.2 Effect of Cell lysis on lipid extraction

To demonstrate the effect of cell lysis on lipid yield, we tested different methods of cell disruption, they include; microwave radiation, sonication and autoclaving. The autoclaving method (for 5 minute) produced the highest lipid content of 62%, followed by microwave with 41%, then sonication with 12%. Lipid yield from non-ruptured cells was also calculated to make a comparative analysis and was 4%, (Figure 3.38), proving the effectiveness of cell lysis. Yap et al., have also reported that as degree of lysis increases, instantaneous lipid yield also increases in *Chlorella* cells (Yap *et al.*, 2014). The reason for higher yield from autoclaving could be the complete rupturing of algal cells as shown in the Figure 3. 39.

3.4.3.3Factors affecting lipid extraction

3.4.3.3.1 Solvent biomass ratio

Increase in the volume of solvent increases the rate of the lipid extraction from the algal cells, due to greater concentration gradient created across the cell membrane. However addition of large volumes of solvent rises the production cost, therefore experiment were conducted to find out an optimum solvent volume, required for better lipid extraction. Figure 3.40 shows changes in lipid content with respect to the amount of Folch solvent added. The solvent-biomass ratio (mlg^{-1}) of 13 was found to be optimal for higher lipid extraction. Increase of solvent-biomass ratio from 4 to 7 had increased the lipid of dried *Botryococcus* from 15 to 23% in 3 hours (Ashokkumar *et al.*, 2014), further increase from 7:1 to 8:1 did not yielded more lipid. This study has proven that by maintaining higher solvent biomass ratio it is possible to extract more lipids from dry biomass under limited time.

3.4.3.3.2 Surface Area factor

The increase in the surface area of biomass is known to increase the rate of diffusion of lipid in a microalgae *Ulva lactuca* (Suganya & Renganathan 2012). There are two ways to increase the surface area of biomass for lipid extraction. One is pulverizing the biomass into fine particles thereby increasing granular surface area. Other, as done in this study, is by spreading the biomass in a wider area so that more solvent comes in contact with biomass; but this may also lead to the unwanted loss of solvent due to evaporation. Therefore, a correct balance has to be obtained between extraction vessel size and the time taken for lipid extraction in order to minimize the quantity of solvent required for lipid extraction. Surface area factor (SAF) was used to express the lipid extraction at different surface areas of biomass with the constant solvent concentration and was calculated from the equation 23. The amount of lipid obtained by instantaneous extraction was shown to increase with a rise in SAF (Figure 3.41). At the end of 6^{th} h, peak values of extractable lipid were obtained at SAF=62.8, whereas the extraction process continued in the vessels which were set at lower SAF values. This clearly indicates that higher SAF values, positively influences the rate of lipid extraction.

3.4.3.4 The static vs mixing extraction

Static extraction method involves adding solvent to the biomass and incubating it at room temperature without stirring until lipid extraction is attained to the maximum. There are contradictory reports on the effects of mixing on lipid extraction from microalgae. Some studies have reported that mixing improves lipid extraction (Ehimen *et al.*, 2010; Suganya and Renganathan 2012) while some report that mixing is not mandatory (Kwon & Yeom 2015). Our results showed no significant differences between static lipid extraction and lipid extracted after stirring the biomass at 200 rpm implying that shaking does not significantly influence lipid extraction (Figure 3.42). This is similar to recent report where shaking was proven ineffective for lipid extraction from *Nannochloropsis sp* (Kwon & Yeom 2015).

Table 3.20 Quantitative analysis of fatty acid methyl ester extracted from microalgae using different solvents. (A) Dry biomass (B) Wet biomass. n = 3 ± SE.

A

S.No	Solvent	FAME yield mg g^{-1} dry biomass
1	Folch	49.09± 3.83
2	Hexane	35.39±8.68
3	Toluene:Methanol-3:1	35.35± 1.86
4	Dichloromethane:Methanol-2:1	32.75± 0.75
5	Ethylacetate: Hexane-1:1	14.57± 0.88
6	Hexane : Isopropanol-3:1	13.99± 1.32
7	Bligh Dyer Method	13.34± 0.27
8	Methanol	12.91± 0.98
9	Methanol:Methyl tertiary butyl ether	12.76± 2.28
10	Petroleum ether: Toluene-3:1	12.54± 0.60
11	Dichloromethane:Ethanol-1:1	12.46± 0.98
12	Isopropanol	12.13± 3.24
13	Dichloromethane	10.69± 0.88
14	Dichloromethane:Methanol-1:1	10.56± 0.07
15	Chloroform	10.28± 0.97
16	Ethyacetate: Ethanol-2:1	7.59 ±5.24
17	Ethylacetate	6.19 ±0.58
18	Ethanol	5.52 ±0.68
19	Hexane:Isopropanol-2:3	5.13 ±0.37
20	Hexane: Ethanol-1:0.9	5.05 ±2.08
21	Toluene	4.72 ±0.58
22	Petroleum ether	4.62 ±0.75
23	Ethylacetate: Hexane-2:3	4.10 ±0.55
24	Hexane: Ethanol-1:2.5	2.25 ±0.07
25	Acetone	1.72 ±2.98
26	Methyl tertairy butyl ether	0.74 ±1.29
27	Toluene:Methanol-1:1	n.d
28	Petroleum ether: Toluene-1:2	n.d
29	Butanol	n.d

B

S.No	Solvent	FAME yield mg g^{-1} wet biomass
1	Bligh Dyer Method	46.83± 5.36
2	Butanol	34.06± 1.28
3	Hexane : Isopropanol-3:1	28.16± 0.52
4	Toluene:Methanol-1:1	24± 0.98
5	Hexane:Isopropanol-2:3	22.95± 1.01
6	Acetone	20.69± 1.83
7	Methanol	20.31± 1.35
8	Dichloromethane:Ethanol-1:1	16.93± 0.74
9	Ethyacetate: Ethanol-2:1	14.55± 0.58
10	Toluene:Methanol-3:1	13.51± 2.79
11	Hexane	12.89± 0.42
12	Dichloromethane:Methanol-1:1	12.66± 0.08
13	Folch	12.01± 1.91
14	Petroleum ether: Toluene-3:1	11.92±1.45
15	Dichloromethane	11.73± 0.50
16	Hexane: Ethanol-1:0.9	11.5± 0.91
17	Hexane: Ethanol-1:2.5	11.33± 0.49
18	Toluene	9.94 ±0.31
19	Ethylacetate: Hexane-2:3	9.04 ±4.5
20	Ethylacetate: Hexane-1:1	7.51 ±0.21
21	Ethylacetate	5.83 ±0.68
22	Isopropanol	5.74 ±1.09
23	Methanol:Methyl tertiary butyl ether	5.22 ±0.51
24	Chloroform	4.23 ±0.42
25	Ethanol	3.39 ±0.27
26	Dichloromethane:Methanol-2:1	2.14 ±0.14
27	Petroleum ether: Toluene-1:2	1.88 ±0.33
28	Petroleum ether	1.25 ±2.16
29	Methyl tertairy butyl ether	0.53 ±0.92

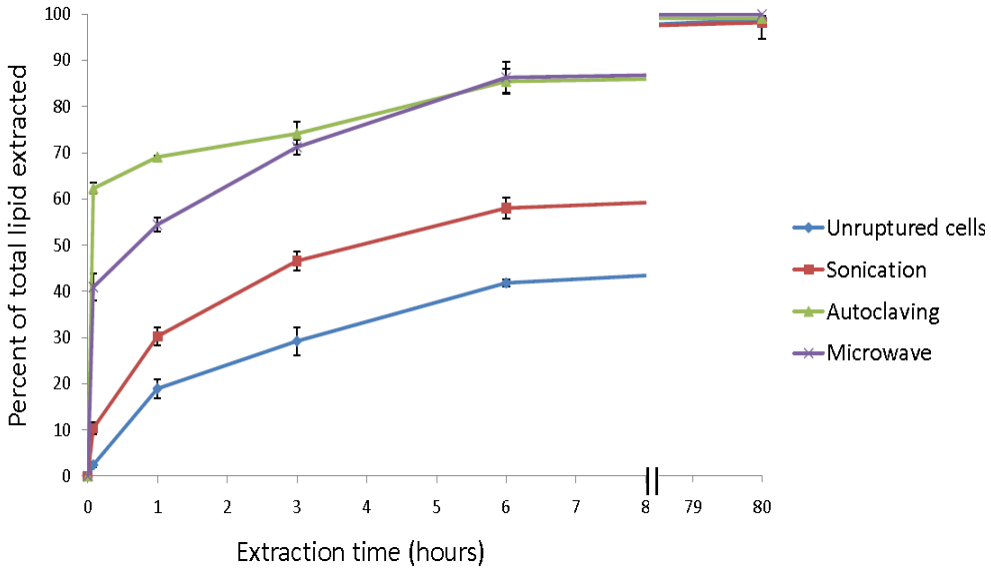

Figure 3.38 Lipid extraction at different time intervals in cells disrupted by different techniques. Data are the mean of triplicates.

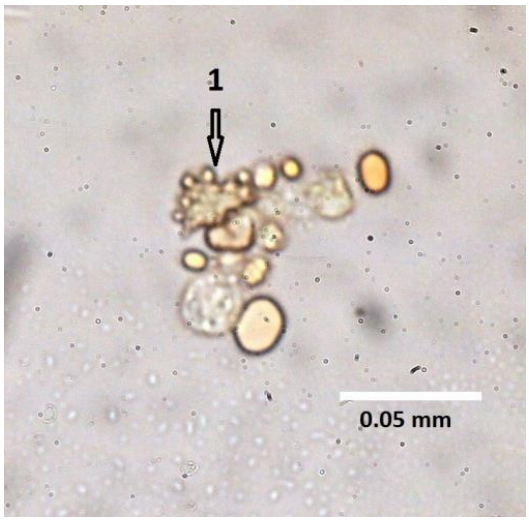

Figure 3.39 Pre-treatment of algal cell by autoclaving. 1 denotes completely ruptured cell.

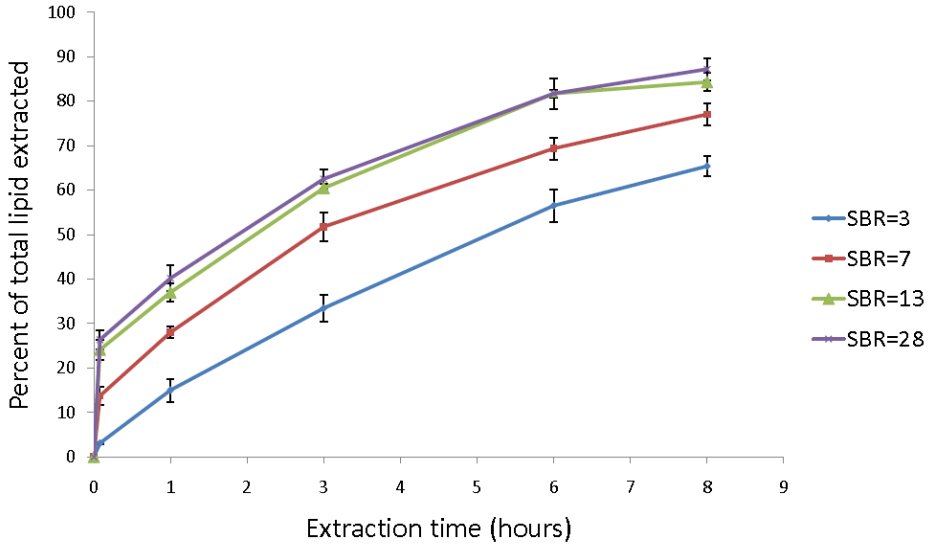

Figure 3.40 Effect of solvent: biomass ratio (ml/g) on lipid extraction. Data are the mean of triplicates.

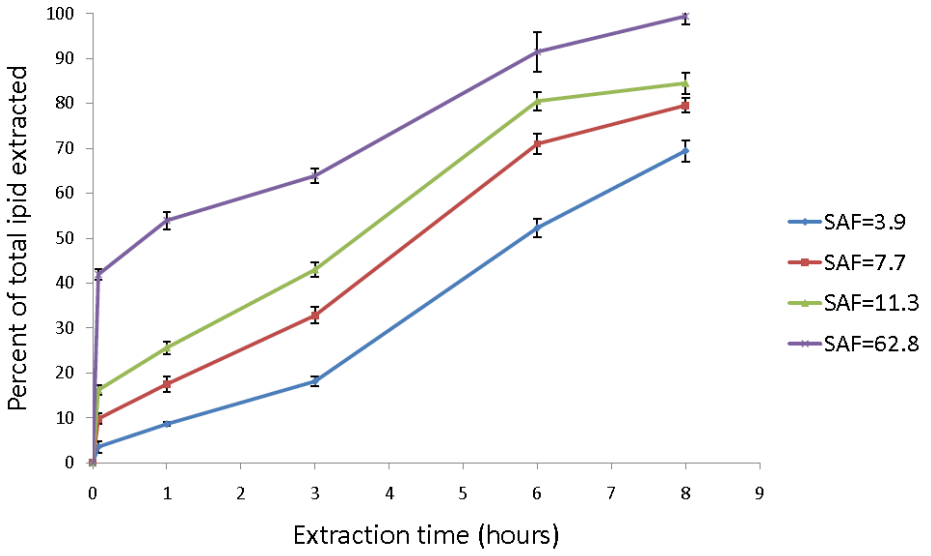

Figure 3.41 Effect of surface area factor on lipid extraction. Data are the mean of triplicates.

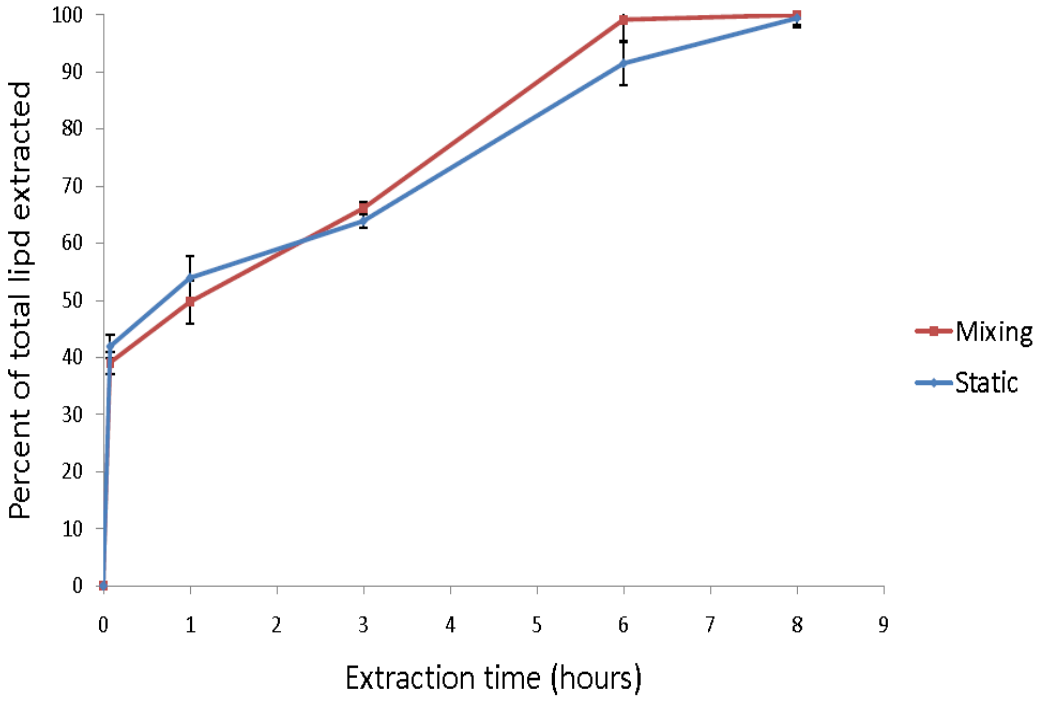

Figure 3.42 Comparison of lipid extraction by mixing and static method. Data are the mean of triplicates.

3.4.3.5 Kinetic Study

All experiments conducted for better lipid extraction, such as, cell lysis, SBR, SAF and biomass stirring were analyzed for different kinetic parameters. The experimental values showed a good fit with model values (R^2>0.97) suggesting the validity of model. The mass transfer coefficient of washing step was found to be nearly 100 times higher than that of the diffusion process suggesting the lipid extraction during washing step is instantaneous. In earlier study, only 19 to 31 times fold differences in mass transfer coefficient between these two steps was reported for olive oil extraction using hexane (Amarni & Kadi 2010).

The results of experiments 1 to 8 (Table 3.21), shows the effect of change in SBR and SAF on the kinetics of static lipid extraction. The kinetic parameters of diffusion process and washing step, k_1, k_2 and M1, increased with increasing SAF and SBR while M2 decreased. This indicates that static extraction with higher SAF and SBR, has higher rate of diffusion as well as higher yield in washing step resulting in higher lipid extraction. The positive effects of increased surface area and solvent volume as reported here corroborates with other literature reports (Ashokkumar *et al.,* 2014; Suganya and Renganathan 2012).

Experiments 9 to 12 contain the effect of various cell lysis treatments on kinetic parameters of static lipid extraction at constant SBR and SAF. The cell lysis treatments had significant effect on parameter M1 ($p<0.05$), with autoclave pretreatment resulting in highest value of 15.5%. The mass transfer coefficient of diffusion process (k_2 min^{-1}) for sonication, microwave and autoclaving method are between 2.59 to 4.11 E-3. The highest k_2 value of lysed cell is similar to the k_2 value of unlysed cell but at higher SBR (28 ml g^{-1}) or SAF (62.8 cm^2 ml g^{-1}). Similar results were observed for the parameter k_1. This implies that by changing SAF and SBR, same rate of lipid extraction could be obtained without cell lysis.

Experiment 13 shows effect of stirring on lipid extraction. There was no significant difference between k_2, k_1, M2 and M1 values in experiment 13 and in experiment 12. Both these experiments have same SBR and SAF, indicating that shaking/stirring did not influence the rate of diffusion and washing step of lipid extraction.

Table 3.21 Kinetic study of lipid extraction from dry biomass of *Desmodesmus* sp. MCC34. The values of M1, M2, k_1 and $k_2$are predicted using equation 22 as described in materials & method section. Data are mean ±standard deviation (n=3)

Expt. No.	Condition	Factors affecting static lipid extraction			M1 (%)	M2 (%)	k_2 E-3(min^{-1})	Correlation coefficient
		Cell lysis treatment	SAF (cm^2 $ml\ g^{-1}$)	SBR ($ml\ g^{-1}$)				
1	Static	_	2.4	3	0.6±0.1	23.3±0.2	1.62±0.21	0.97
2	Static	Sonication	2.4	3	2.4±0.2	21.8±0.5	2.49±0.32	0.97
3	Static	Autoclave	2.4	3	15.4±0.2	8.2±1.1	2.53±0.11	1
4	Static	Microwave	2.4	3	10.4±0.4	13.5±0.6	3.93±0.22	1
5	Static	_	6.8	3	0.7±0.1	23.9±1.1	2.41±0.35	0.97
6	Static	_	15.9	7	3.3±0.1	21.1±0.1	3.26±0.22	0.98
7	Static	_	29.5	13	6.1±0.5	17.6±0.4	3.57±0.13	0.98
8	Static	_	63.5	28	9.4±0.3	14.4±0.3	4.12±0.36	0.97
9	Static	_	3.9	5	1±0.5	22.8±0.6	1.8±0.34	0.98
10	Static	_	7.7	5	2.4±0.2	21.7±0.1	2.61±0.23	0.98
11	Static	_	11.3	5	3.9±0.1	19.9±1.1	3.09±0.61	0.99
12	Static	_	62.8	5	10.5±0.8	13.7±0.7	4.07±0.17	0.99
13	Mixing at 200 rpm	_	62.8	5	10.1±1.1	14.1±0.6	4.11±0.25	0.98

3.4.4 Transesterification reaction

3.4.4.1 Acid catalyzed transesterification

Transesterification process is conversion of lipid and methanol to fatty acid methyl ester (biodiesel) and glycerol. This reaction can be catalyzed by an acid catalyst such as hydrochloric acid or base catalyst such as potassium hydroxide or enzymes such as lipase. The acid catalyzed transesterification is suitable for oil having higher free fatty acid content (Tyagi *et al.,* 2010). Among acid catalyst, hydrochloric acid (HCl) was preferred over sulfuric acid in this study as it does not leave any sulfur residue in biodiesel (Tyagi et al., 2012). In the present study, the effect of various parameters on acid (HCl) catalyzed transesterification was determined.

3.4.4.1.1 Effect of temperature

The effect of temperature on HCl catalyzed transesterification reaction is shown in Figure 3.43. The increase in temperature resulted in increase in FAME yield with highest FAME yield of 1.17 mg g^{-1} biomass was obtained at 70°C. In comparison, a similar trend of increase in FAME yield with increase in temperature during acid catalyzed transesterification was observed for *Spirulina* (Nautiyal et al., 2014). Higher temperature increases the rate of molecular collision of reactants thereby increasing the yield of product, which is FAME in this case (Nautiyal et al., 2014). Further increase in temperature beyond 70°C was avoided in the present study, as earlier investigations have indicated increase of production cost as well as burning of lipid at higher temperatures for acid catalyzed transesterification (Mathimani *et al.,* 2015; Miao and Wu 2006).

3.4.4.1.2 Time duration of HCl catalyzed transesterification

The time required for obtaining maximum FAME under HCl catalyzed transesterification reaction was determined under constant condition of HCl concentration of 2% in methanol, solvent oil ratio (ml/g) of 50 and temperature 50°C. A maximum FAME of 1.1 mg g^{-1} biomass was obtained at the end of 1st hour, after which it did not vary with further increase in time period (Figure 3.44). A similar study involving acidic transesterification of *Chlorella* oil had shown that optimum time for maximal FAME yield is 2 hour (Ahmad *et al.,* 2014), which is longer than the time required for achieving maximum FAME in *Desmodesmsus* sp. MCC34.

3.4.4.1.3 Effect of catalyst amount

Lower concentration of acid catalyst decreases the FAME yield from oil whereas higher amount can burn the lipid (Canakci & Van Gerpen 2001), therefore optimum amount of catalyst for maximum FAME production has to be identified. As shown in Figure 3.45, the highest FAME of 2.99 mg g^{-1} biomass was obtained under catalyst concentration of 2% HCl (w/v of methanol). A further increase of catalyst to 10% did not show any significant difference in FAME yield ($p>0.05$). In a study involving acidic transesterification of lipid from *Chlorella*, a catalyst amount of 3.5% was necessary for maximum FAME yield, which is higher than the amount required for achieving maximum FAME from the lipid of *Desmodesmsus* sp. MCC34 (Mathimani *et al.,* 2015). Beyond the catalyst amount of 10% HCl, the FAME production considerably decreased and total inhibition of FAME production was observed for 40 to 100% HCl.

3.4.4.1.4 Effect of solvent oil ratio

The excess use of solvent, methanol in this case, will not only add to the production cost but also cause problem during purification of biodiesel. With this aim, the optimum solvent to oil ratio for lipid of *Desmodesmsus* sp. MCC34 was determined in the present study. A maximal FAME yield of 9.2 mg g^{-1} biomass was obtained under the solvent to oil ratio (ml/g) of 25 (Figure 3. 46). Also, a decreased yield under lower ratio of 10 and no significant difference in FAME yield between ratio of 50 and 25 were observed ($p>0.1$). In comparison, a maximum FAME yield was obtained under solvent oil ratio of 120 during the acidic transesterification of lipid from a *Chlorella* species (Mathimani *et al.,* 2015).

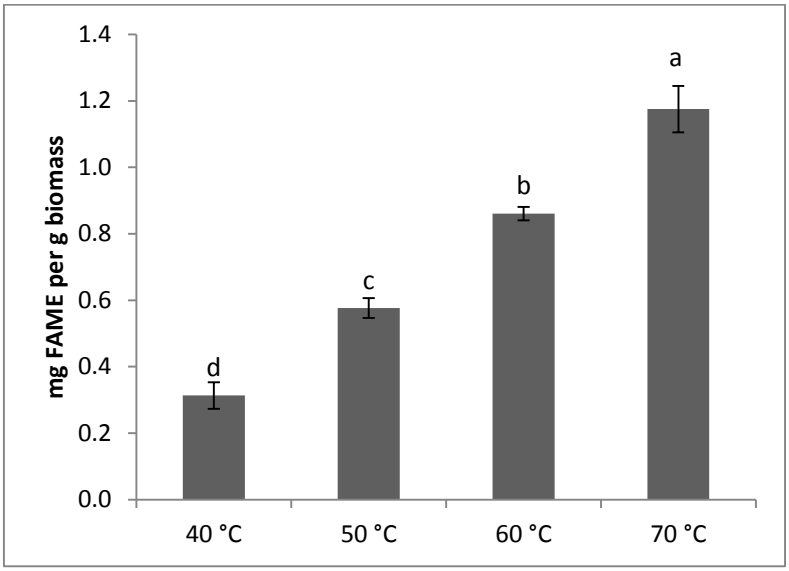

Figure 3.43 Effect of temperature on HCl catalyzed transesterification. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

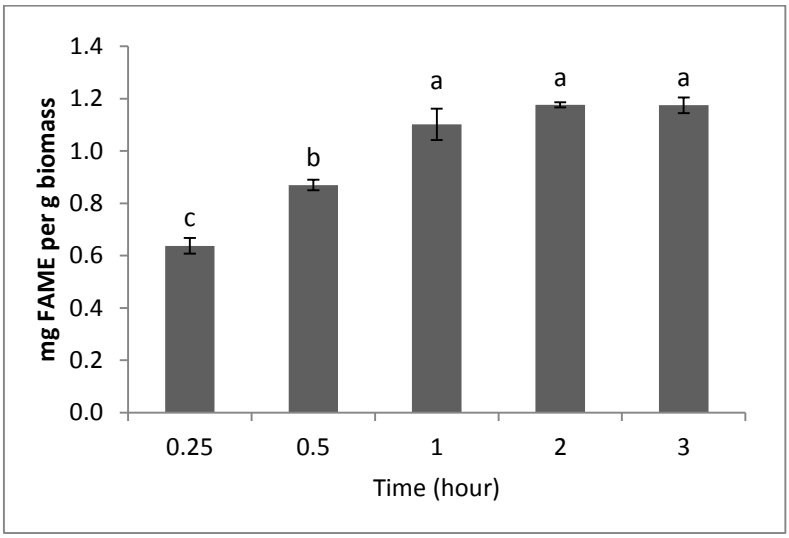

Figure 3.44 Time duration of HCl catalyzed transesterification. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

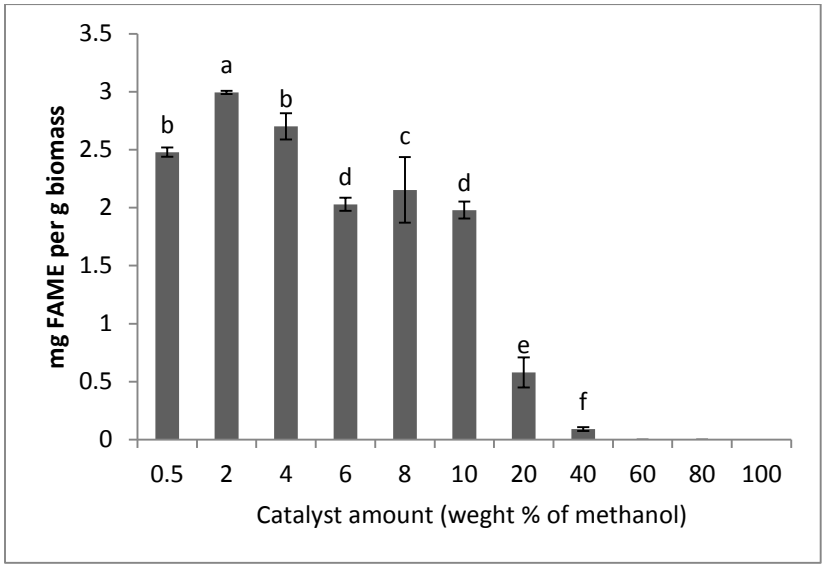

Figure 3.45 Effect of HCl amount on transesterification. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

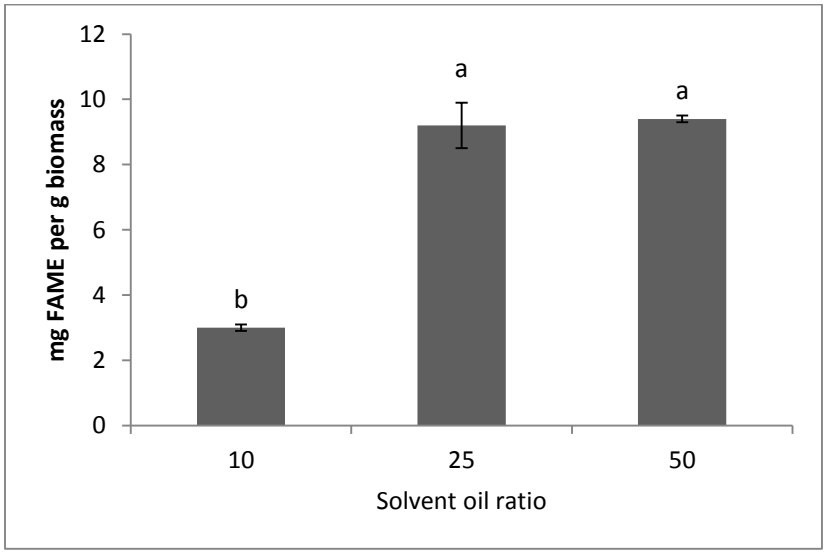

Figure 3.46 The effect of solvent oil ratio (ml/g) on HCl transesterification. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

3.4.4.2 Alkaline catalyzed transesterification

The transesterification reaction catalyzed by alkali such as potassium hydroxide (KOH) is rapid. However the drawbacks of this reaction are formation of soaps and unsuitability for lipids rich in free fatty acid. Formation of soap is primarily due to hydrolysis of fatty acid methyl ester by water (Komers *et al.,* 2002), therefore it becomes essential to remove water from the reaction mixture. It is obvious that this type of reaction is not suitable for wet algae for direct biodiesel production (Tyagi *et al.,* 2010), as water can result in hydrolysis of FAME. In the present study, the effect of various parameters on alkaline (KOH) catalyzed transesterification was studied.

3.4.4.2.1 Effect of temperature

Similar to acid catalyzed reaction, the current experiment was also not conducted above 70°C. Increase in temperature, increased the FAME production, with maximum yield of 8.23 mg g^{-1} biomass was obtained at 70°C under constant conditions of 2% KOH and time period of 15 min (Figure 3.47). This is similar to optimum temperature of 67°C required for achieving maximum FAME production in *Chlorella protothecoides* through statistical optimization of alkaline catalyzed transesterification reaction (Makareviciene *et al.,* 2014).

3.4.4.2.2 Time course analysis

The time required for maximum conversion of lipid to biodiesel through alkaline catalyzed transesterification depends on the composition of lipid (Komers et al., 2002). In this study, within a minute more than 50% of the maximum obtainable FAME was achieved under constant condition of solvent to oil ratio of 500, 2% KOH and temperature of 40°C (Figure 3.48). The maximum FAME yield was obtained in 20 minutes. The difference between mean FAME values of 1, 10 and 20 min were statistically significant ($p<0.05$) whereas the differences between 20, 60 and 120 minutes were insignificant ($p>0.05$). It is reported that by alkaline transesterification, the conversion of FAME from triglyceride occurs within 10 min, phospholipid in 5 min and phosphatidyl choline can be converted to FAME within 1 min, whereas cholester esters take more than 1 h for formation (Komers *et al.,* 2002). In a study, 75 minute was found to be the optimal time for obtaining maximum FAME from oil of *Chlorella vulgaris* (Velasquez-Orta *et al.,* 2012).

3.4.4.2.3 Effect of catalyst amount

In the present study, the optimum amount of KOH catalyst for obtaining maximum FAME from the lipid of *Desmodesmsus* sp. MCC34 was determined. Figure 3.49 shows that the maximum FAME of 9.8 mg g^{-1} biomass was obtained under 0.2% KOH. A further increase in the catalyst amount resulted in a decreased yield of FAME, which is due to hydrolysis effect (Komers et al., 2002). A study had shown that increase of alkaline catalyst, NaOH from 0.08 to 0.5 % in methanol increased the FAME yield in *Chlorella vulgaris*, however a further increase beyond 0.5% did not increased the yield (Velasquez-Orta *et al.,* 2012).

3.4.4.2.4 Effect of solvent oil ratio

The optimum solvent to oil ratio (ml/g) for maximal FAME production under KOH catalyzed transesterification using methanol as solvent was 300 (Figure 3.50). Beyond this ratio, there was no significant difference between the FAME production ($p>0.05$). Although the KOH catalyzed reaction, in comparison to HCl catalyzed transesterification, consumed 12 times more methanol for obtaining the maximum FAME yield from the lipid of *Desmodesmsus* sp. MCC34, it was superior in terms of total FAME yield, temperature and catalyst amount.

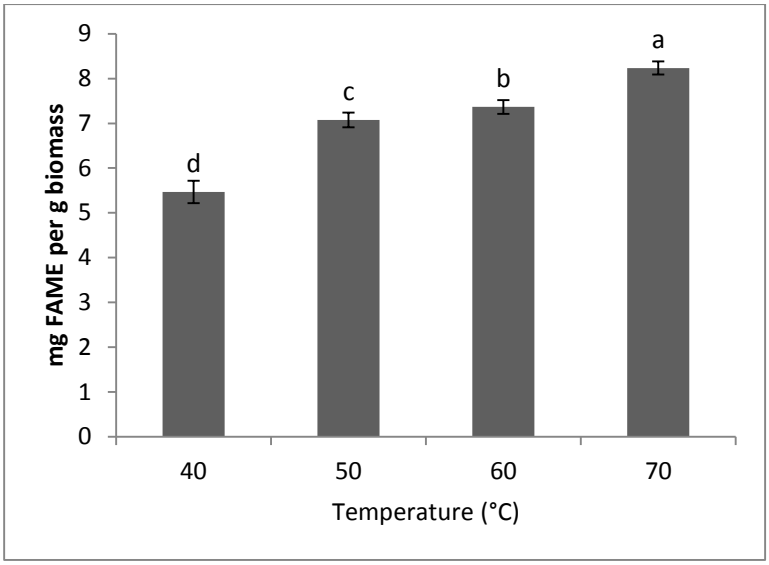

Figure 3.47 Effect of temperature on KOH catalyzed transesterification. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

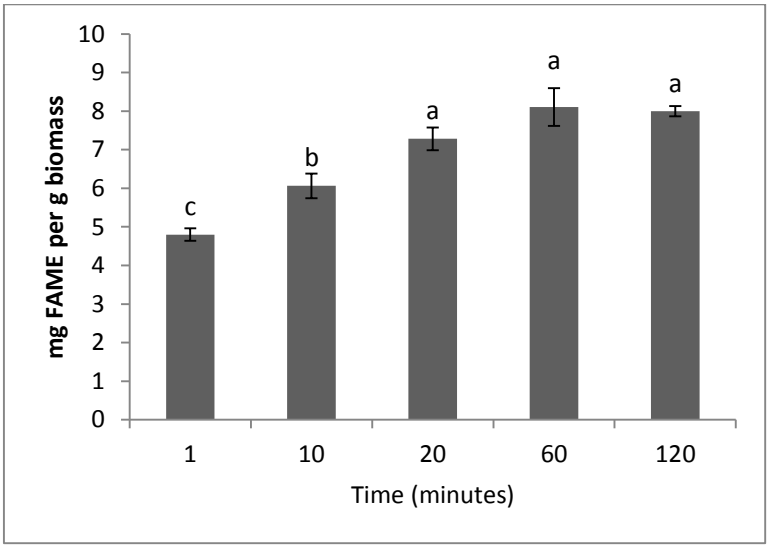

Figure 3.48 Time course analysis of 0.2% KOH catalyzed reaction. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

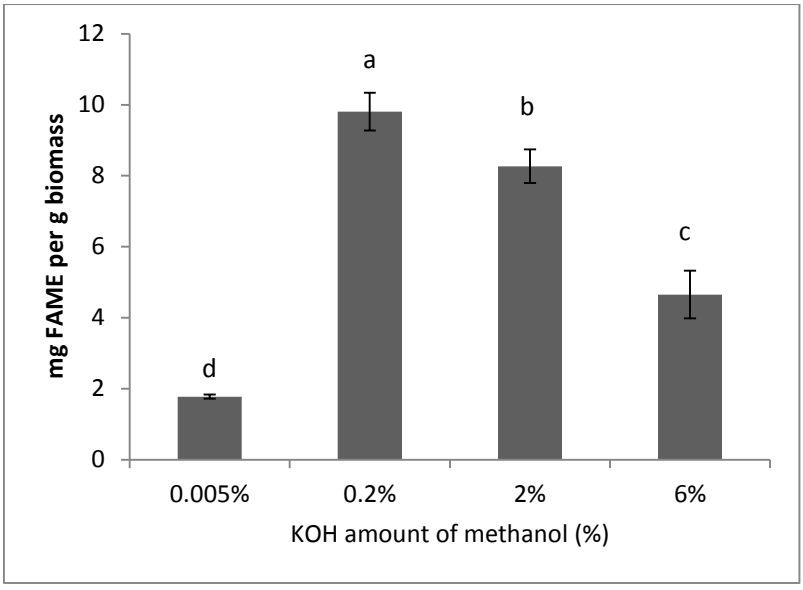

Figure 3.49 Effect of KOH amount on transesterification. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

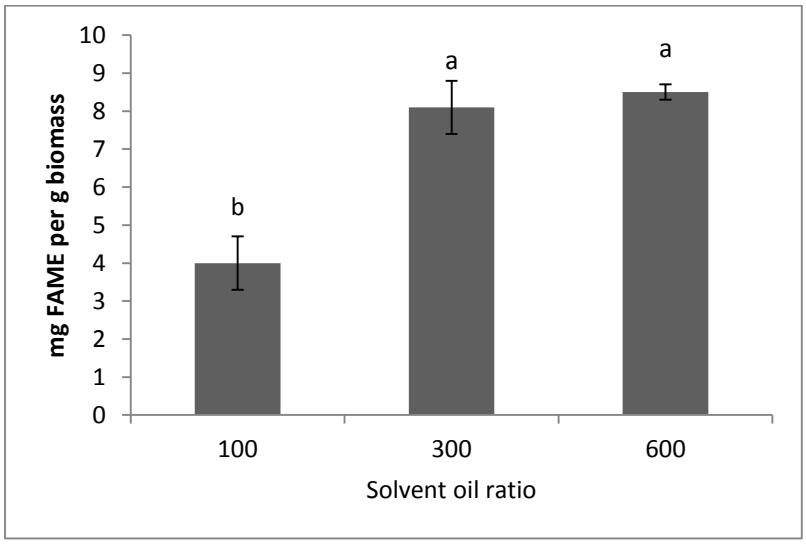

Figure 3.50 The effect of solvent oil ratio on KOH catalyzed transesterification. Data are the mean of triplicates. Bars with different letters are significantly different at $P<0.05$.

Chapter 4: SUMMARY

The present work involves isolation and characterization of lipid rich microalgal species for the production of biodiesel and value added product, alpha linolenic acid, an omega 3 fatty acid. The mass cultivation of promising species was conducted, for which a unique growth model was developed. Further, the process of harvesting, lipid extraction and transesterification were optimized. The major outcomes of this work are as follows.

1. Three microalgal strains, *Desmodesmus* sp. MCC34, *Scenedesmus* sp. MCC32 and *Chlorella* sp. MCC26 were isolated from local water bodies of Pilani, Rajasthan, India (28.37°N and 75.6°E). They were characterized using morphological features such as cell size and shape and rDNA sequencing. After submitting the nucleotide sequences of these three species in Genbank, strains were deposited in Microbial Culture Collection in IARI, New Delhi and obtained accession number.
2. A total of 21 algal species including the three locally isolated species were screened for their specific growth rate, biomass production, lipid content, and lipid productivity. Out of which, *C. minutissima* showed highest lipid content of 23.4%, *Desmodesmus* sp. MCC34 displayed higher growth rate, biomass productivity and lipid productivity of 0.26 day^{-1}, 65.9 mg L^{-1} day^{-1} and 13.4 mg L^{-1} day^{-1} respectively.
3. The *Desmodesmus* sp. MCC34 resulted in higher lipid production when grown under continuous light intensity of 41 µ mol photon m^{-2} s^{-1} at pH 8. The same species was shown to exhibit nearly 10 fold increase in the lipid productivity when subjected to mixotrophic mode of cultivation with 0.5% glucose.
4. The *Desmodesmus* sp. MCC34 produced higher lipid with nitrate or nitrite as nitrogen source than other nitrogen sources such as ammonium and urea. The optimal concentration of key macronutrients such as nitrogen, phosphorus and sulfur for achieving maximal lipid productivity in *Desmodesmus* sp. MCC34 were found to be 2.9 mM, 0.2 mM and 0.08 mM respectively.
5. Lipid was found to accumulate in higher quantities under stress of nitrogen deprivation in *Desmodesmus* sp. MCC34, *C. minutissima*, *D. salina* and *D. tertiolecta*. Under nitrogen deprivation, the protein, carotenoids and chlorophyll content decreased in all these species.
6. The mass cultivation of *Desmodesmus* sp. MCC34 was studied in raceway pond during the months of March and November 2013. Outdoor cultivation yielded areal biomass productivity

of 13946.23 kg ha^{-1} $year^{-1}$ and volumetric biomass productivity of 56.94 mg L^{-1} day^{-1} during March 2013 which decreased to 6262.28 kg ha^{-1} $year^{-1}$ and 25.57 mg $L^{-1}day^{-1}$ respectively during November 2013.

7. A growth model consisting of parameters such as saturating light intensity, molar extinction coefficient, temperature factor and Michaelis-Menten constant of key macronutrients such as nitrate, phosphate and sulphate was developed. The equation,

$$X = X_0 * \exp\left(\mu_{max} * Sn/(K_{Sn} + Sn) * (Sp/(K_{Sp} + Sp) * Ss/(K_{Ss} + Ss)) * (I_Z/I_s * \exp(1-(I_Z/I_s)) * (((T-T_{max})(T-T_{min})^2)/((T_{opt}-T_{min}) * [(T-T_{opt}) * (T_{opt}-T_{min})) - ((T_{opt}+T_{min}-2T)(T_{opt}-T_{max}))]))\right)$$

predicts biomass productivity in outdoor cultivation in raceway pond. The uniqueness of model is that it is developed for biomass production that does not require mixing during cultivation. The model was successfully validated with the experimental results obtained under raceway pond-mass cultivation of *Desmodesmus* sp. MCC34.

8. The biodiesel of *Desmodesmus* sp. MCC34, *C. minussitima*, *D. salina* and *D. tertiolecta* have shown cetane number complying with Indian, European and United States standard. Also, the iodine value of biodiesel from all species falls below 120 g I_2 per 100 g, adhering to the standards except for *Desmodesmus* sp. MCC34. This is due to higher amount of unsaturated fatty acid, predominately alpha linolenic acid (ALAME), which is present in this species.
9. The ALAME purification from *Desmodesmus* sp. MCC34 by silver ion chromatography resulted in 91% purity. The residual FAME purified by the above method yielded more saturated fatty acid compared to the total FAME, with improved cetane value of 54 and iodine value of 94 g I_2 per 100 g, suggesting that removal of ALAME will on one hand improve the biodiesel quality and on other hand, produce commercially important ALAME as byproduct.
10. The harvesting of *Desmodesmus* sp. MCC34, *C. minutissima*, *D. salina* and *D. tertiolecta* when carried out using chemical flocculent ferric chloride at pH 11, the maximum flocculation efficiency of 98%, 99% and 98% were achieved for *Desmodesmus* sp. MCC34, *C. minutissima* and *D. salina* respectively, under $FeCl_3$ concentration of 150 mg L^{-1}. However, *D. tertiolecta* required a lesser concentration of 100 mg L^{-1} $FeCl_3$ for achieving maximum flocculation efficiency. The bio-flocculent chitosan was less efficient in terms of flocculation efficiency, as

only 90% flocculation was obtained under concentration ranging between 60 to 90 mg L^{-1} depending upon the species.

11. The study involving lipid extraction from dried and wet biomass of *Desmodesmus* sp. MCC34 using 31 different solvent and solvent combinations showed that Bligh-Dyer solvent mixture (Chloroform: Methanol: water in ratio of 1:2:0.8) worked efficiently for lipid extraction from wet algae while Folch solvent mixture (Chloroform: Methanol in ratio of 2:1) efficiently extracted lipid from dried algae.
12. To improve process economics, a method of static extraction was developed based upon the finding that the mixing of solvent with the biomass does not influence rate of lipid extraction. The factors affecting microalgal lipid extraction were solvent-biomass ratio (SBR), surface area factor (SAF), type of solvent and cell lysis. The Patricelli model which has been used to describe the lipid extraction kinetics suggests that static extraction, having favorable SBR and SAF, matched the rate of reaction of two processes; one the extraction from lysed cells and other, the stirring of solvent with biomass.
13. The optimal temperature, reaction time, solvent-oil ratio and catalyst amount for acid catalyzed transesterification were 70°C, 60 minute, 25 and 2% (w/v) HCl respectively, whereas the optimal temperature, reaction time, solvent-oil ratio and catalyst amount for base catalyzed transesterification were 60°C, 1 minute, 300 and 0.2% (w/v) KOH. The base catalyzed process is more efficient than acid catalyzed one in terms of temperature, reaction time and catalyst amount but consumed more solvent for biodiesel production.
14. The increase in carbohydrate content in all the four species during nitrogen deprivation shows potential for co-production of bioethanol or biogas along with biodiesel and alpha linolenic acid production.

Future Scope

- The process of separating unsaturated fatty acid from total FAME, as demonstrated in *Desmodesmus* sp. MCC34, improved the biodiesel quality on one hand and on other, produced commercially important ALAME. This technique can be applied for industrial production of microalgal biodiesel. The same technique can be extended for other algal species which are rich in valuable unsaturated fatty acid such as omega-3 fatty acid for co-producing high quality biodiesel and value added omega-3 fatty acid.
- The co-production of biogas or bioethanol along with biodiesel from carbohydrate and lipid rich species such as *Desmodesmus* sp. MCC34, *D. salina* and *D. tertiolecta* presents an additional option to turn the uneconomical microalgal biodiesel production into a process favoring positive net energy ratio.
- The energy less static lipid extraction technique optimized for extracting lipid from *Desmodesmus* sp. MCC34 for biodiesel and alpha linolenic acid production can also be implemented for extracting other highly valuable lipids such as such as decosahexaenoic acid and eicosapentaenoic acid from microalgae.
- The growth model developed in this study for prediction of outdoor biomass productivity by *Desmodesmus* sp. MCC34 can be used for other commercially important microalgae also after validation with experimental results.

References

Adewuyi, A., Oderinde, R.A., Analysis of the lipids and molecular speciation of the triacylglycerol of the oils of *Luffa cylindrica* and *Adenopus breviflorus*, CyTA-Journal of Food (2012). 10, 313-320.

Ahmad, A., Yasin, N.M., Derek, C., Lim, J., Kinetic studies and thermodynamics of oil extraction and transesterification of *Chlorella* sp. for biodiesel production, Environmental Technology (2014). 35, 891-897.

Ahmad, A., Yasin, N.M., Derek, C., Lim, J., Optimization of microalgae coagulation process using chitosan, Chemical Engineering Journal (2011). 173, 879-882.

Akoh, C.C., Chang, S.-W., Lee, G.-C., Shaw, J.-F., Enzymatic approach to biodiesel production, Journal of Agricultural and Food Chemistry (2007). 55, 8995-9005.

Amarni, F., Kadi, H., Kinetics study of microwave-assisted solvent extraction of oil from olive cake using hexane: comparison with the conventional extraction, Innovative Food Science & Emerging Technologies (2010). 11, 322-327.

Amenta, J., A rapid chemical method for quantification of lipids separated by thin-layer chromatography, Journal of Lipid Research (1964). 5, 270-272.

Andersen, R.A., Algal culturing techniques, Academic Press, USA, 2005, pp. 1-578.

Ashokkumar, V., Rengasamy, R., Deepalakshmi, S., Sivalingam, A., Sivakumar, P., Mass cultivation of microalgae and extraction of total hydrocarbons: A kinetic and thermodynamic study, Fuel (2014). 119, 308-312.

Azma, M., Mohamed, M.S., Mohamad, R., Rahim, R.A., Ariff, A.B., Improvement of medium composition for heterotrophic cultivation of green microalgae, *Tetraselmis suecica*, using response surface methodology, Biochemical Engineering Journal (2011). 53, 187-195.

Bajaj, A., Lohan, P., Jha, P.N., Mehrotra, R., Biodiesel production through lipase catalyzed transesterification: an overview, Journal of Molecular Catalysis B: Enzymatic (2010). 62, 9-14.

Batista, A.P., Gouveia, L., Bandarra, N.M., Franco, J.M., Raymundo, A., Comparison of microalgal biomass profiles as novel functional ingredient for food products, Algal Research (2013). 2, 164-173.

Bellinger, E., Sigee, D., Freshwater algae: identification and use as bioindicators, John Wiley & Sons, Newzealand, 2010.

Ben-Amotz, A., Industrial production of microalgal cell-mass and secondary products–major industrial species, John Wiley & Sons, USA, 2004, pp. 273-280.

Benvenuti, G., Bosma, R., Cuaresma, M., Janssen, M., Barbosa, M.J., Wijffels, R.H., Selecting microalgae with high lipid productivity and photosynthetic activity under nitrogen starvation, Journal of Applied Phycology (2014). 21, 1-7.

Bertozzini, E., Galluzzi, L., Ricci, F., Penna, A., Magnani, M., Neutral lipid content and biomass production in *Skeletonema marinoi* (Bacillariophyceae) culture in response to nitrate limitation, Applied biochemistry and biotechnology (2013). 181, 1-13.

Bajhaiya, A.K., Dean, A.P., Driver, T., Trivedi, D.K., Rattray, N.J., Allwood, J.W., Goodacre, R., Pittman, J.K., High-throughput metabolic screening of microalgae genetic variation in response to nutrient limitation, Metabolomics (2016). 12, 1-14.

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

Bonnefond, H., Moelants, N., Talec, A., Bernard, O., Sciandra, A., Concomitant effects of light and temperature diel variations on the growth rate and lipid production of *Dunaliella salina*, Algal Research (2016). 14, 72-78.

Borowitzka, M.A., Beardall, J., Raven, J.A., The Physiology of Microalgae, Springer, USA, 2016, pp. 1-680.

Boyle, N.R., Page, M.D., Liu, B., Blaby, I.K., Casero, D., Kropat, J., Cokus, S.J., Hong-Hermesdorf, A., Shaw, J., Karpowicz, S.J., Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in *Chlamydomonas*, Journal of Biological Chemistry (2012). 287, 15811-15825.

Breuer, G., Lamers, P.P., Martens, D.E., Draaisma, R.B., Wijffels, R.H., Effect of light intensity, pH, and temperature on triacylglycerol (TAG) accumulation induced by nitrogen starvation in *Scenedesmus obliquus*, Bioresource Technology (2013). 143, 1-9.

Breuer, G., Lamers, P.P., Martens, D.E., Draaisma, R.B., Wijffels, R.H., The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains, Bioresource Technology (2012). 124, 217–226.

Cakmak, T., Angun, P., Demiray, Y.E., Ozkan, A.D., Elibol, Z., Tekinay, T., Differential effects of nitrogen and sulfur deprivation on growth and biodiesel feedstock production of *Chlamydomonas reinhardtii*, Biotechnology and Bioengineering (2012). 109, 1947-1957.

Canakci, M., Van Gerpen, J., Biodiesel production from oils and fats with high free fatty acids, Transactions-American Society of Agricultural Engineers (2001). 44, 1429-1436.

Caporgno, M., Olkiewicz, M., Fortuny, A., Stüber, F., Fabregat, A., Font, J., Pruvost, J., Lepine, O., Legrand, J., Bengoa, C., Evaluation of different strategies to produce biofuels from *Nannochloropsis oculata* and *Chlorella vulgaris*, Fuel Processing Technology (2016). 144, 132-138.

Chandra, T.S., Deepak, R., Kumar, M.M., Mukherji, S., Chauhan, V., Sarada, R., Mudliar, S., Evaluation of indigenous fresh water microalga *Scenedesmus obtusus* for feed and fuel applications: Effect of carbon dioxide, light and nutrient sources on growth and biochemical characteristics, Bioresource Technology (2016). 207, 430-439.

Chatsungnoen, T., Chisti, Y., Harvesting microalgae by flocculation–sedimentation, Algal Research (2016). 13, 271-283.

Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J., Chang, J.-S., Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review, Bioresource Technology (2011). 102, 71-81.

Chinnasamy, S., Bhatnagar, A., Claxton, R., Das, K., Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium, Bioresource Technology (2010). 101, 6751-6760.

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

Chisti, Y., Large-Scale Production of Algal Biomass: Raceway Ponds, Algae Biotechnology, Springer, 2016, pp. 21-40.

Choi, W.Y., Kang, D.H., Lee, S.Y., Lee, H.Y., High quality biodiesel from marine microalga, *Scenedesmus* sp. through in situ acid transesterification, Journal of Chemical Technology and Biotechnology (2014). 90, 1245–1252.

Cordero, B.F., Couso, I., León, R., Rodríguez, H., Vargas, M.Á., Enhancement of carotenoids biosynthesis in *Chlamydomonas reinhardtii* by nuclear transformation using a phytoene synthase gene isolated from *Chlorella zofingiensis*, Applied microbiology and biotechnology (2011). 91, 341-351.

Cruz-Hernandez, C., Deng, Z., Zhou, J., Hill, A.R., Yurawecz, M.P., Delmonte, P., Mossoba, M.M., Dugan, M.E., Kramer, J.K., Methods for analysis of conjugated linoleic acids and trans-18: 1 isomers in dairy fats by using a combination of gas chromatography, silver-ion thin-layer chromatography/gas chromatography, and silver-ion liquid chromatography, Journal of AOAC International (2004). 87, 545-562.

Cuellar-Bermudez, S.P., Aguilar-Hernandez, I., Cardenas-Chavez, D.L., Ornelas-Soto, N., Romero-Ogawa, M.A., Parra-Saldivar, R., Extraction and purification of high-value metabolites from microalgae: Essential lipids, astaxanthin and phycobiliproteins, Microbial Biotechnology (2015). 8, 190-209.

Dahlqvist, A., Ståhl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., Stymne, S., Phospholipid: diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants, Proceedings of the National Academy of Sciences (2000). 97, 6487-6492.

Darley, W.M., Biochemical composition, The biology of diatoms (1977). 13, 198-223.

De Godos, I., Guzman, H.O., Soto, R., García-Encina, P.A., Becares, E., Muñoz, R., Vargas, V.A., Coagulation/flocculation-based removal of algal–bacterial biomass from piggery wastewater treatment, Bioresource Technology (2011). 102, 923-927.

Delmonte, P., Kataoka, A., Corl, B., Bauman, D., Yurawecz, M.P., Relative retention order of all isomers of cis/trans conjugated linoleic acid FAME from the 6, 8-to 13, 15-positions using silver ion HPLC with two elution systems, Lipids (2005). 40, 509-514.

Deng, X., Gu, B., Hu, X., Guo, J., Fei, X., Photosynthetic efficiency and lipid accumulation are affected by the concentration of carbon in microalgae *Micractinium pusillum* Y-002, African Journal of Biotechnology (2011). 10, 11600-11610.

Dong, H.-P., Williams, E., Wang, D.-z., Xie, Z.-X., Hsia, R.-c., Jenck, A., Halden, R., Li, J., Chen, F., Place, A.R., Responses of *Nannochloropsis oceanica* IMET1 to long-term nitrogen starvation and recovery, Plant Physiology (2013). 162, 1110-1126.

Dong, T., Knoshaug, E.P., Davis, R., Laurens, L.M., Van Wychen, S., Pienkos, P.T., Nagle, N., Combined algal processing: A novel integrated biorefinery process to produce algal biofuels and bioproducts, Algal Research (2016). doi:10.1016/j.algal.2015.12.021

Doyle, J., A rapid DNA isolation procedure for small quantities of fresh leaf tissue, Phytochemical Bulletin (1987). 19, 11-15.

Du, Z.-Y., Benning, C., Triacylglycerol accumulation in photosynthetic cells in plants and algae, Lipids in plant and algae development, Springer, 2016, pp. 179-205.

Eglinton, G., Hunneman, D., McCormick, A., Gas chromatographic—mass spectrometric studies of long chain hydroxy acids.—III. 1 The mass spectra of the methyl esters trimethylsilyl ethers of aliphatic hydroxy acids. A facile method of double bond location, Organic Mass Spectrometry (1968). 1, 593-611.

Ehimen, E., Sun, Z., Carrington, C., Variables affecting the in situ transesterification of microalgae lipids, Fuel (2010). 89, 677-684.

Eustance, E., Gardner, R.D., Moll, K.M., Menicucci, J., Gerlach, R., Peyton, B.M., Growth, nitrogen utilization and biodiesel potential for two chlorophytes grown on ammonium, nitrate or urea, Journal of Applied Phycology (2013). 25, 1663-1677.

Erdoğan, A., Demirel, Z., Eroğlu, A.E., Dalay, M.C., Carotenoid profile in *Prochlorococcus* sp. and enrichment of lutein using different nitrogen sources, Journal of Applied Phycology (2016). 33, 1-7.

Esakkimuthu, S., Krishnamurthy, V., Govindarajan, R., Swaminathan, K., Augmentation and starvation of calcium, magnesium, phosphate on lipid production of *Scenedesmus obliquus*, Biomass and Bioenergy (2016). 88, 126-134.

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

Fasman, G.D., Sober, H.A., Handbook of Biochemistry and Molecular biology, 3rd ed., CRC press Cleveland, Cleveland, Ohio, 1977.

Fawley, M.W., Fawley, K.P., Hegewald, E., Taxonomy of *Desmodesmus serratus* (*Chlorophyceae*, Chlorophyta) and related taxa on the basis of morphological and DNA sequence data, Phycologia (2011). 50, 23-56.

Fei, Q., Fu, R., Shang, L., Brigham, C.J., Chang, H.N., Lipid production by microalgae *Chlorella protothecoides* with volatile fatty acids (VFAs) as carbon sources in heterotrophic cultivation and its economic assessment, Bioprocess and Biosystems Engineering (2015). 38, 691-700.

Folch, J., Lees, M., Sloane-Stanley, G., A simple method for the isolation and purification of total lipids from animal tissues, Journal of Biological Chemistry (1957). 226, 497-509.

Gardner, R., Peters, P., Peyton, B., Cooksey, K.E., Medium pH and nitrate concentration effects on accumulation of triacylglycerol in two members of the Chlorophyta, Journal of Applied Phycology (2011). 23, 1005-1016.

Gautam, M., Martin, D., Combustion characteristics of higher-alcohol/gasoline blends, Proceedings of the Institution of Mechanical Engineers, Part A: Journal of Power and Energy (2000). 214, 497-511.

Gharagozloo, P.E., Drewry, J.L., Collins, A.M., Dempster, T.A., Choi, C.Y., James, S.C., Analysis and modeling of *Nannochloropsis* growth in lab, greenhouse, and raceway experiments, Journal of Applied Phycology (2014). 190, 1-12.

Go, A.W., Sutanto, S., Ong, L.K., Tran-Nguyen, P.L., Ismadji, S., Ju, Y.-H., Developments in in-situ (trans) esterification for biodiesel production: A critical review, Renewable and Sustainable Energy Reviews (2016). 60, 284-305.

Gong, Y., Guo, X., Wan, X., Liang, Z., Jiang, M., Triacylglycerol accumulation and change in fatty acid content of four marine oleaginous microalgae under nutrient limitation and at different culture ages, Journal of Basic Microbiology (2013). 53, 29-36.

Görl, M., Sauer, J., Baier, T., Forchhammer, K., Nitrogen-starvation-induced chlorosis in *Synechococcus* PCC 7942: adaptation to long-term survival, Microbiology (1998). 144, 2449-2458.

Griffiths, M.J., Harrison, S.T., Lipid productivity as a key characteristic for choosing algal species for biodiesel production, Journal of Applied Phycology (2009). 21, 493-507.

Guarnieri, M.T., Nag, A., Smolinski, S.L., Darzins, A., Seibert, M., Pienkos, P.T., Examination of triacylglycerol biosynthetic pathways via de novo transcriptomic and proteomic analyses in an unsequenced microalga, PLOS ONE (2011). 6, e25851.

Guarnieri, M.T., Nag, A., Yang, S., Pienkos, P.T., Proteomic analysis of *Chlorella vulgaris* : Potential targets for enhanced lipid accumulation, Journal of Proteomics (2013). 93, 245-253.

Guerra, L.T., Levitan, O., Frada, M.J., Sun, J.S., Falkowski, P.G., Dismukes, G.C., Regulatory branch points affecting protein and lipid biosynthesis in the diatom *Phaeodactylum tricornutum*, Biomass and Bioenergy (2013). 59, 306-315.

Guldhe, A., Singh, B., Ansari, F.A., Sharma, Y., Bux, F., Extraction and conversion of microalgal lipids, Algae Biotechnology, Springer, 2016, pp. 91-110.

Guil-Guerrero, J., Campra-Madrid, P., Navarro-Juárez, R., Isolation of some PUFA from edible oils by argentated silica gel chromatography, Grasas y aceites (2003). 54, 116-121.

Guil-Guerrero, J.L., Common mistakes about fatty acids identification by gas–liquid chromatography, Journal of Food Composition and Analysis (2014). 33, 153-154.

Gunstone, F.D., McLaughlan, J., Scrimgeour, C.M., Watson, A.P., Improved procedures for the isolation of pure oleic, linoleic, and linolenic acids or their methyl esters from natural sources, Journal of the Science of Food and Agriculture (1976). 27, 675-680.

Halim, R., Danquah, M.K., Webley, P.A., Extraction of oil from microalgae for biodiesel production: A review, Biotechnology Advances (2012). 30, 709-732.

Hasheminejad, M., Tabatabaei, M., Mansourpanah, Y., Javani, A., Upstream and downstream strategies to economize biodiesel production, Bioresource Technology (2011). 102, 461-468.

Hewes, C.D., The color of mass culture: spectral characteristics of a shallow water column through shade-limited algal growth dynamics, Journal of Phycology (2016). 52: 252–259.

Ho, S.-H., Chen, C.-Y., Chang, J.-S., Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N, Bioresource Technology (2012). 113, 244-252.

Hofstetter, H., Sen, N., Holman, R., Characterization of unsaturated fatty acids by gas-liquid chromatography, Journal of the American Oil Chemists' Society (1965). 42, 537-540.

Hoh, D., Watson, S., Kan, E., Algal biofilm reactors for integrated wastewater treatment and biofuel production: A review, Chemical Engineering Journal (2016). 287, 466-473.

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

Huesemann, M., Van Wagenen, J., Miller, T., Chavis, A., Hobbs, S., Crowe, B., A screening model to predict microalgae biomass growth in photobioreactors and raceway ponds, Biotechnology and Bioengineering (2013). 110, 1583-1594.

Huesemann, M., Crowe, B., Waller, P., Chavis, A., Hobbs, S., Edmundson, S., Wigmosta, M., A validated model to predict microalgae growth in outdoor pond cultures subjected to fluctuating light intensities and water temperatures, Algal Research (2016). 13, 195-206.

Illman, A., Scragg, A., Shales, S., Increase in *Chlorella* strains calorific values when grown in low nitrogen medium, Enzyme and Microbial Technology (2000). 27, 631-635.

Islam, M.A., Brown, R.J., Brooks, P., Jahirul, M.I., Bockhorn, H., Heimann, K., Investigation of the effects of the fatty acid profile on fuel properties using a multi-criteria decision analysis, Energy Conversion and Management (2015). 98, 340-347.

Jeong, G.-T., Park, J.-H., Park, S.-H., Park, D.-H., Estimating and improving cold filter plugging points by blending biodiesels with different fatty acid contents, Biotechnology and Bioprocess Engineering (2008). 13, 505-510.

Jham, G.N., Velikova, R., Nikolova-Damyavova, B., Rabelo, S.C., da Silva, J.C.T., de Paula Souza, K.A., Valente, V.M.M., Cecon, P.R., Preparative silver ion TLC/RP-HPLC determination of coffee triacylglycerol molecular species, Food research international (2005). 38, 121-126.

Khoeyi, Z.A., Seyfabadi, J., Ramezanpour, Z., Effect of light intensity and photoperiod on biomass and fatty acid composition of the microalgae, *Chlorella vulgaris*, Aquaculture International (2012). 20, 41-49.

Kim, D.G., La, H.J., Ahn, C.Y., Park, Y.H., Oh, H.M., Harvest of *Scenedesmus* sp. with bioflocculant and reuse of culture medium for subsequent high-density cultures, Bioresource Technology (2011). 102, 3163-3168.

Kim, K.-B., Nam, Y.A., Kim, H.S., Hayes, A.W., Lee, B.-M., α-Linolenic acid: Nutraceutical, pharmacological and toxicological evaluation, Food and Chemical Toxicology (2014). 70, 163-178.

Kim, S.-H., Ahn, H.M., Lim, S.R., Hong, S.-J., Cho, B.-K., Lee, H., Lee, C.-G., Choi, H.-K., Comparative Lipidomic Profiling of Two Dunaliella tertiolecta Strains with Different Growth Temperatures under Nitrate-Deficient Conditions, Journal of agricultural and food chemistry (2015). 63, 880-887.

Kim, W., Park, J.M., Gim, G.H., Jeong, S.-H., Kang, C.M., Kim, D.-J., Kim, S.W., Optimization of culture conditions and comparison of biomass productivity of three green algae, Bioprocess and Biosystems Engineering (2012). 35, 19-27.

Kleiman, R., Spencer, G., Gas chromatography-mass spectrometry of methyl esters of unsaturated oxygenated fatty acids, Journal of the American Oil Chemists' Society (1973). 50, 31-38.

Komers, K., Skopal, F., Stloukal, R., Machek, J., Kinetics and mechanism of the KOH—catalyzed methanolysis of rapeseed oil for biodiesel production, European Journal of Lipid Science and Technology (2002). 104, 728-737.

Kraft, C., Deplazes, A., Sohrmann, M., Peter, M., Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease, Nature cell biology (2008). 10, 602-610.

Kwon, M.H., Yeom, S.H., Optimization of one-step extraction and transesterification process for biodiesel production from the marine microalga Nannochloropsis sp. KMMCC 290 cultivated in a raceway pond, Biotechnology and Bioprocess Engineering (2015). 20, 276-283.

Lamers, P.P., Janssen, M., De Vos, R.C., Bino, R.J., Wijffels, R.H., Carotenoid and fatty acid metabolism in nitrogen-starved Dunaliella salina, a unicellular green microalga, Journal of biotechnology (2012). 162, 21-27.

Li, Y., Fei, X., Deng, X., Novel molecular insights into nitrogen starvation-induced triacylglycerols accumulation revealed by differential gene expression analysis in green algae *Micractinium pusillum*, Biomass and Bioenergy (2012). 42, 199-211.

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

Liang, C., Cao, S., Zhang, X., Zhu, B., Su, Z., Xu, D., Guang, X., Ye, N., e novo sequencing and global transcriptome analysis of *Nannochloropsis* sp.(eustigmatophyceae) following nitrogen starvation, BioEnergy Research (2013). 37, 1-12.

Lichtenthaler, H.K., Wellburn, A.R., Determinations of total carotenoids and chlorophylls b of leaf extracts in different solvents, Biochemical Society Transactions (1983). 11, 591-592.

Lim, D.K., Garg, S., Timmins, M., Zhang, E.S., Thomas-Hall, S.R., Schuhmann, H., Li, Y., Schenk, P.M., Isolation and evaluation of oil-producing microalgae from subtropical coastal and brackish waters, PLoS One (2012). 7, e40751.

Lin, Q., Lin, J., Effects of nitrogen source and concentration on biomass and oil production of a *Scenedesmus rubescens* like microalga, Bioresource Technology (2011). 102, 1615-1621.

Liu, J., Huang, J., Fan, K.W., Jiang, Y., Zhong, Y., Sun, Z., Chen, F., Production potential of *Chlorella zofingienesis* as a feedstock for biodiesel, Bioresource Technology (2010). 101, 8658-8663.

Liu, J., Yuan, C., Hu, G., Li, F., Effects of light intensity on the growth and lipid accumulation of microalga *Scenedesmus* sp. 11-1 under nitrogen limitation, Applied Biochemistry and Biotechnology (2012a). 166, 2127-2137.

Liu, W., Huang, Z., Li, P., Xia, J., Chen, B., Formation of triacylglycerol in *Nitzschia closterium f. minutissima* under nitrogen limitation and possible physiological and biochemical mechanisms, Journal of Experimental Marine Biology and Ecology (2012b). 418, 24-29.

Liu, T., Li, Y., Liu, F., Wang, C., The enhanced lipid accumulation in oleaginous microalga by the potential continuous nitrogen-limitation (CNL) strategy, Bioresource technology (2016). 203, 150-159.

López, E.N., Medina, A.R., Moreno, P.A.G., Cerdán, L.E., Valverde, L.M., Grima, E.M., Biodiesel production from Nannochloropsis gaditana lipids through transesterification catalyzed by *Rhizopus oryzae* lipase, Bioresource technology (2016). 203, 236-244.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., Protein measurement with the folin phenol reagent, Journal of Biological Chemistry (1951). 193, 265-275.

Lv, J., Guo, J., Feng, J., Liu, Q., Xie, S., A comparative study on flocculating ability and growth potential of two microalgae in simulated secondary effluent, Bioresource Technology (2016). 205, 111-117.

Ma, X., Liu, J., Liu, B., Chen, T., Yang, B., Chen, F., Physiological and biochemical changes reveal stress-associated photosynthetic carbon partitioning into triacylglycerol in the oleaginous marine alga *Nannochloropsis oculata*, Algal Research (2016). 16, 28-35.

Makareviciene, V., Skorupskaite, V., Levisauskas, D., Andruleviciute, V., Kazancev, K., The optimization of biodiesel fuel production from microalgae oil using response surface methodology, International Journal of Green Energy (2014). 11, 527-541.

Martin, G.J., Energy requirements for wet solvent extraction of lipids from microalgal biomass, Bioresource Technology (2016). 205, 40-47.

Martinez, M., Jimenez, J., El Yousfi, F., Influence of phosphorus concentration and temperature on growth and phosphorus uptake by the microalga *Scenedesmus obliquus*, Bioresource Technology (1999). 67, 233-240.

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., Lee, Y.C., Carbohydrate analysis by a phenol–sulfuric acid method in microplate format, Analytical Biochemistry (2005). 339, 69-72.

Mathimani, T., Uma, L., Prabaharan, D., Homogeneous acid catalysed transesterification of marine microalga *Chlorella* sp. BDUG 91771 lipid – An efficient biodiesel yield and its characterization, Renewable Energy (2015). 81, 523-533.

Matyash, V., Liebisch, G., Kurzchalia, T.V., Shevchenko, A., Schwudke, D., Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics, Journal of Lipid Research (2008). 49, 1137-1146.

McConnell, B., Farag, I.H., Kinetics study of the solvent extraction of lipids from *Chlorella Vulgaris*, International Journal of Engineering and Technical Research (2013). 1, 28-37.

Mendoza, H., Martel, A., Del Río, M.J., Reina, G.G., Oleic acid is the main fatty acid related with carotenogenesis in *Dunaliella salina*, Journal of Applied Phycology (1999). 11, 15-19.

Mera, R., Torres, E., Abalde, J., Effects of sodium sulfate on the freshwater microalga *Chlamydomonas moewusii* : implications for the optimization of algal culture media, Journal of Phycology (2016). 52, 75-88.

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

Milledge, J.J., Heaven, S., A review of the harvesting of micro-algae for biofuel production, Reviews in Environmental Science and Biotechnology (2013). 12, 165-178.

Miller, L.T., Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids, Journal of Clinical Microbiology (1982). 16, 584-586.

Mohan, S.V., Devi, M.P., Salinity stress induced lipid synthesis to harness biodiesel during dual mode cultivation of mixotrophic microalgae, Bioresource Technology (2014). 165, 288-294.

Mohan, S.V., Nikhil, G., Chiranjeevi, P., Reddy, C.N., Rohit, M., Kumar, A.N., Sarkar, O., Waste biorefinery models towards sustainable circular bioeconomy: critical review and future perspectives, Bioresource Technology (2016). doi:10.1016/j.biortech.2016.03.130

Monteiro, M.R., Ambrozin, A.R.P., Lião, L.M., Ferreira, A.G., Critical review on analytical methods for biodiesel characterization, Talanta (2008). 77, 593-605.

Morris, L., Separations of lipids by silver ion chromatography, Journal of Lipid Research (1966). 7, 717-732.

Morschett, H., Wiechert, W., Oldiges, M., Automation of a Nile red staining assay enables high throughput quantification of microalgal lipid production, Microbial cell factories (2016). 15, 1-7.

Moutel, B., Goncalves, O., Le Grand, F., Long, M., Soudant, P., Legrand, J., Grizeau, D., Pruvost, J., Development of a screening procedure for the characterization of *Botryococcus braunii* strains for biofuel application, Process Biochemistry (2016). doi:10.1016/j.procbio.2016.05.002

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

Müller, T., Philippi, N., Dandekar, T., Schultz, J., Wolf, M., Distinguishing species, RNA (2007). 13, 1469-1472.

Mus, F., Toussaint, J.-P., Cooksey, K.E., Fields, M.W., Gerlach, R., Peyton, B.M., Carlson, R.P., Physiological and molecular analysis of carbon source supplementation and pH stress-induced lipid accumulation in the marine diatom *Phaeodactylum tricornutum*, Applied Microbiology and Biotechnology (2013). 182, 1-18.

Mutanda, T., Ramesh, D., Karthikeyan, S., Kumari, S., Anandraj, A., Bux, F., Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production, Bioresource Technology (2011). 102, 57-70.

Nagappan, S and Verma, S.K., Growth model for raceway pond cultivation of *Desmodesmus sp*. MCC34 isolated from a local water body, Engineering in Life Sciences (2016). 16, 45-52.

Nakamura, Y., Li-Beisson, Y., Lipids in Plant and Algae Development, Springer, 2016, pp. 1-528.

Nascimento, I.A., Marques, S.S.I., Cabanelas, I.T.D., Pereira, S.A., Druzian, J.I., de Souza, C.O., Vich, D.V., de Carvalho, G.C., Nascimento, M.A., Screening microalgae strains for biodiesel production: lipid productivity and estimation of fuel quality based on fatty acids profiles as selective criteria, BioEnergy Research (2013). 6, 1-13.

Nautiyal, P., Subramanian, K., Dastidar, M., Kinetic and thermodynamic studies on biodiesel production from *Spirulina platensis* algae biomass using single stage extraction–transesterification process, Fuel (2014). 135, 228-234.

Nejat, P., Jomehzadeh, F., Taheri, M.M., Gohari, M., Majid, M.Z.A., A global review of energy consumption, CO_2 emissions and policy in the residential sector (with an overview of the top ten CO_2 emitting countries), Renewable and Sustainable Energy Reviews (2015). 43, 843-862.

Nurra, C., Clavero, E., Salvadó, J., Torras, C., Vibrating membrane filtration as improved technology for microalgae dewatering, Bioresource Technology (2014). 157, 247-253.

Oh, H.M., Lee, S.J., Park, M.H., Kim, H.S., Kim, H.C., Yoon, J.H., Kwon, G.S., Yoon, B.D., Harvesting of *Chlorella vulgaris* using a bioflocculant from *Paenibacillus* sp. AM49, Biotechnology Letters (2001). 23, 1229-1234.

Olmstead, I.L., Hill, D.R., Dias, D.A., Jayasinghe, N.S., Callahan, D.L., Kentish, S.E., Scales, P.J., Martin, G.J., A quantitative analysis of microalgal lipids for optimization of biodiesel and omega-3 production, Biotechnology and Bioengineering (2013a). 110, 2096-2104.

Olmstead, I.L., Kentish, S.E., Scales, P.J., Martin, G.J., Low solvent, low temperature method for extracting biodiesel lipids from concentrated microalgal biomass, Bioresource Technology (2013b). 148, 615-619.

Ördög, V., Stirk, W.A., Bálint, P., Lovász, C., Pulz, O., van Staden, J., Lipid productivity and fatty acid composition in *Chlorella* and *Scenedesmus* strains grown in nitrogen-stressed conditions, Journal of Applied Phycology (2013). 25, 233-243.

Ördög, V., Stirk, W.A., Bálint, P., Aremu, A.O., Okem, A., Lovász, C., Molnár, Z., van Staden, J., Effect of temperature and nitrogen concentration on lipid productivity and fatty acid composition in three *Chlorella* strains, Algal Research (2016). 16, 141-149.

Osanai, T., Oikawa, A., Shirai, T., Kuwahara, A., Iijima, H., Tanaka, K., Ikeuchi, M., Kondo, A., Saito, K., Hirai, M.Y., Capillary electrophoresis-mass spectrometry reveals the distribution of carbon metabolites during nitrogen starvation in *Synechocystis* sp. PCC 6803, Environmental Microbiology (2013). 16, 512-524.

Ota, S., Oshima, K., Yamazaki, T., Kim, S., Yu, Z., Yoshihara, M., Takeda, K., Takeshita, T., Hirata, A., Bišová, K., Highly efficient lipid production in the green alga *Parachlorella kessleri* : draft genome and transcriptome endorsed by whole-cell 3D ultrastructure, Biotechnology for biofuels (2016). 9, 1-8.

Paliwal, C., Ghosh, T., George, B., Pancha, I., Maurya, R., Chokshi, K., Ghosh, A., Mishra, S., Microalgal carotenoids: Potential nutraceutical compounds with chemotaxonomic importance, Algal Research (2016). 15, 24-31.

Parsaeimehr, A., Sun, Z., Dou, X., Chen, Y.-F., Simultaneous improvement in production of microalgal biodiesel and high-value alpha-linolenic acid by a single regulator acetylcholine, Biotechnology for Biofuels (2015). 8, 1-10.

Powell, N., Shilton, A., Chisti, Y., Pratt, S., Towards a luxury uptake process via microalgae–defining the polyphosphate dynamics, Water Research (2009). 43, 4207-4213.

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

Quinn, J.C., Smith, T.G., Downes, C.M., Quinn, C., Microalgae to biofuels lifecycle assessment - multiple pathway evaluation, Algal Research (2014). 4, 116-122.

Rashid, N., Rehman, S.U., Han, J.-I., Rapid harvesting of freshwater microalgae using chitosan, Process Biochemistry (2013). 48, 1107-1110.

Razack, S.A., Duraiarasan, S., Shellomith, A.S., Muralikrishnan, K., Statistical optimization of harvesting *Chlorella vulgaris* using a novel bio-source, *Strychnos potatorum*, Biotechnology Reports (2015). 7, 150-156.

Recht, L., Zarka, A., Boussiba, S., Patterns of carbohydrate and fatty acid changes under nitrogen starvation in the microalgae *Haematococcus pluvialis* and *Nannochloropsis sp*, Applied Microbiology and Biotechnology (2012). 94, 1495-1503.

Rismani-Yazdi, H., Haznedaroglu, B.Z., Hsin, C., Peccia, J., Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights into triacylglyceride accumulation, Biotechnology for Biofuels (2012). 5, 1-16.

Rodríguez, R., Lara, C., Guerrero, M.G., Nitrate transport in the cyanobacterium *Anacystis nidulans* R2: Kinetic and energetic aspects, Biochemical Journal (1992). 282, 639-643.

Ryckebosch, E., Bermúdez, S.P.C., Termote-Verhalle, R., Bruneel, C., Muylaert, K., Parra-Saldivar, R., Foubert, I., Influence of extraction solvent system on the extractability of lipid components from the biomass of *Nannochloropsis gaditana*, Journal of Applied Phycology (2013). 79, 1-10.

Sajilata, M., Singhal, R., Kamat, M., Fractionation of lipids and purification of γ-linolenic acid (GLA) from *Spirulina platensis*, Food Chemistry (2008). 109, 580-586.

Sánchez-Estudillo, L., Freile-Pelegrin, Y., Rivera-Madrid, R., Robledo, D., Narváez-Zapata, J.A., Regulation of two photosynthetic pigment-related genes during stress-induced pigment formation in the green alga, *Dunaliella salina*, Biotechnology Letters (2006). 28, 787-791.

Sánchez, J., Fernández-Sevilla, J., Acién, F., Cerón, M., Pérez-Parra, J., Molina-Grima, E., Biomass and lutein productivity of *Scenedesmus almeriensis*: influence of irradiance, dilution rate and temperature, Applied Microbiology and Biotechnology (2008). 79, 719-729.

Sarwa, P., Verma, S.K., Recovery and recycling of Zn(II) from wastewater by *Scenedesmus* sp. MCC 26 isolated from a heavy metal contaminated site, CLEAN–Soil, Air, Water (2013). 42 1298–1303.

Schmollinger, S., Mühlhaus, T., Boyle, N.R., Blaby, I.K., Casero, D., Mettler, T., Moseley, J.L., Kropat, J., Sommer, F., Strenkert, D., Nitrogen-sparing mechanisms in *Chlamydomonas* affect the transcriptome, the proteome, and photosynthetic metabolism, The Plant Cell (2014). 26, 1410-1435.

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

Sheng, J., Vannela, R., Rittmann, B.E., Evaluation of methods to extract and quantify lipids from *Synechocystis* PCC 6803, Bioresource Technology (2011). 102, 1697-1703.

Singh, A., Nigam, P.S., Murphy, J.D., Mechanism and challenges in commercialisation of algal biofuels, Bioresource Technology (2011). 102, 26-34.

Sinha, S.K., Gupta, A., Bharalee, R., Production of biodiesel from freshwater microalgae and evaluation of fuel properties based on fatty acid methyl ester profile, Biofuels (2016). 34, 1-17.

Solovchenko, A., Verschoor, A.M., Jablonowski, N.D., Nedbal, L., Phosphorus from wastewater to crops: An alternative path involving microalgae, Biotechnology Advances (2016). doi:10.1016/j.biotechadv.2016.01.002

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., Commercial applications of microalgae, Journal of Bioscience and Bioengineering (2006). 101, 87-96.

Suganya, T., Renganathan, S., Optimization and kinetic studies on algal oil extraction from marine macroalgae *Ulva lactuca*, Bioresource Technology (2012). 107, 319-326.

Takagi, M., Karseno, Yoshida, T., Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells, Journal of Bioscience and Bioengineering (2006). 101, 223-226.

Tran, H.-L., Kwon, J.-S., Kim, Z.-H., Oh, Y., Lee, C.-G., Statistical optimization of culture media for growth and lipid production of *Botryococcus braunii* LB572, Biotechnology and Bioprocess Engineering (2010). 15, 277-284.

Tyagi, O.S., Atray, N., Kumar, B., Datta, A., Production, characterization and development of standards for biodiesel — A review, Mapan (2010). 25, 197-218.

Urreta, I., Ikaran, Z., Janices, I., Ibañez, E., Castro-Puyana, M., Castañón, S., Suárez-Alvarez, S., Revalorization of *Neochloris oleoabundans* biomass as source of biodiesel by concurrent production of lipids and carotenoids, Algal Research (2014). 5, 16-22.

Valenzuela, J., Carlson, R., Gerlach, R., Cooksey, K., Peyton, B., Bothner, B., Fields, M., Nutrient resupplementation arrests bio-oil accumulation in *Phaeodactylum tricornutum*, Applied Microbiology and Biotechnology (2013). 179, 1-11.

Valenzuela, J., Mazurie, A., Carlson, R.P., Gerlach, R., Cooksey, K.E., Peyton, B.M., Fields, M.W., Potential role of multiple carbon fixation pathways during lipid accumulation in *Phaeodactylum tricornutum*, Biotechnology and Biofuels (2012). 5, 40-45.

Vargas, M., Rodriguez, H., Moreno, J., Olivares, H., Del Campo, J., Rivas, J., Guerrero, M., Biochemical composition and fatty acid content of filamentous nitrogen-fixing cyanobacteria, Journal of Phycology (1998). 34, 812-817.

Velasquez-Orta, S., Lee, J., Harvey, A., Alkaline in situ transesterification of *Chlorella vulgaris*, Fuel (2012). 94, 544-550.

Wahidin, S., Idris, A., Shaleh, S.R.M., The influence of light intensity and photoperiod on the growth and lipid content of microalgae *Nannochloropsis* sp, Bioresource Technology (2013). 129, 7-11.

Wan, C., Alam, M.A., Zhao, X.-Q., Zhang, X.-Y., Guo, S.-L., Ho, S.-H., Chang, J.-S., Bai, F.-W., Current progress and future prospect of microalgal biomass harvest using various flocculation technologies, Bioresource Technology (2015). 184, 251-257.

Wan, C., Bai, F.-W., Zhao, X.-Q., Effect of nitrogen concentration and media replacement on cell growth and lipid production of oleaginous marine microalga *Nannochloropsis oceanica* DUT01, Biochemical Engineering Journal (2013). 78, 32-38.

White, T.J., Bruns, T., Lee, S., Taylor, J., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, PCR protocols: a guide to methods and applications (1990). 18, 315-322.

Willard, M., Instrumental Methods of Analysis, CBS Publishers and Distributors, London, 1986, pp. 1-623.

Woo, S.-G., Yoo, K., Lee, J., Bang, S., Lee, M., On, K., Park, J., Comparison of fatty acid analysis methods for assessing biorefinery applicability of wastewater cultivated microalgae, Talanta (2012). 97, 103-110.

Wyatt, N.B., Gloe, L.M., Brady, P.V., Hewson, J.C., Grillet, A.M., Hankins, M.G., Pohl, P.I., Critical conditions for ferric chloride-induced flocculation of freshwater algae, Biotechnology and Bioengineering (2012). 109, 493-501.

Xia, L., Ge, H., Zhou, X., Zhang, D., Hu, C., Photoautotrophic outdoor two-stage cultivation for oleaginous microalgae *Scenedesmus obtusus* XJ-15, Bioresource Technology (2013). 144, 261-267.

Xiao, Y., Zhang, J., Cui, J., Feng, Y., Cui, Q., Metabolic profiles of *Nannochloropsis oceanica* IMET1 under nitrogen-deficiency stress, Bioresource Technology (2013). 130, 731-738.

Xie, Y., Ho, S.-H., Chen, C.-N.N., Chen, C.-Y., Ng, I.-S., Jing, K.-J., Chang, J.-S., Lu, Y., Phototrophic cultivation of a thermo-tolerant *Desmodesmus* sp. for lutein production: Effects of nitrate concentration, light intensity and fed-batch operation, Bioresource Technology (2013). 144, 435-444.

Xu, L., Brilman, D.W., Withag, J.A., Brem, G., Kersten, S., Assessment of a dry and a wet route for the production of biofuels from microalgae: energy balance analysis, Bioresource Technology (2011). 102, 5113-5122.

Xu, R., Mi, Y., Simplifying the process of microalgal biodiesel production through in situ transesterification technology, Journal of the American Oil Chemists' Society (2011). 88, 91-99.

Yap, B.H., Crawford, S.A., Dumsday, G.J., Scales, P.J., Martin, G.J., A mechanistic study of algal cell disruption and its effect on lipid recovery by solvent extraction, Algal Research (2014). 5, 112-120.

Yin-Hu, W., Yin, Y., Hong-Ying, H., Effects of initial phosphorus concentration and light Intensity on biomass yield per phosphorus and lipid accumulation of *Scenedesmus sp*. LX1, BioEnergy Research (2014). 7, 927-934.

Zhu, S., Huang, W., Xu, J., Wang, Z., Xu, J., Yuan, Z., Metabolic changes of starch and lipid triggered by nitrogen starvation in the microalga *Chlorella zofingiensis*, Bioresource Technology (2014). 152, 292-298.

Zhang, Z., O'Hara, I.M., Mundree, S., Gao, B., Ball, A.S., Zhu, N., Bai, Z., Jin, B., Biofuels from food processing wastes, Current opinion in biotechnology (2016). 38, 97-105.

Zienkiewicz, K., Du, Z.-Y., Ma, W., Vollheyde, K., Benning, C., Stress-induced neutral lipid biosynthesis in microalgae—Molecular, cellular and physiological insights, Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids (2016). doi:10.1016/j.bbalip.2016.02.008

Appendix I

PUBLICATIONS

- Nagappan, S and Verma, S.K., Growth model for raceway pond cultivation of *Desmodesmus sp*. MCC34 isolated from a local water body, Engineering in Life Sciences (2016). 16, 45-52.
- Nagappan, S and Verma, S.K., The static extraction of lipid from *Desmodesmus* sp. MCC34.The Research journal of Biotechnology (2016). 11, 63-70.

CONFERENCES & WORKSHOPS

- Nagappan, S and Verma, S.K., Investigation of *Chlorella* sp. SSKV1, a new species under normal and stress condition for biodiesel. Symposium on Biotechnology and Stress Biology of Algae and Cyanobacteria. Banarras Hindu University, Varnasi, India (2014).
- Nagappan, S., Verma, S.K., and Upadhyaya, R., Nutritional control of phototrophic and heterotrophic growth of microalgae Scenedesmus sp for biodiesel production. . The 2nd International Conference on Algal Biomass, Biofuels and Bioproducts. San diego, USA (2012).
- Nagappan, S and Verma, S.K., Lipid production from locally isolated Scenedesmus species. National Conference on Contemporary Trends in Biological and Pharmaceutical Research. Birla Institute of Technology and Science, Pilani, India (2011).
- Nagappan, S., Prakash, S., Narendra, V and Verma, S.K., Biomass production by microalgae in industrial effluent. International Conference on Phycology, BHU, Varnasi, India (2010).
- National Workshop on Molecular Techniques: Cell to DNA - The Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, India (2014).
- Training on blue green algae – CCUBGA, IARI, Delhi, India (2011).

Appendix II

Biography of Prof. S. K Verma

Prof. Sanjay Kumar Verma did his Masters in area of Botany, specializing in cyanobacteria and applied phycology before completing Ph.D. in the field of Environmental Biotechnology, both from Banaras Hindu University (BHU), Varanasi. After which, he worked as post-doctoral fellow in the subject of microbial and molecular genetics in University of Hyderabad. In the year 1993, he started his teaching career in BITS Pilani. He had previously held the post of Group leader, now named as Head of the Department of Biological Science and Chief Warden of the Institute. Currently, Professor Verma is the University Dean of Academic Research division (Ph.D. program) of BITS Pilani. He has successfully completed numerous projects granted by various National funding agencies such as UGC, CSIR, DST, DBT, Ministry of Mines, etc. Several symposiums, conferences and workshops at both national and international levels were coordinated by him. He has published several papers in reputed journals and holds two patents. He has guided 7 Ph.D. and large number of graduate and undergraduate students. His areas of research are bioremediation, biosensors and industrial production of algal products.

Biography of A.Senthil Nagappan

A.Senthil Nagappan obtained his Masters in Biotechnology from PSG college of Technology, Coimbatore. In his Master's thesis he studied the soil health of land using biochemical and molecular biology methods. Then in year 2010, he joined BITS Pilani as full time research scholar and worked under a CSIR project of Prof. S.K.Verma. He had received basic scientific research fellowship from UGC. He was actively involved in various workshops and conferences conducted by Department of Biological Sciences such as Cell to DNA, Molecular Techniques (MTCD-2014), etc. He had assisted in conducting laboratory courses in BITS-Pilani such as Instrumental methods of analysis, Biology laboratory, Microbiology, etc.