BIOFUELS FROM MICROALGAE: CULTIVATION, BIODIESEL PRODUCTION AND STORAGE FEASIBILITY

By

AMIT KUMAR SHARMA

COLLEGE OF ENGINEERING STUDIES

Submitted

IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY

TO

UNIVERSITY OF PETROLEUM AND ENERGY STUDIES

DEHRADUN

SEPTEMBER, 2015

Under the Guidance of

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The thesis entitled **"Biofuels From Microalgae: Cultivation, Biodiesel Production and Storage Feasibility"**, being submitted by **Amit Kumar Sharma** to the University of Petroleum and Energy Studies for the award of the degree of Doctor of Philosophy is a bonafide research work carried out by him. He has worked under our supervision, and has fulfilled the requirements for the submission of this thesis, which has attained the standard required for a Ph.D. degree of the University. The content of the thesis, in full or parts have not been submitted to any other Institute or University for the award of any other degree or diploma.

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DECLARATION

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

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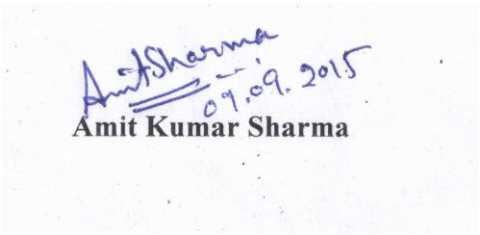

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Executive Summary

Increasing energy demands, predicted fossil fuels shortage in the near future, and environmental concerns such as the production of greenhouse gases have forced the scientists to find alternative and cleaner energy sources. Microalgae based biofuels have received much attention in recent years as potential alternatives because they are green in nature, renewable and sustainable energy sources. Microalgae have many advantages over conventional biofuel feedstocks like high growth rate, ability to fix more CO_2 and higher photosynthetic efficiency. Furthermore, microalgae can grow in both fresh water and saline water. Microalgae can accumulate up to 70% lipids of dry biomass under adverse conditions. Despite these advantages, there are many challenges to scale-up microalgae biofuel production. Literature reveals that significant work had been carried out to enhance microalgae biomass production and lipid production at lab scale. Very few studies are available to scale up microalgae culture system for biodiesel production. Therefore, the present thesis is devoted to scale up microalgae culture system from lab to outdoor conditions for biodiesel production, optimization of lipid extraction & biodiesel production and analysis of fuel properties of corresponding biodiesel according to international standards. Following are the objectives of the thesis:

i. Finding the feasibility of oil content in various microalgae, their culture in photo bioreactor & analysis of corresponding algae oil.
ii. Optimization of reaction conditions for biodiesel production and lipid extraction from microalgae.

iii. Evaluation of physico-chemical properties of algae based biodiesel as per BIS.

iv. Studies the oxidation, storage and thermal stability of algae based biodiesel.

To achieve the objectives, five *Chlorella* strains (*Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.* 1 and *Chlorella sp.* 2) were collected from various sources. All the five *Chlorella* strains were cultured in BBM, BG-11, Fog's medium and M_4N growth medium to select the best and most economical growth medium. After selecting growth media, all the microalgae species were grown using different nitrogen concentrations and sources to enhance lipid productivity. In addition, the effect of organic carbon sources on lipid productivity of all the five *Chlorella species* under mixotrophic conditions was also studied.

Under indoor conditions, artificial light was used for illumination which is energy expensive. To make microalgae biodiesel production more economical, it is necessary to culture microalgae under freely available sunlight. Therefore, feasibility of cultivating microalgae under outdoor conditions was tested in 10 L bubble column photobioreactor and 60 L flat plate photobioreactor. Furthermore, mass cultivation of *Chlorella vulgaris* was also carried out in 1200 L open raceway ponds using BG-11 and commercial grade fertilizer growth medium at batch and semi-continuous mode. Microalgal cells grown in open ponds were harvested by a combined method of flocculation and filtration. Harvested microalgae were dried under sun before lipid extraction.

Lipid extraction was carried out in 100 ml soxhlet extractor unit. Effect of different solvents, pretreatment methods (microwave, autoclaving, freeze drying and ultra-sonication methods), extraction time and solvent to biomass ratio on lipid extraction efficiency was studied. Extracted lipid have 12.39% of free fatty acids (FFA) level, therefore, a two- step transesterification process (acid and base transesterification) were optimized for biodiesel

production using microwave reactor. Obtained biodiesel was evaluated by GC, NMR and FTIR analysis. Various physico-chemical properties like density, viscosity, acid number, flash point, calorific value; pour point was analyzed according to ASTM D6751and IS: 15607, 2005. In addition, storage and thermal stability of microalgae biodiesel was also studied.

Among tested growth media, BG-11 was the most promising to enhance lipid productivity. Maximum biomass concentration was observed as 1.43g/L, 1.65 g/L, 1.48 g/L, 1.44 g/L and 1.39 g/L for *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.1* and *Chlorella sp.*2 respectively in nitrogen rich culture medium. Lipid yield was found maximum in nitrogen deficient conditions (2.21 mM nitrogen concentration). Among different tested nitrogen sources (potassium nitrate, sodium nitrate, urea and diammonium phosphate), urea has shown great potential for economic biodiesel production. Additionally, under mixotrophic condition (using glucose, glycerol, sodium acetate and sucrose as organic carbon source), maximum lipid yield was shown by *Chlorella vulgaris* (490.74 mg/L), followed by *Chlorella minutissima* (369.13 mg/L), *Chlorella sp.1* (325.4 mg/L) and *Chlorella pyrenoidosa* (282.59 mg/l) grown in BG-11 growth medium supplemented with glycerol as organic carbon source. On the other hand, *Chlorella sp.* 2 had maximum lipid yield in case of glucose as carbon source. However, results obtained in mixotrophic condition were much better than control condition (phototrophic condition). When scale up of mixotrophic cultivation system for all the five microalgae species was carried out into 10 L bubble column photobioreactor, *Chlorella sp.* failed to adopt outdoor growth conditions due to contaminations. Hence, these species were grown in phototrophic conditions under outdoor conditions.

Under outdoor conditions, all the five *Chlorella* strains were grown in 10 liter bubble column photobioreactor in BG-11 growth media. Among tested microalgae species, *Chlorella pyrenoidosa* achieved maximum biomass productivity (69.94 mg/L/d), followed by *Chlorella vulgaris* (64.32 mg/L/d), *Chlorella minutissima* (60.24mg/L/d), *Chlorella sp.1* (51.64mg/L/d) and *Chlorella sp. 2* (38.72 mg/L/d). On the other hand, lipid content was found maximum for *Chlorella sp.2* (19.51%) and minimum for *Chlorella sp. 2* (9.60%). However, maximum lipid productivity was observed for *Chlorella vulgaris* (10.07 mg/L/d) among the tested species and it was further increased up to 10.58 mg/L/d by increasing initial culture concentration of 0.300 g/L in microalgae cultivation system. Hence, *Chlorella vulgaris* was identified best species for mass cultivation experiment. For scale up of cultivation system, *Chlorella vulgaris* was grown in 60 liter photobioreactor at batch and semi-continuous mode. Results revealed that maximum lipid productivity of *Chlorella vulgaris* was 23.92 mg/L/d under semi-continuous mode when half culture concentration was harvested at 4^{th} day.

When mass cultivation of microalgae was carried out in 1200 L open raceway ponds, it was observed that commercial grade fertilizer based growth media has good potential to replace BG-11 growth media. At semi-continuous system, *Chlorella vulgaris* achieved biomass productivity of 19.61g/m^2/d with lipid content of 14.91%. Microalgae grown in open raceway ponds were dewatered by combined processes of flocculation and filtration. 92.53% harvesting efficiency was achieved by using 250 mg/L alum at pH 5. Among different drying methods, sunlight drying method for wet microalgae biomass was observed as most efficient method. Lipid extraction was optimized and achieved lipid content up to 22.68% using Chloroform: methanol (2:1) solvent at 8 h soxhlet extraction time. Physico-chemical properties of lipid were analyzed and following results were

obtained: saponification value 194.87 mg KOH/g, ester value 170.19, iodine value 102.32, FFA 12.39% and average molecular weight of the lipids 899.24 g/mole.

Under optimized conditions (volumetric oil to methanol ratio of 10:1, catalyst 1.5% by weight, reaction duration 15 minutes), microalgae biodiesel yield was 84.01%. It was also examined that microwave assisted transesterification was more efficient than conventional water bath assisted reactor. Physico-chemical properties like density, viscosity, acid number, flash point, calorific value and pour point agreed ASTM D6751and IS: 15607, 2005. However, oxidation stability of microalgae biodiesel supported ASTM standards (minimum 3 h) but did not follow European and Indian standards (minimum 6 h). To increase oxidation stability of microalgae biodiesel, different antioxidants [Butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), butylated hydroxyanisol (BHA), propyl gallate (PG), and pyrogallol (PY)] were employed to microalgae biodiesel and PY was observed most efficient antioxidants. Besides this, studies on storage stability of microalgae biodiesel showed that neat microalgae biodiesel (without antioxidant treatment) oxidized within three weeks but pyrogallol (PY) treated biodiesel was stable up to 12 week.

Key words:

Microalgae, *Chlorella sp*., growth conditions, lipid accumulation, photobioreactor, open raceway ponds, harvesting, lipid extraction, transesterification, biodiesel, fuel properties, oxidation stability, and storage stability.

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LIST OF SYMBOLS

%	per cent
η	kinematic viscosity
ρ	Density
μ	Specific growth rate
₹	Rupees
°C	degree Celsius
°F	degree Fahrenheit
Cal	calorie
cm	centimeter
cSt	centistokes
D	diameter of photobioreacor
g	gram
h	hour
j	joule
kCal	kilocalorie
kg	kilogram
KW	kilowatt
Kpa	kilopascal
L	liters
m	meter
min	minutes
MJ	mega joule
ml	milliliters
mM	millimoles
rpm	revolutions per minute
s	seconds

V	volume of photobioreactor
W	Watt

LIST OF ABBREVIATIONS

ASTM	American Society for Testing and Materials
BBM	Bold basal medium
BIS	Bureau of Indian standards
BP	Brake power
CFPP	Cloud filter plugging point
CN	Cetane number
CO_2	Carbon dioxide
CV	Calorific value
DAP	Di ammonium phosphate
DG	Diglyceride
FAME	Fatty acid methyl esters
FB	Fuel blend
FFA	Free fatty acid
FID	Flame ionization detector
FIT	Fuel injection timing
FPP	flat plate photobioreactor
GC	Gas chromatograph
HC	Hydrocarbon
HSD	High speed diesel
IARI	Indian Agricultural Research Institute
IEA	International energy agency
IV	Iodine value
JOME	Jatropha oil methyl ester
KOH	Potasssium hydroxide
LCSF	Long chain saturation factor
MMT	Million metric ton
MNRE	Ministry of New and Renewable Energy
MoPNG	Ministry of Petroleum and Natural Gas
MTOE	Million tones oil equivalent

NCL	National Chemical Laboratory
NO_x	Nitrogen oxides
OD	Optical density
PBRs	Photobiorectors
PL	Propyl gallate
PSII	photosystem II
PY	Pyrogallol
RES	Renewable energy sources
sp.	Species
SV	Saponification value
TBHQ	Tert-butylhydroquinone
TGA	Thermo gravimetric analysis
TGA	Triglyceride
VIAT	Vivekananda Institute of Algal Technology

Chapter 1

Introduction

Energy is essential input for economic development, social growth, human welfare and improving the quality of life. With improving trend of modernization and industrialization, the world energy demand is also increasing continuously. The primary energy consumption of world was 12928.4 MTOE (Million tones oil equivalent) in 2014 which was 0.9% higher than 2013 (BP statistics review 2015). International Energy Outlook 2013 (IEO2013) predicts that world energy consumption will grow by 56% between 2010 and 2040. Since their exploration, petroleum based fuels provide the majority of human energy requirements. According to the report published by World Energy Outlook 2015, fossil fuels will remain the dominant form of energy providing more than 80% of world energy use between 2013 and 2035.

India stands fifth as the world's largest primary energy consumer and fourth as the largest petroleum consumer after United States, China and Japan. Apart from their indigenous production, India has to import crude oil to cope up with their growing energy demand. According to annual report 2013-14 of Ministry of Petroleum and Natural Gas (MoPNG), the import of crude oil was 189.238 MMT, causing a heavy burden of Rs. 8,64,875 crore on foreign exchange. During the year of 2012-13, the import bill was Rs. 7,84,652 crore for 184.795 MMT crude oil, having more than 75% of oil import reliance. Thus, India spent a major chunk of their hard export earnings for procurement of petroleum products. Utilization of petroleum products in India has been expanding rapidly for last few years. The limited reserve of fossil fuels has been the matter of global concern as these are under threat of extinction due to over exploitation. According to prediction of World Energy Forum, fossil fuel, coal and gas reserves will be depleted in less than another 10 decades (Sharma et al. 2009). Worsening environmental conditions have become an issue of world’s concern in present time. Now days, the combustion of fossil fuels is dominant

global sources of CO_2 emission and efforts are going on around the world to save the environment from further deterioration. Factors, such as energy crisis associated with irreversible depletion of traditional sources of fossil fuels, coupled with atmospheric accumulation of greenhouse gases have led to an innovative global search for renewable sources of energy.

1.1 Prospects of Renewable Energy Sources

Renewable energy is energy produced from natural resources that are regenerative, and do not deplete over time They are clean fuels and emit fewer emissions in comparison to fossil fuel (Demirbas et al. 2007). Moreover, being indigenous, they decrease dependency on crude oil imports, thus playing an important role in energy security (Jegannathan et al. 2009). Renewable energy sources such as biomass energy, fuel cell technology**,** geothermal energy, ocean energy solar energy and wind can be used to overcome energy shortage with negligible emissions of air pollutants and greenhouse gasses. It is one of the fastest growing energy source both in developed and developing countries, with increasing consumption from 51.8 MTOE in 2000 to 279 MTOE 2013 (BP Statics 2014). Renewable energy provided an estimated 19.1% of global final energy consumption in 2013, and continued to grow in 2014 (Renewables 2015 Global Status Report).

Renewable energy represents an area of tremendous opportunity for India. Vigorous efforts during the past two decades are now bearing fruit as people in all walks of life are becoming more aware of the benefits of renewable energy, especially decentralized energy which is required in villages and in urban or semi-urban centers. In addition, renewable energy has the potential to create many employment opportunities, accelerate economic development, decrease local air pollution, improve public health, and reduce carbon

emissions. However, in India, renewable energy is at the take-off stage and government, industry, and other private sectors have many challenges to address before these technologies could make a real penetration.

1.2 Biofuels: an overview

Biomass has potential to fulfill the requirement of future energy/fuel supply and can play a vital role in decreasing greenhouse gas emissions. Beringer et al. 2011 calculated global bioenergy potential of biomass sources under environmental and agricultural constraints and concluded that bioenergy may provide about130– 270 EJ/year in 2050, equivalent to 15–25% of the world's future energy demand. As an energy source, human beings have been using biomass for domestic purpose for thousands of years, after humankind began to utilize fire. Wood biomass was the most common energy source used for cooking and heating purpose. Even today, nearly 40% (2.6 billion) of the world population (mainly in the rural areas of developing countries in Asia and sub-Sahara Africa) relies on firewood to fulfill their energy needs, with an annual consumption of 1730 million m^3 (IEA, 2013, FAO 2013).

According to Ministry of New and Renewable Energy (MNRE), New Delhi, approximately 500 million tons of biomass is available in India that comes from agricultural residues, agriculture based industries and forest products. MNRE has also indicated that 15–20% of total crop residues could be used for power generation. According to Gain report 2012, agricultural residues and plantation can be used for harnessing 1525 MW of power by the end of the12th five-year plan. Besides getting energy by direct combustion of solid biomass, it can also be converted to various liquid or gas fuels (biofuels) by different methods like thermal, chemical, and biochemical.

Biofuels are classified in four different generations depending on their biomass feedstock: first, second, third and fourth generation biofuels. First generation biofuels belongs to fuels excluded from traditional food and oil crops including corn, sugarcane, wheat grains, rapeseed oil, soybean oil, sunflower oil, other edible oils and animal fats. Main drawback of first generation biofuels is competition with food crops, lower yield, and their sustainability. Second generation biofuel have been developed to overcome the limitations of first generation biofuels and produced from lingo-cellulosic feedstocks including by-products (cereal straw, sugarcane bagasse, forest residues), wastes (organic components of municipal solid wastes), and non-edible oil crops (Jatropha, Karanj, Calophyllum, Mahua etc.). Third generation biofuels are the fuels which are produced from micro algal biomass. On the other hand, fourth generation biofuels are defined as fuels that are produced from captured carbon from the environment by using advanced technologies like petroleum hydro-processing, and advanced biochemistry,

At present, bio-ethanol and biodiesel are the most attractive liquid biofuels which are produced at industrial scale. Sugar beet, maize (corn), sugarcane, sorghum and wheat are the common feedstock used for bio-ethanol production. Biodiesel can be produce from non-edible oil and waste cooking oil. Bio-ethanol can be blended with gasoline to run SI engines, while biodiesel can be used to run CI engines.

Biodiesel is currently being accepted as a green and alternative renewable diesel fuel that has attracted vast interest from researchers. Some of the advantages of using biodiesel instead of fossil diesel are that it is a non-toxic fuel, is biodegradable and has lower emission of GHG when burned in diesel engine. In addition, it can be used in existing vehicle engines without modification. There have been numerous reports indicating that significant emission reductions are achieved with these blends. In additions, biodiesel has

no aromatic compounds and other chemical substances which are unsafe to the environment and human health. Moreover, biodiesel is better than diesel fuel in terms of flash point and biodegradability.

Biodiesel is a mixture of mono-alkyl esters and can be produced from vegetable oils such as soybean, rapeseed, sunflower, canola, peanut, palm, corn, Jatropha, and cottonseed oil. In addition, biodiesel can also be made from animal fat, waste cooking oil, greases (trap grease, float grease) and microalgae.

In India, attempts are being made for using non-edible and under-exploited oils for production of esters. But the sustainability of biodiesel from these crops is major bottleneck towards their commercialization. Recent studies on microalgae biofuels have shown a new road ahead that can be promising sustainable feedstock for biodiesel production.

1.3 Microalgae: An emerging feedstock for biodiesel production

. Microalgae are one of the most primitive forms of plants (thallophytes), i.e. lack roots, stems and leaves, have no sterile covering of cells around the reproductive cells and are microscopic photosynthetic organisms with chlorophyll-a as primary photosynthetic pigment. Microalgae structures are primarily for energy conversion without any development beyond cells and their simple development allows them to adapt to prevailing environmental conditions and prosper in the long term. While the photosynthesis mechanism in algae is similar to other plant material, they are sunlight-driven cell factories that convert carbon dioxide to various types of renewable biofuels. Microalgae are advantageous over higher plants as a source of biofuels as they:

- can synthesize and accumulate up to 20-50% lipids of dry cell weight.
- have high growth rate (e.g. 1–3 doublings/day).

- possess year-round production, exceeding the oil yield per unit area of other oilseed crops.
- can grow both in fresh and salty waters, and do not fight for agricultural land with food crops.
- can produce 30–100 times more energy per hectare compared to terrestrial crops.
- utilize minerals such as nitrogen, phosphorus, iron and copper from a variety of wastewater sources (e.g. agricultural run-off, industrial waste, dairy waste and municipal wastewaters), providing additional benefit of wastewater bioremediation.
- having photosynthetic efficiency ranging from 3 to 8% as compared to 0.5% for terrestrial plants (Lardon et al. 2009).
- can sequestrate CO_2 from flue gases emitted from fossil fuel-fired power plants and other sources, thereby reducing emissions of a major greenhouse gas.
- 1 Kg of algal biomass requires about 1.8 kg of CO_2 (Rodolfi et al. 2009).
- are not seasonal crops like other terrestrial energy crops and can grow in suitable culture vessels (photobioreactor) or open raceway ponds throughout the year with higher annual biomass productivity on an area basis.
- can further be processed for the production of bioethanol or biogas from the residual biomass obtained after oil extraction.

1.4 Potential of microalgae for biodiesel production

To replace all petroleum diesel fuel used in transportation sector, India will need more than 200 billion gallons of biodiesel annually at the current rate of consumption. Jatropha is most suitable and emerging crop for biodiesel production now days, but land area of 952 million acres (384 million ha) is needed for Jatropha cultivation to fulfill above biodiesel demand (Khan et al. 2008), which is more than 100% of all geographic area of

the India. Based on these calculations, it is clear that edible or non-edible crops would not be able to replace fossil fuels in the future. This situation will however be different if microalgae are utilized as a feedstock for biodiesel. Assuming biomass productivity of 1.535 kg/m^3/day and an average oil content of 30% dry weight, oil yield per hectare of total land area is around 123 m^3 for 90% of the calendar year (About 10% of the year is unproductive, because the production facility must be shut down for routine maintenance and cleaning.). This will result in 98.4 m^3/ha microalgae biodiesel yield. Therefore, for producing 200 billion gallon of biodiesel, India would need an area of 13 million acres or 5.4 million ha (only 2% of the India's geographical area) to cultivate microalgae. This is a feasible scenario even if the algal biomass contains only 15% oil by dry weight. No other potential sources of biodiesel come close to microalgae in being realistic production vehicles for biodiesel fuel is to be replaced with biodiesel.

The productivity of algal cultivation per area is 2 to 5 fold higher as compared with traditional agricultural crops and fast growing energy corps. A microalgal cell commonly doubles their biomass within 24 hours. Biomass doubling time during exponential growth is commonly as short as 3.5 hours (Chisti, 2007). Oil content in micro algae can exceed 80% by weight of dry biomass (Chisti, 2007, Sharma 2001). Oil levels of 20-50% are quite common in microalgae biomass (Sharma, 2001). Microalgae can produce up to 250 times the amount of oil per acre as soybeans, and 7 to 31 time greater oil than palm oil (Hossain et al., 2008). In fact, algae are the highest yielding feedstock for biodiesel and producing biodiesel from algae may be the only way to produce enough automotive fuel to replace current petroleum diesel.

1.5 Significance of the study

Based on biofuel production potential from microalgae, five microalgae species *Chlorella vulgaris*, *Chlorella minutissima Chlorella pyrenoidosa,* and two native *Chlorella* species *(Chlorella 1 and Chlorella 2)* are chosen for present investigations to evaluate the feasibility of efficient biofuel production. In order to achieve above objective, all the microalgae species were cultured in laboratory to optimize and select best species, nutrient media and growth parameters with enhanced lipid productivity. Suitable microalgae species (*Chlorella vulgaris)* was grown at large scale in open raceway ponds using commercially available fertilizer with minimum contamination. Microalgae biomass was harvested using potassium alum and dried under sunlight. Dried biomass was used to optimize the parameters affecting lipid extraction and biodiesel production process. In this thesis, emphasis has also been laid down on physico–chemical analysis and storage stability of biodiesel derived from microalgae. In addition, this investigation also focuses on storage and thermal stability of microalgae biodiesel. Based on these emphases, a comprehensive literature survey was carried out, detailed in the next chapter.

Chapter 2

Literature Survey

2.1 Introduction

Microalgae are one of the most primitive forms of plants (thallophytes), i.e. lacking roots, stems and leaves. They are sunlight driven cell factories that convert carbon dioxide to special chemicals like carbohydrates, proteins, lipids, vitamins and pigments through cellular activities. Microalgae are microscopic photosynthetic organisms and depending on species, their size varies from micrometers to millimeter (Graham et al. 2009). There are about 200,000-800,000 algal species, of which around only 50,000 species have been defined (Richmond, 2004). Microalgae species use light energy (i.e. solar energy) to produce chemical energy by photosynthesis with the natural growth cycle of just few days. Microalgae can be cultivated anywhere in the presence of solar visible radiation and minimum nutrient environment. However, the growth rate can be improved with the help of species-specific nutrient and environmental conditions (pH, temperature etc.). Compared to oil seed bearing terrestrial plants, the main advantage of microalgae is the much lower land area demand, easy cultivation, capability to grow in wastewater, and ability to sequestrate carbon from atmosphere. Microalgae are classified as prokaryotes and eukaryotes. The main difference is that the eukaryotic algae contain organelles that are membrane bound, like the nucleus. Prokaryotes lack chloroplast, mitochondria and nuclei (Knothe 2010, Williams P. 2010; Rashid et al.,2014) but they have chlorophyll-a and high protein content (Lopes, 2009).

Based on their taxonomy, microalgae are classified into ten groups i.e. green algae (Chlorophyceae), diatoms (Bacillariophyceae), yellow-green algae (Xanthophyceae), golden algae (Chrysophyceae), red algae (Rhodophyceae), brown algae (Phaeophyceae), dinoflagellates (Dinophyceae), Cyanobacteria (blue-green algae), Prasinophyceae and Eustigmatophyceae (Hoek, 1995). Microalgae can also be categorized on the basis of

carbon source utilization, i.e. autotrophic, heterotrophic and mixotrophic microalgae. Autotrophic microalgae use inorganic carbon such as CO_2 and undergo photosynthesis process using sun light as energy source. On the other hand, heterotrophic microalgae are non-photosynthetic and require an external source of organic compounds as well as nutrients as energy source. There are some microalgae species which are capable to utilize both organic and inorganic carbon sources; these are known as mixotrophs (Ogawa, 1981). Microalgae can be used for different types of biofuel production such as biodiesel, hydrogen, methanol, ethanol and biomethane (Oncel, 2013).

2.2. Biology and biochemical composition of microalgae

All the microalgae comprise of carbohydrates, proteins, and lipids, the percentage of which varies with the type of algae. It can be rich in proteins or rich in lipids or have a balanced composition of lipids, carbohydrate and proteins. On the basis of minimal nutritional requirements, Chisti, 2007 estimate an approximate molecular formula $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ for the microalgal biomass.

I. Carbohydrates

Microalgae rich in carbohydrate may be a good source for bio-ethanol and bio-butanol production. Different classes of microalgae produce specific type of polysaccharides. For example, green microalgae produce starch as energy stock, containing both amylose and amylopectin. The green *alga Tetraselmis suecica* stores11% and 47% of its dry weight as starch in nutrient replete and deplete conditions respectively (Dismukes, 2008). Microalgae based carbohydrates are mainly in the form of starch and cellulose (with the absence of lignin). Microalgae like *Chlorella*, *Chlamydomonas*, *Dunaliella*, *Scenedesmus*, and *Tetraselmis* have been shown to store more than 40% carbohydrates (on dry weight basis) (John et al., 2011).

II. Proteins

Proteins have significant commodity value as animal feed. A number of amino acids (building blocks of proteins) are dietary essentials for human beings as they are unable to synthesize them. In the past few decades, microalgal biomass was directly used in the health food market and more than 75% of the annual microalgal biomass production was used for the production of powders, tablets and capsules. A filamentous blue-green alga (Cyanobacterium), *Spirulina* is cultivated worldwide and used as a dietary supplement as well as food which are available in tablets, flakes and powder form.

III. Lipids

Lipids can be classified as fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides. They have different biomolecules such as fats, waxes, sterols, fat-soluble vitamins, monoglycerides, diglycerides, triglycerides and phospholipids. Like carbohydrates, lipids also work both as energy reserves and structural components (membranes) of the cell. The simple fatty acid triglycerides are significant energy reserves.

Lipid content of microalgal species differ from species to species. Some microalgae species such as *Botryococcus braunii*, *Chlorella emersonii*, *Dunaliella tertiolecta*, *Nannochloropsis sp.*, *Neochloris oleoabundans* and *Porphyridium cruentum* can accumulate up to 60% lipid of dry weight (DW) biomass (Maity et. al., 2013). Lipids extracted from the microalgal biomass can be converted to biodiesel by transesterification process (Chisti 2007)

2.2.1 Biosynthesis of lipid in microalgae

The biosynthesis process takes place through three independent steps: (i) synthesis of fatty acid in the plastid, (ii) assembly of glycolipid in the endoplasmic reticulum and (iii) packaging into the oil bodies.

The carbon source for lipid synthesis is photosynthate i.e. carbohydrate (starch, sugar etc.) formed during the photosynthesis process. In photosynthesis, the plant microalgae fix CO_2 into sugars in presence of photon energy.

Glycolysis is an oxygen independent metabolic pathway that breaks down glucose and forms pyruvate with the production of two molecules of ATP. The pyruvate end product of glycolysis can be converted into carbohydrates via gluconeogenesis, to fatty acids through acetyl co-enzyme A (acetyl-CoA), amino acid alanine, and ethanol. Thus, pyruvate is the key intermediate in several metabolic pathways. Pyruvate releases CO_2, and makes acetyl-CoA (acetyl coenzyme) in the presence of PDH (pyruvate dehydrogenase). Acetyl-CoA functions as the precursor for fatty acid (building blocks of cellular lipid including triacylglycerol) synthesis in the chloroplast (Fan et. al., 2011).

During fatty acid synthesis, malonyl-CoA is produced from acetyl-CoA and bicarbonate. This reaction is catalyzed through multifunctional enzyme complex such as ACCase (acetyl-CoA carboxylase). Malonyl-CoA group is converted to malonyl-ACP (acetyl carrier protein) catalyzed by an acyl carrier protein malonyltransferase. Malonyl-ACP in the role of FAS (fatty acid synthase) - after a series of carbon chain lengthening and desaturation reactions - finally produces mainly C16 and C18 fatty acids. These fatty acids are the synthetic membranes, organelle membranes and Triacylglycerol (Cagliari et al., 2011; Fan et al., 2011).

Triacylglycerol (TGA) are produced by the sequential acylation of G3P (glycerol-3-phosphate) backbone with three acyl-CoAs catalyzed by a group of enzymes such as acyltransferases. The acylation of G3P using GPAT (glycerol-3-phosphate acyltransferase) forms LPA (lyso-phosphatidic acid). LPA, further acylated by the action of LPAT (lysophosphatidic acid acyltransferase) build PA (phosphatidic acid). PAP (phosphatidic acid phosphatase) catalyzes the elimination of phosphate group from PA to make DAG (diacylglycerol). The oil synthesis is catalyzed by the enzyme DGAT (diacylglycerol acyltransferase) from DAG to TAG which is stored in the form of fat body (Lipid Bodies) in the microalgal cells. In this process, PA and DAG can also be synthesized as substrate polar lipids (phosphatidyl choline and galactolipids) (Cagliari et al., 2011; Fan et. al. 2011). TGA is raw material for the production of biodiesel.

The main factors affecting rate of photosynthesis are light intensity, carbon dioxide concentration and temperature. Therefore, these factors also impacts directly or indirectly synthesis of lipid. By varying composition of growth media, lipid yield and lipid composition of algae can be improved. In addition, environmental condition such as light, temperature, also play a major role to increase lipid accumulation of microalgal cell. Therefore, systematic studies are needed to understand the relationship between environmental parameters and microalgae cultivation in order to increase cell density, rapid turnover rate, and higher lipid content.

2.2.2 Microalgae lipid composition

Microalgae have very high lipid accumulation capacity which may reach up to 80% of their dry weight (Chisti, 2007). Generally, microalgae synthesize the fatty acids chain lengths ranging from C16 to C18, similar to higher plants (Ohlrogge et al. 1995). Fatty acids are either saturated, or monounsaturated or polyunsaturated. Furthermore, mono and

polyunsaturated fatty acids may vary in the number and position of double bonds on carbon chain backbone. The fatty acid composition of microalgae lipid is mainly composed of mixture of unsaturated fatty acids, such as palmitoleic (16:1), oleic (18:1), linoleic (18:2) and linolenic acid (18:3) along with some quantity of saturated fatty acids, such as palmitic (16:0) and stearic (18:0) (Halim et al., 2012).

2.3 Effect of various external factors on microalgae growth and lipid syntheses

Several studies have shown that lipid content and lipid composition is highly influenced by environmental factors like light intensity, temperature, pH, cell culture density, alkalinity, nutrient media composition (concentration of nitrogen, phosphate and iron) and contamination by other microorganism.

Being the basic energy source for microalgae growth, availability and intensity of light is one of the key parameters affecting the performance of microalgae biomass production. Average growth rate of the microalgae culture at very low light intensities is zero (compensation point) (Lee, 1997). Growth rates of microalgae biomass can be increased by enhancing light intensity up to a point of light saturation, at which photosynthetic activity becomes maximum (saturation point). Increasing light intensity beyond the saturation point results in lower growth rate and can lead to photo-oxidation, damaging the light receptors and thereby, decreasing photosynthetic rate and productivity (photo-inhibition).

Microalgae cultivated in different light intensities show significant changes in their gross chemical composition, pigment content and photosynthetic activity. Typically, low light intensity encourages the formation of polar lipids, particularly membrane polar lipids associated with chloroplast, while high light intensity decreases entire polar lipid content with increase in the amount of neutral storage lipids, mainly TAGs (Brown et al., 1996;

Khotimchenko et.al., 2005; Napolitano et al., 1994; Orcutt et al., 1974; Spoehr et al., 1949; Sukenik et al., 1989).

Photoperiod also plays an important role in algal growth and cell lipid content. The experimental work on freshwater and marine microalgae was conducted under dark-light ratios of 12:12, 14:10, 10:14 and 16:8 0:24 and 24:0 (George et al., 2014; Ho et al., 2014; Oh et al., 2009; Richardson et al., 1983; Tang et al., 2011). Up to 25% of algal biomass produced can be lost through respiration during night (Chisti, 2008; Ratledge et al., 2008).

A microalgae cultivation system can be illuminated by different sources of light like artificial light, solar light or combinations of different light sources. Artificial light includes cool fluorescent lamps, bulb or LED. However, these sources require power consumption and increase net algal biomass production cost. Solar energy is the cheapest light source available on earth. Most of commercial algae culture systems are carried out using solar light as a light source, but performance is not good and needs improved technologies to enhance the microalgae growth rate under outdoor conditions.

Temperature is another important environmental factor that affects growth rate and biochemical composition of microalgae. Microalgae may be cultured at various temperatures ranges from 20°C to 40°C. For indoor condition the growth temperature is maintained at optimum value. Furthermore, optimum temperature of microalgae cultivation system depends on particular species and strain. Usually sustained temperatures above optimum condition may damage algal cells and temperatures below the optimum condition result in slow growth rate.

Under outdoor cultivation, seasonal temperature variation as well as more rapid daily fluctuations in temperature is responsible for affecting microalgae production efficiency. Lower temperature results in biomass reduction through respiration during the

night. Temperature, light and photo-inhibition are strongly linked with each other. At low temperature, photo-inhibition takes place at high light intensities. Therefore, temperature may be one of the most significant factors influencing algal growth under outdoor conditions during winters. In addition, temperature play a crucial role in reduction of photo-inhibition and it can be significantly decreased by increasing the temperature.

Temperature also affects the fatty acid composition of algae. A general trend towards increasing fatty acid unsaturation with decreasing temperature and increasing saturated fatty acids with increasing temperature has been observed in many algae and cyanobacteria (Lynch et al., 1982; Renaud et al., 2002; Sato 1980). Converti et al. (2009) observed that the lipid content of *N. oculata* increased up to two-times by applying optimized temperature conditions. Sakamoto et al. (1997) measured the doubling time of *Synechococcussp.PCC7002* at different temperatures and found shortest doubling time (3.5h) at 38°C. To control medium temperature, some researchers recommended to culture microalgae species together, having similar growth characteristics but different optimum temperatures (Munoz et al. 2006). *N. oculata* and *C. vulgaris* showed maximum lipid content at 25°C and 30°C respectively (Converti et al. 2009). Doubling time of *Euglena* species was observed by some researchers at 27–31°C (Kitaya et al. 2005). The best growth rate of *Monoraphidium sp. SB2* was observed at 25–35°C (Wu et al 2013).

Oxygen is a by-product of photosynthesis and the high concentration of oxygen inhibit photosynthesis (‘‘Warburg’’ effect). A high concentration of dissolved oxygen in combination with intense sunlight produces photo-oxidative damage to algal cells. To avoid inhibition and damage, the maximum acceptable dissolved oxygen level should not generally exceed about 400% of air saturation value.

pH is one of the most important factors that affects algal growth as it regulates solubility and availability of CO_2 and essential nutrients by affecting algal metabolism (Chen et al.,1994; Goldman, 1973). Maximum microalga growth takes place around neutral pH, although optimum pH is the initial culture pH at which microalgae are adapted to grow. Changing pH in growth media may limit microalgae growth via metabolic inhibition (Goldman et al., 1982). Generally, microalgae show growth inhibition at pH above10–11 (Muñoz et al., 2006; Kumar et al., 2010).

Microalgae require a wide variety of nutrients to grow. Carbon, nitrogen and phosphorous are essential elements, whereas manganese, magnesium, cobalt, calcium, sulfur, iron, potassium, sodium, and hydrogen are used as trace nutrients (Liu et al., 2008; Yeesang et al., 2011). The effect of carbon, nitrogen and phosphorous on microalgae growth and lipid production is described here-

Carbon is an essential nutrient for microalgae cultivation and can be utilized in both, organic and inorganic forms. Carbon fixed by the microalgae can be utilized for three purposes, (a) for respiration, (b) as an energy source, (c) as a raw material in the formation of additional cells. Autotrophs use inorganic carbon (CO_2, carbonate and bicarbonate) and heterotrophs consume organic carbon (glucose, glycerol, sodium acetate etc.) as a food source. The three principal forms of dissolved inorganic carbon (DIC) are carbon dioxide (CO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}),. Microalgae are capable to use both CO_2 and HCO_3^-, as an inorganic carbon source. CO_2 dissolved in water makes a weak acid/base buffer system, namely bicarbonate-carbonate buffer system. Formation of an inorganic carbon species is a function of pH and temperature. Up to pH 10.5, bicarbonate species dominate while at higher pH values carbonate (CO_3^{2-}) species dominate. The

bicarbonate-carbonate buffer system provides carbon for photosynthesis. CO_2 in water may be present in any of these forms depending upon pH, temperature and nutrient content.

However, dissolution of CO_2 in water results in acidification because of carbonic acid. To overcome this problem, high concentration of inoculum should be provided. At high inoculum rate, the microalgae cells utilize all of the CO_2 available during photosynthesis, and CO_2 does not react with water (Chiu et al., 200).

Therefore, CO_2 is most significant factor to regulate microalgal biomass production and fatty acids synthesis. 1 kg of microalgae can fix 1.83 kg of CO_2. However, higher concentration of CO_2 may be harmful to algal growth. However, there are species that can also grow under high CO_2 concentration. Some Chlorophyceae is also able to sequestrate up to 18% dissolved CO_2 in the nutrient medium. *Spirulina sp.* is able to fix CO_2 up to 38% in cultures.

Generally, CO_2 is directly supplied into microalgae culture from the atmosphere. Atmospheric CO_2 (0.03%, v/v) is not enough to support high microalgae growth rate and productivity required for large scale biofuel production. Therefore, CO_2 provided by power plant and industry flue gases (up to 15%) can be utilized for microalgae cultivation system.

In addition to CO_2, some microalgae can use organic carbon sources like glucose, acetate, glycerol, fructose, sucrose, lactose, galactose and mannose as substrate and energy sources. They are called heterotrophs (Ho et al., 2011; Droop, 1974; Schmidt et al., 2005). Literature shows that microalgae species such as *C. vulgaris, Scenedesmus acutus, C. protothecoides, Chlorella saccharophila, Chlorella sorokiniana* can be cultivated in heterotrophic culture (Rashid et. al., 2014). The cost of the carbon source is one of the most important issues in heterotrophic growth that could cause hindrance to promote this technology at industrial scale. Contamination is another issue in heterotrophic cultivation.

Apart from the Carbon, nitrogen is also an important element required for microalgae growth. It controls protein synthesis and growth metabolites of microalgae. Consumption of Nitrogen by microalgae takes place in the form of nitrate, ammonia, and urea. When ammonia is available, microalgae do not use other nitrogen sources until all the ammonia is consumed (Tam et al., 1996). High uptake of ammonia decreases the medium pH (<6.0) at which microalgae cannot grow. Uptake of ammonia produces H^+. Nitrogen uptake changes pH of microalgae culture which affects the biomass production. Nitrate uptake results in higher pH due to production of OH^- (Song et al., 2011). Theoretically, digestion of one mole of nitrate results into one mole of OH^-.

Nitrogen deficient conditions in microalgae culture leads to enhanced biosynthesis and accumulation of lipids and triglycerides (Converti et al., 2009; Demirbas, 2010; Stephenson et al., 2010; Takagi et al. 2000; Thompson, 1996; Wang et al., 2009). Rodolfi *et al.*(2009) presented a detailed and large-scale model of lipid by nutrient deficient conditions (nitrogen, phosphorus) on several diatoms, green algae, red algae, prymnesiophytes and eustimatophytes. Hence, nitrogen deficiency is the most successful technology for lipid accumulation in microalgae. However, high lipid production due to nitrogen stress results in slow growth rates and thus finally affects the total biomass and lipid productivity.

Phosphorous is another essential macronutrient for normal growth and development of microalgae. It plays an important role in cellular metabolic processes by forming many structural and functional components. The favored form in which it is supplied to microalgae is as orthophosphate. Although microalgae biomass consists of about 1% phosphorus, but it is one of the most important growths limiting factors in microalgae biotechnology. Supply of phosphorus also affects the composition of the biomass produced,

especially carbohydrate and lipid accumulation. Smith et al. (2010) have observed that the cell undergo nitrogen limitation at N/P >1 and phosphorous limitation at N/P<1. Microalgae require high N/P value in exponential phase and lower in stationary phase. High N/P in exponential phase assists more microalgae biomass production. Low N/P in stationary phase supports more lipid production (Ratledge et al., 2008). According to Rashid et al. (2014), three times more lipid can be produced by optimizing N/P values in stationary and exponential phase than controlled conditions. In addition, the consumption of nitrogen and phosphorous nutrients is also greatly affected by CO_2 supply. Therefore, C:N ratio, and N:P ratio should be optimized to get maximum biomass and lipid production.

Iron (Fe), manganese (Mn), cobalt (Co), zinc (Zn), copper (Cu) and nickel (Ni) are some essential trace metals required by microalgae for various metabolic activities (Bruland et al., 1991). Deficiency in trace metals result in lower growth rate, whereas high metal concentrations are toxic for growth. Iron is an important trace metal for normal growth and acts as redox catalyst in photosynthesis and nitrogen assimilation and mediates electron transport reactions in photosynthetic organisms. Liu et al.(2008) studied the effect of iron on biomass and lipids production and found 3–7 fold increase in biomass production. Nasr et al. (1970) observed 37.9% increases in cell biomass by using molybdenum. Cobalt improved the cell biomass by 30.8%, manganese 20.7%, and boron 27.6% (Rashid et al.2014).

Composition of the medium also affects significantly microalgae growth and lipid accumulation. Basically, two types of media i.e. natural and artificial are used. Natural medium is inexpensive than the artificial medium, but it is not suggested because natural medium does not have all of the mandatory nutrients up to the desired concentration.

Therefore, artificial medium is mostly preferred for microalgae cultivation as the nutrients composition and their concentrations can be manipulated based upon the type of microalgae species. Most commonly used artificial media, Johnson's medium, Bold basal medium, F-2 medium, Beneck's medium, Fog's medium, BG-11 medium, C medium, ASW medium, and AF6 medium. BG-11, Fog's medium, and Bold Basal medium are the growth media that have been used extensively for freshwater green algae and cyanobacteria. *Spirulina* requires a bicarbonate-rich Zarrouck growth medium. Salinity also affects biomass yield and lipid contents of microalgae. For example, marine microalgae (*Synechococcus sp, Nannochloropsis salina, Chlorococcum littorale*, and *Botryococcus braunii*) need high salinity medium, whereas freshwater microalgae (*Chlorella vulgaris, Microcystisa eruginosa*) grow well in less saline medium.

Microalgae growth is also influenced by cell density of culture system. Both low and high cell concentration results in a loss of biomass productivity. If the cell concentration is low, some of the light is transmitted through the culture (low absorption). On the other hand, if the cell concentration is too high, a dark zone appears in the depth of culture (preferring the light limitation regime). Both situations result in loss of biomass productivity. At high cell density, cell mutual shading takes place and light intensity reduces due to increase in turbidity of the culture, causing a decrease in the photosynthetic activity (Markou, 2011).

Efficiency of microalgae culture systems and cost of photobioreactor construction and operation are significantly affected by the mixing system employed. Optimum mixing or turbulence serves a variety of purpose, including avoidance of cell settling, homogeneous distribution of nutrition, CO_2 and temperature, removal of photosynthetically produced oxygen, and improvement of efficient light utilization. Slow mixing produces a dark

stagnant zone in microalgae suspension, which poses negative effect on the growth. On the other hand, high mixing can damage cells due to shear stress. Mixing takes place by different methods like gas injection, pumping or mechanical stirring. Among these, mechanical stirring is most efficient method but it cause higher hydrodynamic stress than others. This limitation can be overcome by proper designing of baffles, which controls the mixing pattern.

2.4 Growth techniques

Due to ability of microalgae to survive both on organic as well as inorganic substrates, it can also be cultured in photoautotrophic, heterotrophic and mixotrophic growth conditions. Photoautotrophic and mixotrophic growth is highly influenced by light intensity and by carbon source concentration, while heterotrophic growth is influenced by organic substance concentration. In photoautotrophic conditions, microalgae acquire their energy through absorption of light energy for the reduction of CO_2 by the oxidation of substrates, mainly water, with the release of O_2.

In heterotrophic conditions, microalgae utilize directly organic carbon compound in the absence of sun light. However, heterotrophic microalgae do not utilize CO_2 and solar energy directly, but addition of organic compound increases total production cost. Thus, it cannot be accepted for large scale biofuel production.

On the other hand, mixotrophic microalgae cultivation system has several benefits over photoautotrophic and heterotrophic growths. Mixotrophic microalgae can simultaneously drive both photoautotrophy and heterotrophy. In mixotrophic cultivation system, photoinhibition is decreased, growth rate increased, and biomass losses due to respiration are less. Under mixotrophic condition, biomass yield is much more than photoautotrophic condition (Gim et al, 2014; Skorupskaite et al., 2014; Sheng et al., 2011). For example, *S.*

bijuga biomass growth was 5 times more in mixotrophic condition than phototrophic condition (Bhatnagar et al., 2011).

2.5 Microalgae biodiesel production process

The entire process of microalgae based biodiesel production can be discussed in the following steps: strain isolation and selection, cultivation, harvesting, drying, lipids extraction and finally the biodiesel production.

2.5.1 Strain isolation and selection

The collection, isolation and identification of suitable microalgae strain is the most crucial step for biofuel production. As discussed earlier, microalgae have ability to survive in diverse environment like fresh water, brackish water, saline and hyper-saline environment. Microalgae species face many challenges to adopt new environmental conditions. Microalgae species, isolated from local habitat, have high tendency to adjust with environmental changes. The selected microalgae species should have following desirable characteristics: (1) high lipid productivity, (2) high photosynthetic efficiency, (3) production of valuable co-products, (5) high carbon dioxide sequestration (6) ability to grow in environmental stress condition (Chisti, 2008; Brennan et al., 2010).

Some microalgae such as *Chlorella minutissima*, *Chlorella protothecoides*, *Chlorella vulgaris*, *Chlorella sp*. *Crypthecodinium cohnii*, *Dunaliella salina*, *Isochrysis galbana*, *Nannochloris sp*., *Nannochloropsis oculata NCTU-3*, *Nitzschia sp*., *Scenedesmus dimorphus*, *Scenedesmus obliquus*, and *Schizochytrium sp*. achieved higher lipid content (40-60% of dry biomass) in environmental stress condition, whereas the lipid of *Ankistrodesmus sp.*, *Chlorella sorokiniana*, *Cylindrotheca sp.*, *Dunaliella primolecta*, *Euglena gracilis*, *Haematococcus pluvialis*, *N. oculata*, and *Spirulina platensis* content was found less than 40% of dry biomass (Chisti, 2007; Rashid et al., 2014; Brennan et al.,

2010). Lipid quality also plays a major role in the selection of right microalgae species. Microalgae, that produce polar and neutral lipids, are more suitable for biodiesel production. *Chlorella species* are identified as most suitable to produce such lipids (Sharma et al., 2011). However, diatoms can accumulate a high amount of lipids, but they include a significant quantity of phospholipids that cannot be converted to biodiesel by transesterification. Thus, green algae are preferred for biodiesel production.

2.5.2 Cultivation

Cultivation of microalgae is a major step in the production of biodiesel. Cultivation of microalgae, including photosynthesis, growth pattern, cellular metabolism and cell composition, is affected by several cultivation parameters like light supply, temperature, pH, mixing, nutrient supply and media composition (section 2.3 of this chapter).

The growth of microalgae is also influenced by the cultivation system. It can be classified as open system and closed system. At present time, open culture system is the most promising culture system for large scale biomass production of microalgae (Grobbelaar, 2012) due to its cheaper operating cost. Closed culture system includes various types of PBRs (Photobiorectors) with different cultivation scales.

a). Open ponds system

Commonly used open pond cultivation systems include natural ponds, circular ponds, raceway ponds and inclined systems. These systems are usually made of clay or concrete lined with polyvinyl chloride or porcelain tiles to avoid loss of media and nutrients. The depth of these ponds is kept from 0.15 to 0.45 m so that maximum sunlight penetration can takes place (Murthy, 2011). Mixing and circulation is carried out by a paddlewheel or air pumps. These systems have simple designs and are relatively inexpensive to build. They are easier to operate, as they require control over nutrient and water loss. Among various available open culture

systems, raceway ponds are the most common choice for outdoor algae production. However, this cultivation system is susceptive to climate conditions and has no control over culture conditions like temperature and illumination. Moreover, due to seasonal variations in these conditions microalgae productivity varies significantly. Therefore, microalgae biomass production in open ponds relies on regional climatic conditions. In addition, it also has following disadvantage: (1) inefficient mixing, (2) low mass transfer rates, (3) low biomass productivity, (4) loss of significant water due to evaporation, and (6) difficulty in maintaining microalgae monocultures due to attack of contamination like other algal strains and bacteria.

b). Closed system

Photobiorectors **(**PBRs) are closed systems which allow exchange of light and energy but do not give permission to exchange of material with the atmosphere. Therefore, they permit culture of single species of microalgae for prolonged durations with lower risk of contamination (Chisti, 2007). Numerous types of photobioreactors have been designed in attempt to get best productivities. Among them, three types of PBRs are more common: tubular, flat plate, and column PBRs (Ugwu et al., 2008). Mixing within these PBRs usually takes place by airlift or mechanical stirring/pumping. Mixing is also necessary for gas exchange (Brennan et al., 2010). PBRs need smaller area than open ponds to generate same amount of biomass. Furthermore, PBRs are more versatile than open ponds since they are capable to use sunlight, artificial light or various combinations of light sources and thus providing the potential to increase photoperiod and improve low light intensities given by sunlight variation. According to Amaro et al. (2011) the constancy of light intensity and photoperiod supplied by artificial light is able to improve yearly total oil yields up to 25–42%. Additionally, photobioreactor requires small area for the culture of same volume than an open pond system.

Tubular PBRs are considered as one of the most appropriate system for large scale outdoor culture because they have large surface area that is exposed to light and is thus suitable for both indoor and outdoor cultivation (Chisti, 2007). Solar collector tubes are usually made of transparent glass or plastic with a diameter of 0.1 m or less.

Recently, flat plate PBRs received much research interest due to the large surface area exposed to illumination and high densities of culture (Ugwu et al., 2008). These PBRs seems more suitable for large scale culture than tubular reactors due to high photosynthetic efficiency and low accumulation of dissolved oxygen when compared to tubular PBRs. Scale up of these PBRs still has many challenges.

Main benefits of PBRs over open ponds are higher biomass productivity and level of control. They offer the opportunity to optimize light path length, light intensity and wavelengths and thus increase biomass productivity. Major limitations in using PBRs for large scale microalgae cultivation are the capital and operational costs (Amaro et al., 2011; Davis et al., 20110). Maintenance and cleaning is also another issue because a bio-film is formed on the walls of PBRs which reduces photosynthetic efficiency by reducing light intensity penetration. Therefore, development of efficient PBRs with lower building and operating costs for mass cultivation of microalgae are of great importance in microalgae-based biotechnologies.

2.5.3 Harvesting

Harvesting is the process used to recover microalgae biomass from the medium. This is a key step for large scale biodiesel production process because it contributes 20–30% of total biodiesel production cost (Mata et al. 2010). Microalgae cells present in dilute culture medium have density almost equal to water. Therefore, recovery of microalgae biomass form medium is the most challenging step due to their microscopic size (1-10 μm diameters). Many harvesting techniques like centrifugation, coagulation, flocculation,

flotation, filtration, electro-flotation, electrophoresis, and ultrasound have been applied to harvest microalgae.

Harvesting efficiency of 80–90% can be achieved within 2–5 minutes operation by centrifugation. In lab-scale experiments, centrifugation is the most common technique used for harvesting. However, it is quite expensive to centrifuge algal culture in pilot-scale or large-scale cultivations.

Traditional filtrations are not more effective for microalgal separation. Microfiltration and ultrafiltration are some improved techniques for microalgae harvesting (Tang et al., 2010). Fouling is the major issue during filtration process. Fouling is the deposition of extracellular polymer substance (present in microalgae culture media) on surface of filters. Due to fouling, replacement of membrane and back washing are required which increases the overall process cost.

Presently, flocculation is the most efficient harvesting method with a lower energy and capital cost. Microalgae cells are negatively charged in growth medium and repel each other due to electrostatic force. This helps microalgae cells to make a stable system. When flocculants are added into microalgae culture medium, surface charge is blocked so that microalgae cells can adhere to form flocs. The flocculants utilized must be low-cost, nontoxic, and effective in low application doses. A number of chemicals are identified to be utilized as flocculants for this purpose. Inorganic salts containing metal ions (Al^{+3} and Fe^{+3}) are generally used as flocculants. High concentration of metal flocculants for biomass harvesting results in toxicity and also affects biodiesel production process. However, some organic polymers such as chitosan, cationic starch and grafted starch are getting attention to replace inorganic flocculants. Harvesting efficiency of these flocculants is higher and also, they do not contaminate the biomass. Different flocculants were investigated, including chitosan (Xu et al.,

2013), ferric salts (Godos et al., 2011), aluminum salts (Şirin et al., 2011), and titanium tetrachloride (Zhao et al., 2012).

pH also plays a crucial role in the flocculation process. Microalgae cells have natural tendency to flocculate at pH 9 or above. Change in pH of microalgae growth medium tends to auto-flocculation.

In addition, some physical and biological methods are also used for algal harvesting. Bosma et al. (2003) reported that ultrasound is also able to harvest microalgae. Matos et al. (2013) achieved 97% harvesting efficiency of marine *Nannochloropsis* sp. by using electro-coagulation. Bio-flocculation is also induced by utilizing one flocculating microalgae to concentrate non-flocculating microalgae of interest (Salim et al., 2011), or by using microalgae-associated bacteria to enhance algal flocculation (Lee et al., 2013).

2.5.4 Oil extraction

Harvested biomass of microalgae is dried and processed for oil extraction. Oil extraction is the most crucial step in biodiesel production process. Oil in microalgae is encapsulated by inflexible cell wall composed of carbohydrates and proteins. Therefore, interruption of cell wall is essential to extract oil from microalgae. The extraction of oil is again costly and challenging step. There is no single method for oil extraction which can be applied to all the species of microalgae due to variation in microalgae cell structure and size. Method applied for extraction should be fast, scable, effective and should not damage the extracted lipid. Several methods (mechanical, chemical, and biological etc.) can be used for the extraction of oil

Mechanical pressing is commonly used for oil extraction from both edible and non-edible seeds such as *Jatropha, Karanja* and *Mustard*. This method can be applied for microalgae oil extraction at large scale biodiesel production. Cooney et al. (2011) found

that the major drawback of mechanical pressing is the unicellular nature of microalgae cells and rigid cell wall of some microalgae strains. Oil extraction from microalgae biomass using mechanical pressing is challenging as some cells could flow on moisture of many water micro-channels. The choice of best extraction technology is greatly reliant on the microalgae strain selected and needs to be optimized as has been done for many higher plants.

Chemical method for lipid extraction from microalgae is most common. The basic principle behind oil extraction from microalgae is based upon the chemical concept of 'like dissolving like'. Lipid can be extracted from microalgae biomass using non-polar solvents such as hexane, benzene, toluene, diethyl ether, chloroform and polar solvents such as methanol, acetone, ethyl acetate, and ethanol. The non-polar solvents play an important role to disrupt the hydrophobic interactions between non-polar and neutral lipids existing in the microalgae biomass. Folch et al. (1957) extracted lipid from animal tissues using a mixture of chloroform: methanol (2:1 by v/v) and named it the Folch method. In Bligh and Dyer method, mixture of chloroform: methanol (1:2 by v/v) is used for total lipid extraction and purification (Bligh, 1959).

Balasubramanian et al. (2013) categorized the lipids into three types i.e neutral lipids, free fatty acids (FFA) and polar lipids using solid-phase extraction column and examined that lipid extraction from marine microalgae is highly influenced by the factors like biomass drying, moisture content and solvent systems such as chloroform: methanol (2:1) and hexane: methanol (3:2). Wijffels and Barbosa (2010) advocated that microalgae cells should be ruptured physically and then treated with organic solvents to extract total lipid from the microalgae biomass. Autoclaving, microwaves, ultra sonication and osmotic shocks are some physical methods which are applied to disrupt cell wall of microalgae.

Supercritical fluid extraction is one of the encouraging green technology methods, which has potential to replace conventional solvent extraction method. This technology has the efficiency and ability to extract almost 100% of the oils from biomass. The fundamental principle of this technology is that temperature and pressure of carbon dioxide (CO_2) gas is increased until it reaches up to the liquid–gas state or above its critical point. It is then mixed to microalgae biomass and acts as solvent. Temperature and pressure (critical point) at which fluid liquefies varies reliant on the type of solvent used (Sahenaa et al., 2009). High infrastructure and operational cost associated with this technology are the main drawbacks of this process.

2.5.5 Transesterification

Crude microalgae oil is further processed for biodiesel production via transesterification reaction. Microalgae crude oil is highly viscous in nature, thus needs conversion to lower molecular weight compounds in the form of fatty acid alkyl esters. Transesterification is the process in which raw microalgal lipid (triacylglycerols/free fatty acids) is converted into renewable, non-toxic and biodegradable biodiesel. The efficiency of transesterification is highly influenced by FFA of lipid, type of catalyst, lipid to alcohol ratio, temperature and time. Just like plant seed oils, microalgae oil also has phosphorus in the form of phospholipids. Phosphorous affects production yield due to the formation of emulsions in biodiesel refining step (Cvengros, 1999). Furthermore, microalgae lipids are also rich in FFA content. Therefore, degumming is essential to reduce FFA and remove phosphorous content. Significant problems associated with the conventional processes used for microalgae biodiesel production are long extraction & conversion time, low recovery rates, more use of chemical solvents, and difficulty to maximize the biodiesel yield. Microwave and ultrasonic assisted transesterification processes are the most emerging

technologies and able to solve the problems stated above. These methods are most energy efficient with the shorter reaction time due to rate enhancement (Lidstrom et al., 2001; Guerra, 2014). In situ transesterification is also an emerging technique which can be used as alternative to conventional process. This technology has the potential to reduce the cost of fuel processing. It eliminates solvent extraction steps required as compared to conventional method used for biodiesel production. However, direct esterification gave better results at lab scale (Ehimen et al., 2010), but scale up of this technology still has many challenges.

2.5.6 Biodiesel Quality

Acceptability of microalgae based biodiesel as a substitute of fossil diesel fuel is strongly reliant on compliance with existing standards such as ASTM D6751, EN 14214:2008 and IS: 15607. The fuel properties of biodiesel are greatly affected by fatty acid composition of biodiesel. Typical biodiesel fuels have five common fatty esters: palmitic, stearic, oleic, linoleic, and linolenic which vary from feedstock to feedstock. Esters rich in saturated fatty acids have high cloud point, high viscosity and high probability of clogging the nozzle of the engine, while, esters rich in polyunsaturated fatty acid lead to poor oxidation stability and better cloud point.

Miao et al. (2006) determined that density, kinematic viscosity, and CFPP of the microalgae biodiesel were 882g/cc, 4.43cSt and -13^{o}C, respectively. Amin (2009) observed that the density, kinematic viscosity, and CFPP of the microalgae oil biodiesel were 0.864, 5.2, and -11^{o}C, respectively.

2.6 Current challenges and limitation in microalgae biodiesel production process

Biodiesel from microalgae is getting attention throughout the world and has shown great potential as suitable alternative fuel. One of major advantages of microalgae based

biodiesel is that it has no impact on food security over terrestrial crops. Researchers have made a lot of efforts to investigate the cost-effective technology for microalgae biodiesel production for last few years.

1. Isolation and selection of suitable microalgae, cultivation, harvesting, and lipid extraction are the major steps in microalgae biodiesel production process. Innovative development of large-scale culture systems through proper selection of algal strains that lead to high and sustained growth rates of oil- rich biomass have to be studied further Another important issue is to attain cost-effective photobioreactors with high efficiency, to achieve maximum productivity with minimum operation costs.

2. Maximum open pond systems and photobioreactor, available for large scale commercial production, are limited to grow microalgae for high value products and not biodiesel. Some of the parameters need to be optimized for large scale production include strain selection,seed culture preparation, biomass and lipid productivity optimization, configuration of photobioreactor and open ponds.

3. Factor affecting microalgae cultivation system such as light intensity, pH, temperature, oxidation reduction potential (ORP), salinity, conductivity, nutrient composition, and controlling bacterial & fungal contamination are required to optimize before scaling up microalgae cultivation.

4. Investigations on new large-scale culture systems that will allow selected algal strains to achieve high and sustained biomass growth rates and lipid yields is needed to develop a microalgal-based biofuel industry.

5. Microalgae biomass harvesting efficiency is seen as one of the major challenges for economical biodiesel production. The existing harvesting techniques are costly and have low efficiency. Furthermore, they also cause biomass contamination and affect lipid

quality. Auto-flocculation/bio-flocculation/low cost flocculation along with filtration should be optimize and applied at large scale for efficient harvesting technology. Study of efficient harvesting technique can considerably lower the cost of harvesting process.

6. The extraction of oil from microalgae biomass has also been another challenge for pilot scale biodiesel production. However, mechanical pressing works well for oil extraction from seeds, but it is not more successful in case of microalgae lipid extraction. Therefore, development and optimization of the most efficient method for lipid extraction from microalgae should be investigated for large scale biodiesel production. Pretreatment methods such as microwave or ultra-sonication methods combined with solvent extraction and mechanical press may be best choice for maximum lipid extraction.

7. Microalgal biodiesel production is still in the research and development stages and should have been investigated factors that affect transesterification process. Vital steps need to be critically analyzed at each stage. For improved conversion of fatty acid methyl ester (FAME), reaction temperature, stirring speed, reaction duration, oil to methanol ratio, catalyst concentration and replacement of methanol by green solvents like dimethyl carbonate, ethanol should be optimized. This design and modeling could give effective results at industrial scale biodiesel production from microalgae.

8. Other challenge with respect to the use of biodiesel as fuel includes susceptibility to oxidation due to the presence of more polyunsaturated fatty acids. There is need of more research on storage stability and corrosion study of microalgae biodiesel.

2.7 Motivation

Apart from the problem of diesel scarcity and higher fuel costs, there is the growing threat of vehicular pollution. To compensate the shortage of diesel fuel, the adaptation of alternative fuel like biodiesel is getting attention now days. In developed countries,

biodiesel is produced from soybean, rapeseed, and other edible seeds which disturb food chain. Developing countries like India are encouraging non-edible seeds (e.g Jatropha, Pongamia) for biodiesel production. However, these non-edible seeds are not able to fulfill the requirement to replace fossil fuel. Recently, microalgae have shown great potential for biofuel production. The advantages of microalgae as biodiesel feedstock are its possibility of growing either in fresh or marine waters and avoiding the use of agriculture land, more carbon dioxide fixation than terrestrial crops and higher oil production per hectare per year (100 times more than soybean). Hence, there is a greater motivation to utilize microalgae as a feedstock for biodiesel production.

In the proposed thesis, investigations have been focused on optimization of culture condition for enhancing lipid productivity of five *Chlorella* species (*Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa* and two native isolated *Chlorella* sp. (named as here *Chlorella* 1 and *Chlorella* 2) at lab scale. Mass cultivation of selected *Chlorella* species (*Chlorella vulgaris*) was carried out to extract lipid for biodiesel production.

2.8 Need for the present work

India is one of the most rapidly developing countries in the world. It is witnessing increasing industrialization and thus development. Such rapid development requires energy to progress, which further makes India an energy hungry nation. Currently India relies mainly upon fossil fuels and thus has to pay a huge bill at the end of every contractual period. Due to increasing demand and consumption, fossil fuel reserves were depleting continuously. On the other hand, extensive utilization of fossil fuels has resulted in excess anthropogenic greenhouse gases (GHG) discharge and is responsible for global climate change. These reasons necessitated the development and use of RES &Technologies for sustainable economic development of India.

Transportation sector of India is unique in the world and consumes more diesel than gasoline. According to energy statistics 2015, high speed diesel oil accounted for 38.83% of total consumption of all types of petroleum products in 2013-14. This was followed by Refinery (10.15%), Petrol (9.73%), LPG (9.28%) and Naphtha (6.50%). Therefore, biodiesel may be a best alternative and renewable source of energy towards attaining maximum self-reliance for the corner stone of our energy security strategy in India. At present, Jatropha and Karanj plant based oil have been projected as potential feedstocks for the production of biodiesel in India. There is still a long way to go for the plant oil to cater the fulfillment of enormous amount of fuel demand. Microalgae are emerging as a most promising feedstock worldwide. It can give biomass yield of 15–25 t/ha/acre in comparison to soybean (0.4t/ha/year), rapeseed (0.68t/ha/year), palm (3.62t/ha/year) and Jatropha oil (4.14t/ha/year) .

However, several types of microalgae have been successfully cultured for high value products such as animal feed, fine chemicals and pigments for long periods (Spolaore et al., 2006a), but commercial production of microalgae to be used as renewable fuel feedstock has not yet been achieved. Significant challenges are the low-cost and well-organized production of microalgae biomass and biofuels. Moreover, the technology for producing biofuels from microalgae is still in its nascent stage. Extensive studies have to be carried out for microalgae cultivation, enhancing lipid production, and carbon dioxide sequestration, harvesting and lipid extraction at lab scale. But, limited reports are available for comparative study and mass cultivation of Chlorella strains for efficient biodiesel production process. Hence, the present work investigated energy and cost efficient microalgae based biofuel production route.

2.8 Objectives

The objectives of present research work are broadly categorized as follows:

i. Finding the feasibility of oil content in various microalgae, their culture in photo bioreactor & analysis of corresponding algae oil.

ii. Optimization of reaction condition for biodiesel production and lipid extraction from microalgae.

iii. Evaluation of physico-chemical properties of the algae based biodiesel as per BIS.

iv. Study of the oxidation, storage and thermal stability of the algae based biodiesel.

2.9 Statement of the problem

After an exhaustive survey of the available literature indicate the R & D pursuits and results in the production of microalgae biofuels, the statement of the present problem was evolved as described hereunder.

i. Comprehensive literature survey to know about the present status of microalgae biomass production and enhancement techniques with reference to efficient biofuel production.

ii. Screening and optimization of growth media for microalgae cultivation.

iii. Selection of suitable microalgae species on the basis of growth rate, lipid yield, lipid productivity, lipid profile, and fuel properties of biodiesel produced.

iv. Feasibility of microalgae cultivation in pilot scale photobioreactors i.e. 10 liter bubble column and 50 liter flat plate photobioreactor (FPP) under outdoor conditions.

v. Mass cultivation of microalgae species in open raceway ponds for biodiesel production.

vi. Optimization and enhancement of lipid extraction efficiency using different chemical solvents, pretreatments and drying methods.

vii. Process optimization (reaction temperature, reaction duration, amount of catalyst, and volume of methanol) for efficient biodiesel production.

viii. Analysis of all basic fuel properties like ester content, calorific value, density, kinematic viscosity, flash point, fire point, cloud point, and Cetane number of biodiesel and biodiesel-diesel blends according to ASTM and BIS.

ix. Studies on storage and thermal behavior of microalgae biodiesel

In order to achieve the aims and objectives of the present investigations the experimental procedure and techniques are described in the chapter 3. Subsequently the results obtained are discussed in chapter 4 under various headings.

Chapter 3

Material and Methods

3.1 Microalgae strain and pre-cultivation conditions

Pure cultures of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa* and *Chlorella sp. 2* were procured from Vivekananda Institute of Algal Technology (VIAT), Chennai (India); Centre for Conservation and Utilization of Blue Green Algae, IARI New Delhi (India), National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune (India) and Yogi Vemana University, Vemanapuram, Kadapa, Andhra Pradesh, India respectively. A microalgal species was also collected from nearby area of Dehradun, Uttrakhand and purified at agar plates by standard protocol (Guillard, 2005). It was identified morphologically in IARI, New Delhi, as *Chlorella sp.* and is represented here as *Chlorella sp.1*. *Chlorella sp.* 2 obtained from Yogi Vemana University, Vemanapuram, Kadapa, Andhra Pradesh, is represented as *Chlorella sp. 2* in this study. The stock culture of all the strains was maintained regularly on agar slants using sterilized BBM medium (with initial pH of 6.8) under laboratory conditions at $24^{o}C$ ($\pm 1^{o}C$) under (~2500 lux) light intensity and 16/8 light dark cycle (culture on agar slants shown in figure 3.1). Medium was sterilized at $121^{o}C$ for 15 minutes in autoclave. All the strains were transferred from agar slant into a liquid medium and incubated under the same conditions of temperature and light in photobioreactor. Liquid cultures were used as the source of inoculum for the designed experiments. Photobioreactor used for stock culture are shown in figure 3.2. The serial transfer of all agar slants and liquid cultures into new medium was performed in a laminar flow to avoid the possibility of contamination attack (figure 3.3). The morphology of pure strains was regularly examined under an optical microscope. The stained sections were observed under 100× and photographed by a Nikon Eclipse Ci-E microscope (serial no. 59E05).

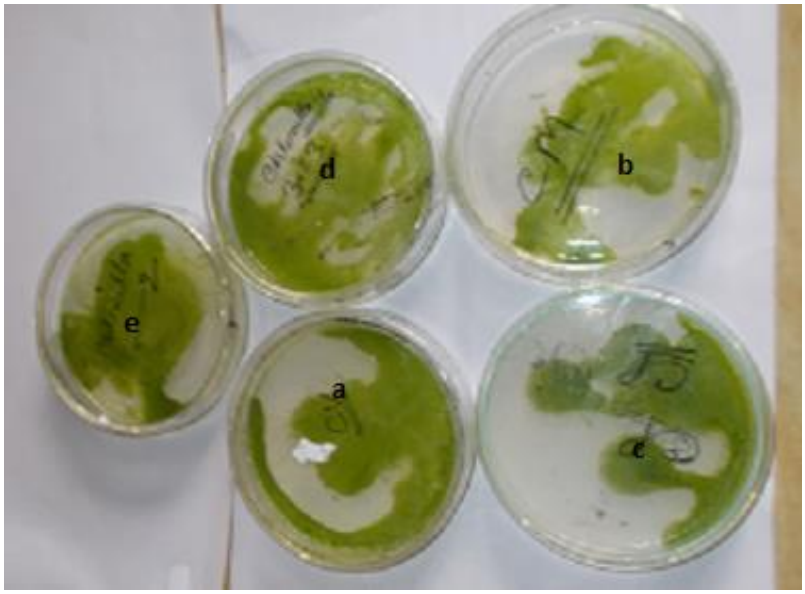


Figure 3.1 Cultivation of all five Chlorella strains at agar slants, (a) *Chlorella Vulgaris*, (b) *Chlorella minutissima*, (c) *Chlorella pyrenoidosa*, (d) *Chlorella sp.1*, and (e) *Chlorella sp. 2*

3.2 Optimization of Culture parameters affecting lipid productivity of microalgae species grown in photobioreactor

3.2.1 Screening and identification of efficient growth media for maximum lipid productivity

The growth performance and lipid productivity of microalgae is highly influenced by composition of medium used for cultivation. Four media that have been used for photoautotrophic culture of *Chlorella* species include Bold's basal medium (BBM), BG-11, Fog's medium and M4N.

Bold's Basal Medium (Andersen, 2005**):** $NaNO_3$ 250 mg/L, K_2HPO_4 75 mg/L, $MgSO_4$.7H2O 75 mg/L, $CaCl_2$.2H_2O 25 mg/L, KH_2PO_4 175 mg/L, NaCl 25 mg/L, Alkaline EDTA solution 1 mg/L (Alkaline EDTA solution: 5g Na_2-EDTA and 3.1 g KOH in 100 ml distilled water), Acidified Iron solution 1 mg/L (Acidified Iron solution $FeSO_4$.7H2O .498g and 0.1 ml H_2SO_4 in 100 ml distilled water) Trace metal solution 1 ml/L (Trace metal

solution: $MnCl_2.4H_2O$ 1.44 g/L, $ZnSO_4.7H_2O$ 8.82 g/L, $(NH_4)_6\ Mo_7O_{24}.2H_2O$ 0.88 g/L, $Co(NO_3)_2.6H_2O$ 0.49 g/L, $CuSO_4.5\ H_2O$ 1.57 g/L).

BG-11 medium (Andersen, 2005)**:** $NaNO_3$ 1500 mg/L, K_2HPO_4 40 mg/L, $MgSO_4.7H2O$ 75 mg/L, $CaCl_2.2H_2O$ 36 mg/L, Citric acid 6 mg/L, Trace metal solution 1 ml/L (Trace metal solution: $FeC_6H_5O_7.NH_4OH$ 6 g/L, Na_2-EDTA 1 g/L, $MnCl_2.4H_2O$ 1.81 g/L, $ZnSO_4.7H_2O$ 0.222 g/L, $Na_2\ MoO_4.2H_2O$ 0.39 g/L, $CuSO_4.5H_2O$ 0.08 mg/L, H_3BO_3 2.86 g/L.

Fog medium (Kothari et al. 2012) **:** KNO_3 2000 mg/L, K_2HPO_4 200 mg/L, $MgSO_4.7H2O$ 200 mg/L, $CaCl_2.2H_2O$ 100 mg/L, Fe-EDTA solution 5ml/L (Fe-EDTA solution: 745 mg Na_2-EDTA and 557mg $FeSO_4.7H_2O$ in 100 ml distilled water), Trace metal solution 1ml/L (Trace metal solution:, H_3BO_3 2.86 g/L, $MnCl_2.4H_2O$ 1.81 g/L, $ZnSO_4.7H_2O$ 0.222 g/L, $Na_2\ MoO_4$-$2H_2O$ 0.39 g/L, $CuSO_4.5H_2O$ 0.08 g/L,).

M_4N medium (Yanagi et al. 1995)**:** KNO_3 5000 mg/L, K_2HPO_4 1250 mg/L, $MgSO_4.7H_2O$ 25000 mg/L, $CaCl_2.2H_2O$ 100 mg/L, $FeSO_4.7H_2O$ 30 mg/L , Trace metal solution 1 ml/L (Trace metal solution:, $MnCl_2.4H_2O$ 1.81 g/L, $ZnSO_4.7H_2O$ 0.222 g/L, $Na_2MoO_4.2H_2O$ 0.39 g/L, $CuSO_4.5H_2O$ 0.08 g/L, H_3BO_3 2.86 g/L).

All the *Chlorella species* were cultured in 250 ml Erlenmeyer flasks containing 125 ml liquid using above four nutrient media composition under cool florescent light (~2500 lux) at 24^{o}C (±1^{o}C) and 16:8 light dark cycle in a photobioreactor. Sterile media was used and sterilization of the medium was carried out for 20 minutes at 15 psi using autoclave (figure 3.3a). To avoid contamination, inoculation of microalgae seed culture in sterile medium was performed in laminar flow hood (figure 3.3b). At stationary phase, *Chlorella species* was harvested using centrifuge and dried at 60^{o}C in hot air oven for dry biomass. Lipid was extracted by modified Folch extraction method (kumari et al., 2011). On the

basis of lipid productivity, and media cost, best growth medium was selected to carry out further experiments.

Figure 3.2 Stock cultures of microalgae strain in photobioreactor

3.2.2 Optimization of growth media (BG-11) to enhance lipid productivity

Among the tested growth media, BG-11 was found suitable for efficient biodiesel production. To enhance lipid productivity, effect of nitrogen concentration, nitrogen sources and organic and inorganic carbon sources was examined. Microalgae species (*Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa Chlorella sp. 1* and *Chlorella sp. 2*) were cultured with different concentrations of sodium nitrate in sterile BG-11 medium to optimize lipid content and biomass production. Sodium nitrate concentration was set 35.30 mM, 17.65 mM, 8.82 mM, 4.41 mM and 2.21 mM. After that, all the microalgae species were cultivated in sterile BG-11 medium replacing sodium nitrate by different nitrogen sources (KNO_3, urea, diammonium phosphate). Nitrogen content was kept same as in BG-11 medium. Calculated amount of nitrogen sources, viz. $NaNO_3$ (8.82 mM), KNO_3 (8.81 mM), urea (4.41 mM) and diammonium phosphate (5.24 mM) was

taken per liter of water nutrient media. Ignoring CO_2 in air, the effect of different organic carbon sources like glucose, glycerol, sodium acetate, and sucrose) was also tested with selected optimal nitrogen sources (4.41mM urea) by adding them in sterile BG-11 media. Carbon content of different organic sources was kept same (0.5 g/L) during the experiment. Sterile media was used during whole experiment.

Figure 3.3 Sterilization techniques for microalgae culture, (a) autoclave for medium sterilization, (b) Laminar hood for culture transferring into new medium

All the experiments were conducted at room temperature (~22-30^{o}C) under cool white, fluorescent light for 14 days. One liter bottle was used as a lab scale photobioreactor as shown in figure 3.4. Working volume of photobioreactor was kept 600 ml with 10% (v/v) inoculum. The photoperiod was set 16:8 light: dark period with fluorescent illumination of ~3000 lux. The culture was aerated (200 ml/min) by aquarium pump to avoid settling of microalgal biomass**.** The experiment was performed for 14 days at batch scale. Initial pH of medium was adjusted to be 7.0. Cell growth of all the strains was estimated by measuring optical density (OD) at 680 nm using a UV–VIS spectrophotometer. The experiment was carried out from October 2011 to November 2012 at room temperature.

Figure 3.4 Microalgae culture in one liter glass bottle photobioreactor

3.2.3 Cultivation of microalgae species in bubble column photobioreactor under outdoor conditions

Outdoor cultivation system is the best choice due to easy access to sunlight (Chen et al. 2011). This approach supports the reduction in overall energy input (mainly for illumination) and subsequently, reduction in the cost of microalgae biomass production. However, there are some limitations to cultivate microalgae under outdoor conditions such as changes of local weather, varying temperature and light intensity. Therefore, only those microalgae species will survive which are able to adopt these extreme cultivation conditions.

To test the feasibility of cultivation of microalgae species under outdoor conditions, all the five microalgae species were grown in bubble columns photobioreactor (diameter of .15m; height of .75 m). The photobioreactor was made of glass and covered with acrylic cap (figure 3.5). BG-11 media was used for microalgae growth. The medium was prepared using hypo-treated tap water. Initial microalgae inoculum density was adjusted at 0.172

g/L. Initial pH was maintained 7.0. Compressed air with 1% CO_2 at about flow of 4 L /minute was supplied in culture media for mixing during day time only. The experiment was carried out from March 2013 to December 2013 at room temperature under natural sunlight. During the experiments, daily temperature variation of medium was between 22^{o}C to 38^{o}C and light intensity was about 1Klux (highest light intensity was about 25-74Klux) at the time of sunrise and sunset. Different growth parameters such as biomass concentration, biomass and lipid productivity, lipid yield and profile were analyzed as described in section 3.3 of this chapter.

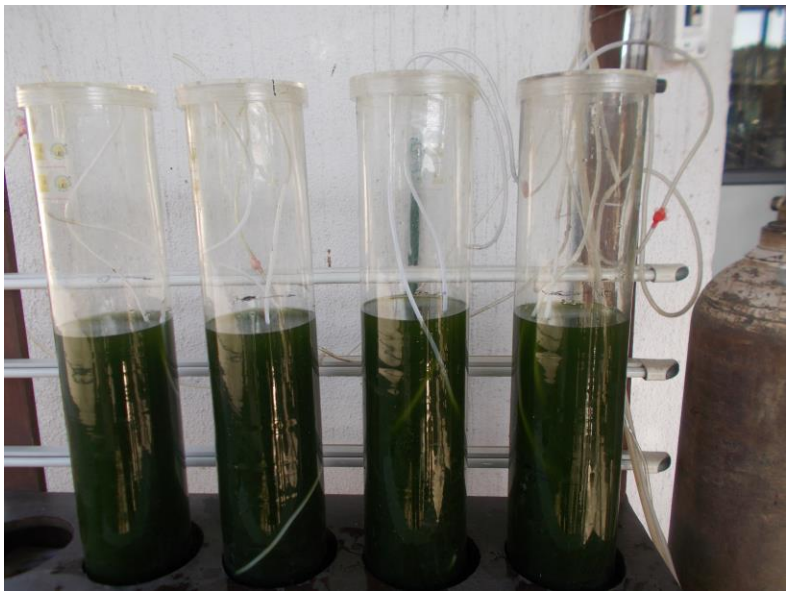


Figure 3.5 Culture of microalgae species in bubble column photobioreactor

A study on mixed culture of *Chlorella vulgaris* with other three species (*Chlorella minutissima*, *Chlorella pyrenoidosa*, and *Chlorella sp.1*) was also performed under outdoor conditions. *Chlorella sp. 2* showed poor growth under outdoor conditions, hence, this species was not included in this experiment. All the experiment with mixed cultures was carried out in 10L bubble column photobioreactor. All the strains were mixed with *Chlorella vulgaris* in 1:1 ratio. The growth kinetics and parameters of pure *Chlorella vulgaris* and its mixed culture such as mixed culture-1 (*Chlorella vulgaris*: *Chlorella*

minutissima), mixed culture-2 (*Chlorella vul*garis: *Chlorella pyrenoidosa*) and mixed culture-3 (*Chlorella vulgaris*: *Chlorella sp.*2) grown under outdoor conditions was analyzed as described in section 3.3 of this chapter. On the basis of biomass & lipid production, lipid profile and biodiesel quality, pure *Chlorella vulgaris* strain was compared with different mixed cultures and found best for biofuel production. Therefore, further experiment was carried out using pure *Chlorella vulgaris* strain.

3.2.4 Effect of initial culture concentration on biomass production and lipid production under outdoor conditions

Initial culture concentration also affects biomass growth and lipid accumulation under outdoor conditions. Hence, the effect of initial cell concentrations (0.175g/L, 0.250g/L, 0.300g/L and 0.350g/L) on growth of *Chlorella vulgaris* (identified best species among tested species for biodiesel production) was studied. The culture medium was BG-11 with 4.41mM urea. The growth parameters of *Chlorella vulgaris* were analyzed as described in section 3.3 of this chapter.

3.2.5 Outdoor cultivation of microalgae species in 60 L Flat plate photobioreactor

To further evaluate its potential for biodiesel production, *Chlorella vulgaris* was grown in 60 L FPP (flat plate photobioreactor) for 15 days under sunlight. Photobioreactor used in the experiments had following dimension: length 1.5 m, height 0.90 m, and thickness 15 cm (figure 3.6). It was made up of transparent glass and acrylic cap. For mixing, compressed air with 1% carbon dioxide was bubbled with flow rate of 16 L /minute at the bottom of the reactor through a silicon aquarium air tube and air stone bubbler. BG-11 (with 4.41mM urea as nitrogen source) was used as culture medium. During the experiments, daily (9:00 am–6:00 pm) temperature variation of medium was between 20^{o}C and 37^{o}C and light intensity was about 1Klux (the highest light intensity was about 80Klux) at the time of sunrise and sunset. The

experiment was conducted from March 2014 to May 2014. The experiment was conducted for 15 days with initial culture concentration of 0.300 g/L at batch scale.

Figure 3.6 Flat plate photobioreactor for microalgae cultivation

An important feature of commercially successful microalgal cultivation system is its capability to develop in continuous or semi-continuous culture for long periods (Moheimani et al., 2006). That can optimize the use of capital intensive culture systems and also decrease labor costs (Sheen et al., 1998). Therefore experiment was also carried out at semi-continuous mode for 60 days using different harvesting concentrations (one third, half and two third culture). To evaluate its feasibility at semi-continuous mode, *Chlorella vulgaris* was grown in FPP for 10 days at batch mode and then, one third (growth phase a), half (growth phase b) and two third (growth phase c) culture was replaced with same amount of freshly prepared nutrient medium at every 4^{th} day. A total of 6 harvests were done in each semi-continuous mode. Growth rate and lipid productivity was analyzed as described in section 3.3 of this chapter.

3.3 Analytical methods

3.3.1 Cultivation parameters

pH was measured by Titrays 352 (Systronics made) and light intensity on the surface of photobioreactor by digital lux meter (Maxtech LX-1010B). Atmospheric temperature and humidity was examined by Digital LCD Temperature and Humidity Meter (HTC-1) respectively

Microalgae growth was determined by measuring optical density at 680 nm (OD_{680}) using UV-visible spectrophotometer (Thermo Scientific) daily and related to algal biomass (g/L). For biomass estimation 10 ml sample containing algae was filtered through pre-weighted Whatman GF/C glass fiber filter and dried in oven at 60^{o}C until constant weight. The dry weight of algae is determined by subtracting from final weight of whatman GF/C glass fiber filter with algae to initial weight. The relationship developed between OD_{680} and biomass (g/L) for BG-11 medium is given as follows:

For *Chlorella pyrenoidosa*

$$y=0.4311\times OD680 +0.0136 \qquad (R^2= 0.996) \qquad (3.1)$$

For *Chlorella vulgaris*

$$y=0.4032\times OD680 +0.0564 \qquad (R^2=0.9624) \qquad (3.2)$$

For *Chlorella minutissima*

$$y=0.414\times OD680 -0.0172 \qquad (R^2=0.9896) \qquad (3.3)$$

For *Chlorella species 1*

$$y=0.3446\times OD680 -0.0196 \qquad (R^2=0.9912) \qquad (3.4)$$

For *Chlorella species 2*

$$y=0.3942\times OD_{680} +0.0188 \qquad (R^2=0.997) \qquad (3.5)$$

where y is algal biomass in g/L and OD_{680} is optical density of culture at 680 nm.

The instrument used for analysis of culture OD is shown below in figure 3.7

Figure 3.7 UV spectrophotometer used for analysis of optical density of microalgae

Microalgal growth curve was expressed in terms of dry cell weight (DCW) (g/L).

The specific growth rate μ day^{-1} at exponential stage was calculated as follows:

$$\mu = \frac{(\ln W_2 / \ln W_1)}{(t_2 - t_1)} \tag{3.6}$$

where, W_1 and W_2 are dry biomass weight (g/L) at time t_1 and t_2 respectively.

The generation time or doubling time T_D (days) was calculated as follows:

$$T_D = \frac{\ln (2)}{\mu} \tag{3.7}$$

The biomass productivity P (mg/L/day) was calculated by following equation:

$$P = \frac{W_2 - W_1}{t} \quad (3.8)$$

where, W_1 was the biomass concentration at the last day of cultivation, W_2 was the initial biomass concentration, t was the cultivation time.

3.3.2 Lipid extraction and measurement

Lipid was extracted by applying folch extraction method (Folch et. al., 1957). According to this method, a weighed amount of biomass (0.250 mg) was dissolved in 5 ml chloroform/methanol (2:1 v/v) and vortex for 30 seconds. This was followed by agitating the mixture for 15-20 minutes at room temperature. The mixture was then centrifuged at 8000 rpm for 10 minutes to separate cell debris from supernatant. This supernatant was washed by 0.9% sodium chloride solution and vortex for few seconds. The mixture was centrifuged at 3000 rpm for 5 minutes. Lower chloroform layer with lipid was removed carefully and collected in 20 ml pre-weighed glass vial. The residue was re-extracted with 2.5 ml chloroform/methanol (1:1, by v/v) thrice as stated above. The supernatant was collected in same vial. The solvent was then dried at 65°C in oven until constant weight of lipid was obtained. The lipid content was calculated gravimetrically. The lipid productivity (mg/ L/ d) was calculated by the following equation:

$$\text{Lipid Productivity} = \frac{\text{Lipid content}_t \times W_1 - \text{Lipid content}_0 \times W_2}{t} \times 1000 \quad (3.9)$$

where Lipid content_t was the lipid content at the last day of cultivation and Lipid content_0 was the initial lipid content in algal cells.

\

3.3.3 Determination of carbohydrate and protein content in microalgae biomass

Total carbohydrate was analyzed through phenol–sulfuric acid method using glucose as standerd (DuBois et al., 1956, Prajapati 2012). Briefly, weighed biomass (100 mg) was hydrolyzed with 2.5 N HCl (5 mL) in boiling water bath for 3 h, cooled at room temperature and neutralized with sodium carbonate. 0.1 mL was pipette out from neutralized sample in a clean test tube and then it diluted to 1 mL by distilled water. After that 1 mL phenol solution and 5 mL 96% sulfuric acid was added to this sample, mixed well and cooled at room temperature. The color intensity of the samples was measured at 490 nm and total carbohydrates were then calculated using standard calibration curve. Protein content was calculated by multiplying the total nitrogen (obtained through CHN analyzer) by 6.25 (Prajapati et al., 2012).

3.3.4 Determination of total pigments in microalgae biomass

Chlorophyll was extracted using 90% methanol. A known amount of microalgal suspension (10 ml) was taken out from photobioreactor or ponds and centrifuged at 5000rpm for 10 minutes. Supernatant liquid was discarded and microalgae pellets were washed thrice with deionized water. The washed microalgae cells were re-dissolved in 90% methanol (v/v) in a tube and keep in water bath at 60^{o}C for 30 minutes. Then sample was cooled at room temperature and again centrifuged at 3000 rpm for 5 minutes. Now, optical density of supernatant was analyzed at OD at 665.2, 652.4 and 470 nm. Chlorophyll a (chl a), Chlrophyll b (chl b) & carotenoid was determined according to the following equations (Ritchie, 2006; Strickland et al.,1968)

$$\textbf{Chl-a (µg/mL)} = -8.0962 \times A652 + 16.5169 \times A665 \quad (3.10)$$

$$\textbf{Chl-b (µg/mL)} = 27.4405 \times A652 - 12.1688 \times A665 \quad (3.11)$$

$$\textbf{Carotenoids (µg/mL)} = 4 \times A480 \quad (3.12)$$

3.3.5 Fatty acid analysis

Extracted lipid was converted to their methyl esters by transesterification (figure 3.8). Lipid was dissolved in 1ml of 1% NaOH in CH_3OH and heated at 55°C for 15 minutes. Then, 2 ml of 5% methanolic HCl was added in mixer and again heated for 15 minutes at 55°C. The obtained product mixture was washed with distilled water. Fatty acid methyl esters (FAME) were extracted using 1 ml hexane thrice and evaporated to dryness (Kumari et al., 2011). FAME was re-dissolved in 200µl hexane and analyzed using a Gas chromatograph (Nucon 5700 series) equipped with Flame ionization detector (FID) using EOX column (serial no 5061; 30 m length, 0.25 mm ID and 0.25 mm outer dia) as shown in figure 3.9. Pure nitrogen (99.999%) was used as carrier gas with a flow rate of 1 ml/min and pre-column pressure of 49.7kPa. The initial temperature was set at 160° C for 2 minutes, followed by 4°C/min ramp up to 240°C and maintained for 40 min. The injector and FID detector temperature was set at 240°C and 220°C respectively. FAME peaks were identified by comparison of their retention time with authentic standard by GC. Methylated heptadecanoic acid was used as an internal standard.

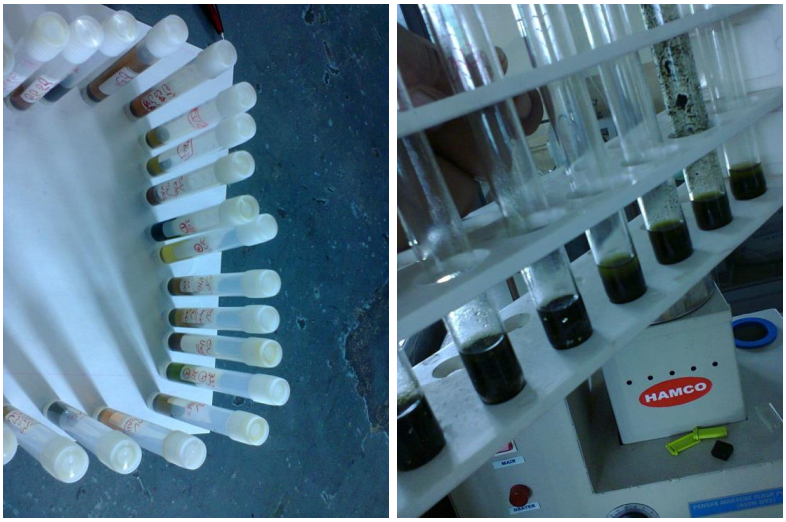


Figure 3.8 Lipid and biodiesel samples extracted from *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa* and *Chlorella sp. 2*

Figure 3.9 Gas Chromatograph for biodiesel analysis

3.3.6 Analysis of biodiesel properties

Biodiesel sample obtained at lab scale experiments was in very less amount and not sufficient to analyses the fuel properties. Therefore, important fuel properties of biodiesel, i.e. saponification value (*SV*), iodine value (*IV*), long chain saturation factor (LCSF) and cloud filter plugging point (CFPP) (Francisco et al., 2010; Ramos et al., 2009), including density, kinematic viscosity, cetane number (*CN*), (Verduzco et al., 2012) oxidation stability (Park et al., 2008) and heating value (Demirbas, 2008) were analyzed using following equations-.

$$\mathbf{SV} = \frac{\Sigma (560 \times \mathrm{N})}{\mathrm{Mw}} \tag{3.13}$$

$$\mathbf{IV} = \frac{\Sigma (254 \times \mathrm{N} \times \mathrm{D})}{\mathrm{Mw}} \tag{3.14}$$

$$\mathbf{CN} = \sum \mathrm{X_i} \times \mathrm{CN_i}$$

$$\mathbf{CN_i} = -7.8 + 0.302.\mathbf{M_i} - 20.\mathbf{N} \tag{3.15}$$

where CN_i is the cetane number of i^{th} FAME

Density ρ at 15°C (kg /m³):

$$\rho_{Biodiesel} = \sum X_i \times \rho_i$$

$$\rho_i = 0.8463 + \frac{4.9}{M_i} + 0.0118 \,.\, N \tag{3.16}$$

where ρ_i is the density of i^{th} FAME at 15°C in kg /m^3

Kinematic viscosity η at 40°C

$$\ln(\eta_i) = -12.503 + 2.496 \,.\, \ln(M_i) - 0.178 \,.\, N \tag{3.17}$$

where η_i is the kinematic viscosity of i^{th} FAME at 40°C in mm^2/s

Long chain saturation factor (LCSF) and the CFPP in °C

$$\mathbf{LCSF} = (0.1 \times C\ 16{:}\ 0) + (0.5 \times C18{:}\ 0) + (1 \times C20{:}\ 0) + (2 \times C24{:}\ 0) \tag{3.18}$$

$$\mathbf{CFPP} = (3.1417 \times LCSF) - 16.477 \tag{3.19}$$

Oxidation stability (h)

$$\mathbf{OS} = \frac{117.9295}{z} + 2.5905 \tag{3.19}$$

Heating valuve (MJ/Kg)

$$CV_i = 46.19 - \frac{1794}{M_i} - 0.21 \,.\, N \tag{3.20}$$

where CV_i is the calorific value of i^{th} FAME in MJ/Kg

3.4 Mass cultivation of *Chlorella vulgaris* under open raceway pond for biofuel production

Mass cultivation of *Chlorella vulgaris* was carried out in a concrete raceway pond (Length 5 m; Width 2 m; Height 0.5 m) having a total working volume of 1200 L (figure 3.10). Seed culture of *Chlorella vulgaris* was prepared in the flat plate photo (FPP) bioreactor and bubble column photobioreactor. Initial concentration of pond culture was adjusted up to 0.300 g/L. To avoid settling and enhancing dissolution of CO_2, algal culture was agitated with a paddle wheel system with a speed of 15 rpm during day time. The experiment was carried out for 21 days until stationary phase was achieved. Microalgal cells were grown in open ponds using optimized BG-11 (4.41mM urea as nitrogen source) and fertilizer based growth media. As biomass production of *Chlorella vulgaris* in open raceway pond using BG-11 media was found very expensive, a cost effective fertilizer based nutrient media was prepared. The composition of commercial grade fertilizer media was as follows: urea 250 mg/L, DAP (Di ammonium phosphate) 250 mg/L, potash (potassium Chloride) 250 mg/L, magnesium sulphate 250 mg/L, sodium carbonate 20 mg/L, Ferric citrate 6mg/L and micronutrient from BG-11 medium (half strength). Both the growth media were prepared using sterilized ground water and culture height in the pond was maintained at 12 cm level. Initial pH of the culture was maintained at 7.0. At every 3 days interval, pH, biomass, pigments, and lipids were analyzed and recorded. Purity of the culture was checked regularly by microscope. The experiment was carried out from April 2014 to November 2014. During the experiment, average minimum and maximum temperature were between 20^{o}C to 34^{o}C while solar light intensity was recorded as 1Klux during sunshine and sun set (maximum sun light intensity was 60-84 Klux). To evaluate the potential of fertilizer based nutrient media, *Chlorella vulgaris* was cultured in semi-

continues mode. During semi-continuous cultivation, microalgae was initially grown for 12 days at batch mode and then 50% of microalgae culture was replaced by freshly prepared nutrient media (commercial grade fertilizer based nutrient media) at every 6^{th} day. This experiment was conducted for a period of 48 days. Total six harvestings were carried out during semi-continuous mode. The culture was agitated continuously during day time using paddle wheel at 15 rpm speed/minute. During the semi –continuous growth mode, the pH was maintained 7-8 by using frequent CO_2 supply in the culture pond. Tape water was used for the cultivation of algae and it was thoroughly treated with sodium hypochlorite.

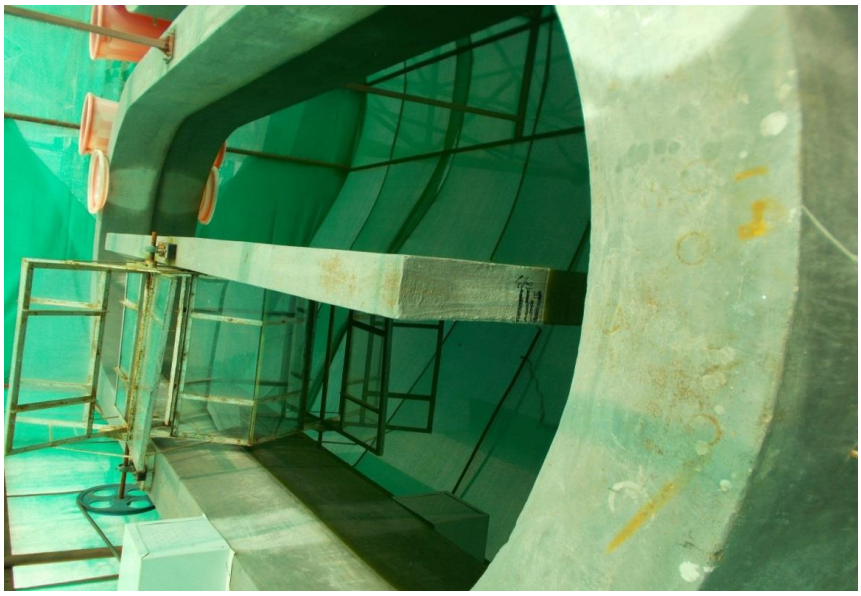


Figure 3.10 Open raceway pond for mass cultivation of microalgae

Growth parameters such as biomass concentration, pH, temprature, humidity, pigments and lipid content was analysed at every three days interval. At the end of experiment piment, carbohydrate and protien content of biomass was also analysed as described in 3.3 section of this chapter.

3.5 Optimization of microalgae biomass harvesting

The algal cells were harvested by a combined process, viz. flocculation and filtration. To optimize suitable dose of flocculation, preliminary study was made in 100 ml test tube (figure 3.11).

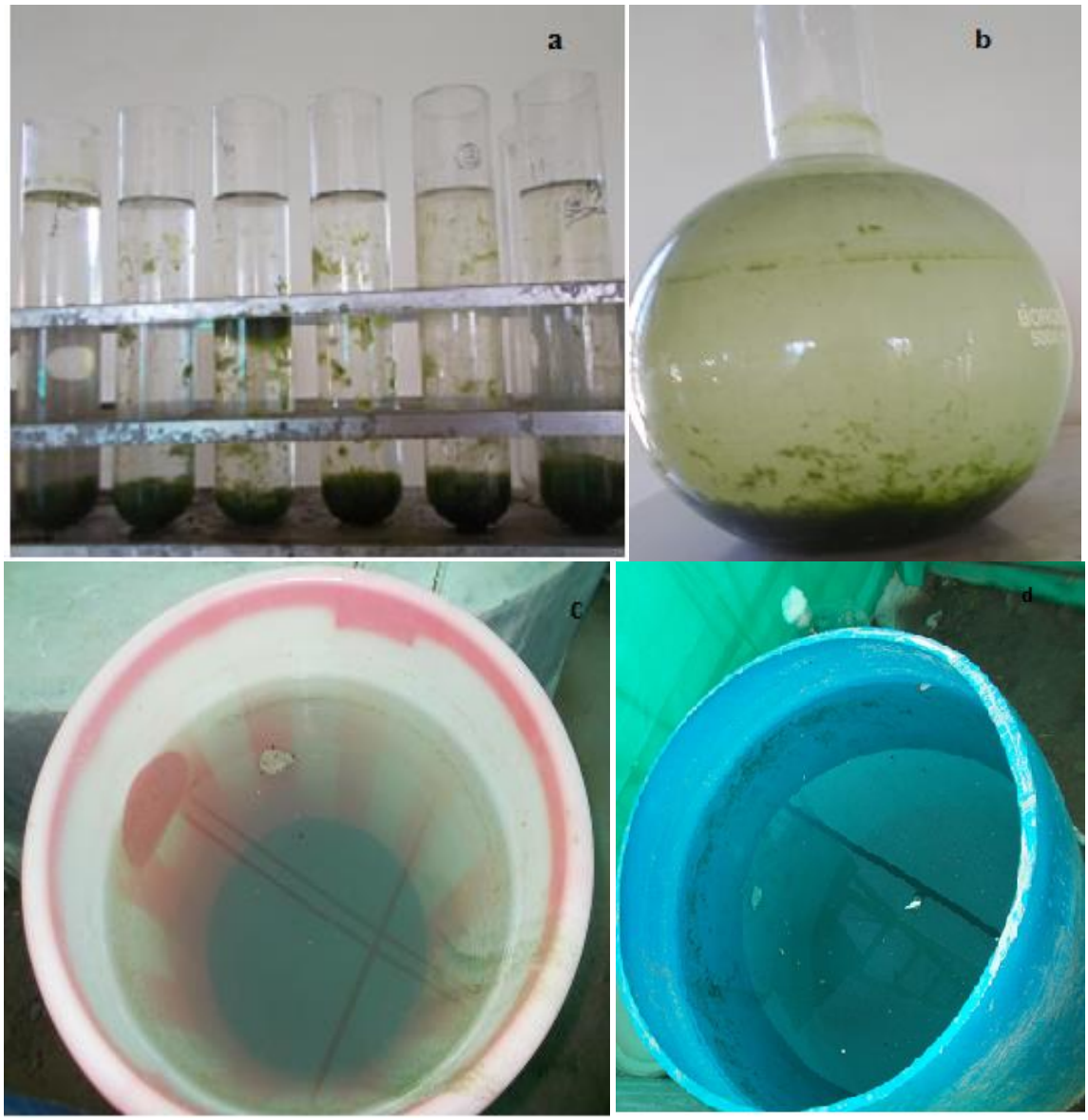


Figure 3.11 Microalgae biomass harvesting in (a) 100 ml test tube, (b) 5 L round bottom flask, (c) 100 L plastic drum and (d) 200 L plastic drum

Flocculation was carried using potassium alum [$(KAl(SO_4)_2 \cdot 12H_2O$]. Effect of concentrations of potassium alum (50 mg/L, 100mg/L, 150 mg/L, 200 mg/L, 250 mg/L,

300mg/L, 400 mg/L and 500mg/L) with time (15minute, 30 minute, 60 minute, 90 minute, 120 minute, 150 minute, 180 minute, 210 minute, 240 minute, 270 minute and 300 minute) on flocculation efficiency was investigated in this study. After optimizing alum concentration, effect of pH on flocculation efficiency was also studied. Optimized conditions were first examined in 100 ml test tube and then applied to the large container (5 liter flask , 100 liter and 200 liter plastic vessel) for harvesting of the algae biomass (figure 3.11). Concentrated microalgae slurry obtained by flocculation, was filtered through filter cloth and washed with fresh ground water 3-4 times to remove excess salts in microalgal biomass (figure 3.12 and 3.13).

Figure 3.12 Filtration of concentrated algae slurry through filter cloth

The flocculating efficiency was determined using following equation:

$$\text{Flocculating efficiency (\%)} = \left(1 - \frac{OD_b}{OD_a}\right) \times 100 \qquad 3.21$$

where, OD_b is the optical density of the algal culture before the flocculation analyzed at 680 nm and OD_b is the optical density of the sample at 680 nm.

Figure 3.13 Microalgae biomass slurry after filtration

Figure 3.14 Drying, powdering and storing of microalgae for lipid extraction

Washed microalgae biomass was dried under sun light followed by oven drying at 60°C for 4 h. After that, biomass was converted into powder form with the help of mortar and pestle and packed in polybags (figure 3.14). This dried algal biomass was stored in freezer under -20°C until lipid extraction experiment was carried out.

3.6. Development of lipid extraction method from microalgae biomass

Development of an effective and energy efficient method for microalgae lipid extraction method is still a challenge for large scale biodiesel production. Therefore, this investigation was tried to examine different lipid extraction methods to enhance lipid extraction efficiency.

Soxhlet apparatus was used for lipid extraction (figure 3.15). After the completion of extraction process, solvent and lipid were separated by fractional distillation process and total lipid yield was analyzed by gravimetric method. To increase the yield, effect of different parameter like type of solvents, pretreatments, drying condition and extraction techniques on lipid extraction efficiency was studied.

Effect of drying technique on lipid extraction efficiency was also investigated. Different drying techniques (solar drying and oven drying at $60^{o}C$, $80^{o}C$, $100^{o}C$) were employed to harvested wet slurry of microalgae biomass to examine the effect on lipid extraction efficiency.

An ideal solvent used for lipid extraction should have following characteristics: high levels of specificity towards lipids, especially acylglycerols and enough volatile nature to ensure low energy distillation to separate the lipid from solvents (Halim et al., 2012). In laboratory, lipid extraction from microalgae biomass was carried out using varieties of solvents ranging from non-polar (hexane, diethyl ether, toluene, di chloromethane, chloroform) to polar (methanol, ethanol, isopropanol, acetone, ethyl acetate, and ethanol) (Mubarak et al. 2014). Efficient extraction of lipids is highly influenced by polarity of the organic solvent or solvent mixture used (Halim et al., 2012; Mubarak et al., 2014). Therefore, screening of different polar and non-polar solvents viz., acetone, diethyl ether, ethyl acetate and acetone, n-hexane, chloroform, methanol, ethanol, dichloromethane

(DCM), toluene and cyclohexane to extract lipid from microalgae biomass was carried out in soxhlet extractor unit. Generally, a mixture of polar and non-polar solvents is more efficient to extract lipids from microalgae biomass (Halim et al., 2012; Mubarak et al., 2014; Ryckebosch et al., 2012). Thus , a mixture of solvents containing a polar and a non-polar solvent such as Chloroform: methanol (2:1), hexane: diethyl ether (1:1), hexane :methanol (3:2), hexane :ethanol (3:2), hexane : isopropanol (3:2), cyclohexane: methanol (2:1), cyclohexane: ethanol (2:1), di chloromethane: hexane (3:1), di chloromethane: cyclohexane (1:1) and ethyl acetate: ethanol (1:1) was examine to enhance lipid extraction efficiency. After optimizing best solvent system for lipid extraction, the effect of moisture content of microalgae biomass (1%, 5%, 10% 20%, 40% and 60%) on extraction efficiency was analyzed. In addition, effect of time (2, 4, 6, 8, 10 and 12 h) on lipid extraction was also studied.

In order to extract intercellular lipids, it is essential to destruct primary cell membrane. These lipids accumulate externally in successive outer walls and can be easily extracted from microalgal biomass with suitable solvents, but it still remains a big challenge to recover total lipid from microalgae biomass because microalgal cell membrane acts as a barrier and reduces the recovery. Hence, different cell disruption processes or pre-treatment methods were applied before extraction in order to achieve high lipid efficiency. In this investigation, following pre-treatment methods were employed:

(i) **Ultrasonication**: In this method, ultrasonication was done using ultrasonic probe at 24 kHz at room temperature for 15 min.

(ii) **Autoclave method**: In this method, microalgal biomass slurry was autoclaved at 121^{o}C and 15 Kpa pressure for 20 min.

(iii) **Deep freezing pre-treatment**: In this method, microalgal biomass was stored in deep freezer at -20^{o}C for 24 h and then utilized for lipid extraction.

(iv) **Microwave pre-treatment**: in this method, the microalgal biomass was treated in microwave oven for 15 minutes at 100^{o}C.

After pre-treatment, microalgal biomass was separated from water by using filtration technique through filter cloth and dried under solar light, followed by hot air oven to maintain specific moisture content. Then pre-treated biomass was used for lipid extraction by applying suitable solvent. In addition, optimization of solvent to biomass ratio (6:1, 8:1, 10:1, 12:1) was also carried out at batch scale to increase extraction efficiency.

Figure 3.15 Soxhlet lipid extraction of microalgae biomass

After completion of extraction process, crude extract was filtered through Whatman 40 filter paper to remove particles, entrained during this process. Mixture of solvent and lipid was separated using rotary evaporator unit, and the yield obtained was expressed in terms of weight percentage and measured gravimetrically. All the experiments were carried out in triplicate form and represented average value with ± standard deviation.

3.7 Process optimization of biodiesel production from microalgae biomass

Transesterification experiments were conducted in a microwave (MW) reactor with temperature and power control functions manufactured by Ragatech (serving industry with Microwave) as shown in figure 3.16a. Microwave-transparent, three-neck custom-fabricated reaction vessels made of borosilicate glass were used as sample vessels. Each experiment was repeated three times to evaluate the reproducibility of microwave effect. After each experiment, microwave reactor was allowed to cool and returned to original conditions with reaction interval. Microwave reactor was equipped with a reflux condenser which condenses and returns back the evaporated solvent mixture into the reaction vessel during experiment. Magnetic stirrer was used for providing uniform mixing with a constant mixing speed during all the experiments.

Crude microalgae lipid extracted by Soxhlet apparatus was taken and re-dissolved in n-hexane, filtered and concentrated. Since, the microalgal lipid has high free fatty acid (FFA) content, biodiesel production was carried out in two steps. First step of biodiesel production process was esterification in which FFA was reduced below 2% because higher FFA leads to soap formation during base catalyst reaction (figure 3.17). This was followed by transesterification.

3.7.1 Acid-catalyzed esterification (Conversion of FFA to Triglyceride)

Acid-catalyzed esterification was carried out at 60^{o}C and atmospheric pressure in microwave reactor with constant agitation using magnetic stirrer. The microalgal lipid and methanol mole ratio was kept 1:10 (v/v) at 60^{o}C, the quantity of methanol used in the reaction was 100 mL, referred to the 10 ml of algal lipid and this amount corresponds to 100% excess relative to the stoichiometric value. Optimization study was carried out by different concentrations of acid catalyst (0–1.5 vol.%) with different reaction time intervals

(5-40 minutes). Mixture was stirred with constant speed and refluxed continuously for proper mixing and to decrease chances of solvent vapor loss during reaction. Progress of reaction was regularly observed by analyzing acid value content at different at pre-determined time intervals. Once acid value was decreased below 1%, reaction was stopped and end products of the reaction were transferred into the separating funnel. Upper layer was collected and concentrated in rotator vacuum. Approximately, 1ml sample was taken from this concentrated solution and washed with hot water to remove the impurities. Refined oil was analyzed by Gas Chromatograph (GC) to examine the biodiesel yield. The above esterified product was used for transesterification process.

3.7.2. Base-catalyzed transesterification

The product of acid esterification stage (pure triglycerides) was transesterified to mono-esters of fatty acids (biodiesel) using alkaline catalyst (KOH). Various experiments were conducted under a matrix of conditions: catalyst concentration of 0.5–2.5 (wt./vol. %), different lipid to methanol molar ratio (1:4–1:14 v/v), different temperatures (45^{o}C-65^{o}C) and different reaction time of 5 to 25 minute. After completion of reaction, biodiesel sample was washed three times with distilled water containing 1% H_3PO_4 acid to remove the unreacted reactants and other impurities. After washing, moisture content of biodiesel was removed using anhydrous Na_2SO_4. Biodiesel sample was centrifuged at 6000 rpm for 10 minutes to remove impurities and collected in a sealed glass bottle. All the experiments were conducted three times, and average value with ± standard deviations was represented as results in graphs. Biodiesel yield was examined gravimetrically as

$$\text{Biodiesel yield (wt \%)} = \frac{\text{wt of biodiesel (g)}}{\text{wt of oil (g)}} \text{X } 100 \qquad (3.22)$$

Obtained biodiesel was analyzed by H NMR, ^{13}C NMR and FTIR.

Figure 3.16 Experimental set up used for transesterification, (a) microwave assisted transesterification reactor, (b) conventional transesterification reactor

3.7.3. Comparison of conventional water bath assisted biodiesel reactor and microwave assisted biodiesel reactor

Biodiesel was also produced in a conventional water bath assisted biodiesel reactor and compared with microwave assisted biodiesel reactor (figure 3.16). The reaction time for esterification and transesterification in conventional reactor was 3.5 h and 3 h respectively. Other reaction conditions like catalyst concentration, lipid to methanol ratio and temperature were kept same as in microwave assisted biodiesel reactor.

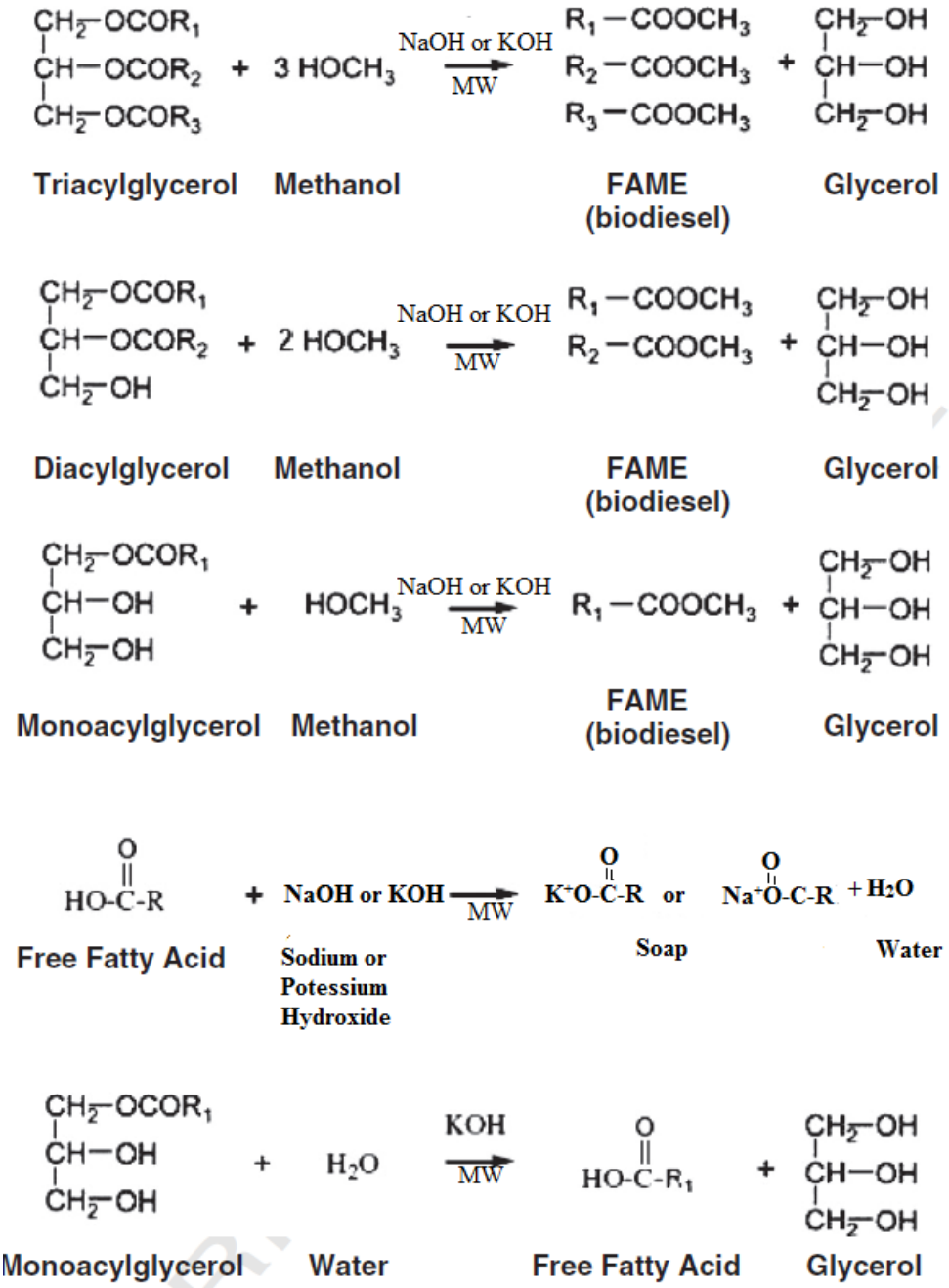


Figure 3.17 Different reactions involved in biodiesel production: conversion of triacylglycerol into FAME (biodiesel), undesirable reactions such as FFA conversion to soap and monoacylglycerol to FFA in presence of moisture (modified from Halim et al. 2012)

3.8 Physico-chemical properties of microalgae oil biodiesel

The physico-chemical properties of the microalgae oil based biodiesel (B100) and their various blends (B2.5, B5, B10, B20 and B100) with diesel were evaluated as per the ASTM and IS: 15607. Various instruments used are shown in Figure 3.18 The fuel

properties data of microalgae oil methyl esters and their different blends with diesel are summarized in chapter 4.

Acid number and moisture content was analyzed by TITRASYS 352 (Systronics made). Viscosity and density was analyzed by viscometer (Fungilab made) and density meter (Lemis made). Flash point was examined by Pensky-Martens Closed Cup apparatus (Hamco made). Pour and cloud point was observed by a digital pour and cloud point apparatus (Hamco made) and calorific value was observed by a digital bomb calorimeter (Rajdhani made).Oxidation stability was studied by Petrotest "PetroOXY(e)-VERSION: 10.08.2011" made in Germany. Fatty acid composition was analysed by Gas chromatograph with FID (Nucon 5765). Corrosion study of microalgae biodiesel and its blends was performed by Copper Strip Corrosion Test Apparatus (Hamco made). Different test method used for analysis of fuel properties of microalgae biodiesel was shown in table 3.1.

Figure 3.18 Instruments used for analysis of biodiesel properties (a). Density meter, (b), Viscometer, (c) Pour and cloud point, (d) Karl fischer Titrator, (e) Fire and Flash point, (f) Bomb calorimeter, (g) PetroOxy for oxidation stability analysis, and (h) Copper strip corrosion method apparatus

S.No.	Properties	unit	Test method	Limit (IS: 15607)
1.	Acid number (mg KOH/g)	mg KOH/g, max	ASTM D664	0.5
2.	Density at 15°C	kg/m^3	ASTM-D 4052	860-900
3	Viscosity at 40 °C	(mm^2/sec) or cSt	ASTM-D 445	2.5-6.0
4	Pour point**	(°C)	ASTM-D 97	-
5	Flash point	(°C) min.	ASTM-D 93	120
6	Cetane number*	Min.	-	51
7	Copper strip corrosion	3 h @ 50°C max	ASTM-D 130	1
8	Calorific value**	Mj/kg	ASTM D 240 - 09	-
9	Water and sediment	ppm	ASTM D-2709	500
10	Methyl linolenate**	(%)	EN 14103	-
11	Unsaturated ester (>4 double bonds)	%	Internal method-GC	1
12	Oxidation stability (IP, at 140 °C,)	(h), min.	EN 14112	6

*Calculated on the basis of fatty acid composition by equation 3.15

**No limit prescribed in Indian standards

Table 3.1 Test methods used for the analysis of fuel properties of microalgae biodiesel

3.9 Storage and thermal stability of microalgae biodiesel

In this study five antioxidants butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), butylated hydroxyanisol (BHA), propyl gallate (PL), and pyrogallol (PY) were employed to microalgae biodiesel to enhance the oxidation stability. Among tested antioxidants, TBHQ, PY and PL was found more suitable to improve

oxidation stability. Therefore, storage stability of biodiesel was investigated using these three antioxidants (TBHQ, PY, PL) over a storage time of 12 weeks. Oxidation stability (induction period *i.e.* IP) of neat microalgae biodiesel were investigated by Petrotest "PetroOXY(e)-VERSION: 10.08.2011" made in Germany. Biodiesel samples (40 mL) were stored in closed Borosil glass bottles of 100 mL capacity for 12 weeks and were kept indoors, at a room temperature. Samples were taken out at every constant time interval of 3 weeks and analysis of fuel properties such as acid value, oxidation stability, density and kinematic viscosity was carried out.

Thermal stability of biodiesel was analyzed by Thermo gravimetric analysis (TGA). The thermogravimetric (TG) thermogram of microalgae biodiesel was analyzed on the thermogravimetric analyzer (Perkin Elmer Pyris 6) using alumina pans in IIT Roorkee. Thermal analysis was carried out at a heating rate of 10°C/minute from 30°C to 700°C in two atmospheres namely dry air atmosphere and nitrogen atmosphere (inert atmosphere) of 200 mL/minute. A sample size of approximately 10 mg was used for TGA analysis.

Chapter 4

Results and Discussion

4.1 Introduction

In this chapter, results obtained from the comprehensive experimental investigations are presented and discussed. Details of the experiments include cultivation of microalgae in photobioreactor and open raceway ponds, lipid extraction, transesterification and physico-chemical analysis of biodiesel along with storage stability study. Growth parameters such as nutrient media, nitrogen concentration, nitrogen sources, and organic carbon sources were optimized to improve lipid productivity under indoor conditions. Feasibility of microalgae species in bubble column photobioreactor (10 L), flat plat photobioreactor (60 L) and open raceway ponds (1200 L) under outdoor conditions were also investigated in this study. Moreover, optimization of lipid extraction and transesterification was carried out for efficient biodiesel production process. In addition, storage and thermal stability of microalgae biodiesel were also studied here. Results have been discussed in the following sequence as described earlier in chapter 3.

(i) Scale up of microalgae cultivation system from lab to pilot scale for biofuel production.

(ii) Development of lipid extraction method from microalgae biomass

(iii) Process optimization of biodiesel production from *Chlorella vulgaris* lipid

(iv) Analysis of physico-chemical properties of microalgae biodiesel.

(v) Storage and thermal stability of microalgae biodiesel.

4.2 Scale up of microalgae cultivation system from lab to pilot scale for biofuel production

4.2.1 Characterization of microalgae strains

Five strains of fresh water microalgae (*Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.* 1 and *Chlorella sp.* 2) selected for the laboratory

experiments belong to the division of Chlorophyta and the class of Chlorophyceae. Figure 4.1 shows the pictures of all the five green microalgae taken by an optical microscope.

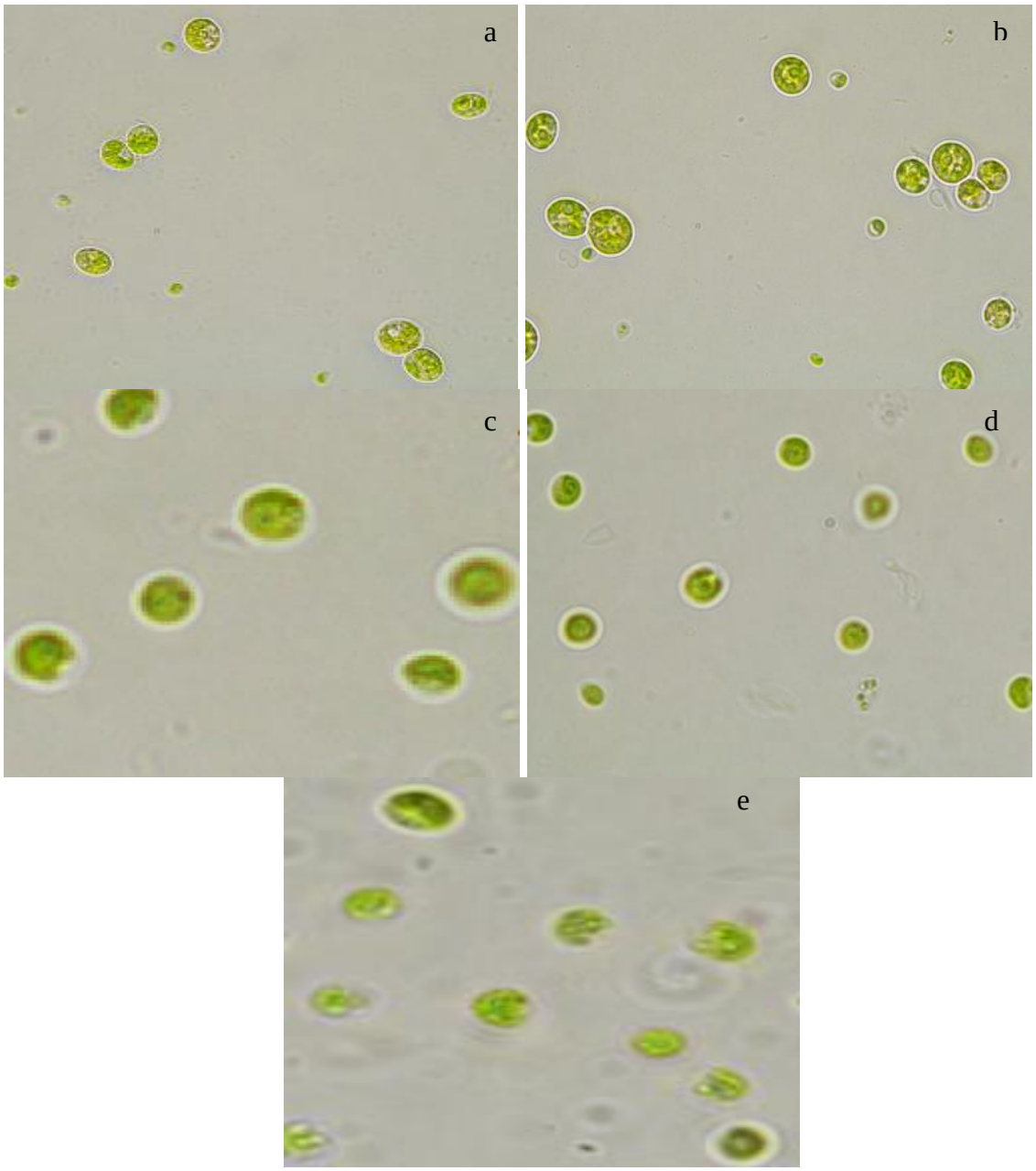


Figure 4.1 Images of five microalgal strains under an optical microscope: (a) *Chlorella Vulgaris*, (b) *Chlorella minutissima*, (c) *Chlorella pyrenoidosa*, (d) *Chlorella sp.1*, and (e) *Chlorella sp. 2*.

Among the tested species, one microalgae species (*Chlorella sp.1*) was isolated from Dehradun region and identified morphologically in IARI, New Delhi. Characteristics and

morphological features of the isolated microalgae have close similarity with genus *Chlorella vulgaris, Chlorella minutissima,* and *Chlorella pyrenoidosa,* and represented here as *Chlorella sp. 1*. The individual cells of the colonies were in the range of 2-10µm. Microalgal cells were green color, unicellular, and spherical in shape.

4.2.2 Screening and identification of efficient growth media for maximum lipid productivity

All the microalgae species (*Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1*, and *Chlorella sp.2*) were cultured in four media i.e. BG-11, BBM, Fog's and M_4N medium in 250 ml Erlenmeyer flasks (conical flask) each containing 125 ml respective medium. Conical flask was kept in photobioreactor having following culture condition: light intensity-2500 lux, light: dark duration-16:8 and culture temperature of 24 $\pm 1^{o}$C. Results revealed that all the microalgal species had approximately 2 days lag period and reached the exponential phase within 4–6 days in all the media. Microalgae cells achieved stationary phase within 15 days and after that cells growth was very slow as shown in figures 4.2. As each microalgal species shows different biochemical composition (e.g., proteins, amino acids, carbohydrates, lipids, fatty acids, chlorophyll and carotenoids) in different media, reflected by different optical density (OD) of samples, biomass dry weight (DW) does not correlate well with OD for different culture media or strains (Loureno et al., 1997). To avoid this error, actual biomass concentration dry weight (DW) was used to study microalgae growth in different media. DW biomass concentration of all the five microalgae species at the stationary phase are shown in figure 4.3. It was examined that highest biomass concentration of *Chlorella vulgaris* was in BG-11 (1.64 g/L), followed by BBM (1.58 g/L), Fog's medium (1.33 g/L) and M_4N (1.30 g/L). Similarly, *Chlorella minutissima* (1.52 g/L) and *Chlorella sp.*1 (1.39 g/L) also showed

maximum biomass concentration in BG-11. But, in case of *Chlorella pyrenoidosa* and *Chlorella sp. 2*, biomass concentration was maximum (1.69 g/L and 1.48 g/L respectively) in Fog's medium. This can be explained by the fact that higher nitrogen concentration is favorable for increasing biomass growth (Li et al., 2008). However, M_4N (having maximum nitrogen concentration) showed poor biomass concentration which is due to deleterious effect of nitrogen at higher concentrations (Li et al., 2008). Lipid content was reported as a percentage of lipids to biomass dry weight. Similar to biomass yield, significant variances in lipid content were found across strains and culture media. Lipid contents and productivity of all the five strains in different culture media are shown in figure 4.4 and 4.5 respectively. Maximum lipid content and productivity was found in BBM media for all the *Chlorella species*. Highest lipid content of 15.87% was found for *Chlorella sp.1*cultivated in BBM, followed by *Chlorella vulgaris* (15.57%), *Chlorella minutissima* (12.95%), *Chlorella pyrenoidosa* (11.43%) and *Chlorella sp.2* (9.64%) in BBM medium. Minimum lipid content was observed in M_4N growth medium. Furthermore, all the microalgae species showed highest lipid productivity in BBM growth media and lowest in M_4N medium. It was observed from above result that with increasing of nitrogen concentration in growth medium, biomass productivity of microalgae increased, while lipid yield decreased. These data are supported by the fact that nitrogen deficient condition favors more lipid accumulation (Converti et al., 2009; Feng et al., 2011; Kumar et al. 2012; Li et al., 2008; Yeh et al., 2011). Among all the tested species, maximum lipid productivity was observed for *Chlorella vulgaris* (12.35 g/L/d), followed by *Chlorella pyrenoidosa (9.56* g/L/d*), Chlorella minutissima* (9.41 g/L/d), *Chlorella sp.1* (9.56 g/L/d) and *Chlorella sp.2* (6.39 g/L/d).

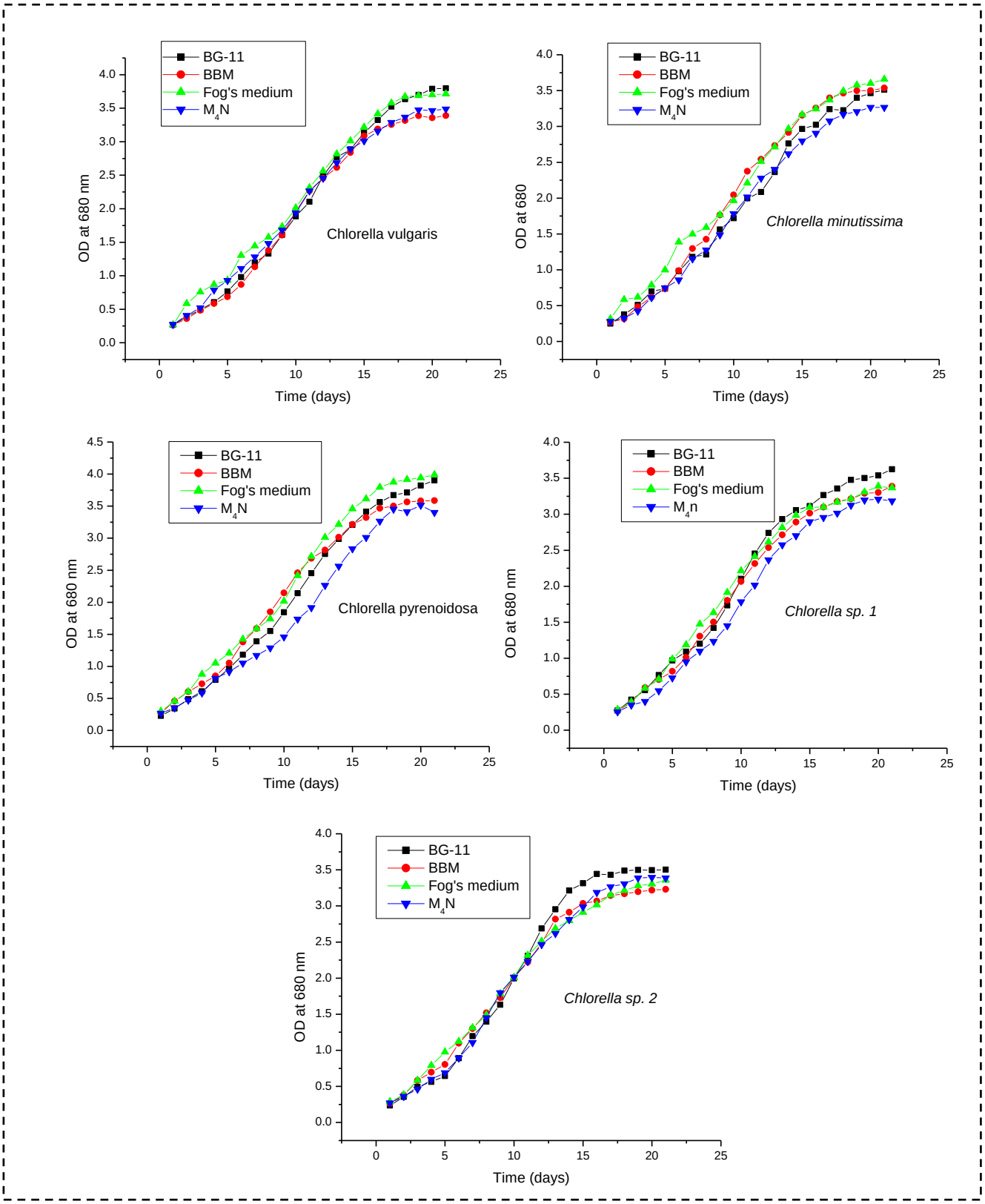


Figure 4.2 Growth rates of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp. 2* in different nutrient media

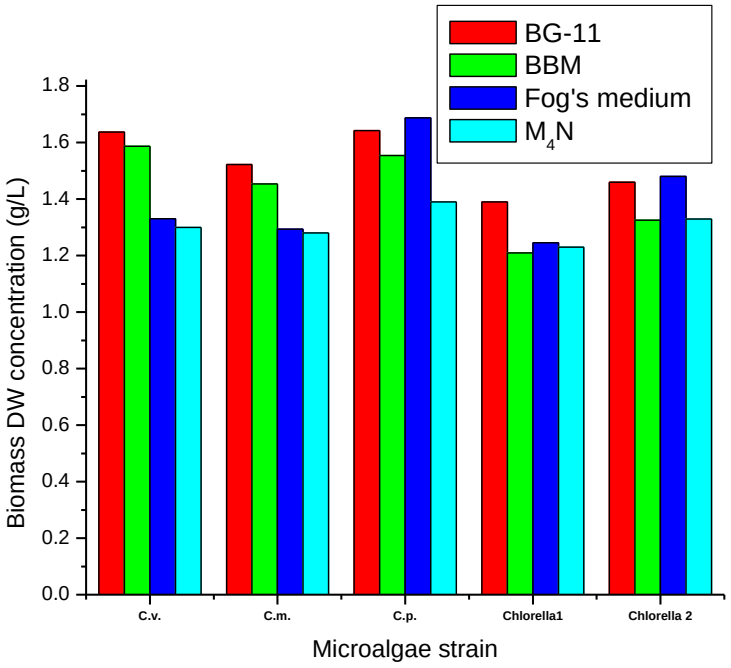


Figure 4.3 Comparison of biomass DW of five microalgal strains in four culture media. Cv=*Chlorella vulgaris*, Cm= Chlorella minutissima, Cp= *Chlorella pyrenoidosa*, Chlorella 1=Chlorella sp. 1and Chlorella 2=Chlorella sp. 2

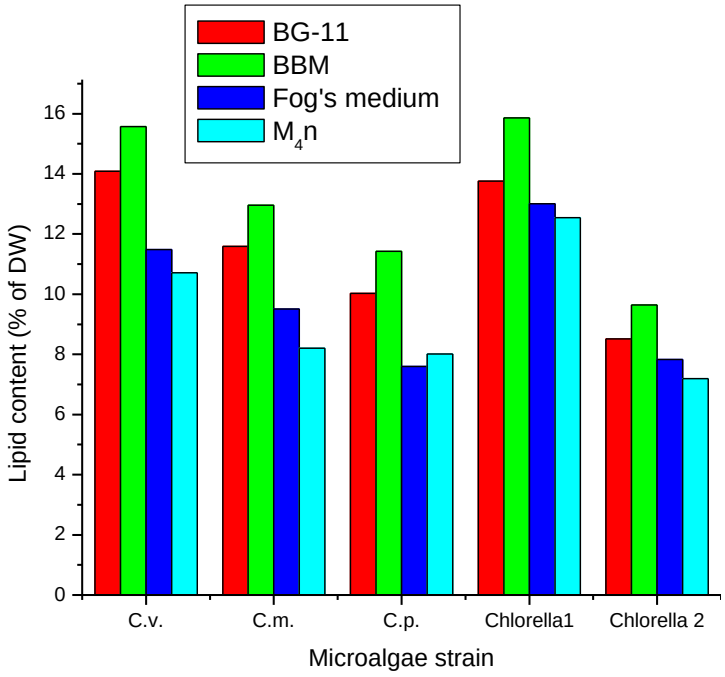


Figure 4.4 Comparison of lipid content of five microalgal strains in four culture media. Cv=*Chlorella vulgaris*, Cm= *Chlorella minutissima*, Cp= *Chlorella pyrenoidosa*, Chlorella 1=*Chlorella sp. 1*and Chlorella 2 = *Chlorella sp. 2*

Selection of culture medium depends upon various factors viz. target product, growth rate, and medium cost. However, nitrogen is the key factor in growth medium and also a limiting nutrient affecting the biomass growth and lipid productivity of various microalgae (Griffiths et al., 2009). Culture media comparison showed that on an average BBM gave highest lipid content and productivity, followed by BG-11, whereas Fog's medium and M_4N had the lowest. Generally, lipid content increases when microalgae are subjected to unfavorable culture conditions, such as nutrient starvation (Converti et al., 2009; Courchesne et al., 2009; Feng et al. 2011; Kumar et al., 2012; Li. Et al., 2008; Yeh et al., 2011). According to Li et al. (2008), lipid yield of microalgae species can be improved by at least 300% under nitrogen limited conditions. Since, lipid production is the main aim of microalgae cultivation in this study, BBM and BG-11 are the preferred growth media. BBM had higher lipid content and productivity due to relatively lower nitrogen and phosphate concentrations, whereas, BG-11 being nitrogen rich medium has good potential to increase lipid productivity by applying nitrogen deficient condition. Furthermore, cost of one liter medium preparation for BBM and BG-11 was Rs. 7.71 and Rs. 4.58 respectively. When applying nitrogen deficient media (suppose half concentration of nitrogen in case of BG-11) to culture media, the cost of BG-11 preparation reduced up to Rs. 4.47 and lipid productivity was increased. On the other hand, BBM is already nitrogen deficient medium and further decrease in nitrogen concentration will result in reduction of total lipid productivity. Therefore, on the basis of potential of growth medium to enhance lipid productivity and cost reduction, BG-11 has been considered as the best medium in this study and used as culture medium for further study.

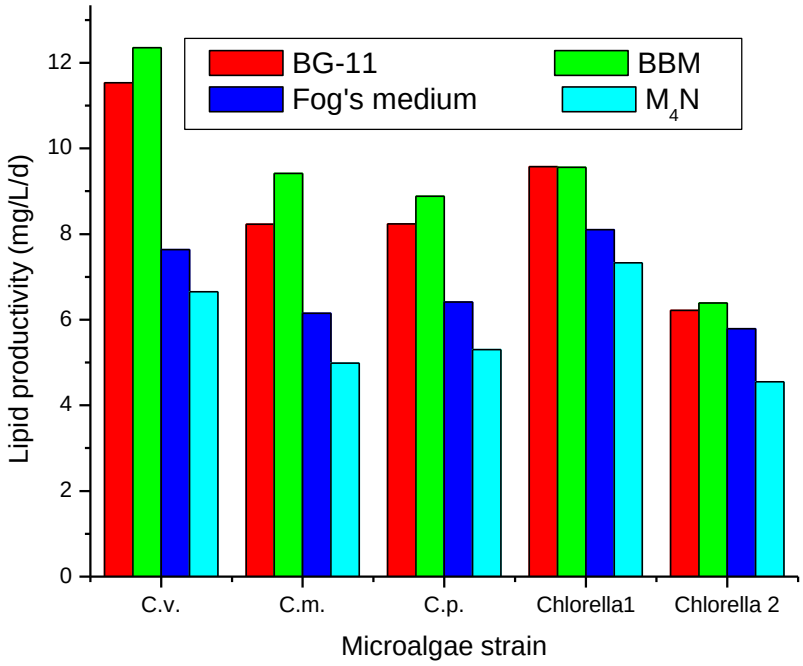


Figure 4.5 Comparison of lipid productivity of five microalgal strains in four culture media. Cv = *Chlorella vulgaris*, Cm= *Chlorella minutissima*, Cp = *Chlorella pyrenoidosa*, Chlorella 1=*Chlorella sp.*1and Chlorella 2=*Chlorella sp. 2*

4.2.3. Optimization of culture condition for microalgae growth with high lipid accumulation

Microalgal biomass has good potential as feedstock for liquid biofuels like biodiesel and bioethanol. The major technical challenge for commercial production is its low productivity. The biomass and lipid productivity of microalgae is highly influenced by different growth media and cultivation conditions. Nitrogen and carbon are the most important elements for microalgae since they are the major components in many biological macromolecules like proteins, lipids, chlorophyll, DNA, etc. and affect biomass and lipid production. Hence, this investigation was carried out to optimize the effect of nitrogen concentration, nitrogen sources and organic carbon sources on biomass growth and lipid productivity of all the five microalgal species.

a.) Effect of nitrogen concentration on microalgae growth and lipid accumulation

In the present study, effect of different nitrogen concentrations on growth and lipid yields of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.*1 and *Chlorella sp.*2 were investigated at room temperature. Microalgae species were grown in BG-11 media for 14 days. Figure 4.6 shows biomass concentration of all the five *Chlorella* species with time at different nitrogen concentrations. The experimental results revealed that the biomass concentration increased with increasing nitrogen concentration and the highest biomass production was found to be 1.43g/L, 1.65 g/L, 1.48 g/L, 1.44 g/L and 1.39 g/L for *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.1* and *Chlorella sp.2* respectively in nitrogen rich culture medium. When nitrogen concentration was decreased up to 2.21 mM, biomass concentration was reduced to 1.0 g/L, 1.12 g/L, 1.03 g/L, 0.99 g/L, and 0.88 g/L while biomass productivity was decreased to 57.09 g/L/d, 67.41 g/L/d, 58.82 g/L/d, 55.83 g/L/d, and 46.17 g/L/d for *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp.2* respectively (table 4.1). Furthermore, Maximum lipid content was recorded as 21.81%, 15.95%, 14.22%, 20.12% and 13.25% at nitrogen concentration of 2.21mM for all the five *Chlorella* species i.e. *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.1* and *Chlorella sp.2* respectively. It was observed that lipid content of all the microalgae species was inversely proportional to nitrogen concentration and decreased with increasing nitrogen concentration. Effect of nitrogen concentration of microalgae lipid accumulation is shown in figure 4.7. This was consistent with the finding that lower nitrogen concentration leads to high lipid production (Dayananda et al., 2007; Illman et al., 2000; Li et al., 2008; Singh et al., 2015).

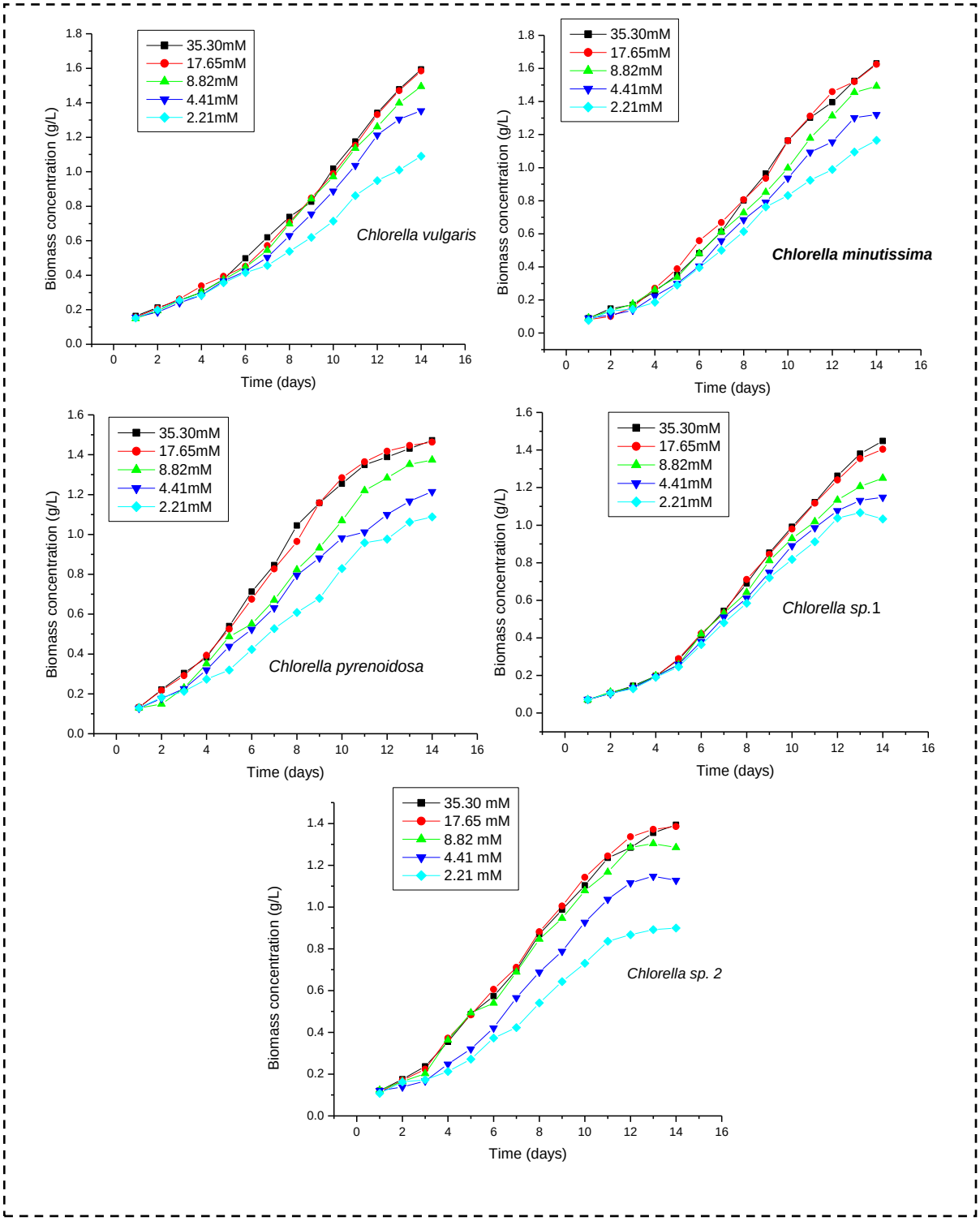


Figure 4.6 Biomass growths of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp. 2* under different nitrogen concentrations

This can be explained by the fact that some microalgae alter their lipid synthesis pathway under stress condition and accumulate more lipids to sustain adverse conditions (Sharma et al., 2012). Nitrogen is essential for protein synthesis. During nitrogen deficient condition, total growth and cell division impairs. During initial phase of nitrogen starvation medium, chlorophyll and protein content of the cells is sufficient and remains unchanged. As a result, carbon dioxide is continuously fixed in photosynthesis process and leads to more carbohydrate production, whereas, no change takes place in fatty acid (FA) content of microalgal cells during early growth stage. At latter point of time, the amount of FAs gradually rises during the growth period. But the *de novo* FA synthesis is not due to newly photosynthetically fixed carbon, since photosynthetic pigments and enzymes, important for carbon fixation are drained at this particular growth period, they are synthesized at the expense of already fixed carbon, which leads to decrease in the total amount of fixed carbon and increase in total FA content (Mandotra et al., 2014; Msanne et al. 2012). Maximum lipid yield and productivity was 223.91 mg/L and 13.98 mg/L/d for *Chlorella vulgaris*, 153.41 mg/L and 9.44 mg/L/d for *Chlorella pyrenoidosa*, and 133.71 mg/L and 7.74 mg/L/d for *Chlorella sp*.2 at 8.82 mM nitrogen concentration, while *Chlorella minutissima* (193.58 mg/L and 11.99 mg/L/d) and *Chlorella sp*.1 (220.35 mg/L and 13.0 mg/L/d) achieved higher lipid yield and productivity at 4.41mM (figure 4.8). This observation is in agreement with previous reports by Li et al. (2008), who examined the influence of nitrogen concentrations between 3 mM and 20 mM $NaNO_3$ on lipid productivity in *N. oleoabundans* and found best lipid yield at 5 mM sodium nitrate concentration. Similarly Hsieh and Wu (2009) reported maximum lipid productivity (124 mg/L/d) in a marine strain of *Chlorella* under nitrogen limiting condition.

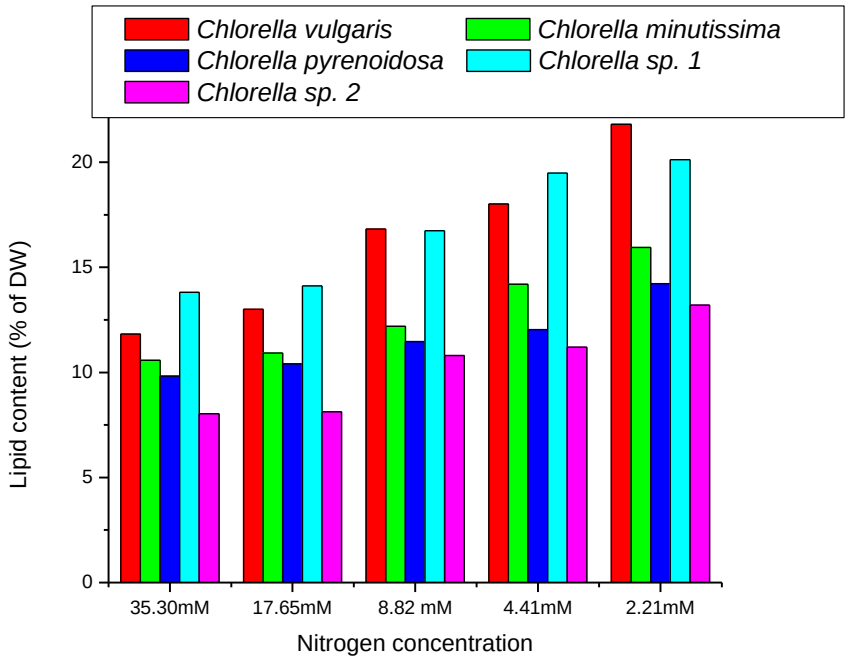


Figure 4.7 Lipid contents of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp. 2* under different nitrogen concentration

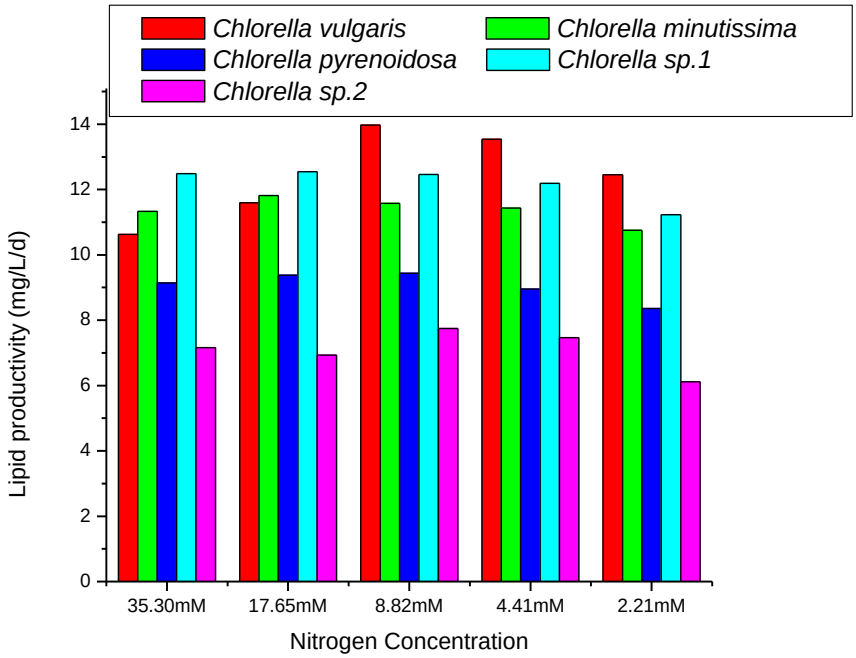


Figure 4.8 Lipid productivities of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp. 2* under different nitrogen concentration

The comparison of lipid productivity under different nitrogen sources shows that 8.82 mM nitrate concentration gave highest lipid productivity on an average for all the strains.

The nitrogen source of BG-11 medium is nitrate which makes the culture medium more expensive. One solution for overcoming the high cost of algal cultivation is to replace the nitrogen source of BG-11 medium with low cost nitrogen source such as agricultural fertilizer (urea or diammonium phosphate). Therefore, effect of different nitrogen sources (sodium nitrate, urea, potassium nitrate, diammonium phosphate) on algae growth and lipid productivity was investigated, but initial concentration of nitrogen content in each source was kept constant (0.123g/l) which is discussed in the next section.

b.) Effect of nitrogen sources on microalgae growth and lipid accumulation

To investigate the effect of nitrogen source on biomass growth and lipid accumulation of microalgae species, *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.*1 and *chlorella sp.*2 were grown in BG-11 having different nitrogen sources i.e. 8.82 mM $NaNO_3$, 8.81 mM KNO_3, 4.41 mM urea and 5.24 mM DAP for 14 days but initial nitrogen concentration (8.8mM) of each nitrogen source was kept same during the experiment. The effect of nitrogen sources on biomass and lipid production is shown in figure 4.9 and 4.10. Results revealed that *Chlorella vulgaris* (1.42 g/L and 88.78 g/L/d), *Chlorella sp.*1 (1.29 g/L and 79.30 g/L/d) and *Chlorella sp.*2 (1.31 g/L and 77.91 g/L/d) showed higher biomass concentration and productivity in urea culture medium while *Chlorella minutissima* (1.40 g/L and 88.04 g/L/d) and *Chlorella pyrenoidosa* (1.44 g/L, 92.59 g/L/d) achieved highest biomass concentration in culture medium supplemented with sodium nitrate. On the other hand, biomass concentration and productivity of all the microalgal species was minimum when DAP was supplemented as nitrogen source.

Microalgae species	Nitrogen concentration/growth parameters	35.30 mM	17.65 mM	8.82 mM	4.41 mM	2.21 mM
Chlorella vulgaris	Biomass concentration (g/L)	1.43 ± 0.07	1.42 ± 0.06	1.33 ± 0.02	1.23 ± 0.05	1.0 ± 0.06
	Biomass productivity (g/L/d)	88.87 ± 4.07	89.09 ± 3.56	83.09 ± 1.43	75.12 ± 3.36	57.09 ± 4.57
	Lipid yield (mg/L)	169.54 ± 7.42	184.34 ± 7.08	223.91 ± 3.29	222.90 ± 8.58	218.07 ± 10.18
Chlorella minutissima	Biomass concentration (g/L)	1.65 ± 0.03	1.64 ± 0.08	1.51 ± 0.06	1.36 ± 0.16	1.12 ± 0.06
	Biomass productivity (g/L/d)	107.10 ± 2.47	106.66 ± 5.32	96.07 ± 3.81	84.48 ± 10.60	67.41 ± 5.24
	Lipid yield (mg/L)	175.24 ± 2.85	179.91 ± 7.49	185.13 ± 6.17	193.58 ± 19.12	180.17 ± 7.89
Chlorella pyrenoidosa	Biomass concentration (g/L)	1.48 ± 0.10	1.44 ± 0.04	1.34 ± 0.15	1.23 ± 0.19	1.03 ± 0.14
	Biomass productivity (g/L/d)	93.07 ± 9.28	90.19 ± 1.81	82.29 ± 8.97	74.40 ± 11.61	58.82 ± 8.56
	Lipid yield (mg/L)	145.42 ± 10.68	150.23 ± 3.41	153.41 ± 14.41	147.56 ± 19.54	146.70 ± 16.78
Chlorella sp.1	Biomass concentration (g/L)	1.44 ± 0.05	1.41 ± 0.02	1.23 ± 0.03	1.13 ± 0.03	0.98 ± 0.09
	Biomass productivity (g/L/d)	90.44 ± 3.62	88.24 ± 1.98	74.50 ± 2.14	66.66 ± 2.41	55.84 ± 5.64
	Lipid yield (mg/L)	198.55 ± 6.24	198.69 ± 2.82	206.30 ± 4.51	220.35 ± 6.46	198.76 ± 15.30
Chlorella sp.2	Biomass concentration (g/L)	1.39 ± 0.06	1.38 ± 0.10	1.21 ± 0.09	1.13 ± 0.21	0.88 ± 0.13
	Biomass productivity (g/L/d)	81.84 ± 1.41	81.67 ± 7.94	70.13 ± 4.72	65.06 ± 11.92	46.17 ± 6.14
	Lipid yield (mg/L)	121.40 ± 4.20	117.10 ± 7.10	133.71 ± 7.85	129.93± 0.06	116.99 ± 4.60

Table 4.1 Growth parameters of *Chlorella vulgaris, Chlorella minutissima, Chlorella pyrenoidosa, Chlorella sp. 1* and *Chlorella sp. 2* under different nitrogen concentration

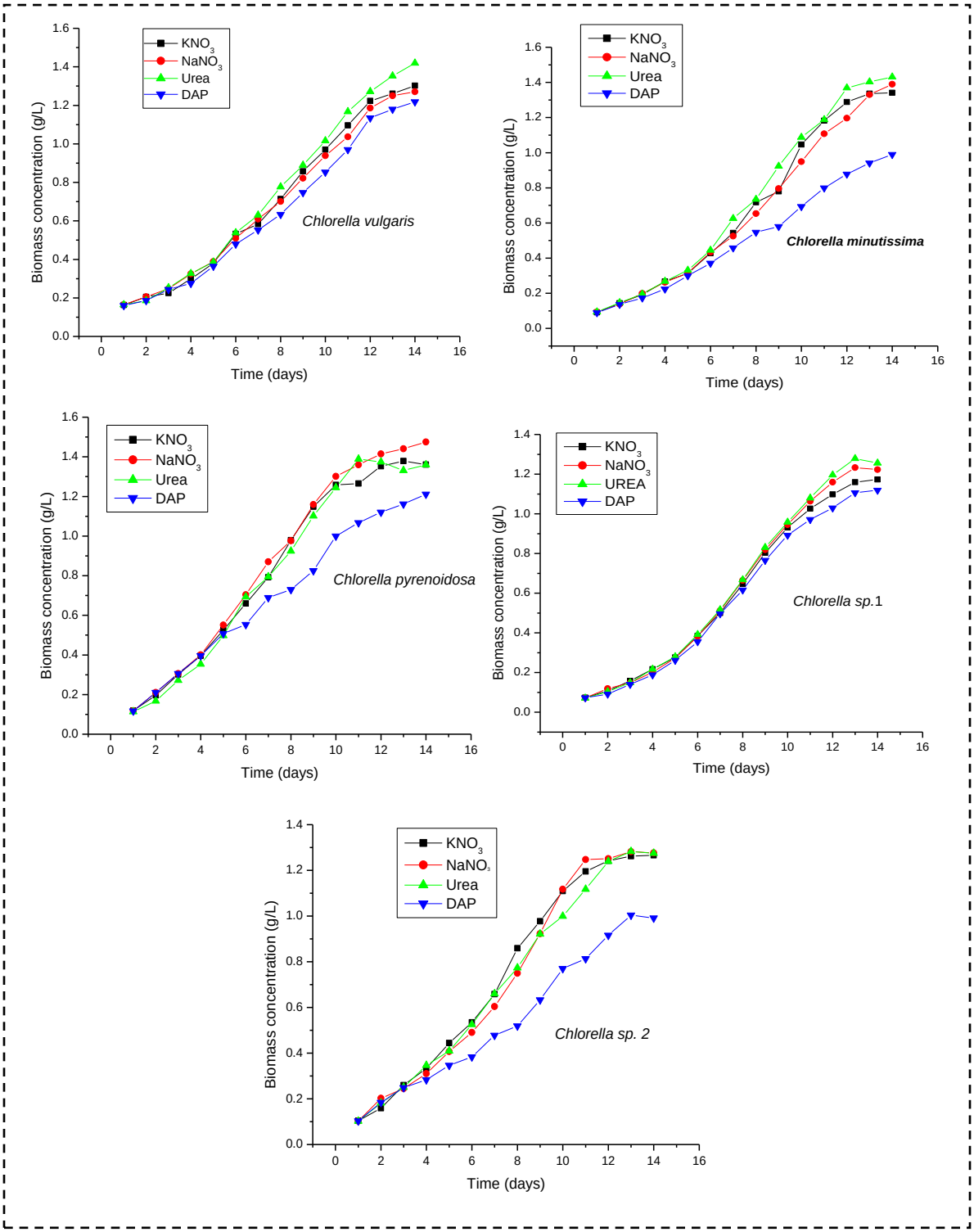


Figure 4.9 Biomass growths of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp. 2* under different nitrogen sources

The study is also supported by various studies which advocate that various nitrogen sources have significant effect on growth of different microalgae species (Arumugam et al., 2012; Campos et. al., 2014; Dyananda et al.2006; Fidalgo, 1998; Hsieh et al., 2009; Hulatt et al., 2012; Lin et al., 2011; Li et al., 2008).

Maximum lipid content was recorded as 16.61% for *Chlorella vulgaris*, 12.66 % for *Chlorella minutissima*, 12.73 % for *Chlorella pyrenoidosa* and 16.88% for *Chlorella sp*.1 in the culture medium supplemented with $NaNO_3$ as nitrogen source while *Chlorella sp.2* shows maximum lipid (11.84 %) in KNO_3 (figure 4.10). Minimum lipid and biomass content was found in case of DAP. Moreover, the lipid yield and productivity was maximum for *Chlorella vulgaris* (220.61mg /L and 14.66 mg/L/d) and *Chlorella sp*.1 (207.47 mg/L and 12.71 mg/L/d) in urea, followed by *Chlorella pyrenoidosa* (183.90 mg/L and 11.79 mg/L/d) *and Chlorella minutissima* (175.69 mg/L and 11.02 mg/L/d) in sodium nitrate and *Chlorella sp.2* (149.65 mg/L and 8.87 mg/L/d) in KNO_3 culture medium (figure 4.11 and table 4.2). DAP showed minimum lipid productivity for all the microalgae strains. This could be due to the fact that microalgal cells are unable to control passive diffusion of ammonia, which is in equilibrium with ammonium ions, across the plasma membrane. At high concentrations, this will reduce tricarboxylic acid (TCA) cycle intermediates, thereby disturbing cellular respiration (Cai et al., 2013; Chen et al., 2011; Ramanna et al., 2014). In case of nitrate utilization by microalgae cells, it first reduced to nitrite by nitrate reductase enzyme and latter to ammonium with the help of nitrite reductase enzyme. The reduction of nitrite to ammonia is an energy consuming process. Therefore, microalgae desire to utilize reduced form of nitrogen e.g. ammonium.

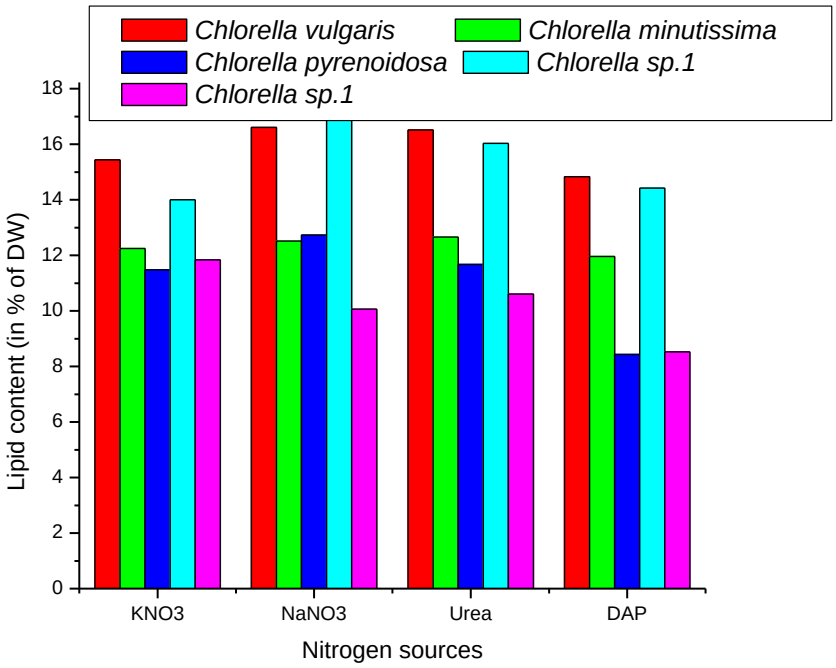


Figure 4.10 Lipid contents *of Chlorella vulgaris, Chlorella minutissima, Chlorella pyrenoidosa, Chlorella sp.* 1 and *Chlorella sp.* 2 under different nitrogen sources

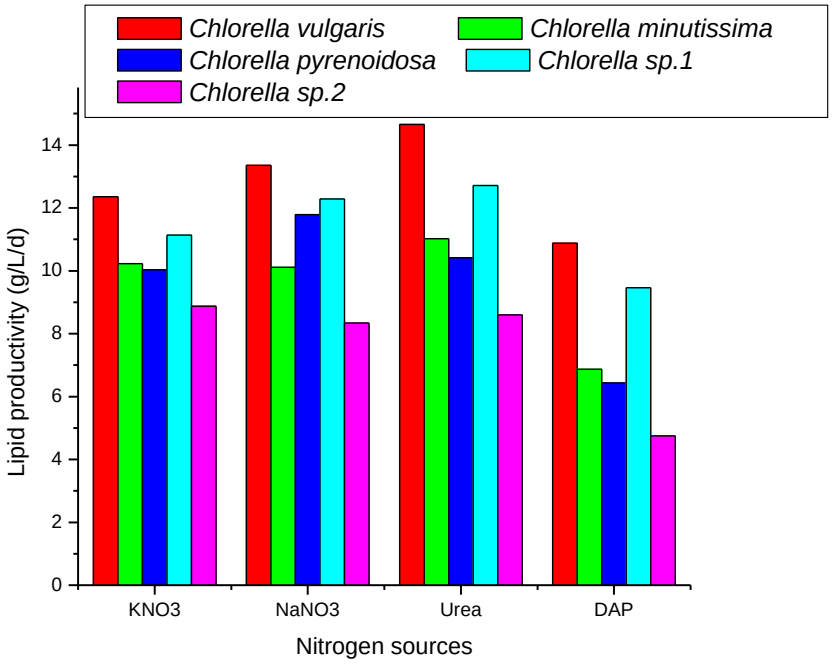


Figure 4.11 Lipid productivities of *Chlorella vulgaris, Chlorella minutissima, Chlorella pyrenoidosa, Chlorella sp. 1* and *Chlorella sp. 2* under different nitrogen sources

During urea assimilation in microalgal cells, it first dissociates to form carbon dioxide and ammonium ion via urea amido hydrolase pathway (urease enzyme). Then, this ammonium is directly absorbed into cell and accumulates to form amino acids, useful in the formation of chlorophylls, which are essential for the photosynthetic process (Kim et al., 2013; Wijanarko, 2011). It was also found that addition of carbon dioxide is responsible for increasing biomass yields. This could be the possible reason for achieving highest biomass concentration and lipid accumulation of some microalgal strains when urea was supplemented as nitrogen source.

This study concludes that sodium nitrate should be replaced by urea as nitrogen source in BG-11 culture medium for economic biodiesel production at large scale as on an average 23.41 mole of urea is sufficient to replace 45.90 mole of sodium nitrate to produce same amount of lipid for all the five *Chlorella* strains. Furthermore, urea is commercially available and cheaper nitrogen source than sodium nitrate and used in agriculture as fertilizer. Because of its universal availability and affordability, it has a great potential to replace sodium nitrate (nitrogen source used in BG-11 media) for economic biodiesel production. This fact is also supported by the work of Hsieh et al. (2009) and Wijanarko (2011) where it is reported that supplementation of nitrogen sources such as urea in microalgae culture medium made biomass production more economical for industrial application purposes.

c.) Effect of carbon sources on microalgae growth and lipid accumulation

In this study, all the *Chlorella species* were grown in optimized BG-11 medium (as optimized in the above section of this chapter) with different organic carbon sources at room temperature and 16:8 light: dark cycle.

Microalgae species	Nitrogen Sources/growth parameters	NaNO3	KNO3	Urea	DAP
Chlorella vulgaris	Biomass concentration (g/L)	1.31 ± 0.05	1.30 ± 0.12	1.42 ± 0.10	1.21 ± 0.09
	Biomass productivity (g/L/d)	80.46 ± 2.06	80.05 ± 6.59	88.75 ± 4.80	73.37 ± 4.63
	Lipid yield (mg/L)	217.31 ± 7.26	200.62 ± 5.71	233.93 ± 13.26	179.95 ± 10.31
Chlorella minutissima	Biomass concentration (g/L)	1.40 ± 0.09	1.32 ± 0.20	1.34 ± 0.16	1.01 ± 0.10
	Biomass productivity (g/L/d)	88.05 ± 5.66	80.80 ± 12.84	82.51 ± 9.51	57.47 ± 5.65
	Lipid yield (mg/L)	175.69 ± 9.32	167.24 ± 12.17	164.72 ± 9.14	121.29 ± 9.70
Chlorella pyrenoidosa	Biomass concentration (g/L)	1.44 ± 0.15	1.37 ± 0.03	1.40 ± 0.04	1.24 ± 0.12
	Biomass productivity (g/L/d)	92.59 ± 9.37	87.44 ± 2.47	89.31 ± 1.99	77.18 ± 7.80
	Lipid yield (mg/L)	183.10 ± 15.97	156.61 ± 3.05	163.38 ± 3.90	104.33 ± 8.15
Chlorella sp.1	Biomass concentration (g/L)	1.22 ± 0.10	1.20 ± .13	1.29 ± 0.04	1.13 ± 0.09
	Biomass productivity (g/L/d)	72.84 ± 8.01	70.89 ± 5.64	79.30 ± 2.21	65.60 ± 5.49
	Lipid yield (mg/L))	206.06 ± 18.28	187.99 ± 11.34	207.47 ± 5.05	162.51 ± 2.72
***Chlorella sp.* 2**	Biomass concentration (g/L)	1.29 ± 0.12	1.26 ± .05	1.31 ± 0.13	1.19 ± 0.16
	Biomass productivity (g/L/d)	75.99 ± 7.71	74.92 ± 3.32	77.91 ± 8.05	55.96 ± 9.10
	Lipid yield (mg/L)	141.22 ± 10.64	149.65 ± 4.89	144.37 ± 11.50	86.73 ± 8.62

Table 4.2 Growth parameters of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp. 2* under different nitrogen sources

Among the tested organic carbon sources, glucose was found to be the best carbon source for mixotrophic growth of all the *Chlorella* strains, followed by glycerol, sodium acetate and sucrose. The study showed that addition of glucose results in maximum biomass production of 2.08 g/L, 2.04 g/L, 1.84 g/L, 1.53 g/L and 1.43 g/L for *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.*1 and *Chlorella sp.*2 at stationary phase (table 4.3 and figure 4.12). However, all the microalgae species achieved maximum biomass concentration within 8-11 days in the culture medium supplemented with glucose and the average highest biomass productivity were found 274.46 mg/L/day for *Chlorella vulgaris*, followed by 239.15 mg/L/day for *Chlorella minutissima*, 191.28 mg/L/day for *Chlorella pyrenoidosa*, 162.52 mg/L/day for *Chlorella sp.*1 and 111.36 mg/L/day for *Chlorella sp.*1 respectively (table 4.3). Biomass concentration of all the microalgae stains was poor in sucrose culture medium. These results are in agreement with previous report that glucose is an efficient trigger to increase biomass productivity of microalgae (Cheirsilp et al., 2012; Garcia et al., 2006; Sun et al., 2014). This can be explained by the fact that glucose is the raw material for photosynthesis and under mixotrophic growth, it can be utilized in presence of light for energy metabolism for ATP and NAD(P)H production, and therefore biomass growth can be accelerated (Yang et al., 2012).

On the other hand, lipid contents of four *Chlorella* strains (*Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.*1) were significantly higher in culture medium supplemented with glycerol as carbon source, followed by sodium acetate, glucose, and sucrose organic carbon sources, while *Chlorella sp.*2 showed maximum lipid content in sodium acetate culture medium. The effect of carbon sources on biomass production and lipid content is shown in figure 4.12 and 4.13.

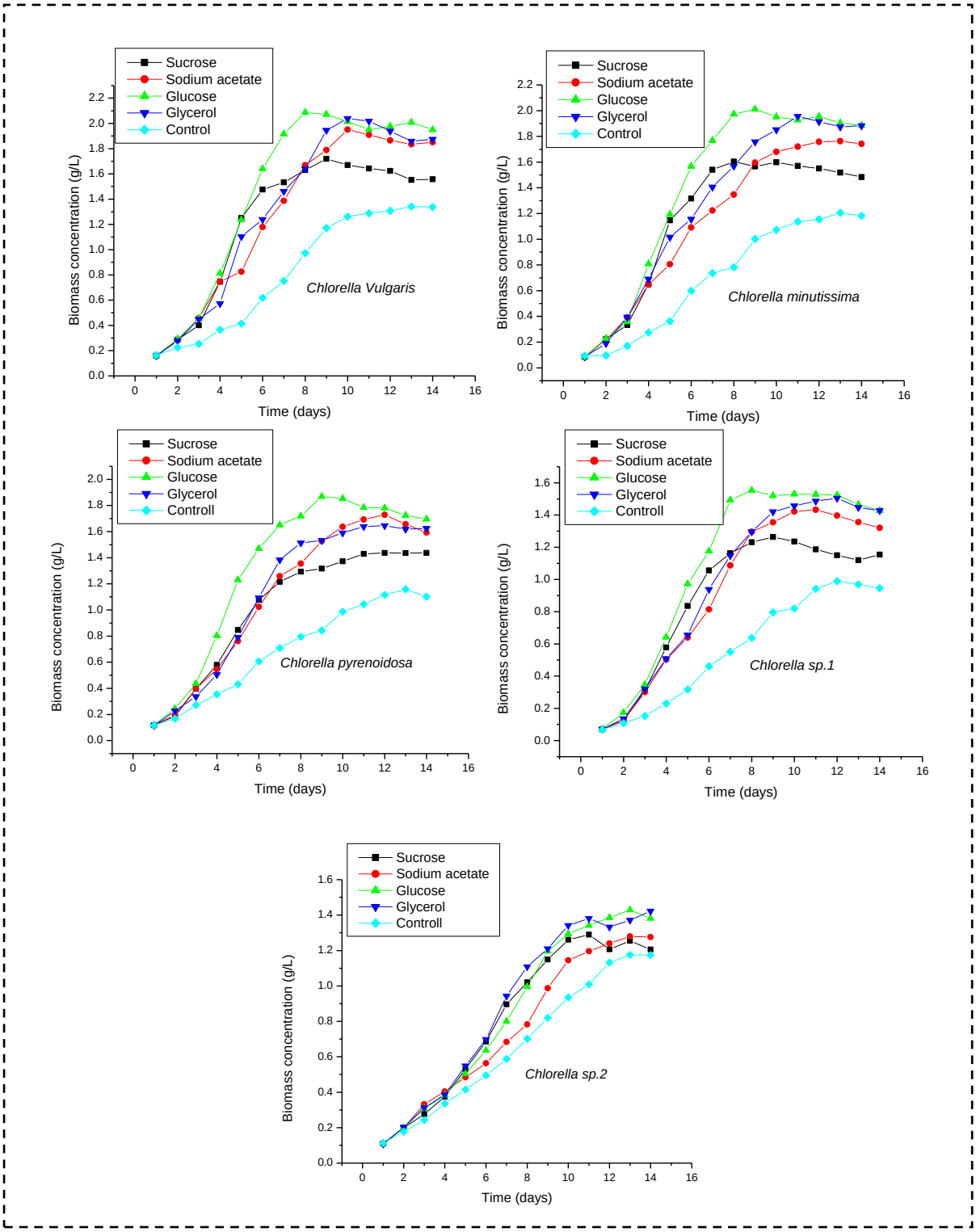


Figure 4.12 Biomass growths of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp. 2* under different carbon sources

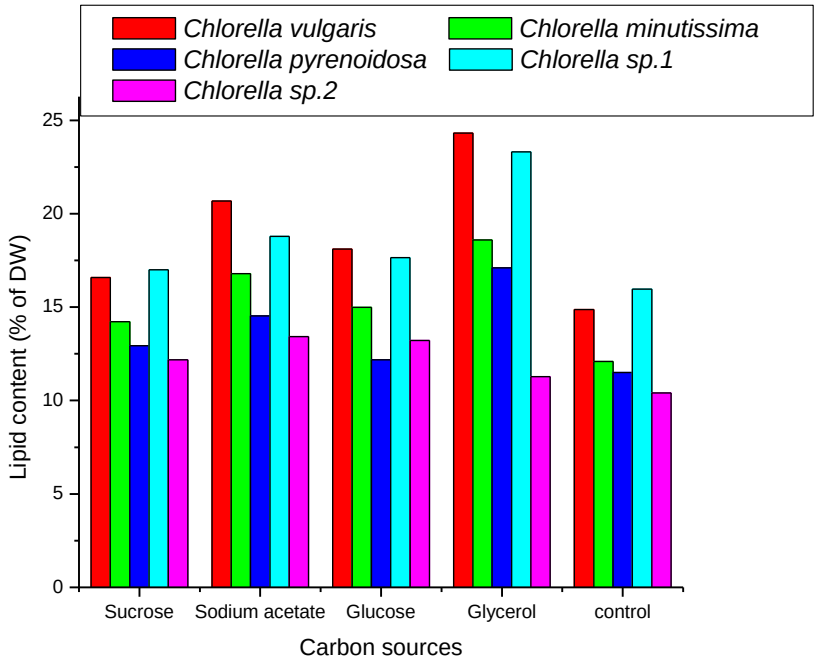


Figure 4.13 Lipid contents of *Chlorella vulgaris, Chlorella minutissima, Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp. 2* under different carbon sources

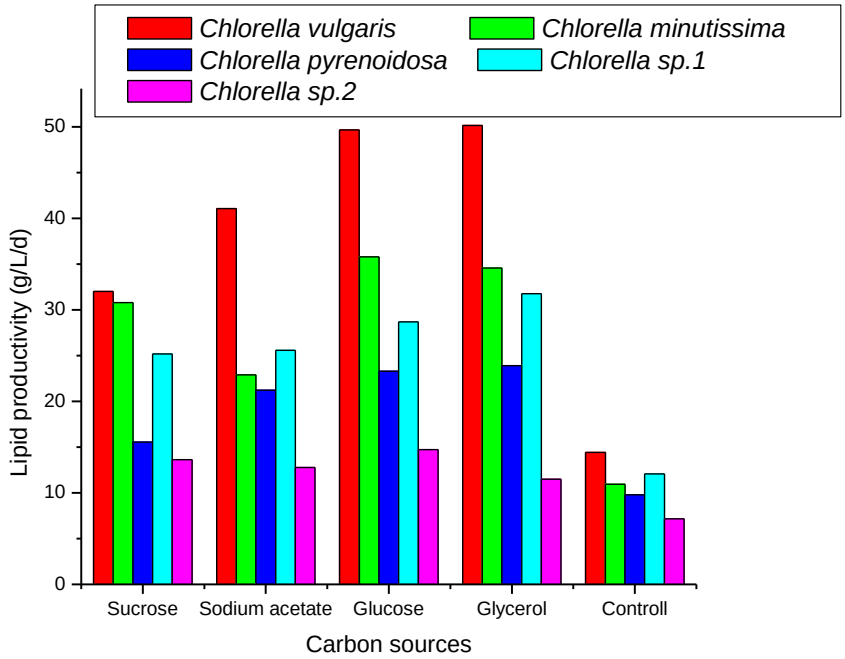


Figure 4.14 Lipid productivities of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp. 2* under different carbon sources

. Maximum lipid production was 24.32% for *Chlorella vulgaris*, 18.59% for *Chlorella minutissima*, 17.10% for *Chlorella pyrenoidosa* and 23.31% for *Chlorella sp.1* in glycerol culture medium while 13.42% *for Chlorella sp.2 in sodium acetate* (figure 4.13). Lipid production was found lowest in case of sucrose carbon source for all the tested microalgal strains except *Chlorella sp.2* which showed exceptional behavior and minimum lipid content of this species was in glycerol. Maximum lipid yield observed for the tested microalgal strains was 490.74 mg/L for *Chlorella vulgaris*, 369.13mg/L for *Chlorella minutissima*, 282.59 mg/L for *Chlorella pyrenoidosa*, and 325.4 mg/L for *Chlorella sp.1* in glycerol culture medium which was more than two folds higher in comparison to control culture (phototrophic culture) for all these species. On the other hand, *Chlorella sp.2* had shown highest lipid yield (188.58 mg/L) in glucose. This can be explained by the fact that glucose is a simple hexose monosaccharide, which is first catabolized into glucose-6-phosphate (important intermediate product for various metabolic precursors) and subsequently to pyruvate through anaerobic glycolysis process, then it enters into tricarboxylic acid cycle (TCA cycle) followed by mitochondrial oxidative phosphorylation for ATPs production (Droop, 1974; Neilson et al., 1974). On the other hand, glycerol enters the cells by simple diffusion without any extra energy, and does not behave as a source of carbons for biosynthesis (Garcia et al. 2011 and Aubert et al. 1994). Pentose phosphate pathway (PPP) is inhibited during glycerol assimilation (Chen et al., 2012). More glycerol would be used in the Embden−Meyerhof pathway (EMP), and then utilized to generate lipids. Furthermore, glycerol is a substrate for triacylglycerol (TAG) synthesis. Overall, it was found that different biomass and lipid productions of all the five microalgae species were due to their different metabolic pathways of carbon and energy sources.

Microalgae species	Nitrogen Sources/growth parameters	Sucrose	Sodium acetate	Glucose	Glycerol	Control medium *
Chlorella vulgaris	Biomass concentration (g/L)	1.71 ± 0.05	1.95 ± 0.10	2.08 ± 0.10	2.02 ± 0.06	1.33 ± 0.12
	Biomass productivity (g/L/d)	193.00 ± 5.26	198.57 ± 8.95	274.46 ± 11.77	206.29 ± 5.64	96.95 ± 7.78
	Lipid yield (mg/L)	283.12 ± 7.04	402.86 ± 16.58	377.05 ± 15.02	490.74 ± 12.30	197.36 ± 14.20
Chlorella minutissima	Biomass concentration (g/L)	1.64 ± 0.11	1.76 ± 0.07	2.04 ± 0.05	1.99 ± 0.10	1.21 ± 0.05
	Biomass productivity (g/L/d)	216.66 ± 12.73	136.44 ± 5.00	239.15 ± 4.69	186.09 ± 8.45	90.63 ± 3.51
	Lipid yield (mg/L)	233.49 ± 12.65	295.85 ± 9.90	305.53 ± 5.64	369.13 ± 15.63	146.75 ± 5.13
Chlorella pyrenoidosa	Biomass concentration (g/L)	1.44 ± 0.06	1.73 ± 0.07	1.84 ± 0.12	1.65 ± 0.05	1.14± 0 .10
	Biomass productivity (g/L/d)	120.34 ± 4.77	146.04 ± 4.92	191.28 ± 10.66	139.87 ± 3.56	85.21 ± 6.50
	Lipid yield (mg/L)	186.39 ± 6.57	250.85 ± 7.72	224.41 ± 11.86	282.59 ± 6.82	131.51 ± 9.18
***Chlorella sp.*1**	Biomass concentration (g/L)	1.26 ± 0.04	1.43 ± 0.11	1.53 ± 0.12	1.44 ± 0.07	0.96 ± 0.09
	Biomass productivity (g/L/d)	148.21 ± 4.18	136.14 ± 8.74	162.52 ± 11.01	124.30 ± 5.26	80.44 ± 6.68
	Lipid yield (mg/L)	213.77 ± 5.50	269.15 ± 16.31	270.83 ± 17.31	325.24 ± 13.40	152.52 ± 11.73
***Chlorella sp.* 2**	Biomass concentration (g/L)	1.27 ± 0.07	1.28 ± 0 .11	1.43 ± 0.10	1.42 ± 0.12	1.18 ± 0.10
	Biomass productivity (g/L/d)	111.85 ± 4.51	95.28 ± 5.63	111.36 ± 4.06	101.94 ± 6.36	90.64 ± 8.46
	Lipid yield (mg/L)	154.89 ± 6.72	171.18 ± 12.25	188.58 ± 10.63	160.66 ± 10.82	103.54 ± 7.01

Table 4.3 Growth parameters of *Chlorella vulgaris, Chlorella minutissima, Chlorella pyrenoidosa, Chlorella sp. 1* and *Chlorella sp. 2* under different carbon sources

Lipid productivity of *Chlorella vulgaris* (50.17 mg/L/d), *Chlorella pyrenoidosa* (23.91mg/L/d) and *Chlorella sp.* 1 (28.69 mg/L/d) was observed best in glycerol medium whereas *Chlorella minutissima* (35.8 mg/L/d) and *Chlorella sp. 2* (14.73 mg/L/d) showed maximum lipid productivity in glucose supplemented culture medium (figure 4.14). But higher cost of glucose is a major hurdle to microalgae biodiesel production economics. Additionally, glycerol is much cheaper than glucose and also a byproduct of biodiesel production; so this study also provided a novel alternative for recycling this crude glycerol. Therefore glycerol is more promising candidate to make algae biodiesel economically viable.

However, all the microalgae strains were successfully grown in the presence of sucrose, sodium acetate, glucose and glycerol with minimum contamination under indoor conditions. Among the tested *Chlorella* strains, *Chlorella vulgaris* achieved maximum lipid content and productivity in glycerol, 1.6 and 3.5 folds higher than control condition. In addition, to reduce costs of microalgal biodiesel production, mass cultivation of microalgae should be carried out under freely available sunlight (Feng et al., 2011; Oh et al., 2010). Hence, the feasibility of cultivating microalgae under outdoor conditions was tested in bubble column photobioreactor and flat plate photobioreactor medium which are discussed in the next section (4.2.4 and 4.2.5) of this chapter.

4.2.4. Cultivation of microalgae species in bubble column photobioreactor under outdoor conditions

The conditions normally required for outdoor mass culture for microalgal lipid production include resistance to contamination, tolerance to a wide range of environmental conditions (such as temperature and solar radiation changes), rapid CO_2 uptake and tolerance to shear force. Moreover, all the lipid-rich microalgae cannot be cultivated under

outdoor conditions in certain area. For example, microalgae *Tetraselmis suecica CS-187* and *Chlorella sp.* were successfully grown for a long-term outdoor cultivation in Victoria, Australia, but *Dunaliella tertiolecta* CS-175 failed to be scaled up even after many trials (Moheimani , 2012). Therefore, to evaluate the feasibility of mass cultivation under outdoor condition in Dehradun, Uttrakhand, all five microalgae strains (*Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.* 1 and *Chlorella sp.* 2) were tried to culture in 10 liter capacity bubble column photobioreactor.

Microalgae strains grew successfully in optimized BG-11 media with glycerol (identified most suitable carbon source for enhancing lipid productivity) as carbon source (mixotrophic growth) under indoor conditions, but when the same cultivation system was employed to outdoor conditions, all the microalgae strains showed poor growth rate due to attack of bacterial, fungal and other biological contamination as glycerol promotes the growth of biological contamination. It was also observed from literature that the risk of contamination in microalgae cultivation system is more in outdoor conditions than indoor conditions (Moheimani et al., 2006). It needs autoclaved or membrane filtered sterile medium and closed environment for photobioreactor or fermenter to utilize sunlight with specific efforts which makes it more expensive. Therefore, outdoor cultivation of microalgae was carried out in 10 liter capacity bubble column photobioreactor under phototrophic conditions. Aeration in bubble column reactor was set at a flow rate of 4 liter/minute. 1% CO_2 in air was supplied to each photobioreactor during the daytime. Optimized BG-11 media (section of 4.2.3 b of this chapter) was used for microalgae growth. The medium was prepared using hypo-treated tap water. A long lag period was observed for all the microalgae species and biomass increased very slowly in first five days. Microalgae cells at such low concentration received too much light energy due to the

lack of self-shading which leads to photoinhibition process. After lag phase, all the microalgae species grew rapidly.

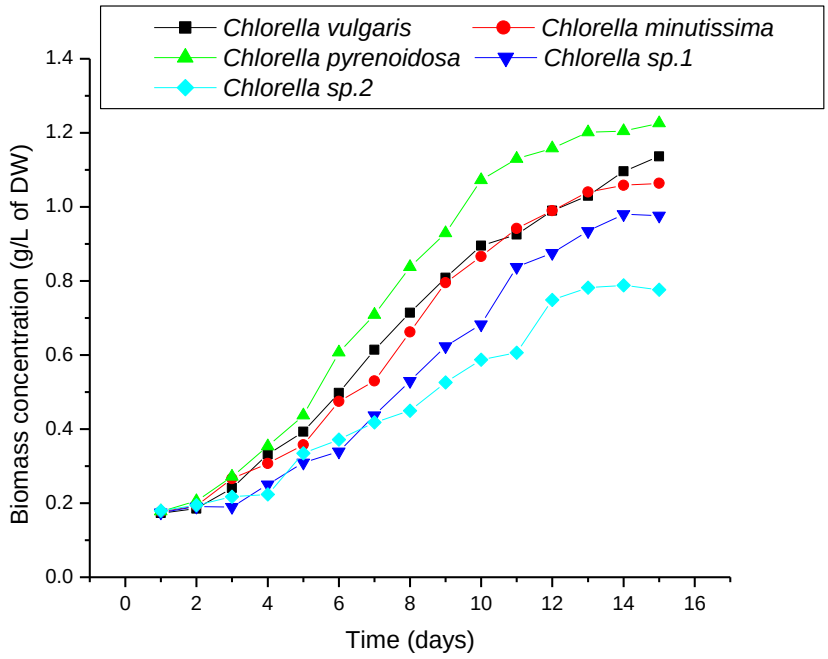


Figure 4.15 Biomass growths of *Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp. 1* and *Chlorella sp. 2* under outdoor conditions

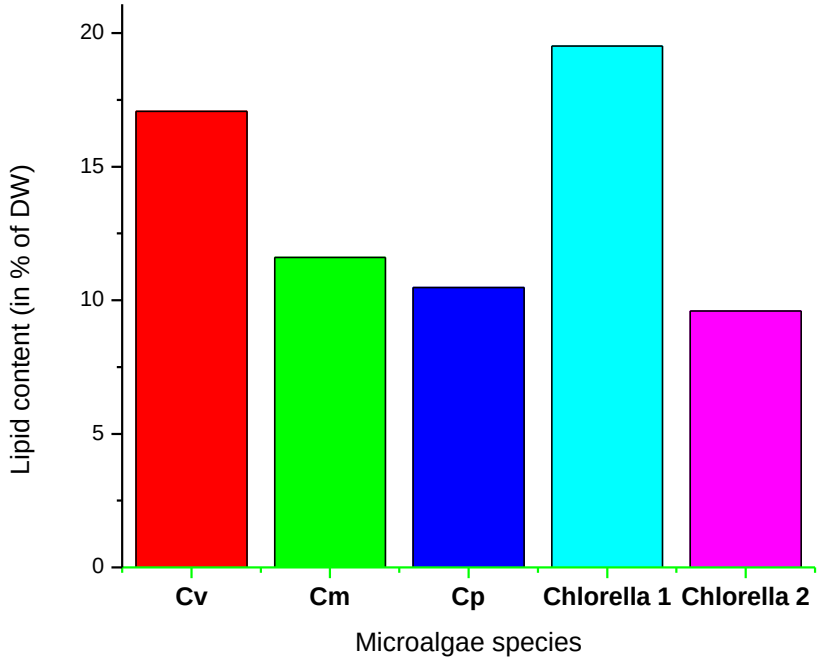


Figure 4.16 Comparison of lipid content of five microalgal strains in outdoor conditions Cv=*Chlorella vulgaris*, Cm= *Chlorella minutissima*, Cp= *Chlorella pyrenoidosa*, Chlorella 1=*Chlorella sp. 1* and Chlorella 2=*Chlorella sp.* 2

S.No.	Microalgae species	Biomass concentration (g/L)	Biomass productivity (g/L/d)	Lipid yield (g/L)	Lipid productivity (g/L/d)
1	*Chlorella vulgaris*	1.13 ± 0.04	64.32 ± 2.66	193.54± 7.12	10.94 ± 0.84
2	*Chlorella minutissima*	1.08 ± 0.06	60.24 ± 4.26	125.07 ± 7.37	7.39 ± 0.4
3	*Chlorella pyrenoidosa*	1.22 ± 0.08	69.94 ± 5.65	128.55 ± 8.80	7.33± 0.97
4	*Chlorella sp. 1*	0.95 ± 0.07	53.16 ± 5.43	190.23 ± 15.43	10.33± 0.95
5	*Chlorella sp.2*	0.75 ± 0.06	38.72 ± 4.25	72.69 ± 6.02	3.76 ± 0.97

Figure 4.4 Growth parameters of microalgae species grown in outdoor conditions

. The growth parameters and lipid contents of all the five *Chlorella* species are shown in figure 4.15, 4.16 and table 4.4. Among the tested species, *Chlorella pyrenoidosa* showed maximum biomass productivity 69.94 mg/L/d, followed by *Chlorella vulgaris* (64.32 mg/L/d), *Chlorella minutissima* (60.24 mg/L/d), *Chlorella sp.1* (51.64 mg/L/d) and *Chlorella sp. 2* (38.72 mg/L/d). However, as shown in table 4.4, lipid yield obtained for *Chlorella vulgaris* (193.54 mg/L/d) was highest among all the tested *Chlorella* strains while *Chlorella sp.2* (97.18mg/L/d) was found with lowest lipid yield. Lipid content and lipid productivity of all the microalgae species are shown in figure 4.16 and 4.17. Lipid content was found maximum in *Chlorella sp.2* (19.51%) and minimum in *Chlorella sp.2* (9.60%). However, *Chlorella sp.1* had shown lower lipid productivity (10.07 mg/L/d) than *Chlorella Vulgaris* microalgae species (10.97mg/L/d). Moreover, lipid productivity was lower for all the microalgae species under outdoor conditions in comparison to indoor

conditions. The unstable environmental conditions (temperature and light intensity) outdoors were the important factors that affected biomass and lipid productivity in this study. In addition, contamination by bacteria, fungus, protozoa and other algae in outdoor cultivation can negatively affect the growth of microalgae leading to low biomass productivity (Moheimani et al., 2006). But the contamination by bacteria and fungi was found minimum during all the experiments in this study.

However, lipid productivity was higher for *Chlorella vulgaris*, but the growth rate and lipid content, as shown in figure 4.15 and figure 4.16, was higher for *Chlorella pyrenoidosa* and *Chlorella sp. 2* respectively in comparison to *Chlorella vulgaris*. Therefore, a study with mixed cultures was carried out to see the effect on lipid productivity. Pure culture of Chlorella *vulgaris* was mixed with other three species (*Chlorella minutissima*, *Chlorella pyrenoidosa*, and *Chlorella sp.1*) in 1:1 ratio and grown in bubble column photobioreactor. *Chlorella sp.2* was not cultivated as mixed culture with *Chlorella vulgaris* because it already had very poor lipid productivity.

The growth parameters of pure *Chlorella vulgaris* and its mixed cultures such as mixed culture-1 (*Chlorella vulgaris*: *Chlorella minutissima*), mixed culture-2 (*Chlorella vul*garis: *Chlorella pyrenoidosa*) and mixed culture-3 (*Chlorella vulgaris*: *Chlorella sp.2*) grown under outdoor condition are shown in figure 4.17 and table 4.5 respectively. The average biomass productivities of mixed culture were 90.12 mg/L/d (mixed culture 1), 94.89 mg/L/d (mixed culture 2), and 77.74 mg/L/d (mixed culture 3) in comparison to pure culture of *Chlorella vulgaris* (79.28 mg/l/d). Lipid content was maximum of Chlorella vulgaris (16.76%), followed by mixed culture 3 (16.72%), mixed culture 1 (13%), and mixed culture 2 (11.96%). Whereas, there was no improvement in lipid productivity and it was 2.53%, 7.33% and 18.88% lesser for mixed culture 1, mixed culture 2 and mixed

culture 3 respectively than pure culture of *Chlorella vulgaris*. The possible reason behind this may be that *Chlorella minutissima* and *Chlorella pyrenoidosa* became dominant over *Chlorella vulgaris* in case of mixed culture 1 and mixed culture 2, due to which biomass growth rate was increased and lipid productivity decreased. In case of mixed culture 3 there was no significant effect on biomass growth and lipid productivity.

It was observed from above studies that among the tested species (pure culture or mixed cultures), *Chlorella vulgaris* had the highest potential for biodiesel production under outdoor conditions. Therefore, pure culture of *Chlorella vulgaris* was selected for mass cultivation in 60 liter Flat plate photobioreactor (FPP) and open raceway ponds.

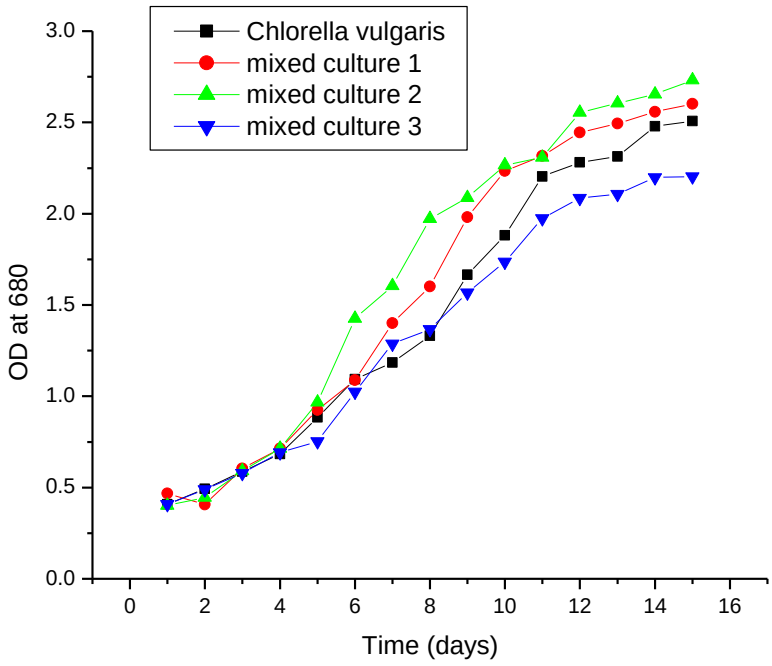


Figure 4.17 Biomass growths of mixed microalgae cultures
Chlorella vulgaris = pure strain, mixed culture 1= Chlorella vulgaris: Chlorella minutissima (1:1), mixed culture 2= Chlorella vulgaris: Chlorella pyrenoidosa (1:1), mixed culture 3= Chlorella vulgaris :Chlorella sp.1(1:1)

S.no.	Microalgae species	Biomass concentration (g/L)	Biomass productivity (g/L/d)	Lipid yield (g/L)	Lipid productivity (g/L/d)
1	*Chlorella vulgaris*	1.18 ± 0.07	79.29 ± 2.15	199.59± 20.86	13.30 ± 1.39
2	Mixed culture 1	1.17 ± 0.02	90.12 ± 1.15	140.30 ± 6.59	10.79 ± 0.50
3	Mixed culture 2	1.23 ± 0.09	94.89 ± 7.29	160.97 ± 23.78	12.38± 1.83
4	Mixed culture 3	1.01 ± 0.10	77.74 ± 6.35	168.60 ± 10.39	12.96± 0.79

Table 4.5 Growth parameters of mixed microalgae cultures
***Chlorella vulgaris* = pure strain, mixed culture 1= *Chlorella vulgaris*: *Chlorella minutissima* (1:1), mixed culture 2= *Chlorella vulgaris*: *Chlorella pyrenoidosa* (1:1), mixed culture 3= *Chlorella vulgaris: Chlorella sp.1* (1:1)**

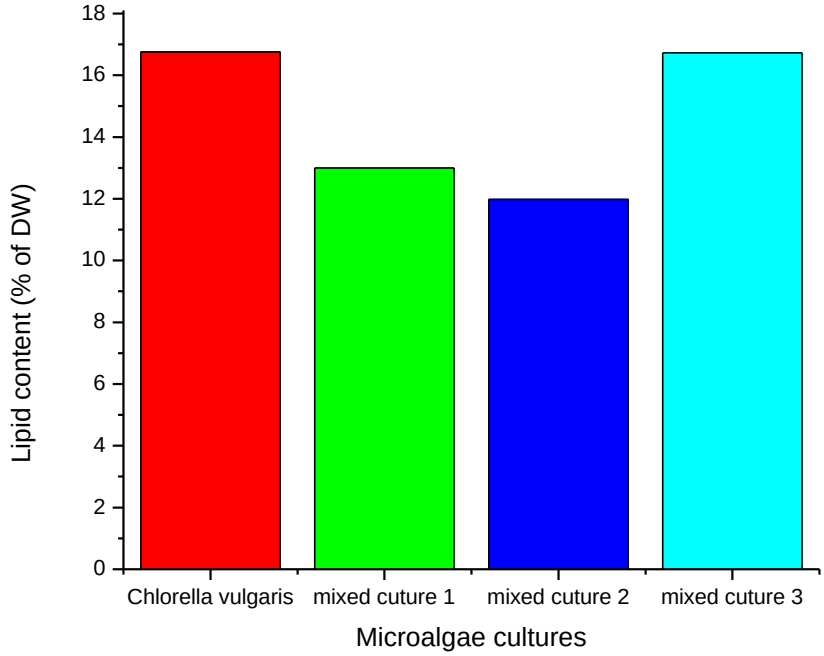


Figure 4.18 Lipid contents of mixed microalgae cultures
Chlorella vulgaris = pure strain, mixed culture 1= Chlorella vulgaris: Chlorella minutissima (1:1), mixed culture 2= Chlorella vulgaris: Chlorella pyrenoidosa (1:1), mixed culture 3= Chlorella vulgaris: Chlorella sp.1(1:1)

Feng et al. (2011) reported that initial cell concentration also greatly influence biomass growth and lipid accumulation under outdoor conditions. Hence, before going to large scale, the effects of initial cell concentrations (0.175g/L, 0.250g/L, 0.300g/L, 0.350g/L) on growth of *Chlorella vulgaris* was also studied. The culture medium was BG-11 with 4.41mM urea (optimized in 4.2.3 b section of this chapter). Growth parameters of *Chlorella vulgaris* are shown in figure 4.19 and 4.20.

However, as shown in Table 4.6, biomass productivity (58.43 mg /L/d) obtained in group A (with lowest initial biomass concentration of 0.172 g/L) was the lowest among all the treatments during the experiments. When initial concentration of microalgae cultivation system was adjusted to 0.250 g/L or above, biomass productivity was observed as 65.58 mg/L/d (at initial biomass concentration of 0.250g/L), 69.27 mg/L/d (at initial biomass concentration of 0.300g/L) and 78.61 mg/L/d (at initial biomass concentration of 0.300g/L), respectively. The cultivations of *Chlorella vulgaris* during the experiments showed that the biomass productivity was directly proportional to initial cell concentration and increased with increasing biomass concentration. While lipid content showed reverse behavior and observed minimum 13.31% at the maximum initial biomass concentration of 0.350 g/L. Maximum lipid (16.65%) was accumulated, when the initial concentration of biomass cultivation system was set at 0.175g/L (figure 4.20). This can be explained by the fact that lower cell concentration allowed each microalgae cell to capture higher light intensity, which supported higher cellular lipid accumulation. This observation is also supported with previous reports by Hu et al. (2008) and Rodolfi et al. (2009), who advocated that lipid storage (mainly triacylglycerol) can be improved by increasing light intensity. Additionally, it is also assumed that the onset of lipid accumulation in microalgae

culture system with higher initial cell concentration was delayed due to remaining intracellular and rudiment nitrogen sources (Feng et al., 2011).

S.No.	Initial culture concentration	Biomass concentration (g/L)	Biomass productivity (g/L/d)	Lipid yield (g/L)	Lipid productivity (g/L/d)
1	0.175g/L	0.99 ± 0.09	58.43 ± 6.92	162.44 ± 13.84	9.73 ± 1.15
2	0.250g/L	1.18 ± 0.05	66.07 ± 3.79	184.59 ± 4.81	10.51 ± 0.60
3	0.300 g/L	1.27 ± 0.10	69.27 ± 7.34	189.45 ± 10.03	10.58± 1.12
4	0.350 g/L	1.44 ± 0.06	78.61 ± 3.44	188.95 ± 4.55	10.46 ± 0.46

Table 4.6 Effect of initial culture concentration on different growth parameters of *Chlorella vulgaris*

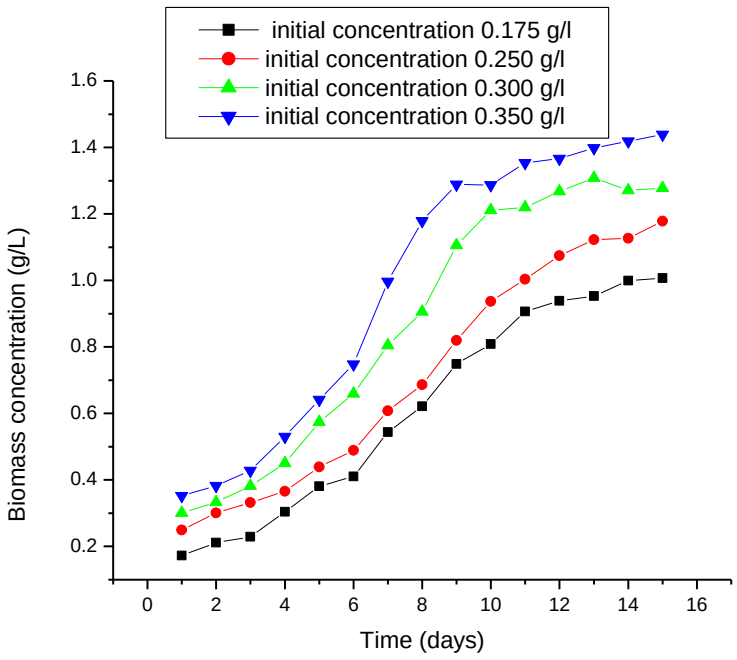


Figure 4.19 Effect of initial concentration on biomass growth of *Chlorella vulgaris*

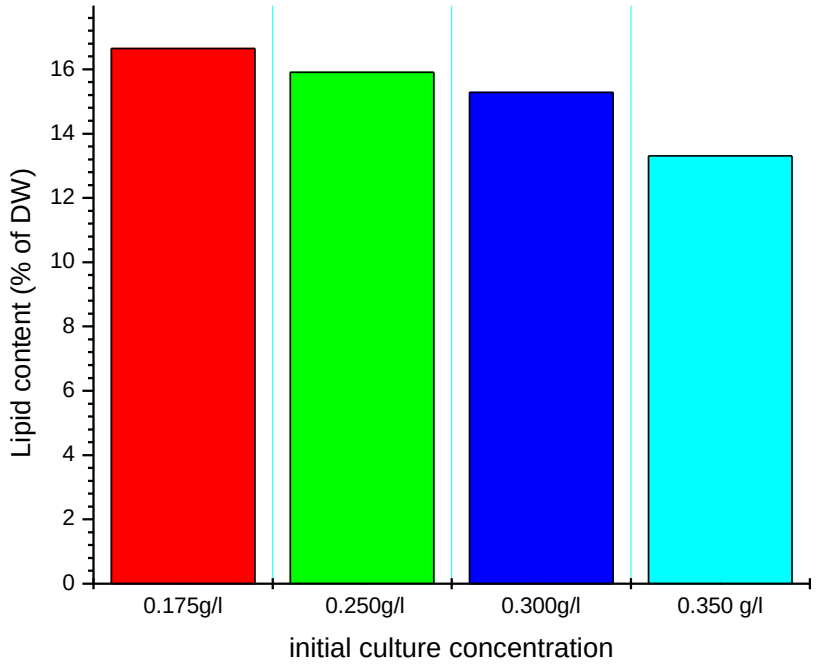


Figure 4.20 Effect of initial concentration on lipid content of *Chlorella vulgaris*

However, lipid productivity of *Chlorella vulgaris* was lowest 9.73 mg/L/d in culture system with initial biomass concentration of 0.172g/L, but maximum lipid productivity (10.58 mg/L/d) was achieved in culture system with initial biomass concentration of 0.300 mg/L/d (table 4.5). Present study indicates that initial concentration of microalgae culture system also affects lipid productivity and *Chlorella vulgaris* grown with initial concentration of 0.300 g/L has the highest potential for biodiesel production.

4.2.5. Cultivation of microalgae species in Flat plate photobioreactor (FPP) under outdoor conditions

To test the feasibility of large-scale cultivation under outdoor conditions, *Chlorella vulgaris* was grown in a 60 L flat plate photobioreactor. The culture medium was BG-11, using 4.41mM urea which was optimized in previous experiment in section 4.2.3 b of this chapter as substitute for $NaNO_3$. The biomass concentration and lipid yield of *Chlorella vulgaris* grown in flat plate photobioreactor is shown in figure 4.21. Microalgal cells grew

well and achieved biomass productivity of 77.84 mg/L/d with 15.96% lipid content in 15 days. However, maximum biomass and lipid productivity (81.64 mg/L/d and 13.51mg/L/d) was achieved at 9 days (figure 4.21). Maximum biomass productivity obtained in current study (81.64 mg/L/d) was higher than the biomass productivity of *Chlorella vulgaris* (40 mg/l/d) and *Chlorella emersonii* (41 mg/L/d) cultured in 230-L pumped tubular photobioreactor under indoors and *Chlorella emersonii* (58 mg/L/d) cultured in 60 liter FPP under outdoors (Feng et al., 2011; Scragg et al., 2002). However, biomass productivity observed in this study was lower than the biomass productivity of *Spirulina platensis* (4300 mg/L/d) reported by Hu et al. (1996). In the current study, light path of the photobioreactor was 15 cm, which could significantly reduce light penetration in the culture, decreasing light availability in the photobioreactor. As a result, photosynthetic efficiency of microalgae cells might be significantly lower (Richmond et al., 2003; Eriksen, 2008). Richmond et al. (2003) found that photosynthetic efficiency of *Nanochloropsis sp.* cultures was nearly doubled when light path length was reduced from 9 cm to 1 cm and biomass concentration increased from 3.9 g/L to 43.5 g/L). Lipid productivity achieved in this study (13.51 mg/L/d) was lower than published data from other microorganisms grown under photoautotrophic cultivation outdoors, such as, 22.3 mg/L/d for *C. emersonii* (Feng et al., 2011), but this can be improved by shortening light path or increasing surface area/volume ratio of photobioreactor, increasing carbon dioxide concentration and applying metabolic and genetic engineering.

An essential aspect of commercially successful microalgae culture is its ability to grow in continuous or semi-continuous mode for long periods (Moheimani et al., 2006), that can optimize the use of capital intensive culture systems and also decreases labor costs. To examine the lipid production potential of *Chlorella vulgaris* under sunlight, cells were

grown outdoors in 60 L Flat Plate Photobioreactor in optimized BG-11 media. The experiments were carried out from March 2014 to May 2014. Effect on biomass growth and lipid yield is shown in figure 4.21.

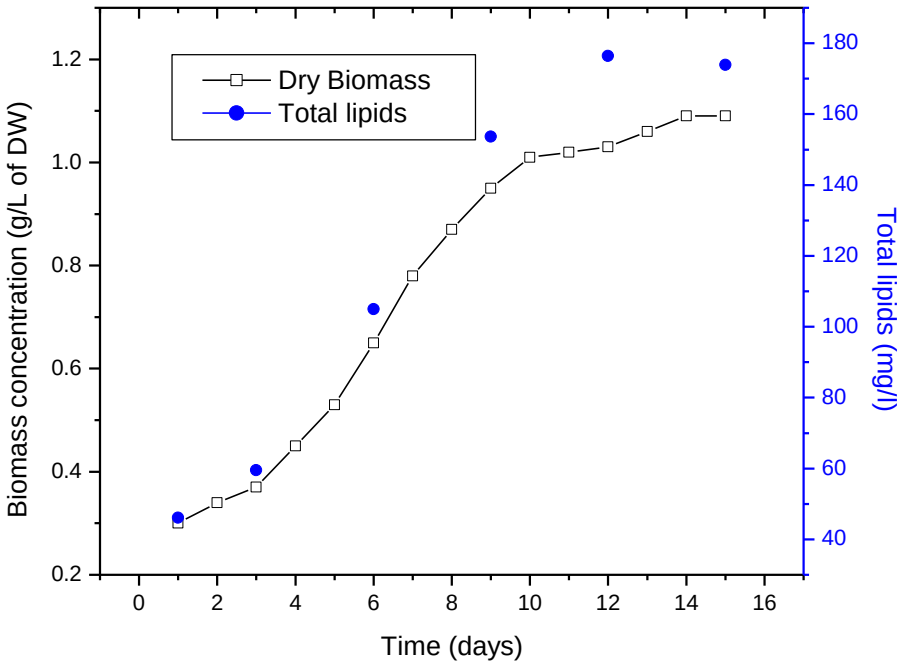
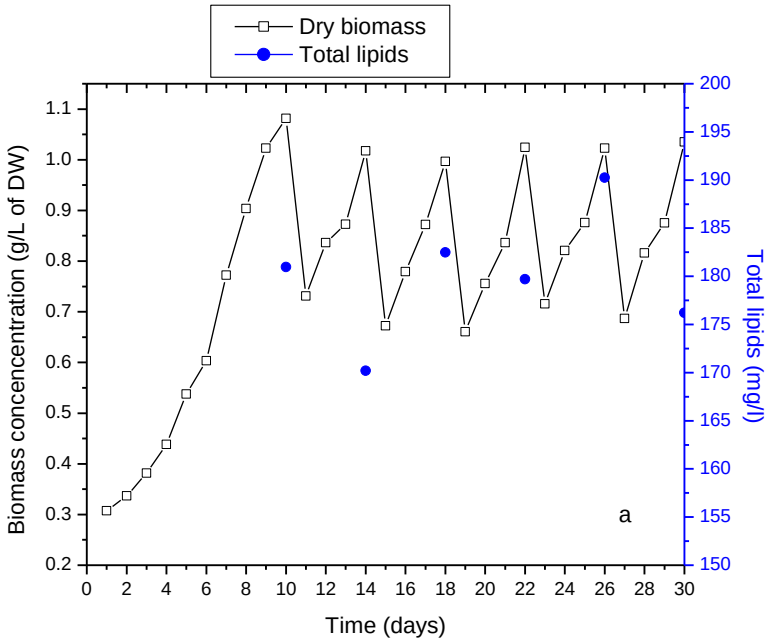
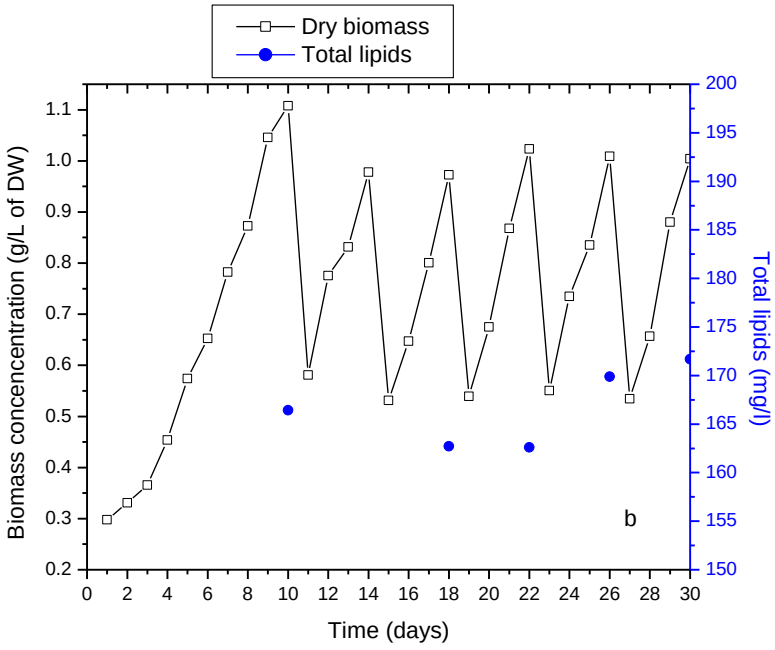


Figure 4.21 Biomass growth rate and total lipid content of microalgae in 60 liter Flat plate photobioreactor

. Microalgae was grown in batch mode for first 10 days and then first harvest was done with one third (growth phase a), half (growth phase b) and two third culture harvesting (growth phase c). A total of 6 harvests were done in each semi-continuous mode. At every harvest, microalgal culture was replaced with same amount of nutrient medium. As shown in figure 4.22, *Chlorella vulgaris* cells grew well in Flat plate photobioreactor in semi-continuous cultivation mode under outdoor conditions. The average biomass productivity was observed 115.21 mg/L/d, 164.37 mg/L/d, 121.65 mg/L/d for growth phase a, b and c respectively (table 4.7). The outcome of this study demonstrates that it is feasible to culture *Chlorella vulgaris* in BG-11 medium for a long period under outdoor conditions. In case of lipid production, it was observed that lipid

content was highest (17.62%) in growth phase 'a' than growth phase 'b' (16.07%) and growth phase 'c' (14.17%). However, lipid productivity was higher in growth phase 'b' (23.92 g/L/d) than growth phase 'a' (20.40mg/L/d) and growth phase c (22.99 mg/L/d) as shown in table 4.7. These results revealed that *Chlorella vulgaris* achieved maximum lipid productivity in growth phase b (when half culture was harvested at 4th day).





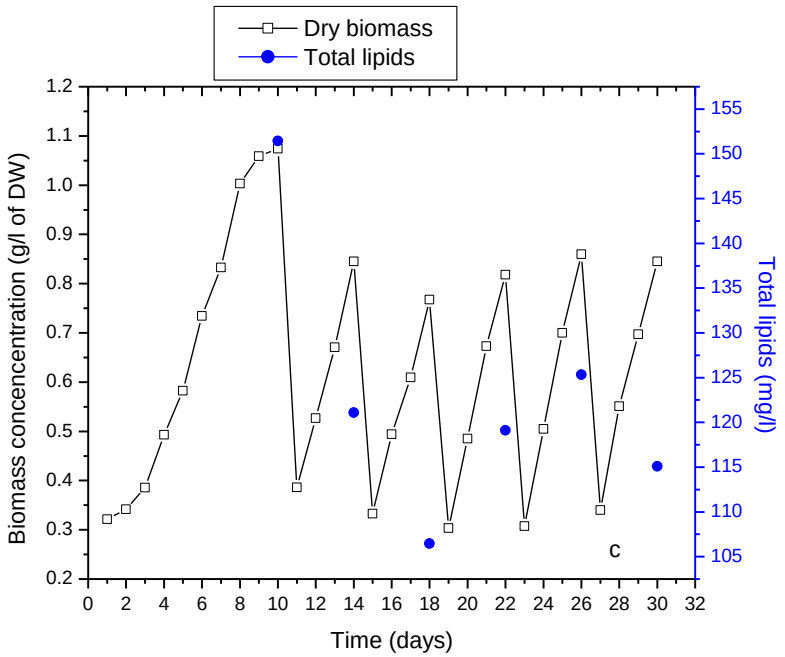


Figure 4.22 Biomass growth and total lipid yield of *Chlorella vulgaris* in three growth phases (a, b and c) of semi-continuous cultivation outdoors, a= one third culture concentration harvesting, b=half culture concentration harvesting, c=two third culture concentration harvesting.

S.No.	Growth phase	Biomass concentration (g/L)	Biomass Productivity (mg/L/d)	Lipid content (%)	Lipid productivity (mg/L/d)
1	a (1/3rd culture harvesting)	1.01± 0.01	115.21± 0.05	17.69 ± 0.73	20.40 ± 1.91
2	b (1/2th culture harvesting)	0.98 ± 0.01	145.93 ± 8.77	16.15± 0.88	23.92 ± 2.37
3	C (2/3rd culture harvesting)	0.827 ± 0.03	164.3 ± 13.84	14.17± 0.38	22.99 ± 1.87

Table 4.7 Growth parameters of *Chlorella vulgaris* in three growth phases (a, b and c) of semi-continuous cultivation outdoors, a= one third culture concentration harvesting, b=half culture concentration harvesting and c=two third culture concentration harvesting. Harvesting time for each growth phase was 4 days.

Although the scale of photobioreactor examined here is far from that of a commercial facility, it seems clear that semi-continuous mass cultivation of *Chlorella vulgaris* is promising for biodiesel production under outdoor conditions.

However, it should be noted that growth and lipid accumulation of *Chlorella vulgaris* in semi-continuous cultivation under outdoor conditions might be influenced by various uncontrollable factors such as geographical variation, circadian rhythms, seasonal changes, weather conditions, bioreactors, scale of cultivation, pollution caused by protozoa, rotifera, other algal species and bacteria etc. (Moheimani et. al., 2006; Rodolfi et al., 2009; Feng et al., 2011).

4.2.6. Comparative analysis of biodiesel derived from *Chlorella vulgaris* grown in Bubble column and Flat plate photobioreactor (FPP) photobioreactor under outdoor conditions

The composition of biodiesel produced from Chlorella vulgaris grown in bubble column and flat plat photobioreactor is shown in figure 4.23.

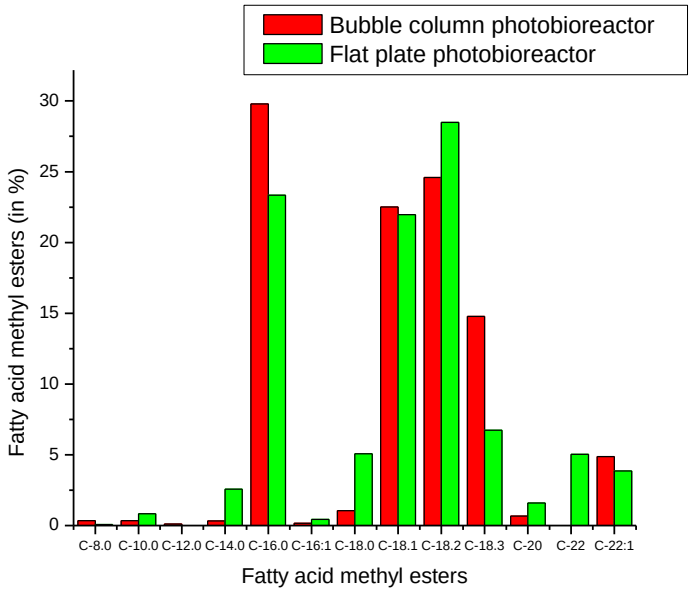


Figure 4.23 Composition of biodiesel derived from *Chlorella vulgaris* grown in bubble column and flat plate photobioreactor

It was observed that biodiesel obtained from Chlorella vulgaris grown in bubble column photobioreactor has 32.63% saturated FAME, 27.54% monounsaturated FAME and

39.36% polyunsaturated FAME in comparison to flat plate photobioreactor grown microalgae biodiesel that have 38.51% saturated FAME, 26.26% monounsaturated FAME and 35.22% polyunsaturated FAME. Physico-chemical properties of biodiesel are highly influenced by its FAMEs composition. Some physico-chemical properties of *Chlorella vulgaris* (grown in both bubble column and FPP) biodiesel are summarized in table 4.8.

Properties*	**Biodiesel produced from microalgae grown in bubble column photobioreactor**	**Biodiesel produced from microalgae grown in Flat plate photobioreactor**
Saponification value	192.21	192.19
Iodine value	103.58	88.36
Viscosity at 40°C, in cSt	4.23	4.55
Density at 15°C (kg/m^3)	0.873	0.875
Calorific value (MJ/Kg)	39.51	39.79
Cetane no.	55.17	60
Oxidation stability.(h)	5.58	5.93

*all the properties are derived on the basis of FAME composition

Table 4.8 Physico-chemical properties of biodiesel produced from microalgae grown in bubble column and flat plate photobioreactor

The presence of more polyunsaturated FAMEs in microalgae biodiesel indicates its poor oxidation stability and good cold flow properties. Bubble column grown microalgae biodiesel have 14.78% of C-18:3 (linolenic acid methyl ester) which does not meet European standards EN14214. According to European standards EN14214, for an ideal biodiesel the percentage of linolenic acid (C18:3) and polyunsaturated FA (>4 double bond) should not increase more than 12% and 1% respectively. Cetane number is the

indication of combustion behavior of fuel in engine. Higher cetane value results in better combustion of fuel lower NOx emission, less knocking and easier start- up of engine. According to ASTM D6751, European (EN 14214) and IS: 15607standards, minimum cetane value of biodiesel should be 47, 51 and 51 respectively. In current study, cetane value of microalgae biodiesel was 55.17 and 60 for microalgae grown in bubble column and flat plate photobioreactor respectively. Iodine value is the measurement of unsaturation of oil. More the number of double bonds in fatty acid chain length more will be iodine value. Higher iodine value of biodiesel may result in the polymerization of glycerides and deposition of lubricant in the engine. Iodine value of biodiesel produced from microalgae grown in bubble column and flat plat photobioreactor was 103.58 and 88.36. Calorific value of microalgae biodiesel grown in FPP was higher than microalgae grown in bubble column photobioreactor (table 4.8). Biodiesel produced from microalgae was more stable in case of FFP than bubble column grown.

4.2.7. Mass cultivation of microalgae species in Open Raceway ponds for biodiesel production

In the recent years, cultivation of microalgae has been widely studied in open raceway ponds than closed photobioreactors for biodiesel application because of its simplicity of operation and construction. Demirbas and Demirbas (2007) suggested that closed systems are capital intensive and its use is vindicated only when high value products are produced. In order to achieve a cost-effective microalgal biodiesel production, it is recommended to culture microalgae in open ponds in which naturally available sunlight can be utilized as energy source (Chisti, 2007). In this context, a concrete open raceway pond (length 5 m; width 2 m; height 0.5 m) having a total working volume of 1200 L was installed at University of Petroleum and Energy studies (UPES), Dehadun, India. Inoculum

preparation (300 L seed culture) was carried out using 10 L bubble column and 60 L flat plate photobioreactor for a period of 6–8 days and then seed culture was added to raceway pond containing 900 L of optimized BG-11 medium. The culture medium was prepared using hypo-treated ground water. This experiment was performed for a period of 21 days at batch mode. To check the purity of the culture, microscopic analysis was carried out daily. The results revealed that total biomass concentration obtained at the end of 21 days of raceway cultivation was 1.41 g/L with a total lipid of 272.35 mg/L (figure 4.24 and 4.25). In addition, daily productivity, specific growth rate, and divisions per day were observed as 0.086 g/L/d or 8.63g/m^2/d, 0.09312 Day^{-1} and 0.134 respectively (table 4.9). Maximum concentration of chlorophyll-a and chlorophyll-b was found as 18.31ml/L and 6.43mg/L at 12^{th} days and after that the concentration of these pigments was decreased with cultivation time. Microalgae composition such as lipids, proteins, and carbohydrates content were 16.65%, 34.33% and 18.24% respectively (figure 4.26). According to Chisti (2013), cost of dry microalgal biomass production should be reduced below Rs.16.20 per dry Kg (0.25$ per dry Kg) to make it an attractive biomass feedstock for biofuel production. In this study, estimated cost of growth medium (optimized BG-11 with urea as nitrogen source) for microalgae biomass production was found to be about Rs. 88.55. However, in case of 0.75g/L sodium nitrate (nitrogen source normally used in BG-11 media), the cost of growth medium was increased up to Rs. 317.16 (4.94$) which is approximately 20 times higher than required. In this study, the cost of growth medium was approximately 5.6 times higher than required which is still very high to make algae biofuels economically competitive with petroleum fuels. Therefore, further research efforts were made to replace BG-11 growth media with commercial fertilizer based growth medium. The algal growth medium was prepared by using commercial fertilizer such as urea 250 mg/L, DAP (Diammonium

phosphate) 250 mg/L, potash (potassium chloride) 250 mg/L, magnesium sulphate 250 mg/L, sodium carbonate 20 mg/L, ferric citrate 6mg/L and micronutrient from BG-11 medium (half strength). The growth medium was prepared by tap water for microalgae cultivation and it was thoroughly treated with sodium hypochlorite. Initial pH of culture medium was adjusted between 7.0-7.5. Results revealed that *Chlorella vulgaris* achieved 0.89g/L biomass and 166.75 mg/L of total lipid after 21 days batch mode cultivation (figure 4.24 and 4.25). However, this medium resulted in lower biomass productivity (up to ~ 47% lesser than optimized BG-11), but total cost of growth medium for same biomass production was also decreased up to ~ 35%.

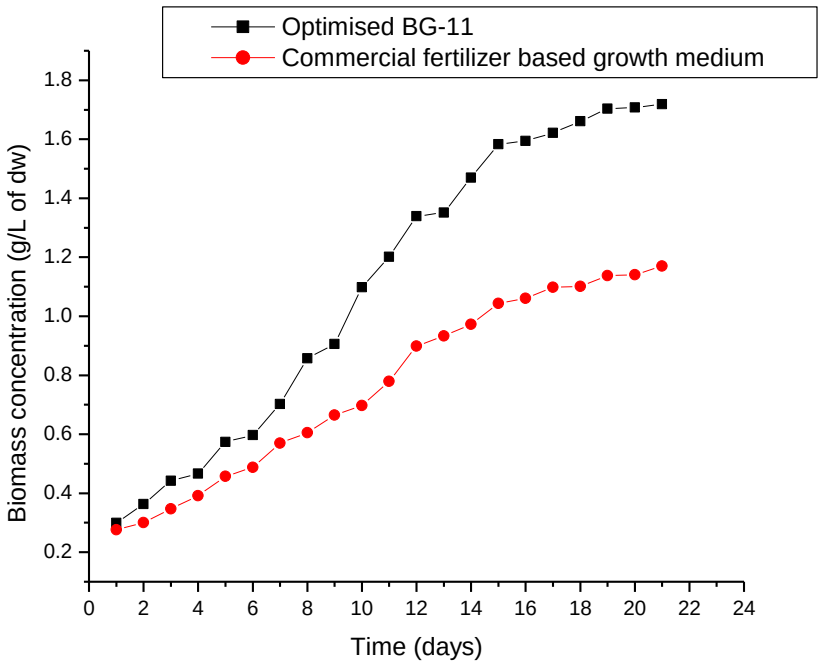


Figure 4.24 Cultivation of *Chlorella vulgaris* in open raceway ponds using optimised BG-11 and comercial fertilizer as growth medium

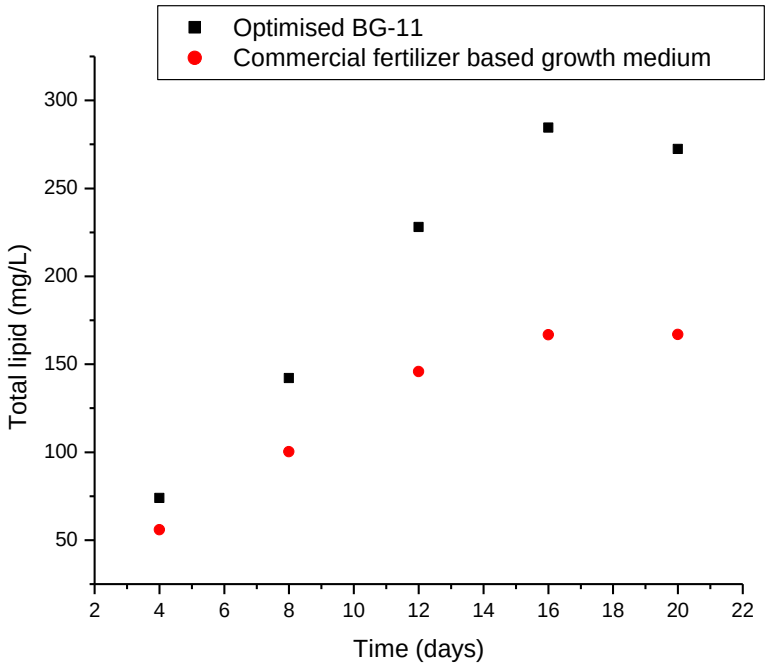


Figure 4.25 Lipid conctent of *Chlorella vulgaris* grown using optimised BG-11 and comercial fertilizer in open raceway ponds

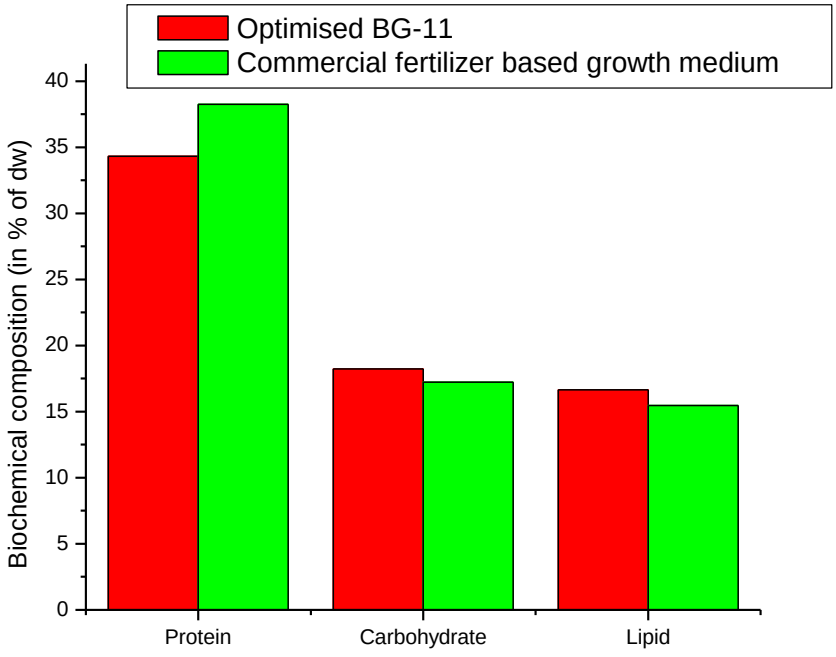


Figure 4.26 Biochemical compostions of *Chlorella vulgaris* grown using optimised BG-11 and comercial fertilizer in open raceway ponds

Growth medium	Biomass concentration (g/L)	Biomass productivity (mg/L/d)	Lipid Productivity (mg/L/d)	Specific growth rate K (Per div)	Division/day
Optimized BG-11	1.41	85	11.67	.0931	0.134
Commercial fertilizer based growth media	0.893	44	6.9	0.0523	.075

Table 4.9 Growth parameters of *Chlorella vulgaris* grown in open raceway pond at batch mode

As already discussed, continuous or semi-continuous mode of algae cultivation is an essential aspect to grow microalgae at commercial scale for long periods. Therefore, the experiment was also carried out in semi-continuous mode. During semi-continuous cultivation, the microalgae was initially grown for 12 days at batch mode and then 50% of algal culture was harvested from the open raceway pond at every six days interval. Harvested culture was replaced by the same volume of nutrition rich growth media, and this experiment was conducted for a period of 48 days. Total six harvestings were carried out during semi-continuous mode. Results are shown in figure 4.27 and table 4.10.

Growth medium	Biomass concentration (g/L)	Biomass productivity (mg/L/d)	Lipid Productivity (mg/L/d)	Specific growth rate K (Per div)	Division/day
Commercial fertilizer	0.921	77.21	11.52	0.1111	0.160

Table 4.10 Growth parameters of *Chlorella vulgaris* grown in open raceway pond at semi-continuous mode

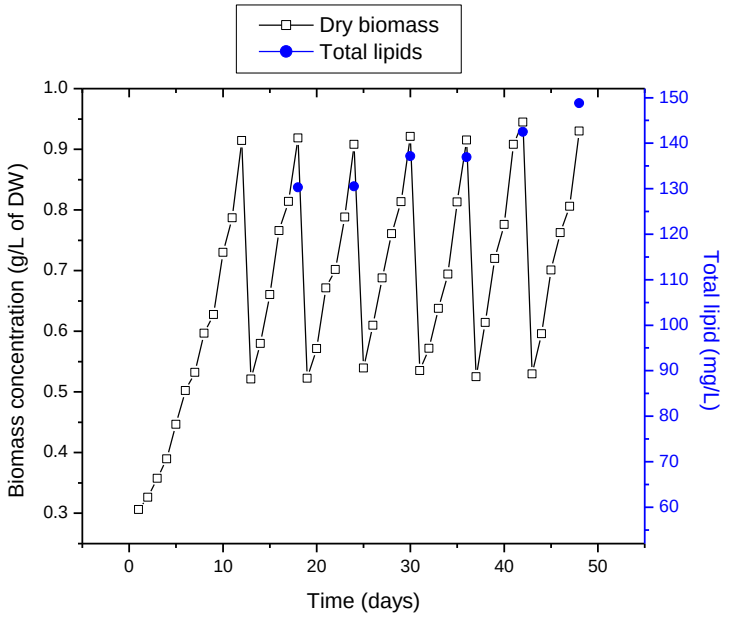


Figure 4.27 Mass culivation of *Chlorella vulgaris* in open raceway ponds at semi-coutinous mode

Optimum volumetric and areal productivity achieved by *Chlorella vulgaris* in the semi-continuous mode was 77mg/L/d and 19.61g/m^2/d respectively. In addition, lipid productivity was 11.52 mg/L/d that was 30.60% higher than batch mode. Moreover, the specific growth rate and division per day were observed 0.1111 and 0.160 respectively for *Chlorella vulgaris*. Veeramuthu et al. (2014) cultured *Microcystis aeruginosa* in 5000 L capacity raceway ponds at semi-continuous mode and observed biomass productivity up to 28 g/ m^2 /day, which is higher than biomass productivity (19.61g/m^2/d) achieved in this study. However, biomass productivity of *Chlorella vulgaris* can be increased by increasing carbon dioxide supplementation, controlling contaminations and applying genetic and molecular engineering. This study concluded that *Chlorella vulgaris* can be grown successfully under outdoor conditions at semi-continuous mode with minimum contamination and has good potential for biodiesel production.

4.2.8. Harvesting of biomass using flocculation and filtration method

After raceway cultivation, microalgal biomass was harvested via flocculation and filtration followed by drying under sunlight. Factors affecting flocculation include cell density, flocculent concentration, temperature, hydrophobicity, pH of culture medium and the type of flocculants used. Alum was used as preferred flocculent in this study as it is less expensive. After flocculation, the dense culture medium was filtered through filter cloth having 2-10µm pores. Experiments were performed with different concentrations of alum (50 mg/L, 100mg/L, 150 mg/L, 200 mg/L, 250 mg/L, 300mg/L, 400 mg/L and 500mg/L) by varying the time (15 minute, 30 minute, 60 minute, 90 minute, 120 minute, 150 minute, 180 minute, 210 minute, 240 minute, 270 minute and 300 minute). Results are shown in figure 4.28 and 4.29. However, maximum biomass was harvested when 500mg/L alum was used as flocculent (82.64%), but 250 mg/l alum concentration was considered as optimized concentration because it also gave approximately same results (82.10%). Furthermore, excess of alum concentration resulted in higher harvesting cost.

pH is also one of the important parameter affecting the harvesting process; hence, effect of pH on biomass harvesting efficiency with 250 mg/L alum was also studied at room temperature. It was observed that alum gives best results in acidic medium and 92.53% harvesting efficiency was achieved at pH 5 by a combined method of flocculation and filtration (figure 4.29). Similarly, Bhowmick et al. (2014) found that *Chlorella variabilis* gives best flocculation results with 160 mg/L alum at pH 4.

After flocculation process, concentrated slurry of biomass was obtained. This slurry was filtered through a filter cloth having 0.10 micron pore size. Filtered biomass was washed 3-4 times with distilled water to remove impurities of inorganic salts or flocculent. After that, algal biomass was spread over polyethylene sheet; thickness of the microalgal paste was

maintained between 3–5 mm and dried under mild sunlight for 6-8 h. Dried biomass was converted into powder form using mortar and pestle and packed in polybags.

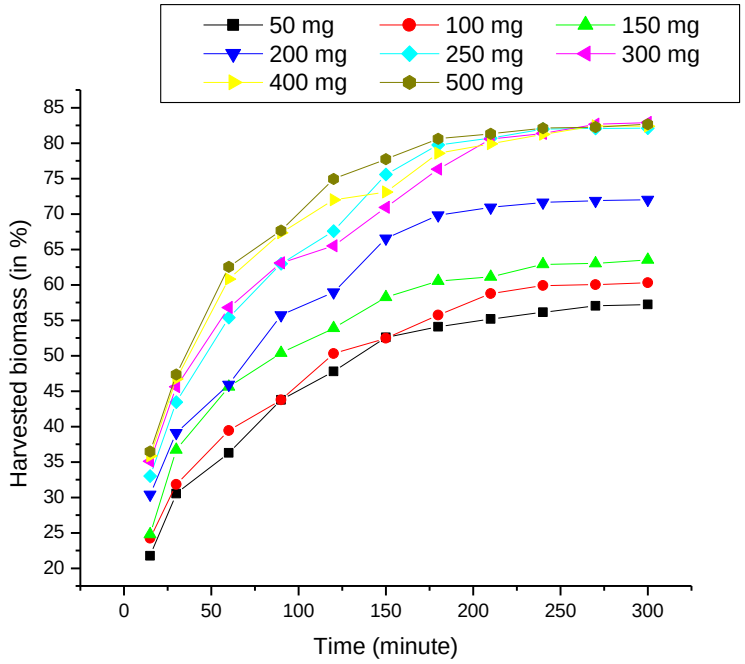


Figure 4.28 Effect of alum concentration on biomass harvesting with time

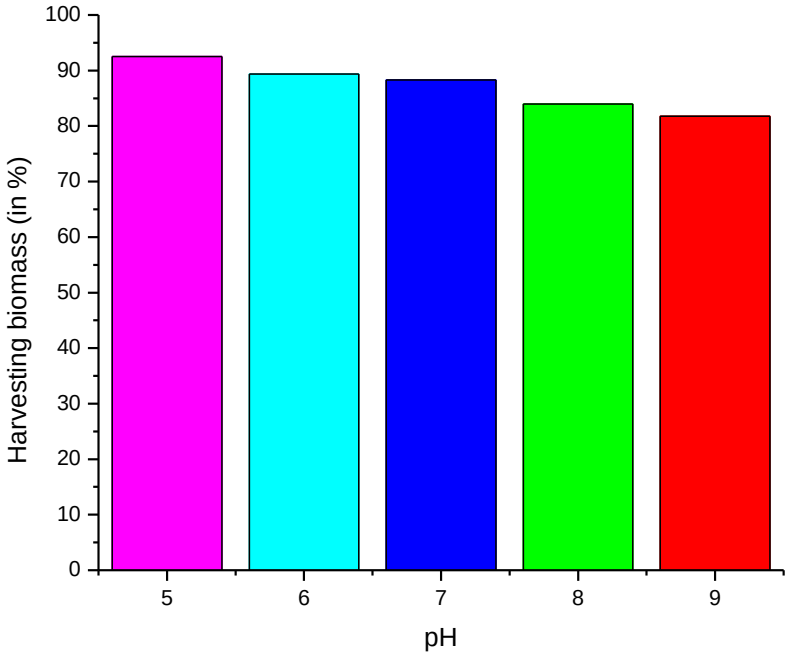


Figure 4.29 Effect of pH on biomass harvesting (250 mg/L alum was used for harvesting biomass)

Microalgae biomass was stored in freezer at -20°C until lipid extraction was carried out. When biomass was employed to lipid extraction, it was dried in hot air oven at 60°C to reduce moisture content less than 5%.

4.3. Development of lipid extraction method from microalgae biomass

Extraction of lipid or oil from microalgae is a crucial step in the production of biodiesel. It depends on different parameters, such as nature of solvents (polar and nonpolar), extraction time, moisture content of biomass, extraction temperature, biomass drying methods and solvent-biomass ratio (Halim et al., 2012). All the parameters were optimized in this study. It also helped in understanding the effect of drying technique on total lipid extraction from microalgae biomass. Lipid extraction was carried out in 100 ml capacity soxhlet extraction unit for 12 h (until the solvent was colorless).

4.3.1 Effect of biomass drying method on lipid extraction

Harvested biomass of *Chlorella vulgaris* grown in open raceway ponds was dried using different techniques, such as sun drying and oven drying (60°C, 80°C and 100°C). Lipid extraction was performed in a 100 ml capacity soxhlet extraction unit with following conditions: solvent to biomass ratio-10:1, time 12 hour at a rate of 8 cycles per hour. Results are shown in figure 4.30. Lipid yields of *Chlorella vulgaris* biomass dried by sun drying and oven drying (60°C, 80°C and 100°C) were found to be 17.07%, 16.88%, 14.37% and 13.66% per g dry biomass respectively. It was observed that there is no significant difference in lipid yield biomass was dried under sunlight or in oven at 60°C. When biomass drying temperature increased up to 80°C and 100°C, the lipid yield was decreased up to 2.7% and 3.7% respectively. The outcome of this investigation is supported by a previous study on the effect of drying temperature on total lipid content (Widjaja et al., 2009). It was observed that there is slight decrease in lipid content when biomass was dried

at 60°C. On the other hand, when the biomass drying temperature was increased up to 80°C or higher, lipid content decreased significantly. However, sun drying took substantially longer time (72 h) to dry microalgal biomass as compared to oven drying (12 h). But oven drying method is an energy consuming process in comparison to solar drying method. Based on energy consumption, it was concluded that solar drying is more economical choice for a large scale production of dry biomass from wet microalgal biomass. But, one limitation of sun drying method is that it needs large land area and enough time for drying the biomass. However, the unutilized land area near the site of harvested microalgal biomass could be used for solar drying of microalgae biomass.

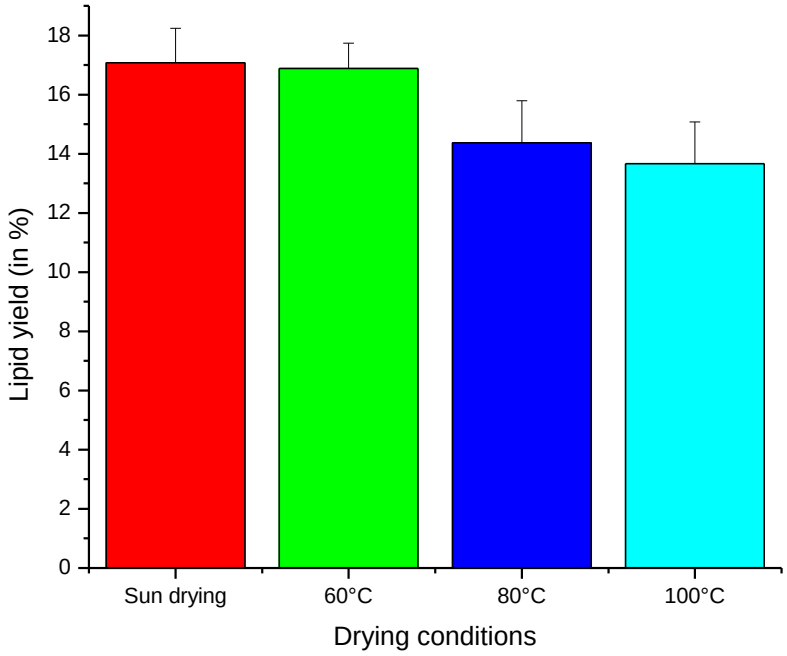


Figure 4.30 Effect of drying method on lipid extraction from *Chlorella vulgaris* biomass

4.3.2 Solvent selection for extraction of total lipids

The choice of solvent for lipid extraction is an important step as the selected solvents should have an ideal extraction capability and low viscosity to increase free circulation. Well-organized extraction needs penetration of solvent into the biomass and to

go with the polarity of the targeted compounds. In this investigation, different solvent systems such as methanol, ethanol, isopropanol, petroleum ether, hexane, dichloromethane, chloroform, diethyl ether, acetone toluene, chloroform: methanol (2:1), hexane: diethyl ether (1:1), hexane :methanol (3:2), hexane :ethanol (3:2), hexane : isopropanol (3:2), cyclohexane: methanol (2:1), cyclohexane: ethanol (2:1), dichloromethane: hexane (3:1), dichloromethane: cyclohexane (1:1) and ethyl acetate: ethanol (1:1) were studied to extract total lipid from solar dried microalgae biomass. Other conditions were kept same as in above section. It was observed that no single solvent (either polar or nonpolar) was able to give maximum yield of lipid content. In case of single solvent system, chloroform appreciate good yield of extracted lipid (13.47%). The combinations of non-polar and polar solvent such as chloroform: methanol (2:1) supported the maximum total lipid extraction (16.27%), followed by cyclohexane: methanol (2:1) with a lipid yield of 14.63% (Table 4.11). The solvents chloroform and cyclohexane are non-polar and highly stable solvents with low oily residual effects, whereas methanol was used to extract all the polar constituents. Chloroform: methanol (2:1) providing maximum yield among tested solvents was selected as an ideal solvent system for lipid extraction and used for the further experiments. These findings were in line with studies of Balasubramanian et al. (2013) and Kumar et al. (2014). However cyclohexane: methanol (2:1) system also has good lipid yield and has the ability to replace chloroform: methanol (2:1) system, but this required further research efforts to optimize ratio of cyclohexane and methanol for maximum lipid recovery from microalgae biomass.

4.3.3 Effect of moisture on lipid extraction on microalgae biomass

Lipid was extracted by soxhlet extractor using chloroform: methanol (2:1) solvent system to study the effect of biomass moisture content on lipid extraction. After

centrifugation at 10,000 rpm for 10 min, the wet algae biomass had 80% moisture by weight. Microalgae with moisture contents of 60%, 40%, 20%, 5% and 1% were then prepared by drying algae in oven. Figure 4.31 shows that when biomass moisture content reduces from 80% to 1%, lipid extraction efficiency was increased from 10.02 % to 15.53%. However, there is no significant difference in the extraction of lipid from microalgae biomass containing 1% and 5 % moisture content. Kumar et al. (2013) examined that lipid extraction efficiency of *B. braunii* AP102 decreased with increasing moisture content of biomass. They found maximum (24.2%) and minimum (15.32%) lipid at 1% and 20.1 wt% moisture content respectively. This is due to formation of hydrated shell around algal cell which acts as barrier to penetrate the non-polar solvent inside the cell and thus hinders lipid extraction (Balasubramanian et al., 2012; Kumar et al., 2013).

4.3.4 Optimization of extraction time for lipid extraction

To optimize the extraction time, soxhlet extraction was carried out for different time periods (2, 4, 6, 8, 10 and 12 h). Other conditions like moisture content, solvent to biomass ratio and nature of solvent were kept constant. The trend of extraction of lipid yield with time is shown in figure 4.32. Results showed that lipid yield increased with increasing extraction time. However, maximum lipid yield (17.74%) was observed at 12 hour, but extraction efficiency was best at 8 hour. As, only 1.51% more lipids was extracted on increasing time from 8 hours to12 hours and led to more energy consumption than energy gain (in terms of extracted lipid). Therefore, it was considered that 8 hour time is sufficient for best extraction and further research work was carried out using 8 hour extraction time.

S.No.	Solvent / solvent mixture	Ratio	Lipid yield, (% of dry wt. of biomass)
1	Methanol	-	6.76 ± 0.87
2	Ethanol	-	7.06 ± 1.35
3	Isopropanol	-	7.54 ± 0.81
4	Acetone	-	4.79 ± 1.66
5	Hexane	-	6.56 ± 0.66
6	Dichloromethane	-	7.32 ± 2.95
7	Petroleum ether	-	4.85 ± 1.55
8	Chloroform	-	13.47±1.30
9	Toluene	-	8.68 ± 0.56
10	Di ethyl ether	-	5.83 ± 2.35
11	Chloroform: methanol	2:1	16.27 ±1.98
12	Hexane: di ethyl ether	1:01	7.80 ± 2.49
13	Hexane : methanol	3:02	12.53 ± 1.73
14	Cyclohexane : methanol	2:01	14.63 ± 1.43
15	Di chloromethane: hexane	3:01	12.14 ± 1.27
16	Hexane: ethanol (2:1)	3:02	10.78 ± 2.18
17	hexane: isopropanol	3:02	11.72 ± 1.85
18	Di chloromethane: cyclohexane		12.170 ± 1.25
19	cyclohexane : ethanol	2:01	11.17 ± 4.07
20	Ethyl acetate: ethanol	1:01	11.01± 2.09

Table 4.11 Effect of different solvent systems on total lipids extraction from *Chlorella vulgaris*

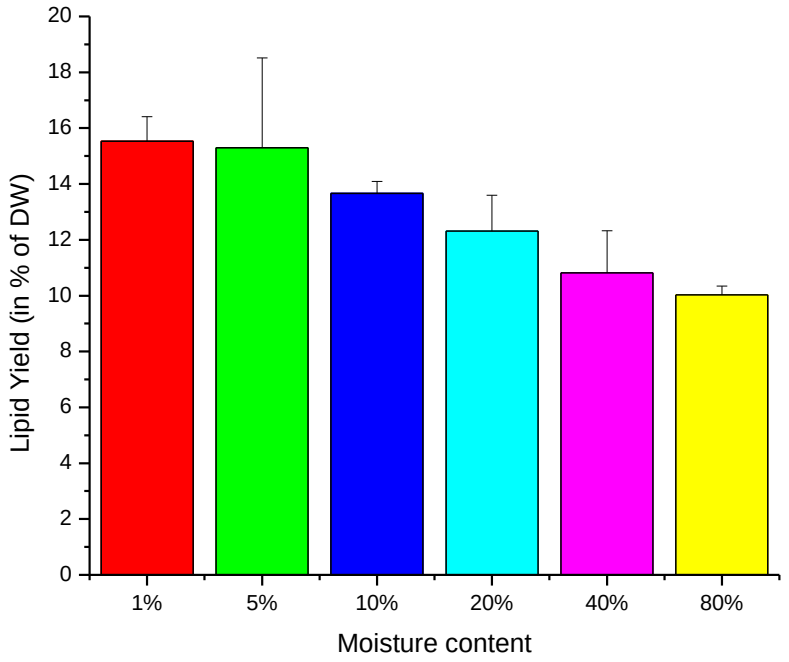


Figure 4.31 Effect of moisture content on lipid extraction from *Chlorella vulgaris* biomass

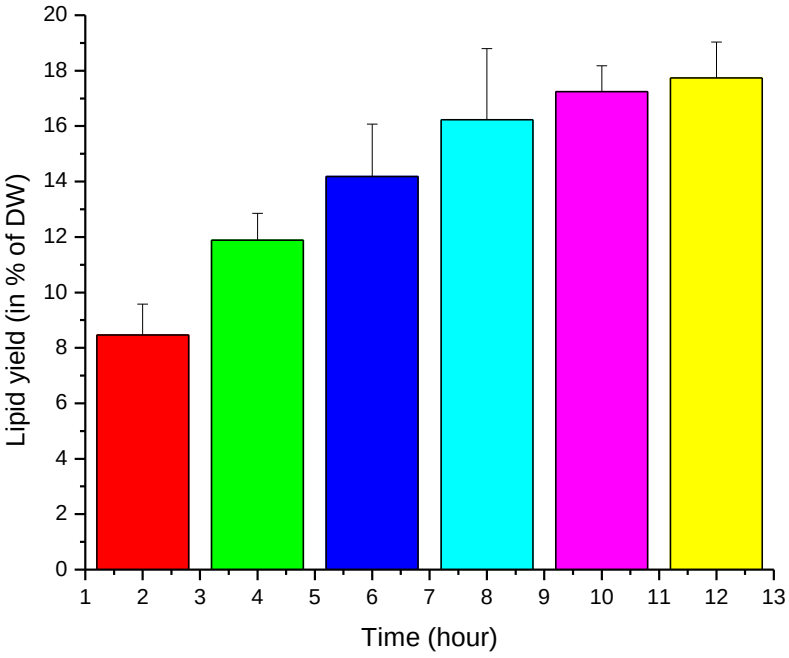


Figure 4.32 Effect of time on lipid yield

4.3.5 Optimization of pre-treatment method for microalgae biomass subjected to lipid extraction

Microalgae biomass was pretreated with different methods such as microwave, autoclaving, freeze drying and ultra-sonication to enhance and improve lipid extraction efficiency. The results revealed that the most effective pre-treatment method was microwaves pre-treatment method with lipid yield of 22.68 % of dry biomass, followed by ultrasonication, freeze drying and autoclaved (figure 4.33). Lee et al. (2010) found microwave pretreatment as most effective method for disruption of algal cells. As microalgae cell is presumed to absorb microwave energy, instantly increase the temperature, causing the cell to break because of the internal pressure exceeding the bearable pressure of the cell wall. Pretreatment method such as autoclave and ultrasound only enhanced the cracking of the cell wall and did not instantly deliver much energy that caused distinct extraction effects.

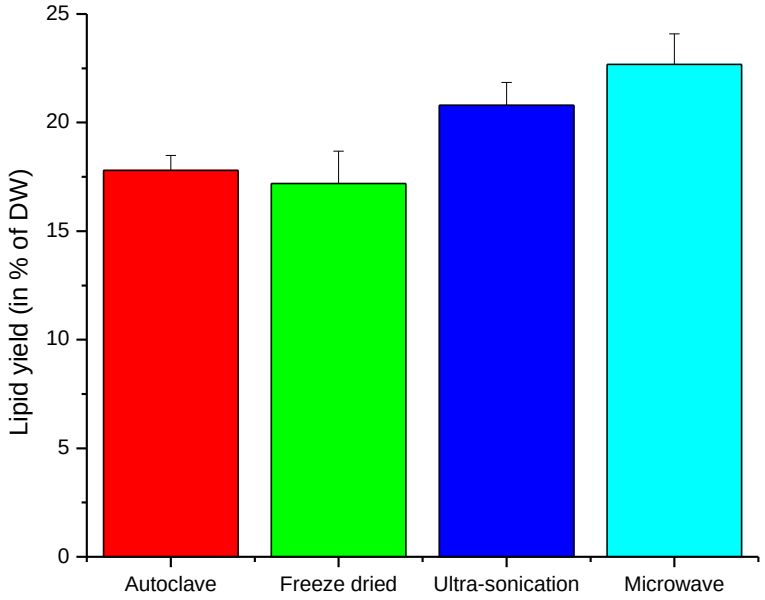


Figure 4.33 Effect of pretreatment methods on lipid extraction

Based on the obtained results, it was decided to use the combination of microwave pre-treatment and chloroform–methanol (2:1) for further experiments to extract lipids.

4.3.6 Optimization of solvent to biomass ratio for lipid extraction at batch scale

Batch extraction of total lipid was studied for optimization of solvent to biomass ratios. The experiment was conducted at 65°C using microwave pre-treated biomass in chloroform: methanol (2:1) solvent system for a maximum time of 3 h. The moisture content of the biomass was 5%. The effect of solvent quantity on extraction yield was studied for solvent to biomass ratio from 6:1 to 12:1 and it was observed that the yield was increased from 17.85% to 22.21% as solvent to biomass ratio increased from 6:1 to 12:1 (figure 4.34). However, there is no significant increase in the yield when more than 10:1 solvent to biomass ratio was applied for lipid extraction. Therefore, 10:1 solvent to biomass ratio was found to be the best condition and used for further study.

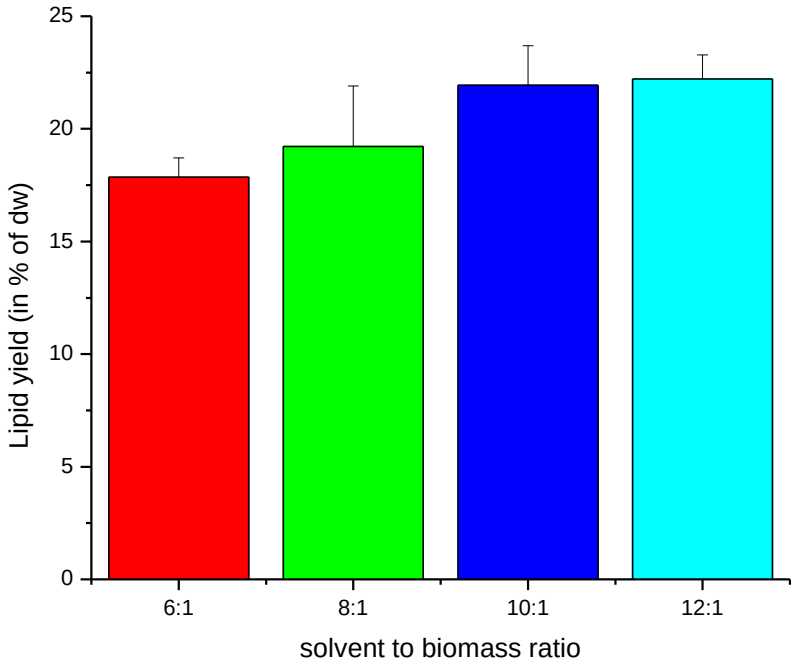


Figure 4.34 Effect of solvent to biomass ratio on lipid extraction yield

4.3.7 Physico-chemical properties of *Chlorella vulgaris* lipid

Results showed that based on above optimization studies, maximum total lipid of 22.68% was extracted from *Chlorella vulgaris* biomass. Different physical and chemical properties of the lipids derived from *Chlorella vulgaris* are shown in table 4.12. The saponification value of microalgae lipid was 194.87 mg KOH/g. Iodine value of the lipid was 102.32. Free fatty acid (FFA) and acid value of lipid was observed up to 12.39% and 24.67mg KOH/g respectively. Gas chromatographic analysis showed the fatty acid composition of algal oil as caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, arachidic acid, and erucic acid. *Chlorella vulgaris* algal lipid contains more unsaturated fatty acids than saturated fatty acids. Average molecular weight of lipid was determined as 899.24 g/mole. Density and kinematic viscosity of the lipid were analyzed as 0.928 kg/m^3 and 31.23 cSt respectively.

Figure 4.35 Images shown microalgae lipid and biodiesel, (a) microalgae lipid/oil, (b) microalgae biodiesel

S.No.	Lipid Properties	values
1	Total lipid content	22.68%
2	Saponification value	194.87 mg KOH/g
3	Iodine value	102.32
4	Free fatty acid (FFA)	12.39%
5	Acid value	24.67 mg KOH/g
6	Ester value	170.19 mg KOH/g
7	Viscosity	31.23 cSt
8	Density	0.928 Kg/m^3
9	Calorific value	38.25 MJ/kg
10	Moisture	0.823%
11	Average molecular weight of total lipid extracted	899.24 g/mole

Table 4.12 Physico-chemical properties of lipids extracted from *Chlorella vulgaris*

4.4 Process optimization of biodiesel production from *Chlorella vulgaris* lipid

The crude lipid was re-dissolved in n-hexane, filtered and concentrated. The FFA content of algal lipid was observed as 12.39%. A two-step process, acid-catalyzed esterification followed by base-catalyzed transesterification was applied for biodiesel production from *Chlorella vulgaris* algal lipid to resolve the problems of soap formation.

4.4.1 Optimization of acid catalyzed esterification

The acid value of *Chlorella vulgaris oil* was 24.67 mg KOH/g. High amount of FFA (12.39%) leads to soap formation during transesterification reaction. Therefore, acid esterification pretreatment was employed to reduce acid value less than 1 mg KOH/g. Optimization study was conducted in microwave assisted biodiesel reactor at atmospheric

pressure under reflux conditions. To neutralize algal oil FFA, experiment was carried out using methanol to oil ratio 1:10 and 1.5% H_2SO_4 (v/v) at 60°C. At different time intervals, sample was taken out from reactor to analyse FFA content. Once FFA level reduced less than 1%, the reaction was stopped. The results revealed that the lipids FFA level was reduced to 0.93% and fatty acid methyl ester yield achieved up to 24.4% within 40 minutes (figure 4.36). After pretreatment of algae lipid, transesterification was carried out using basic catalyst. Similarly, high acid value (34 mg KOH/g) of crude mahua oil was reduced to less than 2 mg KOH/g at 1.26 h of reaction time by its pretreatment with 1.24% v/v sulphuric acid, 0.32 v/v methanol to oil ratio at 60°C (Ghadge et al., 2006). Tiwari et al. (2007) investigated that 1.43% v/v H_2SO_4 acid catalyst, 0.28 v/v methanol-to-oil ratios and 88-min reaction time at 60°C was the optimum condition to reduce the high FFA level of Jatropha oil from 14% to less than 1%.

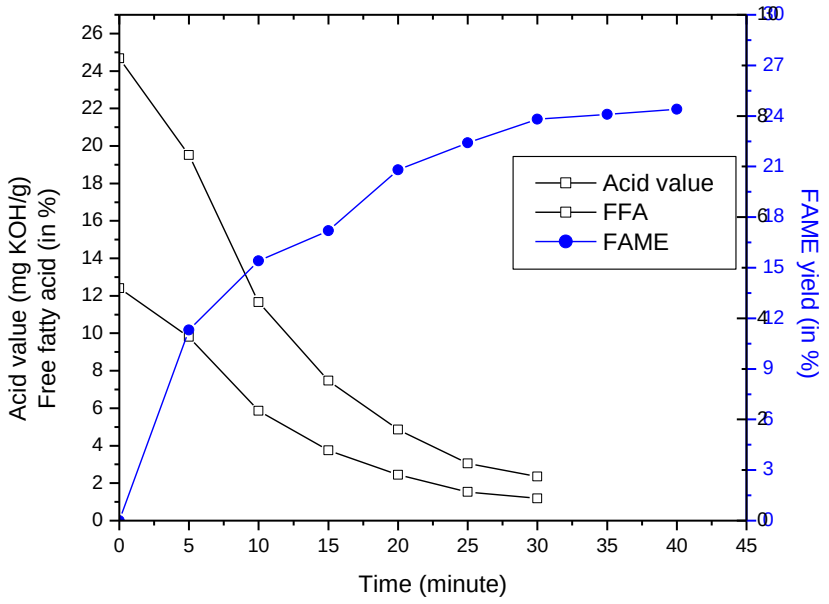


Figure 4.36 Optimization of acid catalyzed esterification with time (catalyst 1.5% v/v, temperature 60°C and oil to methanol ratio (1:10)

4.4.2 Optimization of base catalyzed transesterification

After esterification, pretreated algal lipid was converted into biodiesel (FAME) using KOH as homogenous base catalyst. Different reaction parameters such as catalyst concentration, methanol to lipid ratio, reaction time and reaction temperature were optimized to achieve maximum FAME yield from microalgae lipid. All the reactions were carried out in microwave assisted reactor. Each experiments was carried out in duplicates to get reproducible results.

4.4.2.1 Effect of catalyst concentration

To analyze the effect of catalyst on FAME yield, an alkaline catalyst, potassium hydroxide was used in the range of 0.5% to 2.5% in the present experimental analysis. Other initial parameters were kept as follows: methanol to algal lipid molar ratio 8:1, reaction temperature 55°C and reaction time 20 min. Effect of catalyst amount on FAME yield is shown in Figure 5.37. FAME yield was increased from 41.15% to 63.03% with increase of catalyst concentration from 0.5% to 1.5%. Further increase in catalyst concentration up to 2.5%, decreased the FAME yield to 47.49% (figure 5.37). This is due to the reason that addition of excess of alkaline catalyst caused more triglycerides participation in the saponification reaction which results in higher amount of soap formation and reduction in FAME yields (Guo et al., 2006). It was also observed that insufficient amount of catalyst also gave rise to lower FAME yield.

4.4.2.2 Effect of methanol to lipid ratio

The amount of alcohol added to algal lipid is one of the important factors that affects FAME (biodiesel) yield as well as production cost of biodiesel. Generally, 3:1 methanol/triglyceride molar ratio is required for base catalyzed transesterification. But, in practice this is not sufficient to complete the reaction. Therefore, optimization of

methanol to lipid molar ratio is essential to get maximum FAME yield at faster rate. The effect of methanol to algal lipid ratios (4:1 to 14:1v/v) was examined on biodiesel yield. Other initial conditions were kept constant i.e. catalyst concentration 1.5%, time 20 minute and reaction temperature 55°C. Experimental results are shown in figure 5.38. When methanol to lipid ratio was increased from 4:1 to 10:1, FAME yield was increased from 50.06% to 71.79% at 20 min of reaction time. This is due to high viscosity of algal lipid which needs more volume of methanol to increase the solubility of lipid in it. Further in case of increase in the molar ratio above 10:1, there is no obvious change in conversion efficiency. Moreover, high volume of methanol would also have an impact on biodiesel production cost and interfere with the purification of biodiesel. Hence, methanol to lipid molar ratio 10:1 was found to be the best condition to get maximum FAME yield of 71.79%.

4.4.2.3 Effect of temperature on biodiesel yield

Reaction temperature is another important factor affecting FAME yield. In order to study the effect of reaction temperature, transesterification was carried out at different temperature (45°C to 65°C) for 20 minute. In this study, methanol to lipid ratio of 10:1 and 1.5% potassium hydroxide pellet catalyst (w/v) were used. It was observed that FAME yield increased with rise in temperature (figure 5.39). When temperature was increased from 45°C to 60°C, FAME yield raised from 52.1% to 84.08%. Further increase in temperature does not have significant effect on FAME yield and makes it energy expensive. Hence, 60°C was found to be the optimum temperature condition. Higher reaction temperature raises the reaction rate and decreases the reaction time due to reduction in viscosity of algal lipid. This is advantageous to increase the solubility of algal lipid in methanol and increase contact between oil and methanol molecules, thereby

achieving better yield of FAME. Ehimen et al. (2010) suggested that 60 °C is more favorable for considering total energy consumption and operational cost of biodiesel. Similar trend was reported by Leung and Guo (2006) who investigated the impact of different temperature conditions (30°C to 70°C) on the FAME yield. They found that optimum temperature of 60°C had a positive impact on biodiesel production to obtain maximum yield of 88.8% (Leung et al., 2006). Similarly, Veljkovic et al. (2006) obtained 91% biodiesel yield from tobacco seed oil at 60°C.

4.4.2.4 Effect of reaction duration on biodiesel yield

To increase the efficacy of the reaction, transesterification was carried out with different time durations (2.5 minute to 25 minute) to optimize reaction time for maximum FAME yield. In this part, experiments were conducted with catalyst concentration (1.5%), methanol to lipid molar ratio (10:1), temperature (60°C) and varied reaction time of 3 to 30 minute. Results are shown in figure 5.40. It was observed that FAME yield is strongly influenced by reaction time. On increasing reaction time from 2.5 minute to 15 minute, FAME yield was increased from 41.57% to 84.01%. Beyond 15 minute, even after 17.5 minute there was no obvious change in FAME yield. Hence, time duration of 15 minute is sufficient to achieve efficient yield. Similarly, Kumar et. al. (2011) worked on microwave assisted transesterification of *Pongamia pinnata* seed oil and got maximum yield within 10 minutes.

When the reaction completed, the biodiesel sample was washed three times with deionized warm water containing 1 % acid to remove the unreacted reactant molecules and other impurities. The biodiesel phase was collected and treated with anhydrous sodium sulphate to remove the moisture content. The mixture was centrifuged at 6000 rpm for 10

minute, and the liquid phase was collected and the biodiesel sample was characterized by GC, NMR and FTIR.

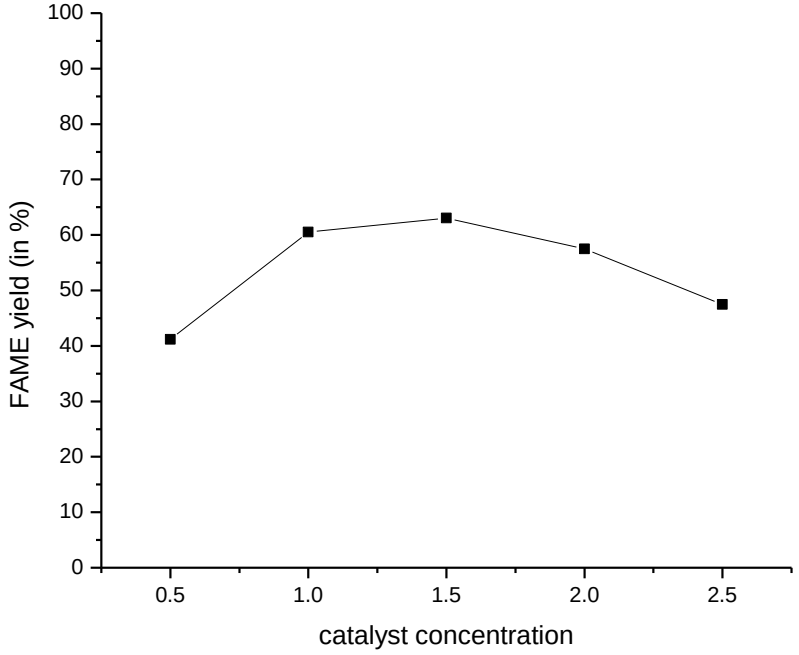


Figure 4.37 Effect of catalyst concentration on biodiesel yield

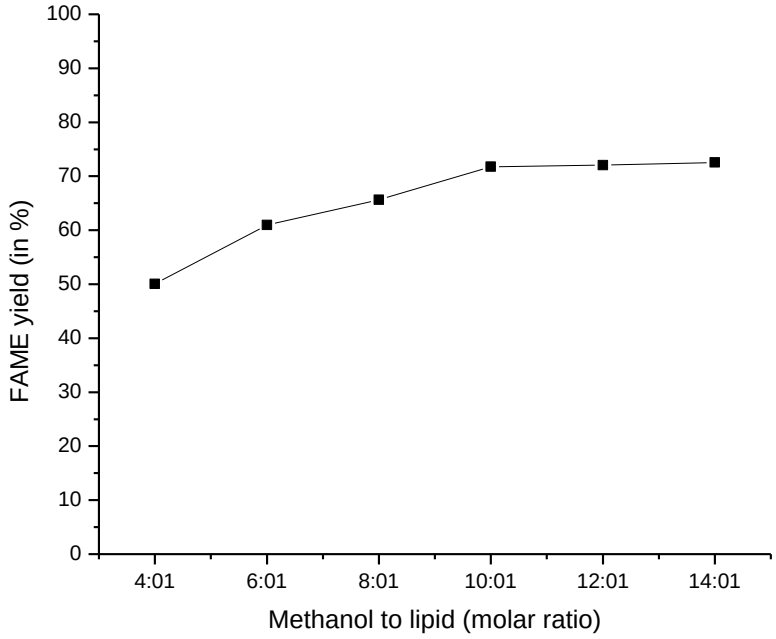


Figure 4.38 Effect of methanol to lipid ratio on biodiesel yield

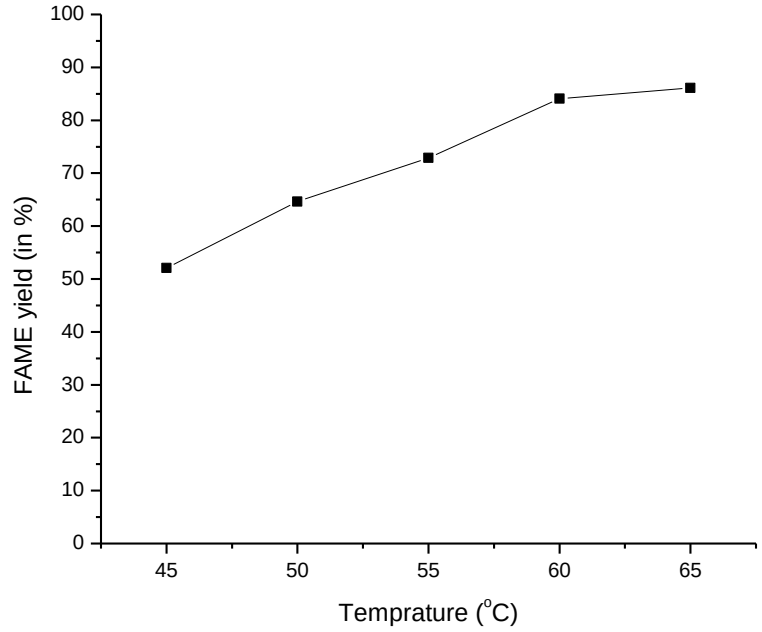


Figure 4.39 Effect of temperature on biodiesel yield

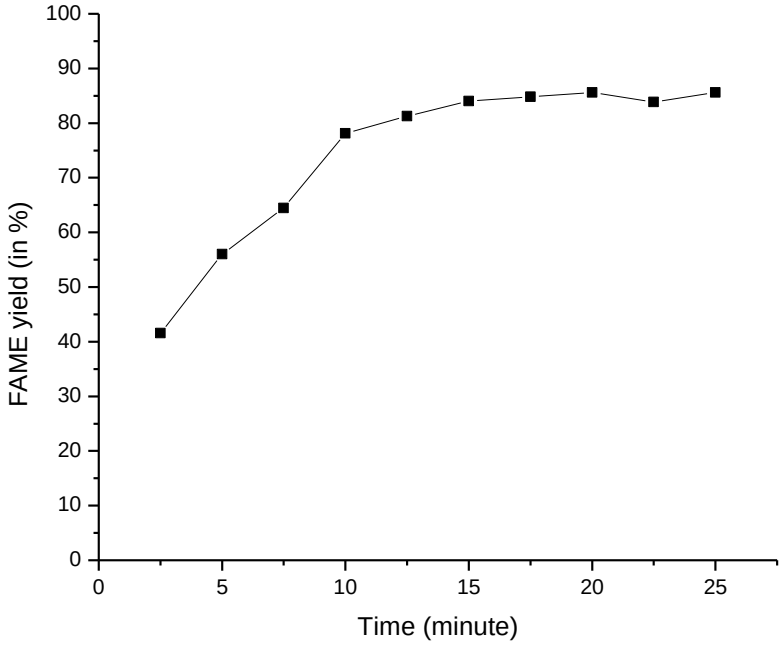


Figure 4.40 Effect of reaction duration on biodiesel yield

4.4.3 Evaluation of biodiesel production using conventional and microwave assisted method

To compare conventional and microwave assisted process, biodiesel was also prepared in a conventional reactor, commonly used for biodiesel synthesis, under same optimized condition as stated in above section. Only reaction duration was changed and the reaction was carried out for 3 h to get maximum yield. Comparison of both conventional and microwave biodiesel reactors are shown in table 4.13. Energy consumption for biodiesel production (esterification and transesterification) was found to be 0.641KWh for microwave reactor which is lesser than conventional heating reactor. Furthermore, reaction time of microwave assisted transesterification was much lower than that of conventional biodiesel synthesis causing the production rate of biodiesel of microwave synthesis at least 12 times higher than that of conventional synthesis. This can be explained by the fact that microwave interaction with the reactants (triglycerides and methanol) leads to large reduction of activation energy due to increased dipolar polarization phenomenon which is achieved due to molecular level interaction of the microwaves in the reaction mixture resulting in dipolar rotation and ionic conduction (Gude et al. 2013, Mazzocchia et al. (2004). Some researchers supported the fact that microwave irradiation is a fast and energy saving method as compared to the conventional transesterification method for biodiesel production from different feedstocks (Mazzocchia et al., 2004, Gude et al., 2013). Lin et al. (2012) carried out microwave-assisted transesterification of soybean oil in the presence of methanol and KOH and found that energy consumption of microwave heating system (0.058-0.21 kWh) was much lower than that of conventional heating system (1.03-3.05 kWh). Conclusively, microwave heating system has good potential to decrease the production cost of microalgae biodiesel.

S.No.	Characteristic/ parameter	Microwave reactor	Conventional reactor (oil bath heating reactor)
1	Reaction duration	15 minutes	3 h
2	Biodiesel yield	84.01%	83.23%
3	Energy consumption (esterification and transesterification)	0.684KWh	0.641 KWh
4	Advantages	Short reaction time, reduce product separation time, cleaner products, and energy efficient	Simple operation, use of low energy source

Table 4.13 Comparative analysis of microwave assisted transesterification and conventional transesterification

4.4.4 Characterization of microalgae biodiesel

4.4.4.1 GC analysis of microalgae biodiesel

Fatty acid composition of *Chlorella vulgaris* algal biodiesel is shown in Table 4.14 and compared with *Jatropha curcas* biodiesel. It was observed that *Chlorella vulgaris* biodiesel had 33.53% saturated fatty acid, 28.68% monounsaturated fatty acid and 37.12% polyunsaturated fatty acid composition in comparison to *Jatropha curcas* biodiesel which had 21.44%, 41.24% and 39.95% saturated, monounsaturated and polyunsaturated fatty acid respectively. GC analysis (figure 4.41) showed that microalgal biodiesel had following fatty acids: caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, arachidic acid, and erucic acid while *Jatropha* biodiesel had palmitic acid, palmitoleic acid, stearic acid, oleic acid and linoleic acid as main fatty acids. According to European standards EN14214, an ideal biodiesel should not have more than 12% and 1% linolenic acid (C18:3) and polyunsaturated FA (>4 double bond) respectively (Gouveia and Oliveira, 2009; Pereira et

al., 2013). In the present study, microalgae biodiesel had 7.54% linolenic acid in comparison to Jatropha biodiesel in which linolenic acid was almost absent (0.07%). This indicates that microalgal biodiesel is less stable than *Jatropha* biodiesel.

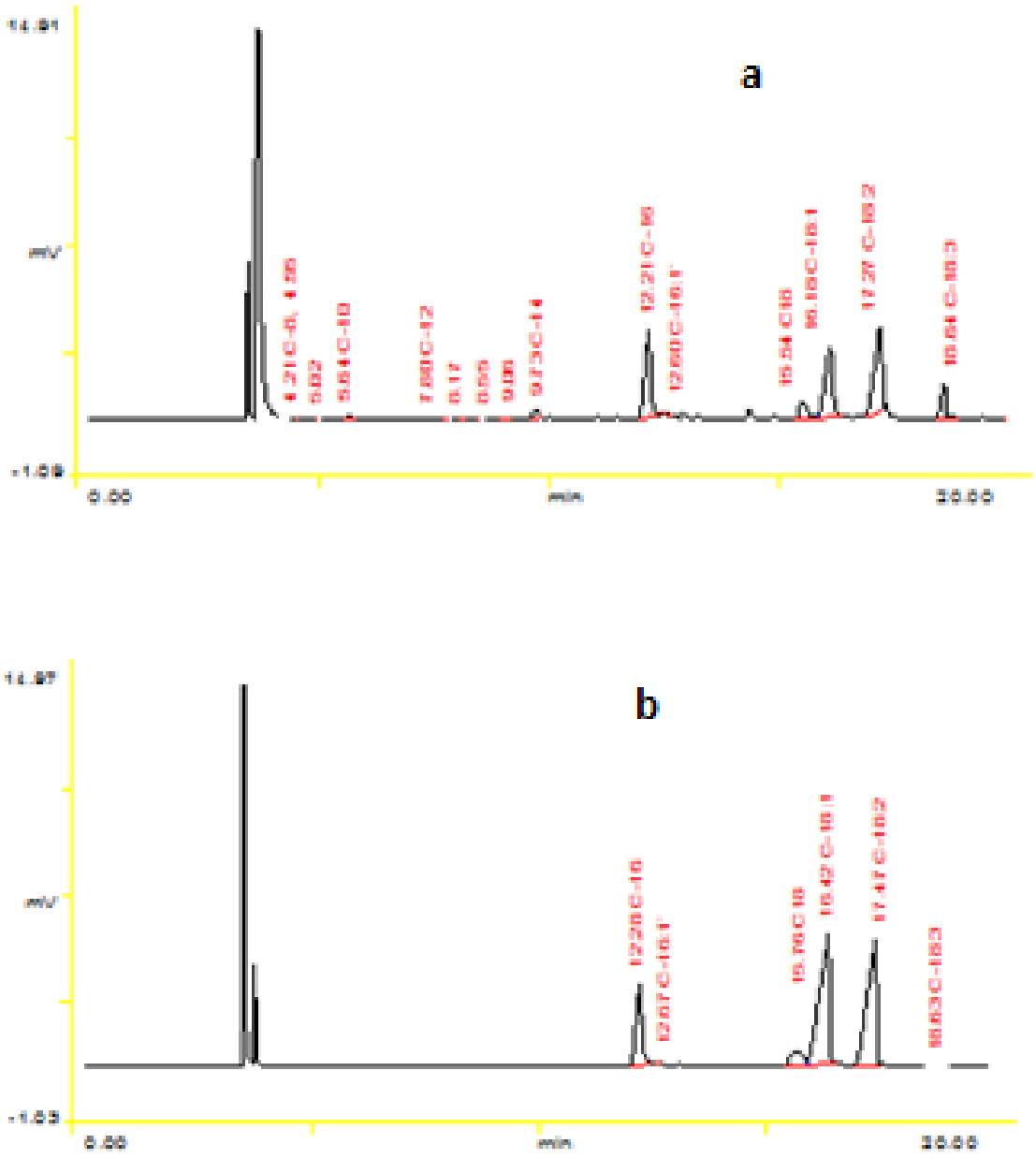


Figure 4.41 Gas chromatograph analysis of biodiesel (a) Chlorella vulgaris biodiesel, (b) Jatropha Biodiesel

S.No.	Fatty acid methyl ester	No. of carbon atoms	Composition of microalgae (*Chlorella vulgaris*) biodiesel	Composition of Jatropha curcas biodiesel
1	Caprylic acid methyl ester	C-8.0	0.0939	nd
2	Capric acid methyl ester	C-10.0	0.7334	nd
3	Lauric acid methyl ester	C-12.0	0.191	nd
4	Myristic acid methyl ester	C-14.0	2.0465	nd
5	Palmitic acid methyl ester	C-16.0	24.3167	15.2922
6	Palmitoleic acid methyl ester	C-16:1	0.6311	0.04024
7	Stearic acid methyl ester	C-18.0	5.9011	6.1507
8	Oleic acid methyl ester	C-18.1	24.5258	41.2007
9	Linoleic acid methyl ester	C-18.2	29.5792	36.8745
10	Linolenic acid methyl ester	C-18.3	7.5421	0.0795
11	Arachidic acid methyl ester	C-20	0.2503	nd
12	Behenic acid methyl ester	C-22	nd	nd
13	Erucic acid methyl ester	c-22:1	3.5251	nd
14	Saturated fatty acid methyl esters		33.5329	21.4429
15	Mono unsaturated fatty acid methyl ester		28.682	41.24094
16	Poly unsaturated fatty acid methyl esters		37.1213	36.954

nd=not determined

Table 4.14 Fatty acid composition of *Chlorella vulgaris* and *Jatropha curcas* biodiesel

4.4.4.2 ^{1}H nuclear magnetic resonance analysis

NMR spectroscopy was used to explain the structure and chemical properties of biodiesel. ^{1}H NMR (proton NMR) spectra of *Chlorella vulgaris* lipid and biodiesel is shown in figure 4.42. A strong singlet signal at 3.66 ppm indicates the protons of the methyl group of *Chlorella vulgaris* biodiesel. This peak was absent in the lipid spectrum. Another multiplet resonance was observed at 0.86-0.88 ppm which represents terminal methyl protons (C-CH_3). Multiplet resonance detected at 5.3 ppm signifies the proton attached to the olefinic carbon (one double bond). Multiplet at 1.61 ppm indicates b-methylene protons to ester bond (CH_2-C-CO_2Me) and a strong resonance at 1.30 ppm was due to the protons of backbone methylenes of aliphatic fatty acid chain. Obviously, absence of glyceride protons at 4.2-4.3 ppm and the presence of methyl resonance at 3.66 ppm confirm conversion of lipid into biodiesel. The present analytical results are consistent with O'Donnell et al. (2013) who had done the similar analytical work with palm oil and soybean oil biodiesel

4.5.3.3 ^{13}C nuclear magnetic resonance analysis

The ^{13}C NMR spectrum of FAME obtained from *Chlorella vulgaris* is shown in figure 4.43. A characteristic peaks at 174.3 ppm represents the ester carbonyl carbon (–COO–). Another peak at 51.4 ppm indicates methoxy carbon (C–O) groups of *Chlorella vulgaris* biodiesel. Cluster of signals at 127.8, 128.0, 128.1, 128.7, 129.6, 129.9 and 130.1ppm designates the carbons of olefinic protons indicating the unsaturation of biodiesel. Some peaks were also observed in the range from 13 to 34 ppm which indicates methyl (terminal) and methylene (backbone of biodiesel) carbons of fatty acid moiety.

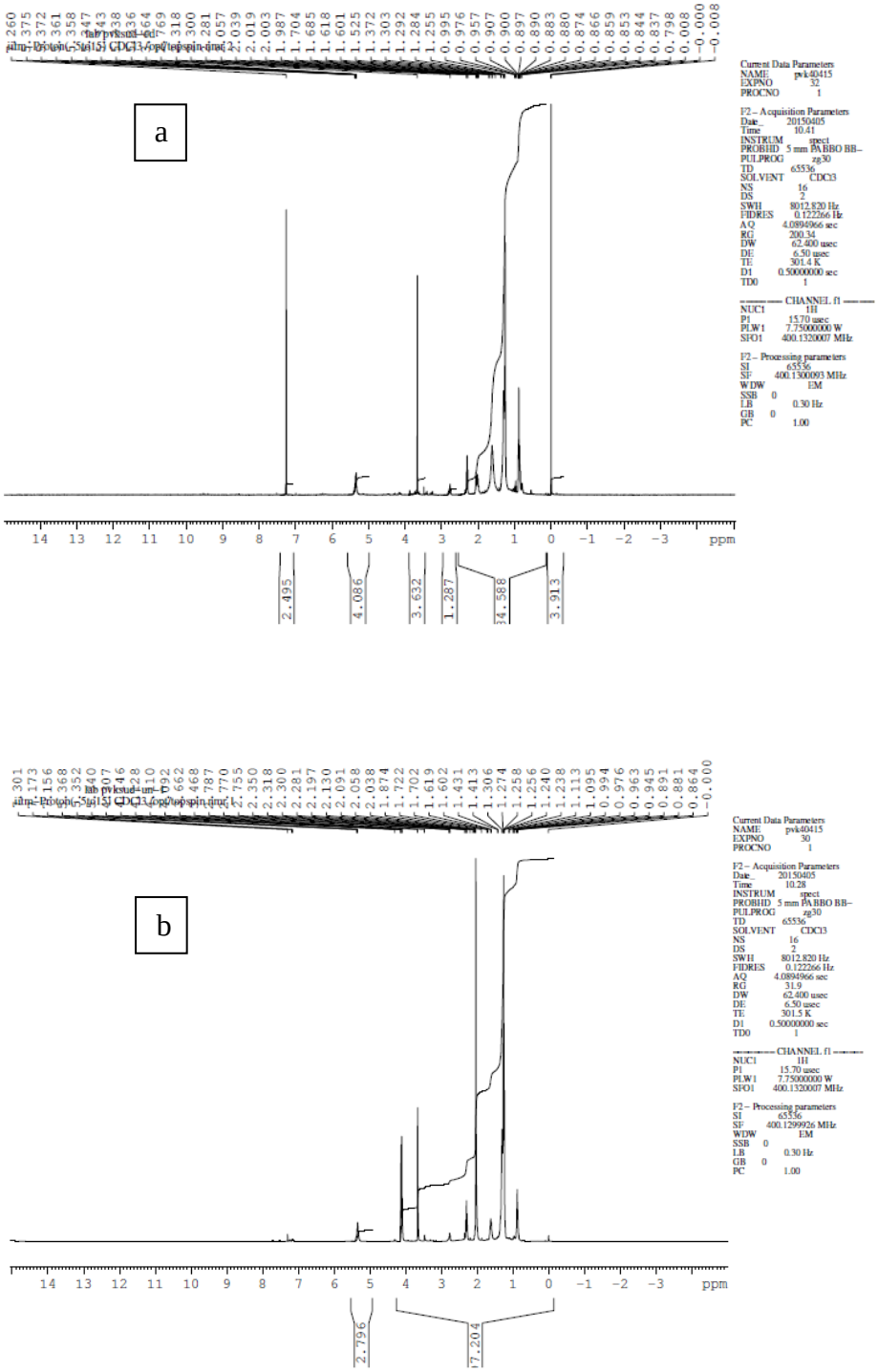


Figure 4.42 ^{1}H NMR spectra of a. *Chlorella vulgaris* lipid, b. *Chlorella vulgaris* biodiesel

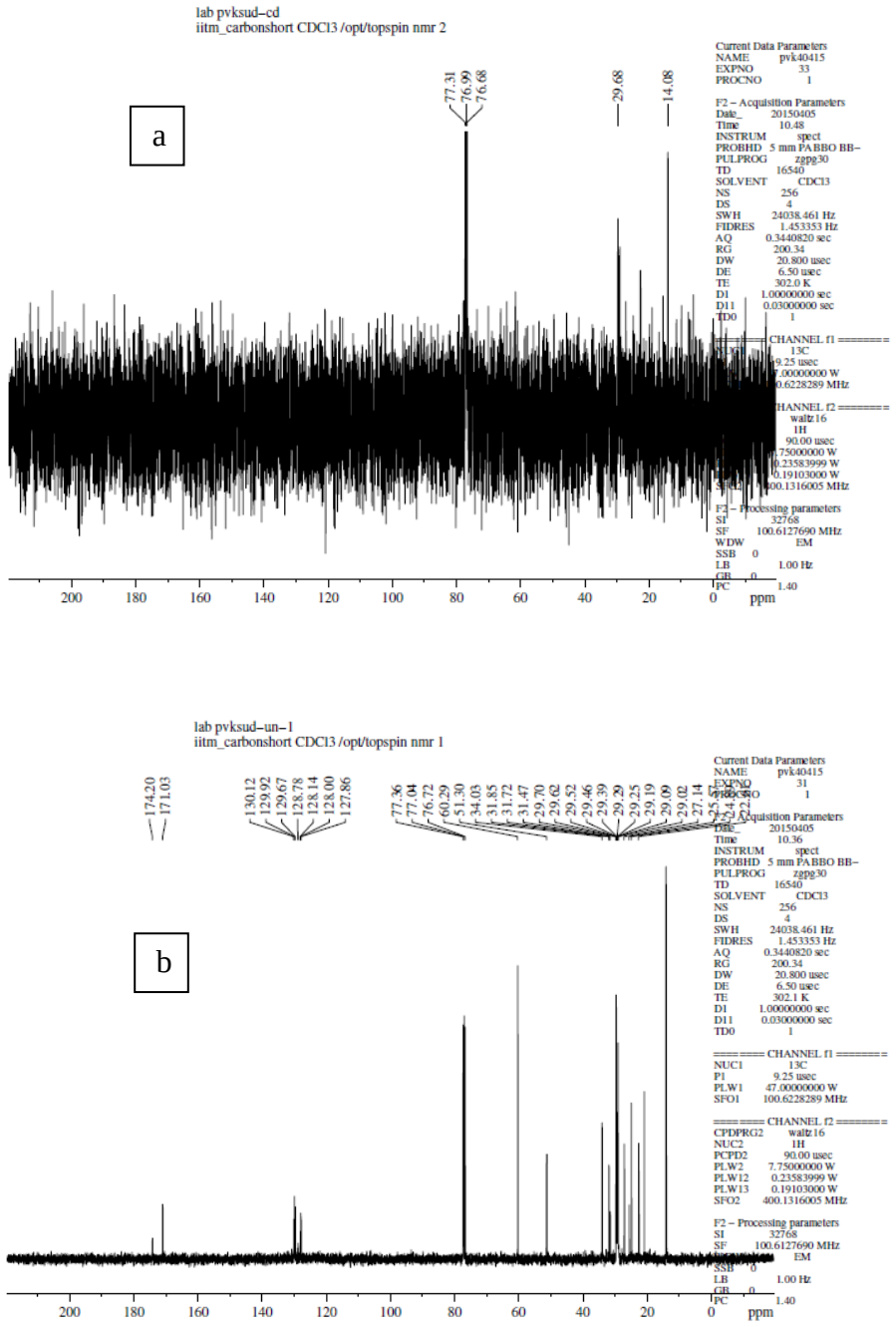


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Figure 4.43 ^{13}C NMR spectra of a. *Chlorella vulgaris* lipid, b. *Chlorella vulgaris* biodiesel

4.4.4.4 FTIR (Fourier transform infrared spectroscopy) analysis

FTIR was used to ascertain the functional groups and their shift in the lipid and biodiesel of *Chlorella vulgaris* (figure 4.44). Peaks observed at 2857.11 and 2924.77 cm^{-1}

represent the symmetrical and asymmetrical -CH_2- stretching and thus confirm the existence of lipid (-CH_2- groups form the backbone in lipids). A strong signal at 1741.22 cm^{-1} indicates the stretching of carbonyl group (-C=O) in biodiesel. A weak signal at 3005.07 cm^{-1} was due to the olefinic group (=CH-) and indicates the presence of unsaturated fatty acids in methyl ester. Peaks observed at 1247.75 cm^{-1} and 1176.71 cm^{-1} represents C-O stretching and absorption at 1368.16 cm^{-1} indicates the methyl group (-CH3-). A sharp absorption peak at 723.46 cm^{-1} is suggestive of -CH_2-rocking.

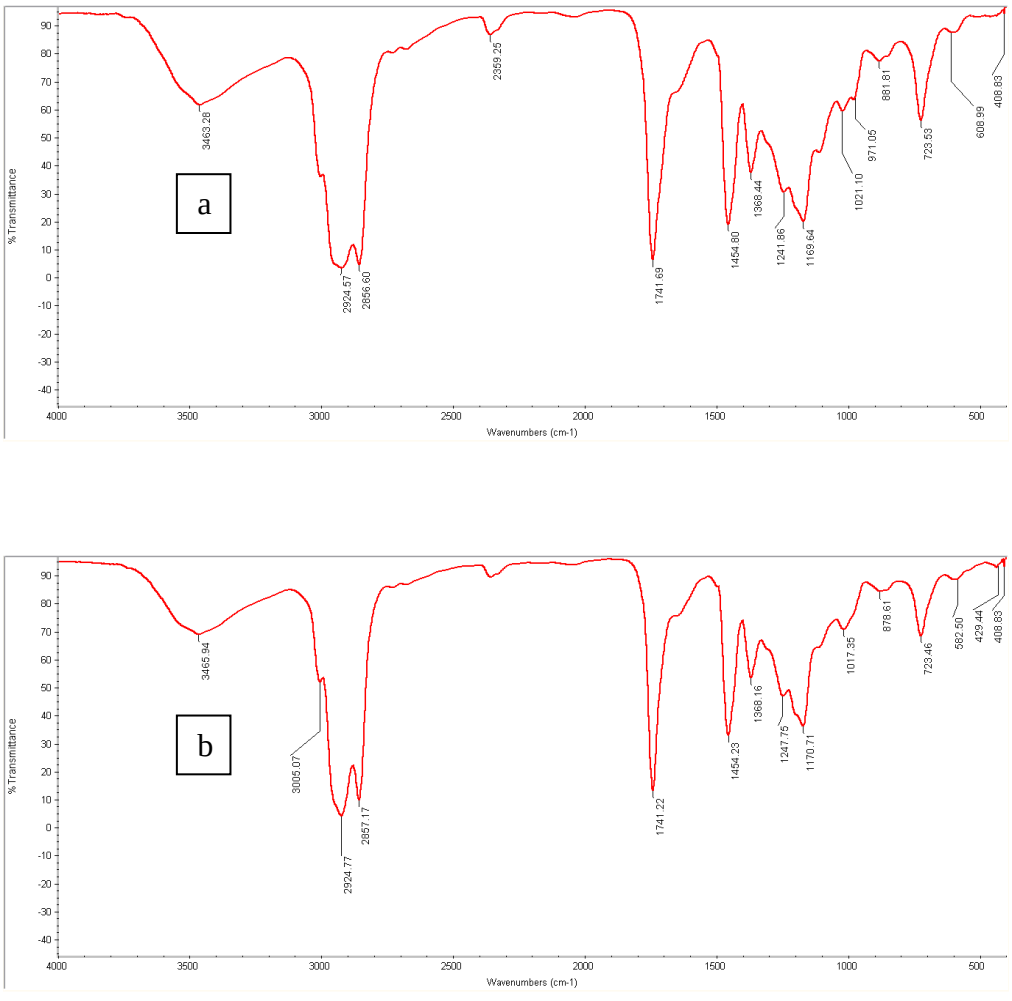

Figure 4.44 FTIR spectra of a. *Chlorella vulgaris* lipid, b. *Chlorella vulgaris* biodiesel

4.5 Analysis of physico-chemical properties of microalgae biodiesel

Biodiesel, an alternate to diesel fuel, could be successfully used in compression–ignition (CI) diesel engines, only if its physical and chemical properties were found to be in accordance with the international standard specifications (e.g. ASTM D6751, EN 14214 and IS: 15607). Microalgae biodiesel was characterized with respect to calorific value, cetane number, density, viscosity, cloud and pour points, water and sediment, flash point, copper strip corrosion, oxidation stability, and acid number according to the ASTM and Indian biodiesel standards.

Fuel properties of the microalgal biodiesel were analyzed and compared with Jatropha biodiesel (Table 4.15). Acid number represents the content of free fatty acids in the sample, which ensure proper aging of the fuel and quality. Acid number of the produced biodiesel was higher (0.49 mg KOH/g) than Jatropha biodiesel (0.44 mg KOH/g), but within the limits of biodiesel standards which indicates that it will cause minimum operational problems, such as corrosion and pump plugging, caused by corrosion and deposit formation (Candeia et al., 2009).

Density at 15^{o}C is a significant fuel property in combustion system because it influences the efficiency of atomization of the fuel (Felizardo et al., 2006). The density of microalgae biodiesel was 889 kg/m^3 in comparison to Jatropha biodiesel (878 kg/m^3). Biodiesel density mainly depends up on its fatty acid ester composition and the remained quantity of alcohol.

Viscosity is an important fuel property indicating the ability of a material to flow. ASTM D445 method was used for detecting the kinematic viscosity according to which biodiesel viscosity should be between 1.9 and 6.0 cST or mm^2/s at 40^{o}C. In the present study, viscosity of microalgae biodiesel was 5.72 cSt which is higher than Jatropha

biodiesel (4.78cSt) but found within ASTM standards. Higher viscosity causes poor fuel atomization, incomplete combustion and carbon deposition on the injector and valve seats, resulting in serious engine fouling method (Sahoo and Das 2008).

S.no.	Parameters	Method	Microalgae biodiesel	Jatropha biodiesel	standard limits
1.	Acid number (mg KOH/g)	D664	0.49	0.44	0.50 max
2.	Density at 15 °C (kg/m3)	D7777	889	878	-
3	Viscosity at 40 °C (mm^2/sec) or cSt	D445	5.72	4.78	1.9–6.0
4	Pour point (°C)	D97	-12	-3	-
5	Flash point (°C)	D93	155	151	>130 min
6	Cetane number*		57.03	55.44	47 min
7	Copper strip corrosion	D130	1	1	No.3. max
8	Calorific value (Mj/kg)	D240	39.45	39.89	-
9	Water and sediment (%)	D2709	.03	.028	0.050 max
10	Methyl linolenate (%)	EN 14103	7.54	.07	12%
11	Unsaturated ester (>4 double bonds) %	Internal method-GC	0	0	1%
12	Oxidation stability (IP, at 140 °C, h)	ASTM-D 7545 & prEN16091	3.08	4.06	3 (min)

Table 4.15 Fuel properties of *Chlorella vulgaris* and *Jatropha curcas* biodiesel

Flash point is the lowest temperature at which a liquid can vaporize to form an ignitable mixture in air. Higher flash point favors the safety during handling, transportation,

and storage. The flash point of microalgae biodiesel was recorded 155°C as compared to Jatropha biodiesel (151°C).

The calorific value of microalgae biodiesel was found to be 39.45 MJ/kg which is approximately equal to Jatropha biodiesel (39.89 MJ/kg) but less than petroleum diesel (44.24MJ/kg).

Two important parameters for low-temperature applications of a biodiesel are cloud point (CP) and pour point (PP). CP is the temperature at which crystallization begins and PP at which fuel no longer pours. Pour point was observed -12°C for microalgae biodiesel in comparison to Jatropha biodiesel (-3°C).

Cetane number (CN) of algae biodiesel was found to be 57.03 which is higher than Jatropha biodiesel (55.44). CN of biodiesel represents the presence of longer fatty acid carbon chains and more saturated molecules in it. High cetane value is the indicator of better combustion, low nitrous oxide (NOx) emission, less occurrence of knocking and easier start-up of engine (Demirbas 2009).

Water content observed for *Chlorella vulgaris* and *Jatropha* biodiesel was 0.03% and 0.028% respectively which was under the maximum value of 0.05% specified in the ASTM D6751 standard. However, biodiesel is hygroscopic in nature and absorbs moisture from atmosphere which can pose a greater negative effect than the presence of FFAs during transesterification reaction (Kusdiana et al., 2004). Also, copper corrosion for both microalgae and Jatropha biodiesel was recorded as class 1, which indicates that engine parts will suffer less corrosion with time.

Oxidation of fuel is one of the important factors that help to judge the fuel quality. Oxidation stability of biodiesel is affected by various factors, such as presence of air, heat, traces of metal, peroxides, light, and unsaturated fatty acid compounds. Generally,

polyunsaturated FAMEs (with more than one double bond) have negative impact on the oxidation stability of biodiesel because they have reactive sites which are susceptible for free radical attack. According to European standards EN14214, an ideal biodiesel should not contain more than 12% and 1% of linolenic (C18:3) and polyunsaturated (having more than 3 double bonds) acids respectively (Gouveia et al., 2009; Pereira et al., 2013). In this study, linolenic acid contributes 7.54% and 0.07% of microalgal and Jatropha FAME respectively, whereas, polyunsaturated FAME with > 4 double bond were found absent in both the cases. However algal biodiesel have more saturated FAME than Jatropha, oxidation stability of microalgal biodiesel was lower (3.08 h) than Jatropha biodiesel (4.06 h). It follows ASTM D-6751 standard (minimum 3 h) but does not meet the minimum limit as required by EN-14112 and IS- 15607 (6 h). This may be due to the presence of more polyunsaturated FAME in microalgae biodiesel.

4.5.1 Fuel properties of microalgae biodiesel blends

Different blends (B0, B2.5, B5, B10 and B20) of the produced microalgal biodiesel and petro-diesel were prepared, and the results of their analysis were compared with international standards (ASTM D7467).

Table 4.16, illustrates the variations of some physico–chemical properties after blending of petroleum diesel with biodiesel. Data revealed that the density and viscosity values decreased with blending and increased with increase in volume percentage of biodiesel. Their values also agree with ASTM standards and may lead to better lubricity. Cold flow properties, such as pour point were improved with increasing blending ratio (from B2.5 to B20). Copper corrosion of all the blends was found to be of class 1 which meets ASTM standards. It indicates that engine parts will be less corrosive with time. Flash point of biodiesel-diesel blends was increased with increasing volume percentage of

biodiesel which leads to better handling and safety during transportation and storage. Calorific value of blends was also improved with increasing biodiesel volume percentage. This will lead to better combustion properties and ignition performance which subsequently will reduce the greenhouse emissions (e.g. CO and CO_2). Oxidation stability of blends decreased with increasing biodiesel volume (by percent). For B2.5, B5 and B10 blends, oxidation stability was observed as 28.66 h, 16.33h and 7.33 h respectively which meets ASTM standards limits (min 6 h for B6-B20). However, B20 blends showed oxidation stability 4.25 h which does not meet ASTM standards. Moreover, oxidation stability can be improved by using different antioxidants.

S.no.	Parameters	Diesel	B 2.5	B 5	B10	B20	Biodiesel B6-B20 ASTM D7467
1.	Density at 15 °C (kg/m3)	830.1	831.4	833.5	835.2	837.2	-
2.	Viscosity at 40 °C (mm^2/sec) or cSt	2.85	2.87	2.91	2.99	3.12	1.9-4.1
3	Pour point (°C)	-2	-2	-3	-3	-4	-
4	Flash point (°C)	57	69	88	101	115	52
5	Copper strip corrosion	1	1	1	1	1	3
6	Calorific value (Mj/kg)	44.24	44.11	44.00	43.50	42.89	-
7	Oxidation stability (IP, at 140 °C, h)	nd	28.66	16.33	7.33	4.25	6 (min)
8	Water content	0.002	0.003	0.005	0.018	0.022	.050 max

Table 4.16 Fuel properties of *Chlorella vulgaris* biodiesel and diesel blends

4.6 Storage and thermal stability of microalgae biodiesel

4.6.1 Screening of antioxidants for oxidation stability of microalgae biodiesel

An important drawback of biodiesel is its susceptible nature to oxidation due to the presence of unsaturated fatty acid acids in FAME. Use of antioxidant plays an important role in improving oxidation stability during storage of biodiesel by delaying the onset of oxidation by their reaction with free radicals to form stable compounds. In this study, five antioxidants viz. butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), butylated hydroxyanisol (BHA), propyl gallate (PL), and pyrogallol (PY) were used as additives. The antioxidants were screened by adding 500 ppm, and 1000 ppm, concentration of each antioxidant in microalgae biodiesel. Results are shown in figure 4.45. Among the tested antioxidants, BHT and BHA antioxidants were found less effective when compared with TBHQ, PY and PL. On the basis of improvement in oxidation stability, effectiveness of the antioxidants used was observed as follows: PY > PL > TBHQ > BHA > BHT. This can be explained based on their molecular structure. Additives such as THHQ, PY, and PL have two OH groups attached to aromatic ring, while both BHT and BHA have one OH group attached to aromatic ring. Thus, based on their electro-negativities, TBHQ, PL, and PY provide more sites for the formation of complex between free radical and antioxidant radical for the stabilization of the ester chain (Karavalakis et al., 2010). Screening study of antioxidant additives also showed that the 1000 ppm concentration of additive was most effective concentration in comparison to the concentration of 500 ppm for each antioxidant. Therefore, further study on storage stability of biodiesel was carried out using 1000 ppm concentration of TBHQ, PY and PL. In addition, PY was observed the best among all with induction period (IP) of 6.45 and 10.58 h for 500, and 1000 ppm, respectively. Similarly, Obadiah et al. (2012) studied the effect of BHT, BHA, PY, GA and

TBHQ on oxidation stability of *Pongamia pinnata (L.)* and observed that pyrogallol (PY) is more effective than other tested antioxidants (Obadiah et al., 2012).

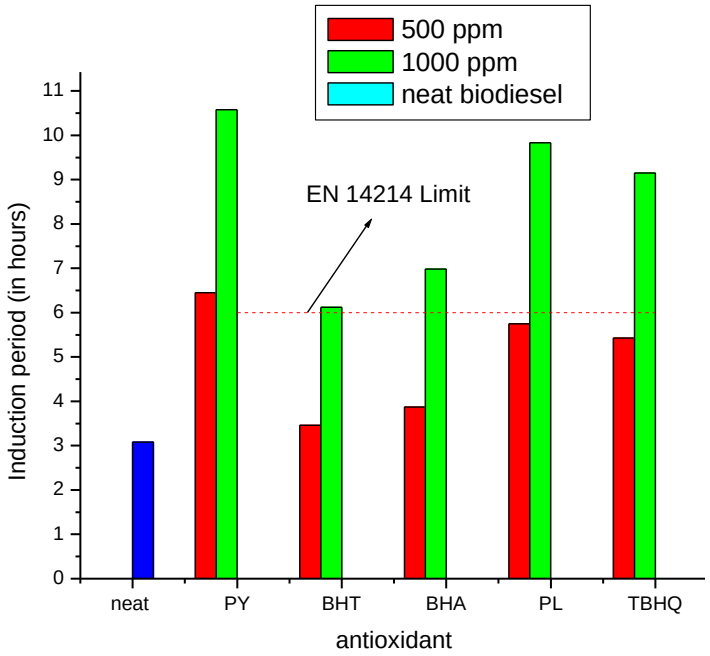

Figure 4.45 Oxidation stability of *Chlorella vulgaris* biodiesel with additives

4.6.2 Storage stability of biodiesel

In order to evaluate the storage stability, 40 ml biodiesel sample of microalgae biodiesel was stored in closed borosil glass bottles of 100 ml capacity for 12 weeks. These sample bottles were kept at a room temperature (20°C to 32°C). 60 mL space in the bottle was occupied by air. Samples were taken out periodically (every three weeks) in order to monitor the oxidation stability, acid value, density and kinematic viscosity.

4.6.2.1 Effect of antioxidants on the storage stability of microalgae biodiesel

Figure 4.46 shows the storage stability for neat biodiesel treated with various antioxidants (1000 ppm concentration of TBHQ, PY and PL each) over a storage time of 12 weeks. The induction period of neat microalgae biodiesel was 3.08 h which could be improved significantly up to 10.6 h, 9.8 h and 9.23 h by the addition of PY, PL and TBHQ

respectively. The oxidation stability of neat biodiesel (without antioxidant) was further decreased for next 3, 6, 9 and 12 weeks storage duration, due to the decomposition of unsaturated fatty acids present in biodiesel. Even after three weeks, the induction period of neat biodiesel was found to be 2.82 h which failed to meet the minimum induction period (3 h according to ASTM D6751 and 6 h according to EN 14214 and IS: 15607 specification). This means that it cannot be stored even for 3 weeks without antioxidants. On the other hand, induction period of biodiesel on addition of antioxidant PY, PL and TBHQ was reduced up to 7.69h, 6.04h and 5.02h during storage of 12 weeks. Gradual reduction in induction period with time was examined for all the antioxidants blended. The order of effectiveness was PY > PL > TBHQ. From the results obtained, it is clear that PY and PL blended biodiesel can be stored for 12 weeks but TBHQ treated biodiesel can be stored only up to 9 weeks. Among all the tested antioxidants, PY was found to be the best additive for improving oxidation stability during 12 hours storage time.

4.6.2.2 Influence of storage duration on kinematic viscosity

During storage, viscosity of microalgae biodiesel increases due to the formation of more polar, oxygen containing molecules and also by the formation of oxidized polymeric compounds. This leads to the formation of gums and sediments that clog filters (Dunn, 2005). The kinematic viscosity of microalgae biodiesel at the initial stage was found to be 5.72mm^2/s at 40°C. When biodiesel was left itself for duration of 12 weeks at room temperature, kinematic Viscosity value was increased up to 6.12mm^2/s (figure 4.47). On employing antioxidants to delay the oxidation process during storage, it was observed that all the tested antioxidants improved storage stability and maintained kinematic viscosity value around the standard value (maximum 6mm^2/s according to ASTM D 445).

4.6.2.3 Influence of storage duration on density

Analysis of density reflects stability and reliability of a fuel sample. The initial density value for neat biodiesel was 0.8892 g/cm^3. Final density was reduced up to 0.8912 g/cm^3 during 12 weeks storage which was due to oxidation of biodiesel. When biodiesel was blended with antioxidants, such as PY, PL and TBHQ, storage stability was improved and reduction of density of microalgae biodiesel increased due to slow down oxidation process. During the storage time of 12 weeks, the final densities of the biodiesel treated with PY, PL, and TBHQ were 0.88904 g/cm^3, 0.8909 g/cm^3 and 0.8912 g/cm^3 respectively. The change in density during 12 weeks storage time is shown in figure 4.48.

4.6.2.4 Influence of storage duration on acid value of microalgae biodiesel

Figure 4.49 shows changes in acid value over the storage duration of 12 weeks. As expected, acid value of biodiesel samples increased with storage time due to hydrolysis of fatty acid methyl esters (FAME) to fatty acids (FA). Acid value of *Chlorella vulgaris* biodiesel initially was 0.49 mg KOH/g but when biodiesel was stored for 12 weeks it was found to undergo oxidation and acid value raised up to 5.37 mg KOH/g. The order of effectiveness of these antioxidants was as follows: PY > PL>TBHQ. Maximum acid values of biodiesel treated with antioxidant PY, PL and TBHQ after 12 days storage were 3.04 mg KOH/g, 3.99 mg KOH/g and 4.24 mg KOH/g respectively. It was concluded that pyrogallol (PY) is the best antioxidant to improve the storage stability of microalgae biodiesel.

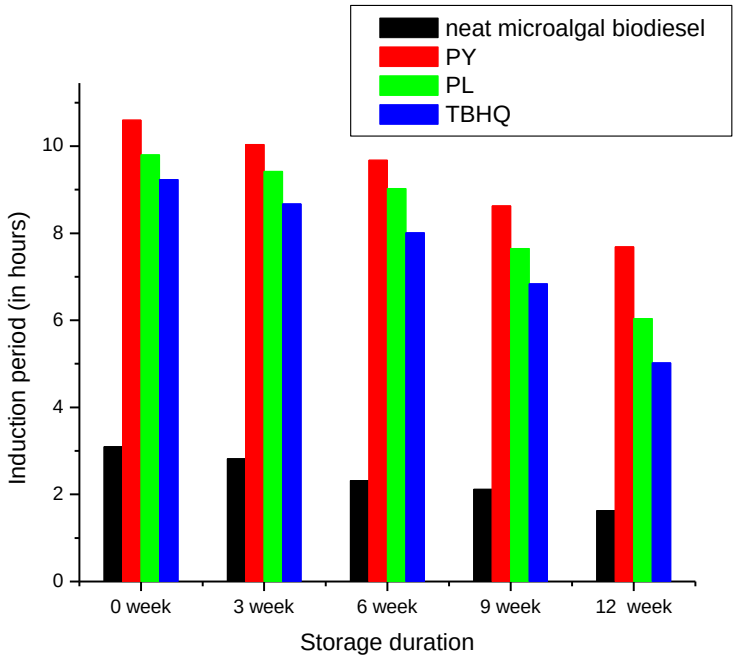

Figure 4.46 Effect of antioxidant on storage stability of microalgae biodiesel

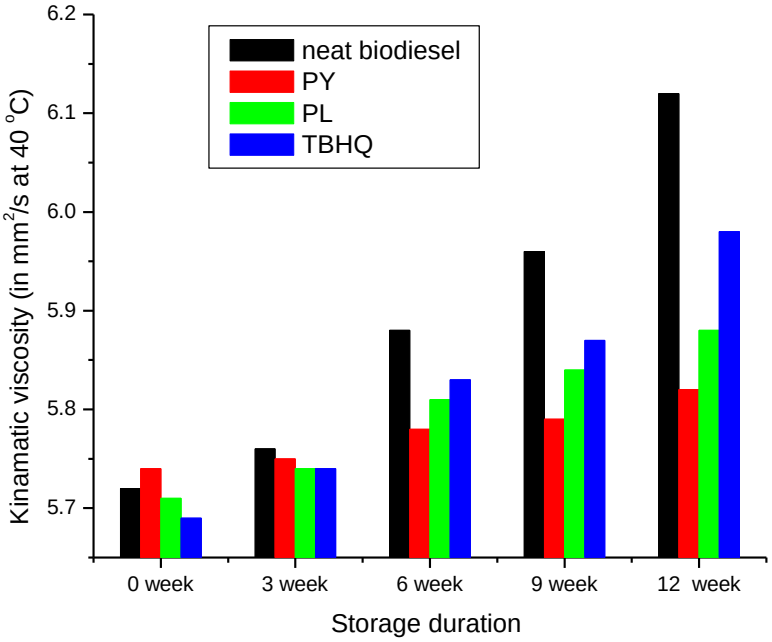

Figure 4.47 Effect of antioxidant and storage duration on Kinematic viscosity of microalgae biodiesel

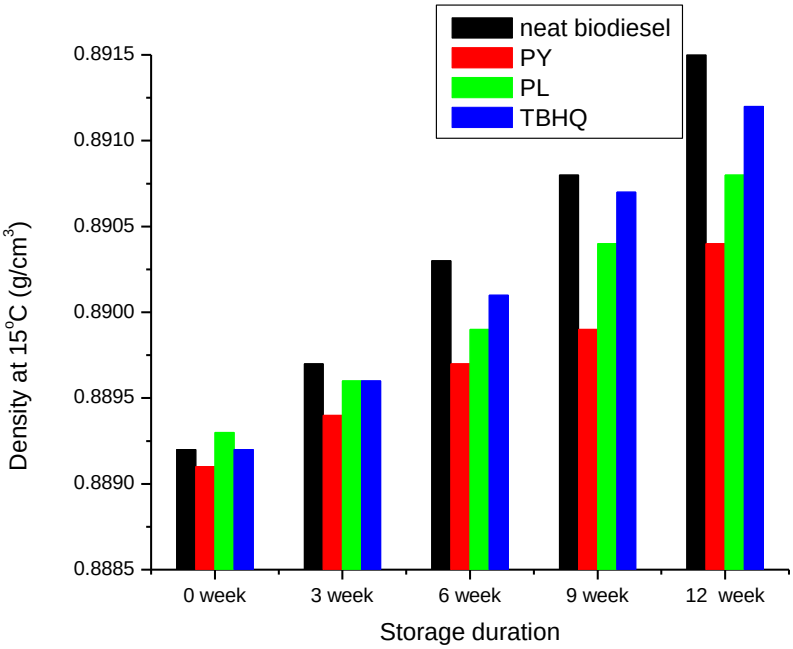

Figure 4.48 Effect of antioxidant and storage duration on density of microalgae biodiesel

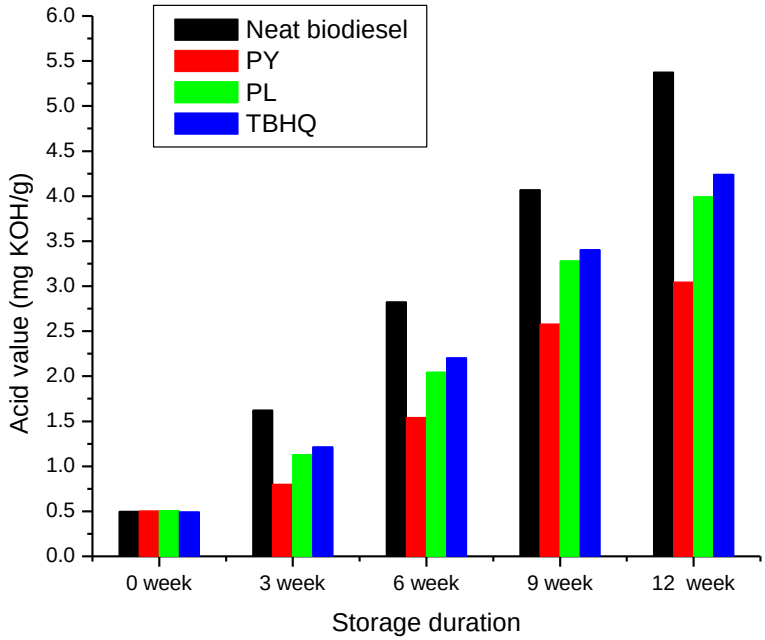

Figure 4.49 Effect of antioxidant and storage duration on acid value of microalgae biodiesel

4.6.3 Thermal stability of microalgae biodiesel

Thermogravimetric analysis has been commonly used to study the thermal stability of different types of materials, such as polymers and oils. Thermal stability of oils is highly influenced by their chemical structures. It was found from literature that oil samples with

more unsaturated fatty acids are less stable than saturated ones. Figure 4.50 and 4.51 show thermogravimetric analysis of microalgae biodiesel in the presence of nitrogen and air which shows decomposition and weight loss of microalgae biodiesel samples and derivative weight loss (DTG) with the corresponding temperature. The changes in weight happened due to evaporation and/or combustion of the methyl esters. Thermogram shows that biodiesel is stable up to 100°C. Approximately 1% weight loss of the fresh microalgae biodiesel was observed at around 150°C and 125°C (T_{on} temperature) when TGA was carried out in the presence of dry air and nitrogen respectively. After that, a rapid weight change was observed up to 271°C. Maximum degradation rate was observed at 234°C (1.44 mg/minute) and 254°C (1.96 mg/ minute) respectively when TGA was employed in the air and nitrogen atmosphere respectively. Degradation rate increased to maximum up to this point. At higher temperature (more than 300°C), rate of weight reduction was found slow.

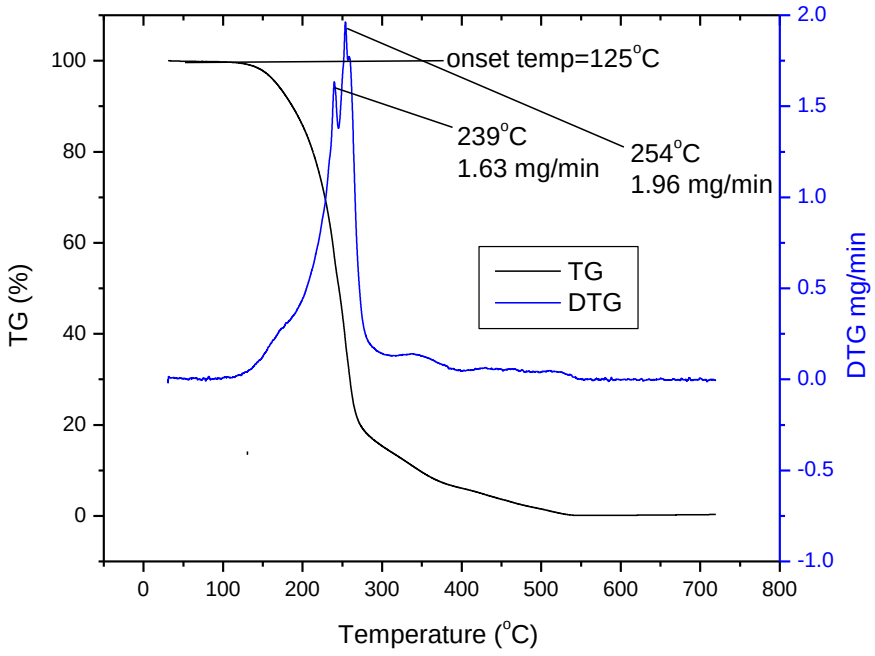

Figure 4.50 Thermogravimetric analysis of microalgae biodiesel in nitrogen atmosphere

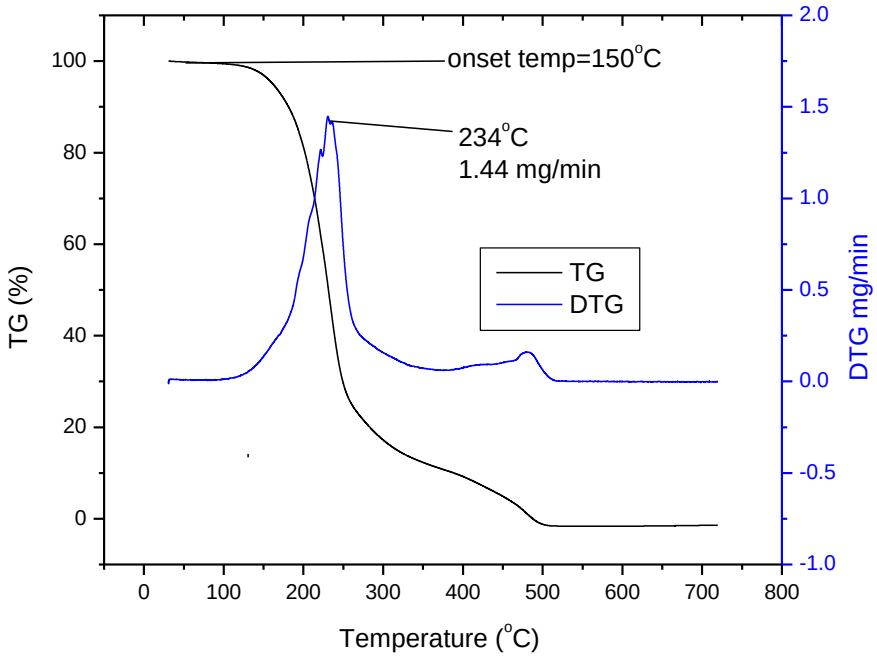

Figure 4.51 Thermo gravimetric analysis of microalgae biodiesel in dry air atmosphere

It can be concluded from TGA analysis that biodiesel obtained from *Chlorella vulgaris* showed as a good promising alternative fuel in terms of engine performance as it showed spontaneous single step of decomposition. Similar study was carried out by Jain and sharma (2011) on *Jatropha* biodiesel and observed T_{on} temperature as 193°C.

4.6.4 Hygroscopic nature of biodiesel

Biodiesel may have small but problematic quantities of water. The solubility of water is reliant on temperature and composition of biodiesel. Due to hygroscopic nature of biodiesel, water content increases during storage. Water absorbance capacity of biodiesel is more than petroleum diesel since fatty acid methyl esters (biodiesel) are hygroscopic compounds that make biodiesel much more hydrophilic than regular diesel. To evaluate its hygroscopic nature, microalgae biodiesel was kept under different humidity condition (RH) of (66 and 79) at room temperature for 10 days. After 10 days, absorption of moisture in samples attained their corresponding equilibrium. Results revealed that water absorption

nature of biodiesel increased with increasing humidity. Under relative humidity of 79%, water content of B100 microalgae biodiesel was increased from 0.04% to 0.57% while only 0.099% of water content was observed for diesel samples. Under Relative Humidity of 66%, there was an increase of 25% of water content for B100 microalgae biodiesel while only 8 % of water content for diesel samples.

Chapter 5

Conclusions

Conclusions and future prospects

5.1 Conclusions

This thesis investigated the potential of five *Chlorella* species (*Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.* 1 and *Chlorella sp.* 2) for biofuel production under indoor and outdoor conditions. Among the tested species, *Chlorella vulgaris* was identified as the most suitable species for biodiesel production and therefore, scale up of *Chlorella vulgaris* cultivation system was carried out in 10 L bubble column photobioreactor, 60 L flat plate photobioreactor and 1200 L open raceway ponds. Harvesting of microalgae was optimized using alum as flocculants. Different drying techniques (sunlight and hot air oven drying) were employed to harvested microalgae. Optimization of lipid extraction and transesterification was also carried out in this study. Physico-chemical properties of microalgae biodiesel was analyzed according to ASTM and Indian standards. In addition, this thesis also focused on the storage and thermal stability of microalgae biodiesel.

Major conclusion of this thesis was that among the tested microalgae species (*Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.* 1 and *Chlorella sp.2*), *Chlorella vulgaris* showed best potential for biofuel production, utilization of commercial grade fertilizer as growth medium for economical biomass production, enhancement of lipid extraction efficiency by employing microwave pretreatment method and improving storage stability of microalgae biodiesel using pyrogallol as antioxidant.

Specific conclusions are as follows

- Five strains of fresh water microalgae (*Chlorella vulgaris*, *Chlorella minutissima*, *Chlorella pyrenoidosa*, *Chlorella sp.* 1 and *Chlorella sp.* 2) were grown in BG-11, BBM, Fog's and M_4N growth medium. On the basis of lipid productivity and growth

media cost, BG-11 was found the best growth medium for all the five microalgae species.

- The impact of different nitrogen concentrations, nitrogen and carbon sources on biomass and lipid productivity of all the five *Chlorella* species was studied using BG-11 as growth medium. Among the tested species, *Chlorella vulgaris* achieved maximum lipid yield and productivity (223.91 mg/L and 13.98 mg/L/d) at 8.82 mM nitrogen concentration. Additionally, nitrogen source commonly used in BG-11 media (sodium nitrate) was replaced by urea due to its lower cost and commercial availability which leads to decrease in microalgal culture cost.
- When all the microalgae species were cultured in BG-11 in the presence of different organic carbon sources (glucose, glycerol, sodium acetate and sucrose) under mixotrophic condition, glycerol was found cheap and most promising carbon source to enhance lipid productivity of all the microalgae species. Utilization of glycerol (a byproduct of transesterification) for microalgae culture provides novel alternative method for recycling crude glycerol.
- All the five microalgae species were successfully cultured phototrophically in 10 liter bubble columns photobioreactor under outdoor conditions and *Chlorella vulgaris* showed maximum lipid productivity (10.94 g/L/d). When *Chlorella* vulgaris was grown as mixed cultures i.e. mixed culture-1 (*Chlorella vulgaris*: *Chlorella minutissima*), mixed culture-2 (*Chlorella vul*garis: *Chlorella pyrenoidosa*) and mixed culture-3 (*Chlorella vulgaris*: *Chlorella sp.*2) and compared with pure *Chlorella vulgaris* culture, it was found that pure *Chlorella vulgaris* has more lipid productivity than mixed culture (section 4.2.4 of chapter 4).

- *Chlorella vulgaris*, cultured in large scale photobioreactor (60 L flat plate photobioreactor) under semi-continuous culture mode, achieved lipid productivity of 23.92 g/L/d when half culture concentration was harvested at 4^{th} day. The algal biomass obtained in this study was found to have good composition of saturated, monounsaturated and polyunsaturated fatty acids which makes it good feedstock for biodiesel production.
- Additionally, study on mass cultivation of *Chlorella vulgaris* in 1200 L open raceway ponds using BG-11 and commercial grade fertilizer as culture growth medium showed that *Chlorella vulgaris* achieved biomass concentration of 8.63 $g/m^2/d$ and 5.6 $g/m^2/d$ in BG-11 and commercial grade fertilizer based growth medium respectively. The use of commercial grade fertilizer resulted in ~ 35 % reduction in total cost of culture media for the production of same quantity of biomass. Therefore, replacement of BG-11 growth media by commercial grade fertilizer provides a better option for cost-effective biodiesel production.
- Biomass harvesting was carried out using a combined method of flocculation and filtration. 250 mg/L concentration of alum at pH 5 was found the optimum condition to get more than 90% harvesting biomass.
- Microalgae lipid extraction is a very crucial step and depends up on various parameters, such as nature of solvents (polar and non-polar), extraction duration, moisture content of biomass, extraction temperature, biomass drying methods and solvent-biomass ratio. All these parameters were studied under this investigation. Harvested algal biomass was dried using different techniques such as sun drying and oven drying (60^oC, 80^oC and 100^oC). It was found that sun dried biomass results in maximum yields of 17.07% while oven dried biomass at 100^oC showed only

13.66% lipid yield. Additionally, sun drying method consumes no electricity and decrease biodiesel cost. Therefore, sun drying method can be preferred over oven drying method. Among the different tested solvents, chloroform: methanol (2:1) had shown best performance and extracted lipid yield was 16.27%. To increase extraction efficiency different pretreatment methods were applied to sun dried algal biomass and the optimal yield of total lipid was extracted up to 22.68% under microwave pretreatment. It was also observed that 10:1 solvent to biomass ratio (v/w) was sufficient to extract maximum lipid. Results show that this investigation will help to develop lipid extraction technique at large scale and useful for biodiesel production.

- Extracted lipid from microalgae had higher FFA up to 12.39%. Therefore, a two-step transesterification for microalgae lipid was employed to convert high FFA lipid to its methyl esters. In First step (acid catalyzed esterification), FFA content of microalgae lipid was reduced up to less than 2% under the following reaction conditions: lipid to methanol ratio 1:10, H_2SO_4 1.5% (v/v), temperature 60°C and time 40 minute. Second step (base catalyzed transesterification process) converts products of the first step to its mono-esters and glycerol. Factors affecting transesterification such as catalyst amount, lipid to methanol ratio, temperature, and reaction duration was optimized. Results showed that molar ratio of 10:1 of alcohol favors completion of base catalyzed transesterification process at 60°C within 15 minutes for microalgae biodiesel production in microwave reactor, which is sufficient to give 84.01% yield of ester. Obtained biodiesel was agreed by GC, NMR and FTIR analysis.

- It was also found that microwave assisted transesterification is more energy efficient and time saving process than conventional water bath heating assisted transesterification.
- Fuel properties of microalgae biodiesel obtained in this work were in good agreement with ASTM D6751, EN 1424 specifications and Indian IS-15607 specification.
- Oxidation stability of microalgae biodiesel was 3.08 h which meets ASTM D-6751 (minimum 3 h) but does not follow EN-14112 and IS- 15607 standards (minimum 6 h). Oxidation stability of biodiesel was improved by the application of different antioxidants. Pyrogallol (PY) was found the most effective antioxidant to increase oxidation stability of microalgae biodiesel.
- Storage stability studies showed that oxidation stability of neat biodiesel was reduced up to 2.82 h within 3 weeks while biodiesel treated with different antioxidants was stable for 12 weeks. Among the tested antioxidants, pyrogallol (PY) was observed the best antioxidant during storage study.
- Thermogravimetric Analysis (TGA) showed that more than 80% wt. of biodiesel was lost at 271°C.
- Hygroscopic study of microalgae biodiesel shows that it is highly hygroscopic and an increase of moisture content from 0.04% to 0.57% was observed at 79% Relative Humidity.

5.2 Future prospects:

Although, present research provides a basis for mass production of microalgae in photobioreactor and open raceway ponds, efficient lipid extraction and transesterification for biodiesel production, there are few issues which still need to be adduced.

- Light penetration in dense algal cultures, light saturation effect and photoinhibition caused by strong light intensity can be solved by proper designing of sophisticated PBRs. Investigations on designing of suitable PBRs according to different outdoor environmental conditions are required.
- Commercial grade fertilizer based growth media provide good potential for large scale biodiesel production. But, fertilizers or chemicals used to prepare this growth media should be optimized to get maximum lipid productivity.
- Extracting lipid from dry algae biomass using solvent is very energy intensive. Hence alternative processes of extracting lipid from the wet biomass must be developed to make algal biofuel feasible.
- Lipid extracted biomass (algal de-oiled cake) should be utilized for biofuel production to make it more energy efficient.
- Producing microalgal biofuel needs various processes, out of which, few were studied in this thesis. All these processes consume energy and total energy input should be less than energy content of the biofuel. In addition, effect of all the processes on biofuel production cost should be evaluated through their Life Cycle Assessment. However, neither the energy balance, nor the Life Cycle Assessment was made in this study, because of limited data availability.

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DATE OF BIRTH : November 8th, 1982

EDUCATIONAL / PROFESSIONAL QUALIFICATIONS:

Name of the course/ Diploma/Degree	School/College/Institution	Year of Passing	%age / Grade
HSC/Equivalent	Barla inter college, Barla, Muzaffar nagar	1997	71.1%
+2 science	Barla inter college, Barla, Muzaffar nagar	1999	63.6%
B.Sc. (CBZ)	DAV college, Muzaffar nagar	2005	64%
M.Sc. (organic chemistry)	DAV college, Muzaffar nagar	2007	66.6%
PhD (Science)	Institute for alternative energy research, University of Petroleum and Energy Studies, Dehradun	**	**

AWARDS/RECOGNITIONS, if any:

i) Awarded Junior Research Fellowship from DST for 2 years

ii) Awarded Senior Research Fellowship from Ucost for 2 years

ii) Worked as research assistant in Biofuel Research Laboratory from June 2013 till now

WORK EXPERIENCE:

August 20, 2008 to Nov. 30, 2008

- Junior Research Fellow, DST funded Project Titled "Feasibility of blending of SVO in petro-diesel and its utilization in IDI diesel engine." University of Petroleum & Energy Studies, Dehradun (U.K.)

Dec. 1, 2008 to Oct 31, 2010:

- Junior Research Fellow (JRF), DST funded Project Title "Technical performance analysis of diesel ethanol blends in DI diesel engine". University of Petroleum & Energy Studies, Dehradun (U.K.).

Nov. 1, 2010 to April 31 2013:

- Senior Research Fellow (JRF), UCOST funded Project Title "Establishment of Jatropha De-oiled cake based Biogas plant.".University of Petroleum & Energy Studies, Dehradun (U.K.)

May 1, 2013 to till now

- Research assistant (Biofuel research laboratory), University of Petroleum & Energy Studies, Dehradun (U.K.).

ACHIEVEMENTS & AWARDS

Professional

- Awarded best student award at Barla Inter college Barla M.Nagar

- Get seven days training on microalgae culture under Dr sunil Pabbi (Principal Scientist IARI)

Academic

- Gold Medal awarded by Barla Inter College Muzzafar nagar in high school and intermediate
- Honored by the Principal Mr Harpal singh of Barla Inter College as a best student of the college.
- Scholarship during Secondary education in1997-1999.

SKILLS

- ***Research and Synthesis skills:*** I have good experience to synthesize biodiesel from edible and non-edible oils, waste cooking oil and microalgae oil. In addition, I have also learnt how to analysis metal contamination in lubricant oil. Besides this, I have also worked on kitchen waste and non-edible based biogas reactors (floating drum type, fixed drum type and CSTR type). I have also worked on endurance characteristics for a diesel engine runs on jatropha oil and diesel-ethanol blends.

- ***Instruments handled***: I have good exposure in the analysis of various petroleum and Biofuel products with respect to the following parameters:

- Calorific value by Bomb calorimeter
- Flash Point by Pensky Martin Closed Cup Tester
- Density by Digital Density Meter and manually
- Kinematics viscosity by fungilab viscometer
- Corrosion by copper strip corrosion
- Oxidation Stability by PetroOxy meter
- Distillation of petroleum product

- Pour and Cloud Point of petroleum products
- Ultrasonic velocity by Ultrasonic Interferometer
- Acid-base titration by Potentiometric Titrator
- Moisture analysis by Karl Fischer for
- microalgae culture in Photobioreactor
- metal (Na and K) analysis by Flame photometer
- UV visible spectrophotometer
- Biodiesel and hydrocarbon composition analysis by Gas chromatograph with FID
- Biogas and producer gas analysis by Gas chromatograph with TCD
- Smoke meter and Gas analyzer for emissions analysis of CI and SI engines
- Operation of software based single test rig DI diesel engine
- Operating of 100 liter batch biodiesel plant
- Operating of 6 m^3 and 85 m^3 biogas plant

- ***Research interest*:** Biodiesel production from non-edible oil and microbes, bioethanol production**,** biogas production from wastes, conversion of waste biomass into fuel grade hydrocarbon, pyrolysis, gasification, synthesis of different fuel additive such as lubricity enhancer, antioxidants, corrosion behavior of different fuels and lubricant oils, green routes for organic synthesis, performance and emission study of CI and SI engines with different fuels, endurance characteristics for a diesel engine,

CONFERENCES

1. AK Sharma, R. Mahajan, PK Sahoo.**"Feasibility of biogas production from jatropha de-oiled cake with a modified floating dome anaerobi digester''.** Poster presentation in international Conference GTER, Gurukul Kangari University, Haridwar

2. A. K. Sharma, R. Mahajan, S. pandey, P. K. Sharma and P. K. Sahoo. **"Biogas production potential of Jatropha de-oiled with cattle dung from anaerobic digestion under psychrophilic and mesophilic temperature conditions''** Oral presentation, 7th Uttarakhand State Science and Technology Congress.

3. A. K. Sharma, P. K. Sahoo, S. Singhal, 2015.**"Feasibility of biodiesel production from Chlorella vulgaris microalga under outdoor conditions''** Oral presentation, NCBB 2015.

4. A. K. Sharma, P. K. Sahoo and S. Singhal, **Impact of various nitrogen and organic carbon sources on biomass growth and lipid production of *Chlorella spp.*** ETE 2015- Second International Conference on Environment Technology & Energy 2015 22-23 November, 2015, Colombo, Sri Lanka, (**oral ppt accepted**)

PUBLICATIONS

1. P K Sahoo, R. K. Tripathi & AK Sharma, 2011. "**Long term testing of indirect injection diesel engines fueled with diesel, neat jatropha oil and their blends**" *International journal of emerging science and technology* Vol. 4, number 1, pp. 103-120.

2. Shyam Pandey, Amit Sharma, P. K. Sahoo 2012. "**Experimental Investigation on the Performance and Emission Characteristics of a diesel engine fuelled with Ethanol, Diesel and Jatropha based Biodiesel blends**" in *International Journal of Advances in Engineering & Technology* ,Vol. 4, Issue 2, pp. 341-353.
3. Rajeswari B, Pradeep Kumar S, Amit Sharma, Pradeepta Kumar Sahoo and Sha Valli Khan P.S. "**Effect of C-5 and C-10 Fuel Blends of Euphorbia caducifolia haines on IC Diesel engine emissions,** International Journal of Advances in Engineering & Technology Vol. 6, Issue 3, pp. 1177-1186
4. S. Mondala , A. K. Sharma , P. K. Sahoo. **Solar Thermal Biomass Pyrolysis-A Review Paper,** International Journal of Scientific & Engineering Research, Volume 5, Issue 11, November-2014
5. A. K. Sharma, P. K. Sahoo and S. Singhal. 2015 "**Influence Of Different Nitrogen And Organic Carbon Sources On Microalgae Growth And Lipid Production"** *IOSR Journal of Pharmacy and Biological Sciences Volume 10, Issue 1*
6. A. K. Sharma, P. K. Sahoo and S. Singhal, 2015 **Screening and Optimization of culture media for *Chlorella sp.*as a raw material for Biodiesel Production Int J Pharm Bio Sci ; 6(3): (B) 251 – 262.**
7. A. K. Sharma, P. K. Sahoo and S. Singhal, 2015, **Feasibility of Biodiesel production from *Chlorella Vulgaris* grown in flat plate photobioreactor under outdoor conditions,** International Journal of ChemTech Research (accepted).

MISCELLANOUS

- Citizenship: Indian
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