# Studies on Cultivation Strategies and Biodiesel Production from Selected Microalgae Species of North-East India

*Thesis submitted in partial fulfillment of the requirements for the degree of*

**DOCTOR OF PHILOSOPHY**

*By*

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**September, 2015**

# Dedicated to my family.....

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# STATEMENT

I do hereby declare that the matter embedded in this thesis is the result of investigations conducted by me in the Centre for Energy, Indian Institute of Technology Guwahati, Guwahati, Assam, India, under the supervision of Prof. K. Mohanty and Dr. V. V. Goud, Department of Chemical Engineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described is based on the findings of other investigators.

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# CERTIFICATE

It is certified that the work contained in the thesis entitled "Studies on Cultivation Strategies and Biodiesel Production from Selected Microalgae Species of North-East India", by Miss Amrita Difusa (Roll No. 09615107), for the award of degree of Doctor of Philosphy, has been carried out under our joint supervision and that this work has not been submitted elsewhere for a degree.

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# Abstract

Microalgae have attracted much attention in recent times as a potential source for alternative biofuel production. In particular, microalgae derived biodiesel has been widely developed as an alternative energy source. Microalgal biomass has certain potential over the other feedstock due to their faster growth rate, higher oil yielding capacity, $CO_2$ capture and wide range of tolerance towards various environmental conditions. Besides cultivation and processing techniques for efficient production of microalgae derived biofuel, it also depends on selection of the potential strain. Biomass recovery also plays a challenging role in microalgae derived biofuel production system. Subsequently, a lot of research has been directed towards the biodiesel production focusing on various area of cultivation to downstream processing of microalgae. As a contribution to the research on microalgae derived biodiesel production, this thesis aim to select a potential microalgae species from existing 'microalgae resources' of the region and their potential aspects towards biodiesel production. From the collected 30 water samples, ADIITEC-III microalgae strain was selected for potential utilization as renewable biomass feedstock for biodiesel production. The selection was based on dominancy, growth and biochemical compositions of the microalgae. Moreover, *Scenedesmus* sp (GUBIOTJT116) was also taken as a reference species from the Department of Biotechnology, Gauhati University. The microalgal isolate ADIITEC-III was molecularly characterized by 18S rRNA gene sequence analysis and sequence of the strain was submitted to NCBI GenBank under the accession number KF471125.1. Under the optimum physiological condition, total lipid yield of *Chloromonas* species (ADIITEC-III) and *Scenedesmus* species (GUBIOTJT116) was recorded to be 31.8 ± 0.9% and 35.2 ± 0.72% respectively.

Nitrogen supplementation for algal growth was studied to address the importance of nitrogen which plays an imperative role in microalgal lipids and fatty acid metabolism.

# Abstract

Four different nitrogen sources i.e. ammonium nitrate ($NH_4NO_3$), potassium nitrate ($KNO_3$), urea (U) and sodium nitrate ($NaNO_3$) were employed for cell growth and lipid accumulation in a batch mode. Among the studied nitrogen sources, ammonium nitrate supports the lipid yield 35.86% in ADIITEC-III and GUBIOTJT116 showed high lipid yield (37.2%) with urea. Therefore, based on high lipid yield, the oil recovered from the isolates was transesterified into methyl esters (FAME) and was characterized by $^1H$ NMR spectrophotometer, thermogravimetric analysis (TGA), Fourier transform infrared (FTIR) spectroscopy, acid value, iodine value, bomb calorimeter and differential scanning calorimetric (DSC) analysis. Thereafter, the FTIR spectrum of the oil sample of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 obtained under different nitrogen sources were investigated in detailed using the chemometric techniques of discriminate analysis and multivariate calibrations.

Other than the nutrient supplement for microalgae cultivation, induction of stress is also an important element for lipid enhancement. The studied strains were further subjected to salt (NaCl) stress at two different culture conditions such as single stage saline condition and two stage cultivation strategies to enhance the lipid yield of ADIITEC-III and GUBIOTJT116. In single stage saline condition, NaCl concentrations were varied over the range such as 10, 30, 50, 70, 90, 110, 130, 150 and 200 mM respectively. Among the studied concentrations, 200 mM salt (NaCl) stress showed better results in ADIITEC-III with maximum lipid yield of 38.8 ± 0.5%. Therefore, results of the first stage cultivation strategy revealed that ADIITEC-III can be considered for two stage cultivation than GUBIOTJT116. From the two stage cultivation, ADIITEC-III showed high biomass productivity (1.5 ± 0.3 g $L^{-1}$) compared to control condition (0.45 ± 0.03 g $L^{-1}$) and increased lipid content up to 40.8 ± 0.2%. Moreover, *Chloromonas* species grown in high salinity stress condition (NaCl) showed better

biodiesel properties than control condition. Further, the thesis includes the utilization of cow dung and piggery waste as an effective alternative media for microalgae biomass production. For large scale cultivation, synthetic nutrient inputs deals with high expense which can be replaced by animal waste. The cow dung and piggery waste were characterized for their elemental composition and based on the compositions a range of concentrations i.e. 5%, 10% and 15% were considered for the experimentation. Increased lipid yield was noticed at 5% cow dung and piggery waste concentrations for ADIITEC-III and GUBIOTJT116 compared to the culture grown in BG11 medium. The physico-chemical characteristics of synthesized methyl esters (FAME) of ADIITEC-III and GUBIOTJT116 were evaluated and were found to be within the limits of ASTM D6751 biodiesel standards.

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# List of abbreviations

| | |
|---|---|
| ASTM | American Standards for Testing Materials |
| ATP | Adenosine tri phosphate |
| BBM | Bolds basal medium |
| BECCS | Bio-energy coupled with Carbon Capture and Storage |
| BG-11 | Blue-green 11 medium |
| BLAST | Basic Local Alignment Search Tool |
| BSA | Bovine Serum Albumin |
| CCS | Carbon Capture and Storage |
| CD | Cow dung |
| CEM | Continuous electrolytic microalgae |
| CP | Cloud point |
| CFPP | Cold filter plugging point |
| CV | Calorific value |
| DAF | Dissolved air flotation |
| DCW | Dry cell weight |
| DSC | Differential scanning calorimetry |
| DTG | Differential thermogravimetric |
| EIA | Energy Information Authority |
| EU | European Union |
| FAME | Fatty acid methyl esters |
| FESEM | Field Emission Scanning Electron Microscopy |
| FFA | Free fatty acid |
| FGBs | First generation biofuels |

# List of abbreviations

| | |
|---|---|
| FTIR | Fourier transform infrared spectroscopy |
| IEA | International Energy Agency |
| GDP | Gross domestic product |
| GHG | Green house gas |
| HCA | Hierarchical Clustering Analysis |
| HiPUFA | Highly polyunsaturated fatty acids |
| IONPs | Iron oxide magnetic nanoparticle |
| iP | Inorganic phosphate |
| IPCC | International Panel on Climate Change |
| IV | Iodine value |
| LCA | Life-cycle assessment |
| Ln | Linolenic acid |
| MDS | Multidimensional scaling |
| MUFA | Monounsaturated fatty acids |
| MTOE | Million tons of oil equivalent |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NMR | Nuclear magnetic resonance |
| PBR | Photo-bioreactor |
| PCA | Principal Component Analysis |
| PP | Pour point |
| PUFA | Polyunsaturated fatty acid |
| PW | Piggery waste |
| SFA | Saturated fatty acids |

# List of abbreviations

| | |
|---|---|
| SGBs | Second generation biofuels |
| TAG | Tri acyl glycerol |
| TFF | Tangential flow filtration |
| TGA | Thermogravimetric analysis |
| TGBs | Third generation biofuels |
| WEO | World Energy Outlook |
| WTW | Well-to-wheel |

# CHAPTER 1

# Introduction

# 1. INTRODUCTION

## 1.1. Rationale and significance

The entire world is facing a formidable fuel crisis due to the declining non-renewable energy resources popularly known as 'Peak Oil' while the energy demand is exploding (Campbell, 2008). Energy has been distinguished as a '*strategic commodity*' which plays a decisive role in functioning of the economy. The inevitable role of energy drives directly or indirectly the socio-economic development and human welfare of any nation (Singh *et al*., 2010). However, the soaring energy demand has also generated a huge energy gap poised by diminishing fossil fuel reserves. Global energy demands are attributed to the mounting global population and a growing economy around the globe. Driven by such demands, fuel prices have gone up a sky high in recent times, which has ominously threatened the world's economic security (Abou-Shanab *et al*., 2010). Also, the strong economic growth in response to the growing population has escalated a potential threat to environmental security. Nevertheless, to establish sustainable economic development, energy and economic security must be reconciled with environmental security (Figure 1.1).

Today due to the projected uprising trend in world population growth and energy demands, the international community is facing challenges in dealing environmental security *vis-à-vis* to attain sustainable economic development. The strong and determined political commitment is highly needed for a fundamental change in our approach to the production and consumption of energy **(**Subhadra *et al*., 2010).

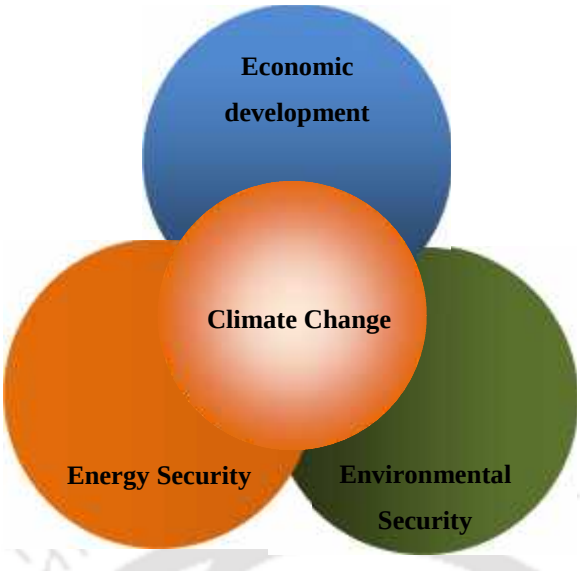


Figure 1.1 The 3E's security linkage for sustainable development (Talukdar *et al*., 2011)

The development of recent technology and increased exploitation of unconventional fossil reserves may let the availability of fossil fuel for a considerable period of time. Besides clean alternative fuels from renewable biomass, the assessment of new technologies, such as Carbon Capture and Storage (CCS) may implement the '*negative emissions*' technology to halt the rise in emissions from the energy sector (IEA 2013). The '*negative emissions*' implies the amount of $CO_2$ sequestered from the atmosphere generated through the growth of biomass is larger than the $CO_2$ emitted during the production of biomass (IEA, 2013). It is also associated with the emissions released during the conversion of biomass to final products. The continuous use of fossil fuel may create uncertainty regarding the sustainability of current fossil fuel because of depleting supplies and environment problems. From the climate change perspective, Bio-energy coupled with Carbon Capture and Storage (BECCS) could be a promising technology with wider range of applications (Gough *et al*., 2010). However, to attain the anticipated *'net negative emissions'* with BECCS, it is essential to employ biomass that can be sustainably converted to product.

## 1.2. Current scenario of world energy demand and oil price

The world energy market is mostly driven by fossil fuels in which oil, coal and gas are major contributors. According to EIA, (2013), these fossil fuels are projected to meet about 80% of the world energy demand by 2040. In addition, along with the increasing world energy demand, world total energy consumption is estimated to rise by 54% over the next 30 years (EIA, 2013). The reports also suggest the rise of world energy consumption from 505 quadrillion British Thermal Units (quads) in 2008 to 524 quads in 2010, with many probabilities to jump over 824 quads in 2040 (Fig. 1.2). Much of this growth in energy consumption is contributed by countries outside the Organization for Economic Cooperation and Development (non-OECD), where demand is due to the strong long-term economic growth. However, energy consumption in OECD nations increased by 18%, as compared to an increase of 85% for the non-OECD economies (EIA, 2013). According to the report published by Energy Information Agency (EIA), the world may need almost 60% more energy by 2030, of which 45% will be accounted for China and India. At this increased state of consumption, the world fossil oil reserve will be exhausted in less than 45 years (Ahmad *et al*., 2011). Among the various energy sources, total primary energy needs are being met by liquid fuel (oil) and consumption increases at an average annual rate of 1.0% from 2008 to 2035, whereas total energy demand increases by 1.6% per year.

According to an estimate, transportation sector alone contributed to 27% of the total liquid fuel consumption in 2008, with an annual increase of 1.4% per year between 2008-2013 (EIA, 2013). In recent times, as a result of such escalating energy demands supported with insufficient supplies, oil prices have increased to over US$ 100 per barrel (EIA, 2013). The impact of such fuel prices and aided supply disruptions has poised substantial uncertainty to the pace of economic progresses of the Nation. Therefore, the

clean alternative fuels from renewable sources, such as biomass could play a pivotal role to environmentally sustainable development (EIA, 2013).

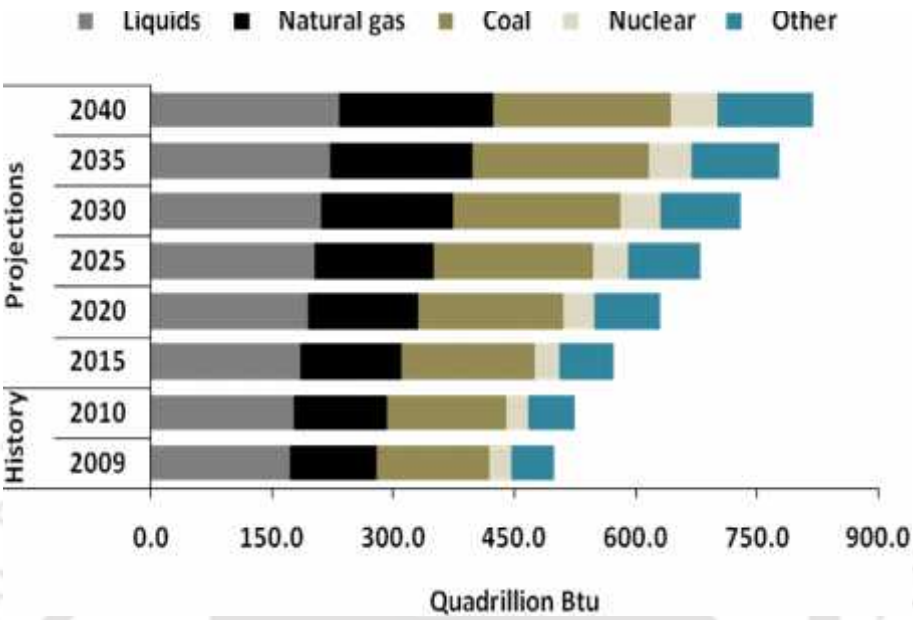


Figure 1.2 World total energy consumption, 1990-2040 (EIA, 2013)

### 1.2.1. Current status of India's energy basket

In past few decades, India has been developing very rapidly achieving constant upward industrial growth and ranks the ninth largest economy in the world (World Energy Statistics, 2013). The Indian economy has experienced an unprecedented economic development with an impressive gross domestic product (GDP) growth of about 8.0%. The rapid economic growth has placed massive demands on its indigenous energy resources. India ranks as the world's seventh largest energy producer accounting for about 2.49% of the world's total annual energy production and placed as the world's fourth largest energy consumer with about 3.6% of the global primary energy demand (Fig. 1.3). However, India also depends on the huge import of about 76% of the energy demands and only 30% of India's total primary energy needs are being met by petroleum oil (The Planning Commission Govt. of India, 2007). The pervasive imbalance between

energy demand and supply requires serious efforts to supplement the supplies as India undergoes an imminent energy supply constraint (IEA, 2013).

India's energy basket has a mix contribution of non-renewable fossil-based energy and renewable alternatives. Coal remains the dominant source of primary energy and accounted for 54% of energy consumption and is likely to be continued in the foreseeable future (BP World Energy statistics, 2013). Other forms of energy such as nuclear energy and hydroelectricity represent 5% and 6% of total primary energy consumption, respectively. The increasing demand for coal is expected to reach 980 MT, but domestic production will be about 795 MT by 2017, imposing a huge supply and demand gap, which needs to be met through imports (BP World Energy statistics, 2013). The Twelfth Plan document of the Planning Commission of India projected that the total domestic energy production of 669.6 million tons of oil equivalent (MTOE) will be scored by 2016-17 and 844 MTOE by 2021-22. The expected production will meet only around 71% and 69% of the energy demand respectively, while the remaining supplies could be met from imports.

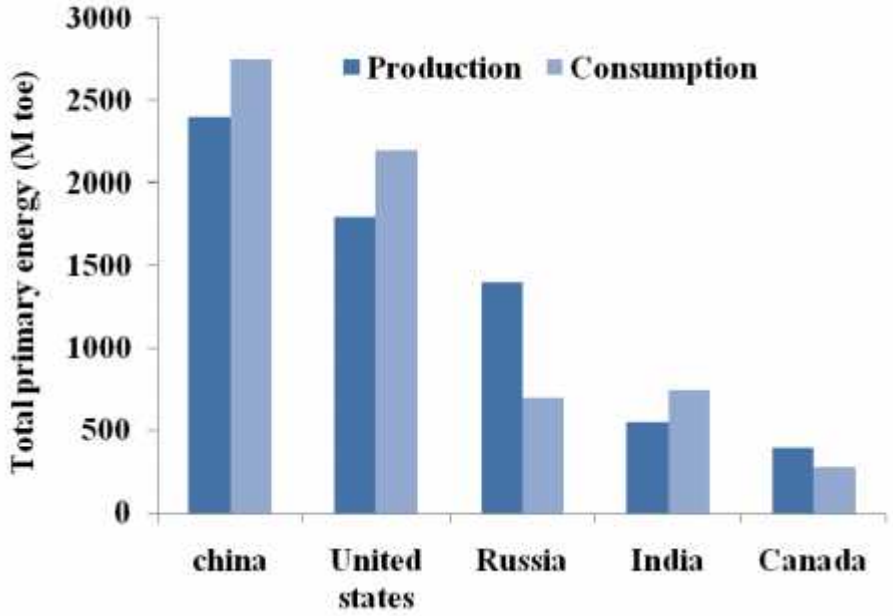


Figure 1.3 India's total primary energy production and consumption

(Source: Data from BP World Energy Statistics, 2013)

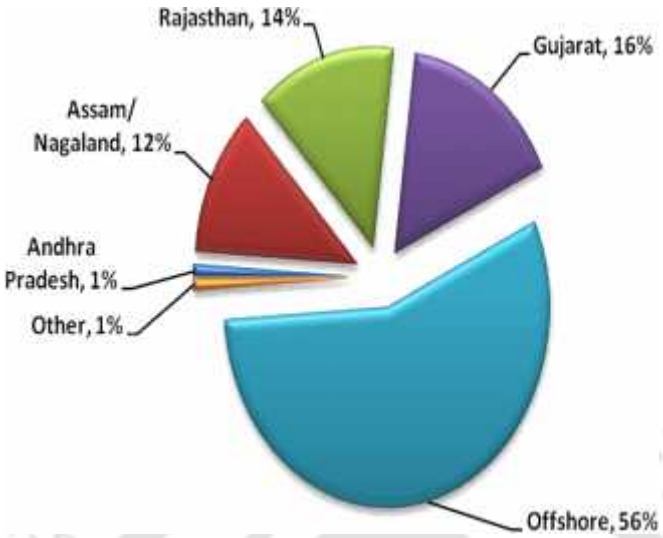


Figure 1.4 India's crude oil production by region (EIA, 2013)

To meet the envisaged growth of economies at 9% of GDP, the indigenous energy resources of India will not be sufficient enough to sustain for a long run (The Planning Commission, Govt. of India, 2007). Despite of its exhausting reserves, India continues to be dependent on fossil fuels and being the second-largest (behind China) energy producer among the Asia-Pacific region, had 5.7 thousand million barrels of proven oil reserves as of 2012, which can sustain the current level production only for about 30 years (BP World Energy Statistics, 2013) and most of these oil reserves are located in the Western part of India, particularly in Gujarat and Rajasthan (Figure 1.4). The Assam-Arakan basin is another important oil-producing region that contributes 10% of the country's oil reserves (The Planning Commission, Govt. of India 2007; EIA, 2013). The major oil reserves are from onshore resources which contribute about 53%, whereas 47% are offshore reserves.

### 1.3. Environmental issues

The growing energy demand and consumption of fossil fuels are causing serious environmental problems. The growing anthropogenic activities and industrialization has

been responsible for accumulating green house gases (GHG) in Earth's atmosphere, causing severe global impact, i.e., global warming and climate change. The post-industrialization has evident the largest change in atmospheric $CO_2$ concentration that attributed to rapid warming of the globe (Fig. 1.5) (IPCC, 2007). Fossil fuel combustion, over the last 100 years has increased $CO_2$ concentration into the atmosphere, currently it was recorded around 350-380 ppm and would increase to about 450 ppm by 2020, if certain control measures are not taken to reduce global $CO_2$ emissions (Kraan, 2013). The World Energy Outlook (WEO) (2013) projected the increase in $CO_2$ emissions in India by 3.4% per year from 2011 to 2035, which accounts 10% of global $CO_2$ emissions (IEA, 2013). In the European Union (EU), transportation and energy sectors are the major anthropogenic sources generating more than 20% and 60% of greenhouse gas (GHG) emissions respectively. Agriculture is the third largest anthropogenic source, contributing about 9% of GHG emissions, where nitrous oxide ($N_2O$) and methane ($CH_4$) are the most important gases (Mata *et al*., 2010). Although carbon dioxide is the most abundant anthropogenic greenhouse gas in the atmosphere and it accounts for up to 68% of total emissions (Ho *et al*., 2009). Atmospheric concentrations of carbon dioxide have been rising at a rate of about 0.6% annually in recent years, with further increase in the growth rate. As a result, by the middle of the 21$^{st}$ century, carbon dioxide concentrations in the atmosphere could be double than their pre-industrialization level (EIA, 2008). Fossil-fuel-fired power plants contribute approximately one third of the total $CO_2$ contributed by anthropogenic activities. Increased carbon emissions are expected unless energy systems reduce the carbon emissions in the atmosphere. Necessary strategies need to employ to stabilize and reduce concentrations of $CO_2$ gas in the atmosphere like carbon sequestration – carbon capture, separation and storage or reuse (Demirbas, 2011).

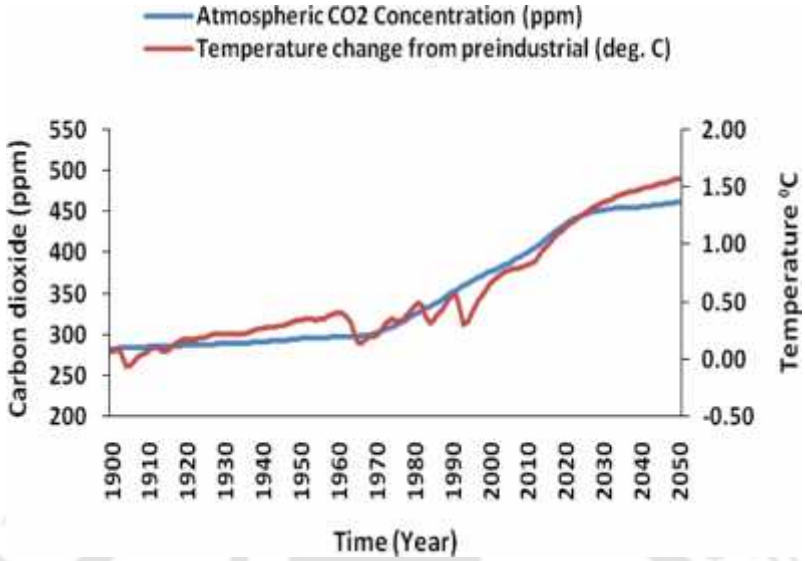


**Figure 1.5** World atmospheric concentration of $CO_2$ and an average global temperature change (Sources: Temerature data from NASA, (2013); $CO_2$ concentration from NOAA)

## 1.4. Development of biofuel resources

The growing concern over global warming and energy crisis has turned many countries towards the effective mitigation strategies and development of new, clean and sustainable energy sources. Today, various sources of renewable energy are coming into existence and among all biofuels are attaining most interest and are expected to play a crucial role in the future global energy infrastructure. The term biofuel is referred to as solid, liquid, or gaseous fuels that are predominantly produced from renewable feed stocks. Biofuels can be classified based on their production technologies: first generation biofuels (FGBs); second generation biofuels (SGBs); third generation biofuels (TGBs); and fourth generation biofuels (Demirbas, 2009). First generation biofuels have been mainly extracted from food and oil crops, including rapeseed oil, sugarcane, sugar beet, and maize as well as vegetable oils and animal fats using conventional technology (Brennan *et al*., 2010). Second and third generation biofuels are also called advanced biofuels. Second generation biofuels include bioethanol made from cellulosic material,

hemicelluloses, sugar, starch, and waste, as well as biomass-based biodiesel, biogas, biohydrogen, and other fuels made from cellulosic biomass or nonfood crops. Third generation biofuel include algal fuel. On the other hand, fourth generation biofuels are based on the conversion of vegetable oil and bio-diesel into bio-gasoline using most advanced technology. Overall, the production and consumption of liquid biofuels have certain limitations towards meeting the overall energy demands in the transport sectors due to: food security, technological, economical, supply and storage, safety, and policy barriers (Demirbas, 2009). However, to achieve environmental and economic sustainability, fuel production processes are required that are not only use renewable resources, but also capable to mitigate atmospheric carbon dioxide ($CO_2$). Having potential to reduce GHG emissions, it significantly contributes energy security by expanding the supply source for transport. The efficacy of biofuels in reducing GHG emissions also varies based on its sources. The comparison of a reduction in GHG emissions efficacies of biofuels is done on the basis of well-to-wheel (WTW) life cycle analysis with respect to conventional fossil fuels. The analysis signifies the total GHG emissions from production to the consumption stage of the product (IEA, 2013). For example, based on the analysis GHG reduction efficacy of first generation biofuels such as, corn derived ethanol is in the range of 10-70% with respect to conventional gasoline, while sugarcane derived ethanol showed much higher reduction efficacy (i.e. 70-120%). Likewise, oilseed-derived biodiesel in comparison to conventional petroleum diesel provide GHG reduction efficacy of only 30- 60% (IEA, 2013).

Currently, microalgae have grabbed much attention in terms of its potentiality towards the sustainable production of fuel, as well as able feedstock to mitigate the emitting carbon dioxide, which is one of the major GHG's to the atmosphere. Whereas,

production of the microalgae derived biofuel could favor the current emission reduction and therefore make a significant contribution towards $CO_2$ bio-mitigation.

### 1.5. Microalgae as a contemporary source of biofuels

Microalgae are emerging as the most versatile biomass source and suitable alternative feedstock for biofuels owing to their higher oil content, a very short harvesting cycle (~1-10 days depending on the process), greater light capture and conversion efficiencies, can be cultivated in waste water or marine water and on marginal or non-arable land (Chisti, 2007). Microalgae possess high photosynthetic efficiency and potentially have high oil yield per area than that of the best oilseed crops (Fig. 1.6). Microalgae from mini 'cell factories' is turn into a superior biomass due to the unique combination of higher photosynthetic efficiency poised with high yield of controlled microbial cultivation which prevailed the microalgae as promising organisms for economical, an industrial-scale production process in the 21$^{st}$ century (Rosenberg *et al*., 2008; Hu *et al*., 2008). Due to the huge diversity of microalgae together with various applications entail them to search for a suitable candidate for biofuel production. For successful microalgae derived biofuel production, the crucial step is to search, collect and identify hyper-lipid yielding strains.

Several microalgae strains such as *Nanochloropsis* sp., *Secenedesmus* sp., *Chlorella* sp., *Chlorococcus* sp., *Tetraselmis* sp., *Monoraphidim* sp., *Botryococcus braunii*, etc. have already been studied for their suitability as biofuel feedstock. However, many other potential microalgae strains are still remain to be explored and promises new approach into their biotechnological applications. Therefore, bio-prospecting is significant in potential introspection of microalgae strain in terms of biomass generation and lipid accumulation (Mutanda *et al*., 2011). Isolation and

selection of native strains is currently considered as a promising strategy to achieve desired strains with high growth rates under the respective climatic conditions (Mutanda *et al*., 2011; Talukdar and Kalita, 2011).

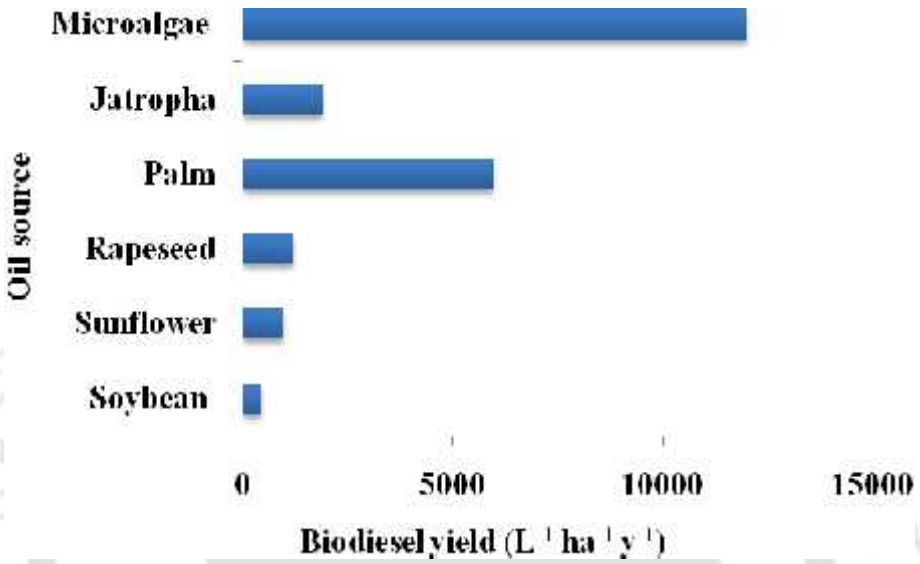


Figure 1.6 Average productivities of some common oil seed crops compared to microalgae (Schenk *et al*. 2008)

Large scale cultivation of microalgae for biomass production could play an immense role in mitigation of $CO_2$ along with the production of biofuels and other value-added products. Biological utilization of $CO_2$ using microalgae is an economically beneficial method to employ greenhouse gas emissions to generate value-added products. Microalgae have a higher capacity for $CO_2$ utilization through photosynthesis than any higher order plants. Utilization of $CO_2$ for biomass production can be accomplished in fully contained photo-bioreactors or in open systems, such as open ponds and channels. The concept of using outdoor microalgae cultures in ponds, lakes, oceans or land (using photobioreactors) can be applied as a method to reduce $CO_2$ emissions. However, it is difficult to optimize algal growth in an open pond, resulting in low productivity of the process (Campbell, 1997; Chisti, 2007 & 2008; Talukdar *et al*., 2011). Such advantages

of second generation biofuel production from algal biomass have significant benefits as a technology to $CO_2$ Capture and Storage (CCS), which would not only mitigate $CO_2$ besides reducing other GHGs concentration in the atmosphere, but also could replace fossil fuels currently being used (Moomaw, 2008). Third generation biofuel production by microalgae have the potential to overcome many of the limitations and target an emerging clean energy market, which is predicted to expand rapidly to a value of US $500 bn by 2050, or more (current oil market value the US $2.65 Trillion) (Schenk *et al*., 2008).

## 1.6. Organization of the thesis

The study conducted in the thesis has been represented in the following chapters:

**Chapter 2** is a comprehensive review of literature entirely based upon the objectives chosen in the study.

**Chapter 3** includes the detailed list of material and methods applied during the thesis work.

**Chapter 4** deals with the results and discussion of the entire study of the thesis.

**Chapter 5** represents the highlights of important findings and overall conclusion of the thesis.

# CHAPTER 2

# REVIEW OF LITERATURE

# 2. REVIEW OF LITERATURE

## 2.1. The microalgae diversity

Algae are aquatic photosynthetic organisms that occur in most habitats, with varied size and structure ranging from small, single-celled forms to complex multicellular forms. They are mainly distributed in the aquatic habitats with a wide range of reproductive strategies, from asexual cell division to complex sexual reproduction (APHA, 1998). Photosynthesis in microalgae is similar to higher plants but lacked stems, leaves and conducting vessels (xylem/phloem) (Croft *et al*., 2006). Microalgae are unicellular or multicellular photosynthetic microorganism that includes prokaryotes and eukaryotes also. Examples of prokaryotic microorganisms are Cyanobacteria (Cyanophyceae) and eukaryotic microalgae are, for example, green algae (Chlorophyta) and diatoms (Bacillariophyta) (Kraan, 2013). Based on storage compounds, life cycle and basic cellular structure microalgae can be classified as given in (Table 2.1).

Table 2.1 Classification of microalgae based on storage compounds, life cycle and a basic cellular structure of microalgae

| **Algal group** | **Important storage compounds†** |
|---|---|
| Cyanophytes (blue green algae) | Glycogen |
| Chlorophytes (green algae) | Starch, lipids |
| Bacillariophytes (diatoms) | Lipids, chrysolaminarin |
| Chrysophytes (golden-brown algae) | Lipids, chrysolaminarin |
| Haptophytes | Lipids, chrysolaminarin |
| Euglenophytes | Paramylon |
| Rhodophytes (red algae) | Floridean starch |
| Phaeophytes (brown algae) | Laminarin |

†According to Sheehan *et al*., (1998); Wang *et al*., (2012); and Hildebrand *et al*., (2013)

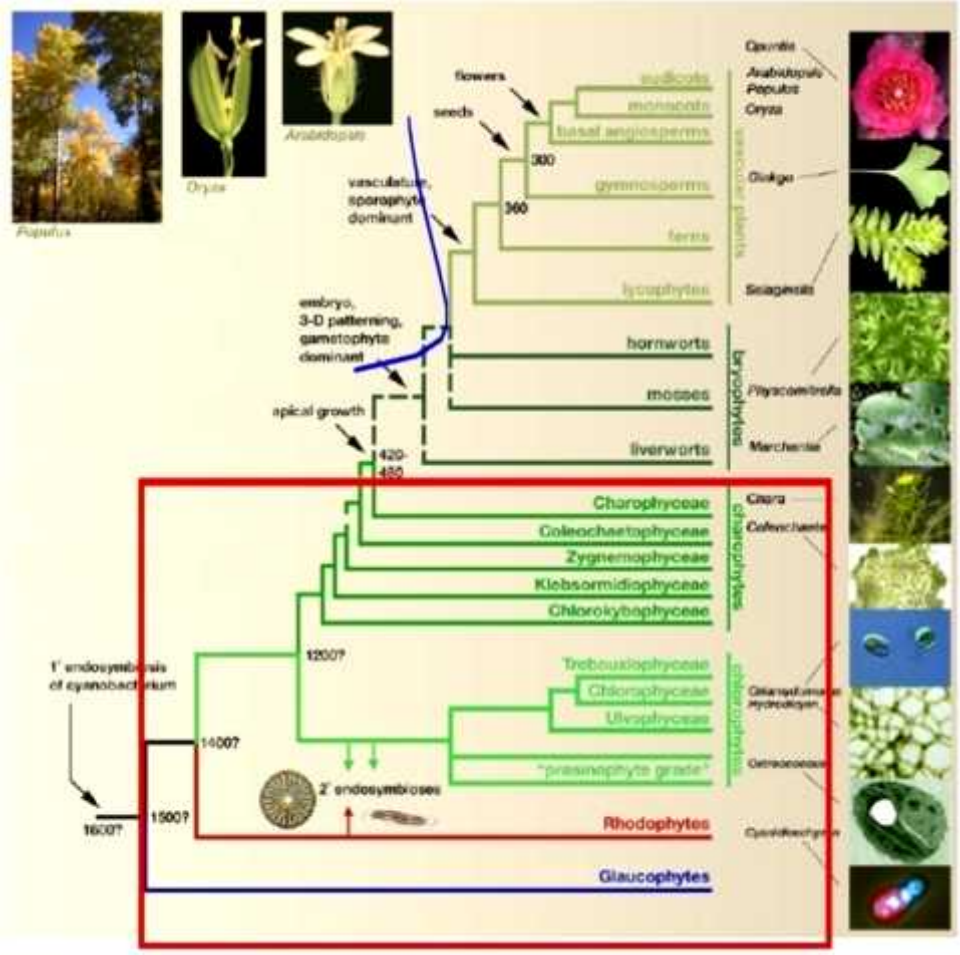


Figure 2.1 Phylogenetic relationships among algae and higher plants. The three lineages of algae, including Glaucophytes (freshwater algae; blue line), Rhodophytes (red algae; red line), Chlorophytes and Charophytes (green algae; green line) are marked within the box (Bowman *et al*., 2007)

The microalgae diversity is currently investigated in various genome projects and RNA sequencing studies. In fact, diverse microalgae storage compounds becomes very important in relevance to microalgae biofuel productions. The microalgae are able to produce considerable amounts of triacylglycerols (TGA) in comparison to other energy feedstocks such as canola or soybean (Chisti, 2007; Mata *et al*., 2010; Sivakumar *et al*., 2010).

## 2. 2. Microalgae as a potential source of biofuel production

Microalgae as a potential renewable source of bioenergy has long been discussed as the most efficient feedstock for oil production and a versatile biomass source, microalgae may soon be one of the Earth's most important renewable fuel crops (Campbell, 1997). These versatile photosynthetic microorganisms may be exploited either as biomass for gasification or combustion, or as a source of oil (hydrocarbons/lipids). They are also potential for biological hydrogen production or in a direct bio-photovoltaic electricity generation. The advantage of using microalgae is that they have very high growth rates, utilize a large fraction of the solar energy (up to 10% of the solar energy), and can grow in conditions that are not favorable for terrestrial biomass growth (Corma *et al*., 2006). Microalgae are capable of producing more amount of oil per unit area of land in comparison to that of all other known oil producing crops (Campbell, 2008; Christi, 2007; Schneider, 2006; Hagg, 2006). The annual yields of microalgal oil per unit area are in order of magnitude greater than those of higher plants. The per hectare yield of microalgal oil has been estimated between 58,700–136,900 liters per year, which is 7–31 times higher than the next best land crop i.e. palm oil (5950L $ha^{-1}y^{-1}$) or jatropha (1892 L $ha^{-1}y^{-1}$) (Chisti, 2007). According to an estimate algae can produce about 10 tons of biodiesel per acre per year (Mitchell, 2008) and only about 4.52 mha of land will be required to meet India's fossil diesel requirement by 2020 (Talukdar *et al*., 2007). Gouveia and Oliveria, (2009) screened six potential microalgae species (*Chlorella vulgaris*, *Spirulina maxima*, *Nannochloropsis* sp., *Neochloris oleabundans*, *Scenedesmus obliquus* and *Dunaliella tertiolecta*) for biofuel production. Among them, *Neochloris oleabundans* (fresh water microalgae) and *Nannochloropsis* sp. (marine microalgae) proved to be suitable source for biofuel production, due to their high oil content (29.0 and 28.7%, respectively). Each microalgae species produces different ratios of lipids,

carbohydrates, and proteins. Nevertheless, these tiny organisms have the ability to manipulate their metabolism through simple manipulations of chemical composition of the culture medium (Behrens and Kyle, 1996), thus high lipid productivity can be achieved.

## 2.3. Criteria for species selection

The first step in developing an algal process is to choose the right algal species. The selection of right species/strain(s) with relevant properties for specific culture conditions and products is the key factor for a successful biotechnological application of algae (Pulz and Gross, 2004). A variety of desirable characteristics reported for large-scale algal culture are summarized in Table 2.2 (Griffith and Harrison, 2009). A single algal species is unlikely to excel in all categories, hence prioritization is required. Environmental conditions, available resources and choice of culture system influence species choice. Some algae are most productive at high temperatures and bright light, while growth of others is retarded by full sunlight (Sheehan *et al*., 1998). Certain algal species cannot be grown outdoors as they are quickly dominated by fast growing microalgae species. However, those with slower growth rates could potentially be maintained in a closed photo-bioreactor (PBR) to facilitate more accumulation of desired products.

The selection of fast-growing, productive strains, optimized for the local climatic conditions is of fundamental importance to the success of any algal mass culture, and particularly for low-value products such as biodiesel. The fast growth encourages high biomass productivity. High biomass density increases yield per harvest volume and decreases cost. The high growth rate also reduces contamination risk owing to out-competition of slow growers in planktonic, continuous culture systems. A high content of the desired product increases the process yield coefficient and reduces the cost of

extraction and purification per unit product (Borowitzka *et al*., 1992). Choosing a species well suited to the bio-refinery approach, for example, producing valuable co-products such as fine chemicals, nutraceuticals or a nutrient-rich biomass, contributes to both economic success and environmental sustainability.

Table 2.2 Desirable characteristics of microalgae selection for mass culture.
Adapted from (Griffith and Harrison, 2009)

| **Characteristic** | **Advantages** | **Reference** |
|---|---|---|
| Rapid growth rate | Competitive advantage, require less area for culture | Borowitzka *et al*., (1992) |
| High product content | Higher value of biomass | Borowitzka *et al*., (1992) |
| Growth in extreme environment | Reduces contamination and predation | Borowitzka *et al*., (1992) |
| Large cell size, colonial or filamentous morphology | Reduces harvesting and downstream processing cost | Borowitzka *et al*., (1992) |
| Wide tolerance of environment conditions | Required less control of culture conditions, growth over range of seasons & ambient weather conditions | Borowitzka *et al*., (1992) and Grobbelaar, (2000) |
| $CO_2$ tolerance & uptake | Greater potential for $CO_2$ sequestration & use of waste $CO_2$ | Grobbelaar, (2000) |
| Tolerance of contaminants | Potential growth in polluted water & on flue gases with high $CO_2$, $NO_x$ & $SO_x$ | Zeiler *et al.*, (1995) |
| Tolerance of shear force | Allows cheaper pumping & mixing methods | Borowitzka *et al*., (1992) |

## 2.4. Cultivation condition for microalgae growth

To obtain optimal growth, microalgae culture medium must be provided with nutrients in an adequate amount (Borowitzka, 1988), which include macronutrients such as carbon, nitrogen, phosphorus, potassium, sulfur, and silicon (for diatoms), and trace elements such as minerals (*e.g.* Co, Mo, Mn) and vitamins (e.g. Cyanocobanlamin, thiamin). Besides quantities, right proportions among the nutrients (*e.g.* N: P; N: Si) must be maintained (Kaplan *et al*., 1986).

### 2.4.1. Macronutrients

The three most primary macronutrients are carbon, nitrogen and phosphorus.

**Carbon:** A large group of algae can utilize both inorganic ($CO_2$ and $HCO_3^-$) and organic carbon. Depend on the species the algal culture systems may utilize the organic carbon either by photo-heterotrophically near the surface, or heterotrophically in the deeper parts of the pond. According to Abeliovich, (1980), an alga utilizes about 20% to 50% of organic carbon heterotrophically.

The modes of organic carbon nutrition vary greatly with algal species (Kaplan *et al*., 1986). Algae which are able to grow mixotrophically (photo-heterotrophically) can grow faster in the light when both inorganic and organic carbon sources are available (Marquez *et al*., 1995; Neilson and Lewin, 1974). Several reports are available in the literature on number of species of genera *Chlorella* and *Scenedesmus*, to investigate the organic carbon nutrition with respect to biomass production (Burell *et al*., 1984; Martinez *et al*., 1987). These species shows the dual nature of obligate autotrophy and obligate heterotrophy i.e. they can shift rapidly and reversibly between growth in darkness on organic substrates and growth in light on carbon dioxide ($CO_2$). Although very scanty work has been done to study the photo-heterotrophic potential of microalgae

and appears that these algae would be very efficient at organic carbon utilization (Cid *et al*., 1992; Marquez *et al*., 1995; Neilson and Lewin, 1974).

**Nitrogen:** Besides carbon, nitrogen is quantitatively the most important element affecting the biomass, growth and lipid productivity of various microalgae (Arumugam *et al*., 2013). The theoretical C: N: P values required for balanced growth of an algal population is 106:16:1 (Healy, 1973). McCarthy *et al*., (1997) demonstrated that phytoplankton prefers nitrogen in the form of ammonium ($NH_4$). The order of preference observed in their study was $NH_4^+$ > urea, followed by nitrate, while nitrite only used in small quantities. However, in a study conducted on *Scenedesmus bijugatus* revealed that nitrate forms ($KNO_3$ and $NaNO_3$) of nitrogen favored the algal growth over ammonium (Arumugam *et al*., 2013). As per the report, biomass production of *Botryococcus braunii* was also supported by potassium nitrate as a nitrogen source over the other sources (Dyananda *et al*., 2006). Therefore, nitrogen supplied in any form promotes algal growth. Studies on algal growth, biomass and lipid productivity in response to nitrogen supplementation are also reported for many microalgae strains such as *Porphyridium purpureum* (Becker, 1994), *Scenedesmus dimorphous* (Benider *et al*., 2001), *Tetraselmis suecia*, *Skeletonema costatum* and *Thalassiosira pseudonana* (Griffiths and Harrison, 2009; Rodolfi *et al*., 2009). Nitrogen limitations are also reported as an efficient environmental stress to increase lipid accumulation (Zhang *et al*., 2013). During insufficient nitrogen, the energy required for protein synthesis and excess carbon from photosynthesis is converted into storage molecules such as triglyceride or starch (Scott *et al*., 2010). According to the reports, *Chlorella* species could double their lipid content under insufficient nitrogen conditions and showed a linear relationship between the nitrogen source concentration and lipid content (Converti *et al*., 2009; Zhang *et al*.,

2013). It has been found that the lipid composition gradually changes from free fatty acid-rich lipid to mostly triglyceride-containing lipid when the media culture is changed from normal nutrient to the N depleted media (Takagi *et al*., 2000).

**Phosphorus:** Phosphorus is one of the major elemental nutrients required for normal growth of algae. The concentration of organic phosphate in natural waters often varies and exceeds that of inorganic phosphate (iP), the major form that microalgal cells acquire P is as iP. Generally, optimum N:P ratio for phytoplankton growth is ~15:1; and high ratios (*i.e.*, ~30:1) indicates P - limitation, whereas low ratios of ~5:1 suggest N - limitation (Darley, 1982).

### 2.4.2. Micronutrient

Micronutrients are those elements required in much lower concentrations (usually in mg $L^{-1}$) than the major elements for the normal growth and metabolism of a living organism, most importantly are vitamins or minerals including S, K, Na, Fe, Mg, Ca and trace elements such as B, Cu, Mn, Mo, Co, V and Se, as reflected in the elementary composition of algae (Grobbelaar, 2000). Usually three vitamins - cyanocobalamin (vitamin $B_{12}$), thiamine (vitamin $B_1$), and biotin (vitamin H) are used in the algal culture, but very few algae need all three vitamins (Provasoli and Carlucci, 1974). The general order of vitamin required for algae is vitamin $B_{12}$> $B_1$> H. For a large-scale culture of single species, prior determination of the type of vitamin(s) required for the species is necessary. Algal growth in both axenic and non-axenic condition is influenced by a number of abiotic and biotic factors, which are summarized in Table 2.2 (Borowitzka, 1997).

## 2.5. Utilization of animal waste for microalgae cultivation

Generally, microalgae cultivation requires nutrients (e.g., N and P) in large quantities, which are significantly associated with the cost and environmental impact of its production (Levine *et al*., 2011). Utilization of wastewater as a nutrient source for microalgae cultivation emerges as a promising option in terms of minimum energy input (Chinnasamy *et al*., 2010). The wastewater promotes microalgae growth due to high content of nitrate and phosphate. Microalgae also have a potential to remove nutrients from wastewater before discharging to water sources (Levine *et al*., 2011). However, challenges associated in using wastewater are susceptible to contamination and inconsistency of nutrients compositions which directly retard the growth of microalgae. On the other hand, organic fertilizers which are obtained through composting and refining processes from waste materials offers an alternative and a convenient way to minimize the level of contamination (Marzouk *et al*., 2010). During the composting process, waste materials such as manure, sewage sludge, biomass and food tends to release $CO_2$, however, the amount of $CO_2$released during this process is low compared to processes used in the production of inorganic fertilizer (Lam and Lee, 2012). Nevertheless, $CO_2$ generated from the composting process can be recycled and utilize as a carbon source for microalgae cultivation.

Several reports are available on utilization of animal waste for microalgae cultivation. The utilization of organic fertilizer was investigated on *Chlorella vulgaris* where cells grown favorably at initial nitrate content of 26.67 mg $L^{-1}$ and 24 h of continuous illumination (Lam and Lee, 2012). According to the reports, *Chlorella* and *Scenedesmus* species could grow better at high concentration of nitrates or ammonium solutions compared to planktonic algae (Syrett *et al*., 1962). It has been reported that an animal digested effluent supports the growth of non-filamentous green algae due to the

excess ammonium-N content in animal digested effluent (Lam and Lee, 2012). However, excessive ammonium content in the culture could inhibit nitrate uptake and algal photosynthesis (Ohmori *et al*., 1978). In fact, growth inhibition was observed in *Scenedesmus subspicatus* with increased phosphate concentration (< 50 $gm^{-3}$), but phosphorous has less influence on algal growth compared to excessive nitrate (Hund, 1997). The waste water nutrient removal i.e. nitrogen, phosphorus, calcium and inorganic carbon from piggery wastewater by microalgae cultivation was also investigated by Abou-Shanab *et al*., (2013). The study showed maximum removal of nutrient by *C. Mexicana* and *C. vulgaris.* Another study with *Chlorella* sp. showed significant reduction in total nitrogen and phosphorous content from 132 to 13 mg $L^{-1}$ and from 215 to 45 mg $L^{-1}$, respectively (Li *et al*., 2011). De la Noue and Basseres, (1989) also reported removal of ammonium-N (99.5%) and phosphate-P (88.2%) from AD effluent containing high concentration of 102 $gm^{-3}$ TN and 8.31 $gm^{-3}$ TP.

Altogether reported literature suggest that digested manure perform better than the raw manure as anaerobic digestion helps in reducing the suspended solids and coloration of the manure (De la Noue and Basseres, 1989). However, there are some factors that negatively affect the algal growth, such as: variable in compositions of digested swine waste, it has high turbidity and strong coloration, high free ammonia (FA) and heavy metals (As, Cu, Zn) (Martin *et al*., 1985). Normally, dilution of organic compositions is usually needed before feeding to algae to avoid high $NH_3$ inhibition and turbidity. In fact, beyond certain pH condition unionized form of ammonia showed toxicity towards algal growth (Abeliovich, 1980). On the other hand, *P. bohneri* grew adequately better on raw manure and ammonium toxicity level has been recorded slightly higher than the normal toxicity level reported for the algal species (Abeliovich, 1980).

## 2.6. Factors influencing the growth of microalgae

The growth of microalgae in both axenic and non-axenic condition is influenced by a number of abiotic and biotic factors, which are summarized in Table 2.3 (Borowitzka, 1997).

### 2.6.1. Light

The intensity of light has the most profound effect on microalgae growth as it directly relates to the amount of energy supplied to the photosynthetic apparatus which influences biochemical reactions such as the rate of nutrient uptake (Curtis and Megard, 1987; Meseck *et al*., 2005; Terry *et al*., 1983) and the degree of lipid saturation (Fabregas *et al*., 2004; Klyachko-Gurvich *et al*., 1999; Thompson *et al*., 1990). Changes in net gas exchange with increasing irradiance follows a well-defined pattern, which is described by Blackman or P-E (Photosynthetic rate *vs* Irradiance) curve (Rabinowitch and Govindjee, 1969) and a similar trend in the growth rate with respect to irradiance is typically observed (Goldman, 1980; Terry *et al*., 1983). One very important aspect of light intensity is its effect on the photosynthetic apparatus at high irradiances. The physiological and biochemical responses to photo-inhibition include photo-protection, photo-inactivation and photo-acclimation. Photo-protection prevents excessive light absorption through dissipation of excess excitation energy (thermal dissipation) and through the removal of active oxygen species such as superoxide anion radical $O_2^-$ and singlet oxygen $^1O_2$ (Demmig-Adams and Adams, 1992; Franklin *et al*., 2003).

Besides light intensity, spectral (light) quality also plays a vital role in the process of microalgae photosynthesis. Nevertheless, its effect has not been widely investigated with respect to outdoor cultivation of microalgae, where it could be of highly significance, as the use of full solar energy is already challenged by inefficient capture

and unequal distribution through the water column (Richmond, 2004). Photosynthetic and metabolic responses appear to be species specific, owing to the complement of pigments inherent of microalgae species. In general, white light results in an intermediate photosynthetic response, whereas appreciable inter-specific differences can be observed at either blue-green or red wavelengths. Aidar *et al.,* (1994) in their study showed that photosynthesis and growth rate in *Tetraselmis gracilis* was enhanced in red light compared to white and blue lights, whereas both blue and red light stimulated photosynthesis in diatom *Cyclotella caspia*, while blue light resulted in higher growth rate. Likewise, faster growth and higher photosynthetic rates in *Dunaliella tertiolecta* and *Cyclotella nana* was achieved in blue light, which lead to increased protein/carbohydrate ratios as compared to white light (Wallen and Geen, 1971). Usually the optimum N: P ratio is influenced by varied light spectra, as shown for *Dunaliella tertiolecta, Phaeodactylum tricornutum, Prymnesium parvum and Thalassiosira pseudonana* (Wynne and Rhee, 1986). Ultraviolet (UV-A and UV-B) radiation has been shown to reduce photosynthesis (Franklin *et al*., 2003); growth rates (Bothwell *et al*., 1993); lipid contents and can cause damage to DNA (Karentz *et al*., 1991).

Table 2.3 Factors influencing algal growth

| **Abiotic factors** | **Biotic factors** | **Operational factors** |
|---|---|---|
| Light (quality & quantity) | Pathogen | Mixing, Dilution rate |
| Temperature, pH, $O_2$, $CO_2$ | Predator | Depth, Addition of |
| Nutrients (N, P, C), | Competition | bicarbonate |
| Salinity, Toxic chemicals | | Harvest frequency |

### 2.6.2. Temperature

Temperature influences the growth of microalgae primarily through: (a) controlling the enzyme kinetics (Davison, 1991; Raven and Geider, 1988), (b) metabolite degradation or synthesis, and (c) changing the conformation of vital structures such as cell membranes (Fogg, 2001). Microalgae growth rates with respect to an increase temperature is either exponential or linear in case of nutrient-limited cultures (Thompson *et al*., 1993). While most microalgae are able to adjust and adapt to short as well as long-term changes in temperature, each species and/or strain has a characteristic optimum level of temperature (Eppley, 1972; Oliveira *et al*., 1999; Payer *et al*., 1980; Renaud *et al*., 2002), and either display stenothermal (tolerance to a narrow temperature range) or eurythermal (broad temperature tolerance) properties (Marrè, 1962).

Adaptation to changes in temperature involves various responses in both the physiology and biochemistry of microalgae species. A widespread occurrence of the effect of temperature on algal lipids is an increase in the degree of unsaturation of fatty acids at low temperature and an increase in fatty acid saturation at high temperature (Thompson, 1993). The higher proportion of polyunsaturated fatty acids at low temperatures, which could be also the result of higher oxygen concentration, is believed to serve as a mechanism to maintain membrane fluidity and function (Hitchcock and Nichols, 1971; Singh *et al*., 2002).

Temperature also influences species dominance and succession in natural phytoplankton populations particularly in outdoor microalgae cultures (Andersson *et al*., 1994). In mixed phytoplankton assemblages of outdoor cultures, *Skeletonema costatum* tends to dominate at temperatures below 12°C, whereas *Phaeodactylum tricornutum* prevails between 12-20°C (de Pauw, 1984). Otherwise, cyanobacteria typically dominate at temperatures beyond 30°C (Regan and Ivancic, 1984). Temperature can also influence

the dominance of different species or strains of the same genera. For example, *Spirulina platensis* has optimal temperatures at 35-37°C whereas *Spirulina minor* grows best between 22 to 27°C (Richmond *et al*., 1990). Knowledge of species dominance at different temperatures is thus of critical importance in the management of unialgal outdoor cultures.

### 2.6.3. pH condition

pH determines the extent of ionization of chemical compounds and biochemical metabolites, which consequently has a non-negligible influence on their uptake and reactivity. Specific pH-dependent processes which are of prime importance in microalgae cultivation are: speciation and differential availability of inorganic carbon species ($CO_2$/$HCO_3$-/$CO_3^{2-}$) in the culture medium (Azov, 1982; Moss, 1973); at high carbonate levels precipitation of phosphate with calcium, magnesium and carbonate; low solubility and availability of trace metals at high pH, intracellular pH regulation, and changes in the uptake of essential nutrients such as nitrate and phosphate (Smith and Meseck, 2004; Meseck *et al*., 2007).

### 2.6.4. Oxygen

One of the major constraints encountered in microalgae cultivation systems is the build-up of oxygen (up to 4 - 5x saturation with respect to air) in actively growing cultures maintained at high cell densities (Tredici, 2004). It represents a critical hurdle in closed photobioreactors where it accumulates to excessive levels and cannot be out gassed as efficiently as in open cultures (Camacho Rubio *et al*., 1999; Richmond *et al*., 1993; Weissman *et al*., 1988). High oxygen level is counter-productive because it promotes photorespiration and can lead to irreversible photo-oxidative damage (Richmond *et al*.,

1993). Photorespiration is a process by which organic carbon is converted to $CO_2$ and other compounds without any metabolic gain. High oxygen promotes the oxygenase activity of RuBisCO (ribulose 1, 5- biphosphate carboxylase/oxygenase) and results in the preferential uptake of $O_2$ rather than $CO_2$ (Beardall *et al*., 2003).

### 2.6.5. Biotic factors

Even if abiotic conditions are optimally fulfilled, a number of biological problems can arise in the mass cultivation of microalgae. The common biotic factors that hinder the mass cultivation of microalgae include contamination, grazing, premature collapse and lack of species control (Becker and Venkataraman, 1982; De Pauw *et al*., 1984; Richmond, 1986). Control measures for avoiding contamination by bacteria and other algal species are sterilization and ultrafiltration of the culture medium (Ukeles, 1976). Grazing by protozoan and fungal diseases can eventually be treated chemically (Becker and Venkataraman, 1982). Larger zooplanktons and insects can be removed mechanically by screening, eradicated chemically or by changing the culture conditions (Lincoln *et al*., 1983). Premature collapse of pure algae cultures during scaling up can be avoided by establishing balanced growth conditions. However, most problems in aquaculture are due to protozoans such as ciliates, rhozopods and zoo flagellates, which can devastate algal culture in less than 24 h, despite sterilization and ultrafiltration of culture medium and the culture devices (De Pauw, 1981).

### 2.6.6. Operational factors

Algal culture is necessary to prevent sedimentation of algal cells, to ensure that all the cells are equally exposed to the light and nutrients, minimize thermal stratification and to improve gas exchange between culture medium and air (Borowitzka, 1998). However, it should be noted that not all algal species can tolerate vigorous mixing, high turbulence

may, however, be damaging to the algae (Mitsuhashi *et al*., 1995). Vigorous mixing may increase the flashing light effect, removal of excess dissolved oxygen, and proper supply of $CO_2$ which results in higher productivity (Laws *et al*., 1983; Richmond and Vonshak, 1978). Richmond and Becker, (1986) stated that, when the nutritional requirement of mass algal cultured algae are satisfied and the environmental conditions are not growth-limiting, mixing designed to create a turbulent flow constitutes the most important requisite for consistently obtaining a high yield of algal mass. Such turbulence is crucial not only for enhancing an exchange rate of nutrient and metabolites between the cultured cells and their growth medium, but the increase light and dark frequency result in increased productivity and photosynthetic efficiency (Grobbelaar, 1994).

## 2.7. Salt stress for lipid production

The lipid content of microalgae can be increased by a number of factors such as nitrogen (Illman *et al.*, 2000) and silicon (Lynn *et al.*, 2000) deficiency, phosphate limitation (Reitan *et al.*, 1994), high salinity (Rao *et al.*, 2007). Salt stress has always been an important factor as salinity alters the intracellular compositions of microalgae lipid (Kirrolia *et al*., 2011). Several reports are available in the literature on salt stress to enhance lipid accumulation for various microalgae like *Chlamydomonas* sp. JSC4 (Ho *et al*., 2014), *Desmodesmus abundans* (Xia *et al*., 2014), *Nannochloropsis* sp. (Pal *et al*., 2011) etc. The studies to enhance lipid content are mainly carried out on marine microalgae and very few reports are available on freshwater microalgae (Pancha *et al*., 2015). Among the microalgae, *Dunaliella* species are known to tolerate high salt concentrations. This species has the potential to proliferate over the saturation range of salinities (Takagi *et al*., 2006). It has been reported that, *D. salina* showed significant increase in C18 (mostly unsaturated) to C16 (mostly saturated) ratio when transferred

from 0.5 to 3.5 M (29 to 205 g $L^{-1}$) NaCl concentration culture media (Azachi *et al*., 2002).

However, an increase in intracellular lipid content and higher percentage of TAG is recorded in *Dunaliella tertiolecta* when NaCl concentration increased from 0.5 M (29 g $L^{-1}$) to 1.0 M (58 g $L^{-1}$) followed by further increased to 2.0 M (117 g $L^{-1}$) (Takagi *et al*., 2006). With further increase in salt concentration from 0.4 M to 4 M (23 to 234 g $L^{-1}$), showed increased proportions of total saturated and monounsaturated fatty acids, while PUFA content decreases (Xu *et al*., 1997). Similarly, another study on salt stress indicates that with an increased NaCl concentration, the percentage of saturated fatty acid decreased, while unsaturated fatty acid content increases (Fujii *et al.*, 2001). Moreover, high salinity is responsible for the higher degree of saturation thought to be the result of a protective and adaptive mechanism to decrease membrane permeability to higher ion fluxes (Thompson, 1993 and 1996). The salt might have a direct effect on electron transport and/or photophosphorylation which causes a hindrance in the quantum efficiency of photosynthesis (Seeman and Critchley, 1985). In this connection, a study conducted in *Chlorella vulgar* is suggested that salt stress affect the ATP, but does not affect the NADPH (El-Sheekh and Omar, 2002).

Despite the potential in enhancing microalgae lipid accumulation, salt stress conditions are also associated with relatively low biomass productivity, and in all low lipid productivity (Xia *et al*., 2014). Hence, to build a bridge between biomass yield and lipid production, the two stage cultivation systems are developed where appropriate conditions can be used in each phase of culture to improve overall biomass and lipid productivity (Su *et al*., 2011; Mujtaba *et al*., 2012; Xia *et al*., 2013). In this cultivation strategy, the first stage is subjected to optimal condition for highly concentrated biomass production followed by stressful conditions in second phase to induce lipid biosynthesis

(Xia *et al*., 2014). The two stage cultivation of *Desmodesmus abundans* showed highest lipid productivity (67.08 mg $L^{-1}d^{-1}$) and better biodiesel quality when supplemented with 20 g $L^{-1}$ NaCl, and biomass recovery of 1.79 g $L^{-1}$ at stage two (Xia *et al*., 2014). Interestingly, salt stress also showed a significant effect in FAME profile. *Desmodesmus abundans* showed a remarkable increase of monounsaturated FAs (44.08%) at high salinity in comparison to control conditions (19.12%) (Xia *et al*., 2014). The similar response was also reported in freshwater *Chlamydomonas mexicana* and *S. obtusus* XJ-15 under high salt condition (Salama *et al*., 2013; Xia *et al*., 2013). This dramatic change in FA profile in response to high salinity is to keep the membrane fluid in order to prevent its destruction (Xia *et al*., 2014).

## 2.8. Downstream processing of algal biomass

The biggest challenge regarding process modeling and the cost estimation of microalgaeproducts, is perhaps the uncertainty regarding downstream processing (Williams and Laurens, 2010). Currently, the major cost in processing algal biomass contributes to 'dewatering' step (Wyatt *et al.,* 2012) which account for almost 30% of the production cost (Parmar *et al.,* 2011). This is a consequence of dilute and neutrally buoyant nature of microalgae cultures, which typically reach a cell density of between $10^3$-$10^8$ cells $mL^{-1}$ (Pulz, 2001), or 0.1-4 wt% (Wyatt *et al.,* 2012). According to the reports, approximately 1000 kg of water is processed to recover each kg of raw algal biomass, (Parmar *et al.,* 2011). The production of concentrated biomass with current technology requires a large energy input, significantly adding to the costs and disturbing the energy balance (Uduman *et al.,* 2011).

### 2.8.1. Harvesting/dewatering

The harvesting of microalgae biomass involves various techniques which require one or more solid-liquid separation steps. The processes used for recovery of microalgae have significant problems in separating the algal cells from water because of its typical cell size ranges from 0.2-30 $\mu$m and large volume of dilute culture need to be handled (< 0.5 kg $m^{-3}$ dry biomass) to recover the biomass. The biomass recovery has been estimated to contribute about 20-30% to the total cost of biomass production (Molina *et al*., 2003). The involvement of harvesting steps to the system is not only costly, but also has some effects on downstream processing. Though the process of biomass recovery is an essential part of the system and needs to perform for further product processing. Whereas, lowering the cost of algal biomass recovery with an appropriate harvesting technique, which allows the recovery of feasible biomass, remains a challenge.

#### 2.8.1.1. Centrifugation

Centrifugation is a preferred method for harvesting microalgae compared to other harvesting techniques such as dissolved air flotation and drum filtration (Harun *et al*., 2010). The process is efficient with a biomass recovery of 80-90% within 2-5 min (Chen *et al*., 2011). Heasman *et al*., (2000) reported that centrifugation of algal cells at 13,000 rpm provides maximum harvesting efficiency of 95-100% and the cell viability of 88-100%, whereas its efficiency decreases to 60% at 6000 rpm and 40% at 1300 rpm. The method is suitable for a laboratory scale operation when the culture concentration is above 30 mg $L^{-1}$. The method of centrifugation and cell viability depend significantly on the algal species. Basically, centrifugation process is reliable for recovery of metabolites (Harun *et al*., 2010). However, the recovery of algal biomass under a rotational velocity of 3000 rpm is evident with 84% removal efficiency of 0.2 g $L^{-1}$ algal cultures at the

flow of 100 gal $min^{-1}$ (Dassey *et al*., 2013). The harvesting of microalgae cells is accompanied by the drying process and it responds effectively when the processed algal biomass is increased to at least 20% dry weight during the dewatering stage (Dassey *et al*., 2013). Although the processes is very rapid, but at the same time it is very energy intensive. The technique involves high gravitational and shear forces which can damage the cell structure (Chen *et al*., 2011).

**2.8.1.2. Filtration**

Filtration to separate the algal cells is one of the simplest traditional methods, but with the advancement of modern techniques it has become the most competitive method compared to other harvesting options. The filtration process can range from simple screening or micro-strainers to dewatering up to complex vacuum or pressure filtration systems. There are many different forms of filtration, such as dead end filtration, microfiltration, ultra filtration, pressure filtration, vacuum filtration and tangential flow filtration. These processes involve separation of a suspension through a permeable medium and it retains the algae while passing through the filter (Harun *et al*., 2010). Filtration processes operating under pressure are much efficient for recovering the large microalgae while the recovery of algae species, such as *Scenedesmus, Dunaliella* and *Chlorella* are not suitable for the process due to their small dimensions (Uduman *et al*., 2010). However, tangential flow filtration (TFF) is one of the high rate microalgae harvesting method and reported to recover about 70-89% of biomass using this approach (Chen *et al*., 2011). Membrane technology for recovering algal biomass is also a possible alternative to conventional filtration. Usually membrane technology is used to recover algal cells from small aquaculture farms for feeding shellfish larvae. But the same technique is inefficient for harvesting algal biomass from large scale cultivation

processes (Molina *et al*., 2003). The membrane technology also have a prevailing problem of fouling due to the formation of a algal layer causing blockages in pore and decrease the permeability (Ladner *et al*., 2010). However, frequent backwash of the membrane helps in controlling fouling. Understanding of certain drawbacks with higher efficacy and lowering the fouling of membrane filtration processes still remains a challenge. In terms of economic feasibility, the membrane technologies are not cost effective for harvesting the algal biomass in large scale. Although, harvesting of algal biomass with microfiltration in small volumes (e.g., < 2 $m^3$ $day^{-1}$) are more cost-effective than the centrifugation (Molina *et al*., 2003).

**2.8.1.3. Flocculation**

Flocculation is considered to be an effective process for harvesting microalgae culture in large scale (Wu *et al*., 2012). The process is based on negative surface charge carried by the microalgal cells that prevents self aggregation in suspension. The surface charge can be countered by the addition of flocculants such as multivalent cations and cationic polymers to amass the biomass from the culture (Harun *et al*., 2010). Flocculants must be inexpensive and non-toxic to culture. In addition, flocculent should not have any adverse effect for further downstream processing. Multivalent metal salts such as ferric chloride ($FeCl_3$), aluminum sulfate ($Al_2$ $(SO_4)^3$, alum) and ferric sulfate ($Fe_2$ $(SO_4)^3$) are widely used for flocculation (Molina *et al*., 2003). Microalgae also have a tendency for self-flocculation known as auto-flocculation. The process is sensitive to pH and it increases due to consumption of dissolved carbon dioxide. Increase in pH enhances the super saturation of calcium and phosphate ion of the culture and further the precipitant get neutralized with the algal cells which are negatively charged. The optimum pH range for the auto-flocculation was found to be 8.5-9.0 (Christenson *et al*., 2011).

Polyelectrolytes which are cationic polymers are also used as an alternative flocculent. These are found to be better than the non-polymerized metal flocculants and are effective over a wide range of pH conditions (Molina *et al*., 2003). An increase in pH condition plays a major role in flocculation. As reported among the four different flocculants, (i.e. sodium hydroxide, potassium hydroxide, calcium hydroxide, magnesium hydroxide and sodium carbonate) the flocculation conducted with lime showed the maximum efficiency compare to other base flocculants (Vandamme *et al*., 2012).

Even though, the method is easy and very effective for harvesting microalgae culture, but the process is not cost effective and not sustainable for large scale harvesting. The involvement of chemicals for flocculation needs to be washed off before it can be re-used leading to extra operational cost and can be significantly hazardous to the environment (Salim *et al*., 2011).

**2.8.1.4. Flotation**

Flotation methods are based on gravity separation in which air or gas is bubbled through a solid-liquid suspension and gaseous molecules are attached to the solid particles (Chen *et al*., 2011). The dispersed micro-air bubbles are used to trap algal cells. In flotation technique, the size of particles plays an important role, smaller the size it is more likely that the particle move up by the bubbles. The process is dependent on the size of particle and size less than 500 $\mu$m can be used during the process (Uduman *et al*., 2010). Some microalgae also have a tendency to float naturally on the water surface due to its high lipid content (Brennan and Owende, 2010). The process of flotation is divided into three types based on the bubble generation i.e. dissolved air flotation (DAF), dispersed flotation and electrolytic flotation (Chen *et al*., 2011) (Table 2.4).

Table 2.4 Comparison of water removal and energy use for various microalgal dewatering techniques. Adapted from Christenson *et al*., (2011); Xu *et al*., (2011); Buckwalter *et al*., (2013)

| **Methods** | **Recovery** | **Scale** | **Energy consumption ($kWh/m^3$)** | **Benefits** | **Limitations** |
|---|---|---|---|---|---|
| Centrifugation | 90% | Bench | 8 | Reliable, high solids conc. | Energy intensive, expensive |
| Flocculation | 80%-95% | Pilot | 14.81 - 0.33 | Good recovery | Flocculants can be expensive, may cause contamination issues |
| Tangential flow filtration | 70-89% | Bench | 0.38 - 2.06 | Reliable, high solids conc. | Membrane fouling, high cost |
| Dissolved air flotation | 80-90% | Pilot | 7.6 | Proven in large scale | Use of flocculants may create problem |
| Electroflotation | >95% | Bench | - | Efficient recovery | High cost, electrodes need to be replaced periodically |
| Forward osmosis | 80%-92% | Pilot | 0.3 | Low-energy input | Slow and problem in reverse solute flux |
| Magnetic separation | 90-98% | Bench | - | Rapid, less energy intensive and environmental friendly | Complex fabrication and expensive |

**2.8.1.5. Electroflotation**

Electroflotation process involves the applications of electric field to separate the algae. In this method, inactive metal (electrochemically non-depositing) act as cathode that generates hydrogen bubbles from water electrolysis. The bubbles produced adhere to the microalgal flocs and carries them to the surface (Chen *et al*., 2011). The electrolytic flotation method in batch and continuous reactors is studied for microalgae harvesting (Alfafara *et al*., 2002). The batch study reveals that with an increase in the input of electrical power, removal rate of chlorophyll increases and electrolysis time decreases (Uduman *et al*., 2010). The disadvantages of electrolytic flotation are scaling of the cathode and high cost. Sandbank *et al*., (1987) investigated the different microalgal recovery techniques and concluded that electroflotation is not the effective method, but it may be preferred for harvesting marine microalgal species (Uduman *et al*., 2010). However, a new technique has been introduced for continuous harvesting of microalgae via electro-coagulation-flotation which termed as ‘‘continuous electrolytic microalgae harvest’’ (CEM harvest). This technique generates the polarity exchange with simultaneous harvesting and cultivation of microalgae. This technique is efficient in terms of cost, but requires further optimization for its commercial feasibility (Kim *et al*., 2012).

**2.8.1.6. Magnetic separation**

Among the various harvesting processes, magnetic separation have been introduced as a simple technique for efficient recovery of cells and biomolecules from liquid solution with the help of functional magnetic particles driven by an external magnetic field (Wang *et al*., 2007). Recently magnetic separation has been used in removal of harmful microalgae by functionalized magnetic particles. This technique still limits the practical

applications due to its complex fabrication and cost (Liu *et al*., 2009). A recent study on magnetic separation for recovery of *Botryococcus braunii* and *Chlorella ellipsoidea* with naked $Fe_3O_4$ nanoparticles has been investigated. The recovery efficiency of both the species was above 98% within 1 min. The magnetic nanoparticles can be reused and recovered easily from the harvested microalgae biomass with efficient biomass recovery (Xu *et al*., 2011). Magnetophoresis has also been applied in separation of microalgae blooms in commercial fish production ponds. A study conducted using iron oxide magnetic nanoparticle (IONPs) for microalgae separation in aquaculture industry showed recovery efficiency of 90% in less than 3 min (Toh *et al*., 2012). This magnetic separation technology provides efficient microalgae recovery with less energy input and time.

### 2.8.2. Algal oil (lipid) extraction

The subject of (oil) lipid analysis has been extensively reviewed in the literature (Christie, 1982; Parrish, 1999), and many different preparative and analytical techniques have been published for lipids and fatty acids (Folch *et al*., 1956; Bligh and Dyer, 1959; Kattner and Fricke, 1986). However, the classical works on lipid extraction efficiency have been carried out on relatively large samples of animal tissue (Folch *et al*., 1956; Bligh and Dyer, 1959). Given the absence of cell walls in animal material, blending or mixing samples during the extraction sufficed for quantitative lipid extraction. In contrast, the extraction procedures and efficiencies for plant material, especially for algae, are less well established (Wiltshire, 2000). Indeed, the literature on fatty acid and lipid content of microalgae contains no standard extraction method. Published extraction methods include the addition of solvents and subsequent incubation (Ackman *et al*.,

1968), agitation (Cartens *et al*., 1996), stirring (Whyte, 1988), homogenization (Ben-Amotz *et al*., 1985), grinding (Rai *et al*., 1997), and sonication (Napolitano, 1994).

In general, oil extraction from biological materials is performed by chemical means, physical means, or a combination of two. The process of oil extraction from microalgae is usually accomplished with mechanical cell disruption followed by solvent extraction. The mechanical disruption is commonly performed with either a bead mill or ultrasonication. A bead mill operates by a vertical or horizontal cylindrical chamber that houses a series of mechanically driven agitating elements. Grinding of the cells is performed by plastic or glass bead that occupy about 80% of the chamber's volume, and this has been successfully used to disintegrate algal cells (Richmond, 2004). Ultrasonication, another main technique uses an ultrasonic probe to disrupt microalgae cells. The probe uses a transducer to generate sound waves which in turn cause small bubbles to form. The formation and cavitation of these bubbles produces shock waves that rupture the cell wall. It has been found that at higher working volumes higher acoustic power is required which can cause larger bubble formation and decrease effectiveness. Thus, for larger scale use, specially designed disruption vessels, with a continuous flowing stream of material to be disrupted are used (Richmond, 2004).

The solvent extraction of oil which typically follows mechanical disruption of algae cells can be performed with a two solvent system. At the lab scale, extraction can be carried out by Bligh and Dyer method (1959), where often no mechanical disruption precedes. This process uses a polar and a non-polar solvent to extract the corresponding lipid fractions from the cells. The drawbacks of using solvent extraction on a commercial scale are that it requires extra energy input (to distill solvents) and it has the potential to contaminate algal solids, thereby limiting the end product usages. Although many methods have been found in the literature for extraction and estimation of lipids from

microalgal samples, only two, originally proposed over two decades ago, have met with great acceptance because their use presumably allows quantitative isolation of lipid and free of non-lipid contamination. These two methods are: (a) Folch *et al*., (1957) method and (b) Bligh and Dyer, (1959) method. Both these two methods require the use of mixtures of chloroform ($CHCl_3$) and methanol ($CH_3OH$) as the extraction solvent. However, both these methods have certain limitations (Marmer and Maxwell, 1979):

- use of toxic chemicals like $CHCl_3$, which is suspected to be carcinogenic in nature and $CH_3OH$, which is a potent toxic chemical
- required excessive amounts of solvent
- the procedures are tedious and time-consuming, especially for multiple extractions
- required the use of expensive tissue homogenizing equipment
- emulsion problems are often encountered
- separation of the lipid into its subclasses requires subsequent chromatographic procedures

## 2.9. Algal biomass-to-biodiesel

Biodiesel is a mixture of the monoalkyl esters of long chain fatty acids (FAME) derived from renewable feedstocks (i.e. vegetable oil or animal fats) by a transesterification process (Clark and Deswarte, 2008; Demirbas, 2009). Generally methanol is preferred for transesterification because it is less expensive than ethanol. The transesterification process in the presence of catalyst produces mono-esters that are termed as biodiesel which involves a chemical reaction between triglycerides and alcohol (Sharma and Singh, 2009). In following section the transesterification process for synthesis of

biodiesel (homogeneous and heterogeneous), their challenges and importance are discussed.

### 2.9.1. Conventional transesterification process

The conversion of microalgae lipid to methyl esters/biodiesel can be carried out by both homogeneous and heterogeneous catalysis. Homogeneous alkaline catalysis is widely used for transesterification process because it catalyzes the reaction at low temperature, atmospheric pressure and complete conversion can be achieved in a short time (Park *et al*., 2015). The most common example of alkali catalysts are NaOH, $CH_3ONa$, and KOH (Gemma *et al*., 2004); however it is not suitable for the oil containing high free fatty acid (FFA) (<3%), because FFA content in the oil reacts with the alkaline catalysts to forms soaps (Ramadhas *et al*., 2005). Therefore, for alkali-catalyzed transesterification reaction without extensive pre-treatment, only pure vegetable oil feeds are appropriate. In this regard, acid catalyst is applicable when the content of free fatty acids is higher than 1% (Park *et al*., 2015). The most used acid catalysts are sulfuric acid ($H_2SO_4$) and hydrochloric acid (HCl). The acid catalyzed reaction is much slower process and requires high temperature than base catalyzed reaction (Vyas *et al*., 2010; Hidalgo *et al*., 2013; Vonortas and Papayannakos, 2014). Therefore, a more efficient method would be via two step transesterification approach that consists of acid catalyzed reaction before preceding the transesterification with alkaline catalyst. In the first step acid catalyst is used to reduce the FFA content to less than 1 wt% via esterification. Meanwhile, in the second step, alkaline catalyst is used to transesterify the esterified product to biodiesel (Park *et al*., 2015). Despite the high efficiency in conversion by homogeneous catalysts, there is always a loss of catalyst after the reaction. In that case, heterogeneous catalysts are advantageous as they can be recovered and reused (Park *et al*., 2015). The study on

transesterification of *Nannochloropsis oculata* using CaO and MgO supported on alumina catalyst showed highest yield of 97.5% (Umdu *et al*., 2009). Whereas, *Dunaliella tertiolecta* and *Nannochloropsis oculata* on conversion to biodiesel by metallic oxides composed of ZrO, TiO, and $Al_2O_3$ showed simultaneous esterification and transesterification of free fatty acids and triglycerides under supercritical conditions with conversion yield of 85% (Krohn *et al*., 2011).

### 2.9.2. Direct transesterification process

The direct transesterification process includes reaction of dry biomass with sulfuric acid and methanol; wherein methanol used as both the extraction solvent and as the esterification reagent (Park *et al*., 2015). During the process, contact of microalgae oils with the esterification reagent can be enhanced by using some additional solvent such as hexane or chloroform so as to help easy extraction of oils within microalgae cells (Cao *et al*., 2013). In a study conducted by Sathish *et al*., (2014), it was reported that decrease in methyl ester yield could be the result of lower methanol concentration with increased water content. Moreover, water might react with methyl ester by hydrolysis reaction which converted biodiesel back to fatty acids and methanol. During the transesterification reaction, water content of wet microalgae biomass inhibits the process and therefore requires an economically efficient process for utilization of wet biomass. In this regard, recently many wet oil extraction technologies have been developed in which the technology can be combined with a direct process to overcome water inhibition problem (Park *et al*., 2015). Few microalgae species such as *Chlorella* sp. and *N. oculata* having moisture contents of 0%, 1.5%, and 10% have been transesterified using acid and alkaline catalysts (sulfuric acid, sodium hydroxide, and sodium methoxide). In this study,

high FAME yield of 73% for *N. oculata* and 92% for *Chlorella* sp was observed with Sulfuric acid as a catalyst (Velasquez-Orta *et al*., 2013).

Recently *in-situ* transesterification method, in which the oil-bearing material contacts with alcohol directly instead of reacting with pre-extracted oil, has received serious attention for algae-based biodiesel production (Wahlen *et al*., 2011). According to the reports, conversion yield of biodiesel is proportional to methanol loading and inversely proportional to water content (Park *et al*., 2015). It has been reported that direct transesterification of wet microalgae such as *N. oceanic* yield 91.1% biodiesel (Im *et al*., 2014). Direct transesterification of *C. pyrenoidosa* (90% moisture content) also showed biodiesel yield of 92.5% (Cao *et al*., 2013). The reported studies on direct transesterification suggested, high requirement of methanol and sulfuric acid for the process. However, the use of these solvents requires a large reactor and the risk of reactor corrosion is quite high by sulfuric acid (Park *et al*., 2015). On the other hand, pentane and diethyl ether solvents are often used, as it reduces the volume of methanol by enhancing the reaction yield. These solvents improve the diffusion of microalgae oils across the cell walls which help in extraction of microalgae oil in conjugation with methanol. During the process, selectivity and solubility of the extraction media provide greater availability of oils for biodiesel conversion (Ehimen *et al*., 2012). The study conducted in a one-step reaction using *S. limacinum* (80% moisture content), showed higher biodiesel yield with methanol (3.4 mL) compared with methanol (7.4 mL) (Johnson and Wen, 2009). Therefore, study indicates the possibility of using minimum amount of methanol which requires necessary development in reducing the amount of solvent. A study on combination of sonication and co-solvent for *Chlorella sp*, showed significant decrease in oil to methanol molar ratio. In this process, conversion yield recorded to be 99.9% at 1:52 molar ratio (Ehimen *et al*., 2012).

## 2.10. Challenges

The economic viability of the liquid biofuel production process is a decisive challenge for establishing microalgae based biofuel industry. To confront the issues, bio-refinery approaches are suggested (Campbell *et al*., 2011; Chisti, 2007; Stephens *et al*., 2010) in which single or a selection of feedstock are converted into single or multiple products based on their categorization (Srirangan *et al*., 2012). Nutrient recycling is also an important factor besides the optimization of each individual step of biofuel production process (Figure 2.2) (Mata *et al*., 2010).

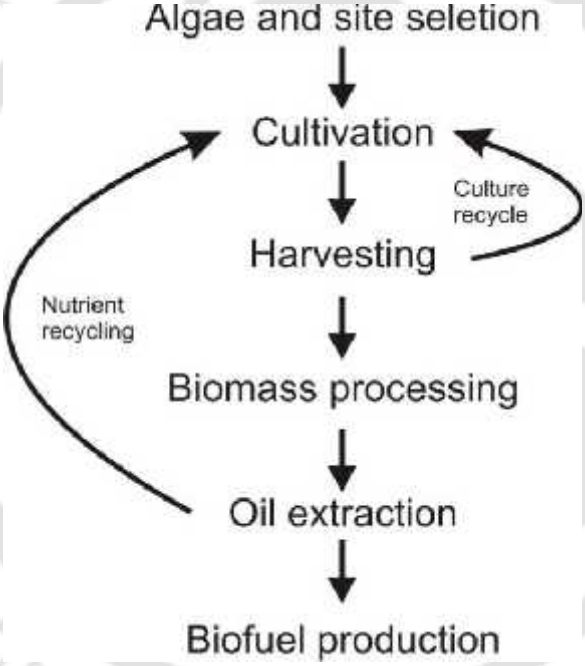


Figure 2.2 Value chain stages of the process to use microalgae for liquid biofuel production (Adapted from Mata *et al*., 2010)

The use of microalgae biomass as a feedstock for bio-refinery allows a self-subsidizing model on co-production of several high value products such as beta-carotene (Stephens *et al*., 2010). However, market size and saturation need to be taken into consideration while addressing the high value products (Stephens *et al*., 2010). The residual biomass after lipid extraction can be used for biogas production by anaerobic

digestion (Chisti, 2007; Yang *et al*., 2011). The produced biogas can be used for heat and electricity generation and as transportation fuel (Srirangan *et al*., 2012), therewith focuses on bioenergy generation concept. This approach can be applied for another biofuel production such as hydrogen as indicated previously (Schenk *et al*., 2008).

The commercialization of those biofuels are highly cost intensive and the possibility of their blending with fossil-derived fuels is dependent on the first generation feedstock until the following generations enter the scope of economic viability (Srirangan *et al*., 2012). Therefore, as stated by Sheehan *et al*., (1998), inexpensive feedstocks are regarded as a major challenge (Srirangan *et al*., 2012) in terms of large scale production systems. Depending on the cultivation method, the cost of algal material varies and currently reported to be about 15 – 30 € $kg^{-1}$ which is almost tenfold higher than the average price for other biomass feedstock such as rapeseed ranging between 0.25 and 0.45 € $kg^{-1}$ (Kröger and Müller-Langer, 2012). The major bottleneck deals with the selection of organisms with high biomass productivities potential for cultivating in outdoor pond systems (Sheehan *et al*., 1998). Main challenges in terms of economic viability also include optimization of harvesting and oil extraction processes (Mata *et al*., 2010).

## 3. Objectives of the thesis

Based on the literature survey, the study conducted in the thesis includes the following objectives:

- Isolation, screening, identification and establishment of unialgal culture of the selected freshwater microalgae
- Optimization and characterization of selected microalgae strains in terms of growth, biomass and lipid production

- Determination of growth, biomass and lipid yield of selected microalgae strains under different nitrogen sources and characterization of derived methyl esters
- The effect of saline (NaCl) salt stress and a two-stage cultivation of selected microalgae strains on growth, biomass, lipid yield and characterization of methyl ester
- Cultivation of selected microalgae strains with cow dung and piggery waste as cheap media compositions to produce biodiesel and its property assessment

# CHAPTER 3

# Materials and methods

# 3. MATERIALS AND METHODS

## 3.1. Chemicals

The chemicals used in this study were procured from M/s Sigma Aldrich, M/s Merck India Pvt. Ltd., and M/s Rankem. All the other reagents used for analysis were analytical grade and were used without any further purification.

## 3.2. Algal culture media

Based on the available literature, number of autotrophic media supplemented with all the basic nutrients for the growth of freshwater microalgae and their modified form were initially used to ascertain their suitability for optimum growth and major biochemical components. Unless otherwise stated, BG11 medium was used throughout this study for isolation and maintain the selected microalgae strains for cultivation and improvement. Nevertheless, in addition to these, specific modifications of the same by altering their compositions as well as few other media were formulated for specific use in different experiments. Following is the list of various autotrophic media used in this study.

List of autotrophic nutrient media used throughout this study

1. Blue Green algal (BG11) medium (Boussiba and Vonshak, 1991)
2. BG11 + NaCl
3. Bold's Basal medium (BBM) (Nichols and Bold., 1969)
4. BG11 + Ammonium nitrate (Used in this study)
5. BG11 + Potassium nitrate (Used in this study)
6. BG11 + Urea (Used in this study)
7. Chu 13 (modified) (Yamaguchi *et al*., 1987)

The various components of culture media are listed in Table 3.1.

### 3.2.1. Preparation of media

To prepare 1 L of a medium, the respective components were first weighed in required amounts and dissolved in 500 mL of double distilled water. Then the final volume was made up to 1 L with distilled water. The culture medium pH was adjusted to 7.5 either with 0.1 N HCl or 0.1 N KOH followed by sterilization using an autoclave at 121°C and 14.1 MPa for 15 min.

For isolation, agar plates were prepared by adding 1% (w/v) of agar to preferred media. After autoclaving the molten agar - media was poured into 90 mm Petri dishes.

Table 3.1 Culture compositions of BG11, BBM and Chu 13 media

| Compositions | BG11 (mod) | BBM | BG11 + NaCl | BG11+ $(NH_4)(NO_3)$ | BG11+ $KNO_3$ | BG11 + Urea | Chu13 |
|---|---|---|---|---|---|---|---|
| $NaNO_3$ | 1.5 | 0.2465 | 1.5 | 0.22 | 0.3 | – | – |
| $KNO_3$ | – | – | – | – | 1.779 | – | 0.371 |
| $(NH_4)(NO_3)$ | – | – | – | 1.408 | – | – | – |
| Urea | – | – | – | – | – | 1.06 | – |
| $KH_2PO_4$ | – | 0.175 | – | – | – | – | – |
| $K_2HPO_4$ | 0.040 | 0.0749 | 0.040 | 0.040 | 0.040 | 0.040 | 0.080 |
| $MgSO_4.7H_2O$ | 0.075 | 0.0700 | 0.075 | 0.075 | 0.075 | 0.075 | 0.200 |
| $CaCl_2.2H_2O$ | 0.036 | 0.025 | 0.036 | 0.036 | 0.036 | 0.036 | 0.107 |
| NaCl | – | 20.1 | 0.58/1.75/2.9/ 4.1/5.26/6.42/ 8.8/9.9/11.68 | – | – | – | – |
| EDTA-FeNa | 0.001 | 0.005 | 0.001 | 0.001 | 0.001 | 0.001 | – |
| $NaCO_3$ | 0.020 | – | 0.020 | 0.020 | 0.020 | 0.020 | |
| Citric acid | 0.006 | – | 0.006 | 0.006 | 0.006 | 0.006 | – |
| $NaHCO_3$ | – | 0.0497 | – | – | – | – | – |
| Ammonium ferric citrate | 0.006 | – | 0.006 | 0.006 | 0.006 | 0.006 | – |
| Ferric citrate | – | – | – | – | – | – | 0.020 |
| $H_3BO_3$ | 0.061 | 1.13 | 0.061 | 0.061 | 0.061 | 0.061 | 2.86 |
| $ZnSO_4.7H_20$ | 0.287 | 0.82 | 0.287 | 0.287 | 0.287 | 0.287 | 0.02 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| $CuSO_4.5H_20$ | 0.003 | 0.095 | 0.003 | 0.003 | 0.003 | 0.003 | 0.08 |
| $MnSO_4.5H_2O$ | 0.169 | 0.001 | 0.169 | 0.169 | 0.169 | 0.169 | – |
| $Na_2MoO_4.2H_2O$ | 0.013 | 0.11 | 0.013 | 0.013 | 0.013 | 0.013 | 0.039 |
| $Co(NO_3)_2.6H_2O$ | – | 0.049 | – | – | – | – | 0.05 |

Apart from the above listed autotrophic media, enriched organic media compositions were also used to test growth response under specific objectives.

### 3.2.2. Preparation of cow dung (CD) and piggery waste (PW) extract

The cow dung and piggery waste (pig dung) were used to formulate the low-cost nutrient medium for mass cultivation of microalgae species. Both the manures were properly sundried and then ground into powdered form. A definite amount (i.e. 500 g) each organic composition was suspended in 1000 mL of distilled water by stirring with a magnetic stirrer for 24 h at room temperature (28 ± 2.0ºC). The suspension was centrifuged, and the supernatant was collected by filtering through filter paper (Whatman No.1) and filtrate collected was used as a stock solution. From the stock solution, different concentration such as 5%, 10% and 15% was prepared to perform specific objectives in the thesis.

## 3.3. Sample collection and isolation of microalgae strains

### 3.3.1. Sampling sites

The water samples were collected from IIT Guwahati campus, Amingaon and Jalukbari area of Kamrup district, Assam, India. The site used for sample collection includes the freshwater reservoirs and roadside water clogged area. The sample collection was carried out in between September 2010 to February 2011.

### 3.3.2. Sample collection and processing

Samples were randomly collected from various fresh water habitats as stated above. During this period, over 30 water samples were collected for isolation of the microalgae strains. Ecological parameters such as water temperature and pH of the sampling sites were recorded at the site.

Collected samples were subjected for microscopic observations in the laboratory for further processing and isolation. Based on microscopic observations, samples were selected for isolation of selected species.

### 3.4.3. Isolation and establishment of a monoculture

The selected water samples based on microscopic observations were subjected to isolation of microalgae strains and establishment of monoalgal cultures. Isolations of microalgae strains were carried out according to the procedure described by Kawai *et al*., (2005). Microalgae strains were isolated by serial dilution up to $10^{-5}$ (For instance, if the sample contained approx. $5.4 \times 10^5$ cells $mL^{-1}$, after 5 dilutions, the last tube contained ~5 cells) followed by repeated streaking on enriched agar plates. From each tube 100 $\mu$L of culture was transferred on a sterilized solidified BG11-agar plate (BG11 medium + agar-agar 1%, w/v). The whole procedure was performed in a laminar hood, and the plates were placed at controlled conditions to monitor the algal growth. After the incubation period (~1 week), various algal colonies were observed under an inverted microscope. The targeted colony was picked up using a sterilized needle, and placed in a screw capped vial containing 1 mL of sterilized liquid BG11 medium. The sample was mixed gently by hand shaking, and the whole steps of a serial dilution were repeated again as described above. This dilution and plating procedures were repeated until only one type of algal colony was formed, and took at least 6 months to yield axenic cultures.

### 3.4.4. Algal growth conditions

The algal growth conditions inside the culture room were strictly maintained, unless otherwise stated. The temperature was maintained at 25 ± 2ºC. Incident light intensity of 40-47 $\mu$mol $m^{-2}s^{-1}$ (PAR) was provided by cool white florescent tubes and was measured using a photometer (Lux meter: Metravi 1330, India). The light and dark phase of 12/12 h (photoperiod) in each culture rack was maintained using automatic electronic timer. The stated growth conditions were kept constant for maintaining the microalgae isolates.

### 3.4.5. Establishment of culture, culture expansion and maintenance

The unialgal cultures of selected microalgae isolates were inoculated in Erlenmeyer flask (100 mL) containing 50 mL growth media and grown for 3-4 weeks under the stated growth conditions in the laboratory to maintain the cultures. Based on the preliminary observation, isolates with fast growth were used for culture expansions and further experimentation. The cultures were shaken manually twice a day and not provided with additional $CO_2$ supply or aeration. Sub-culture was done after every 2-4 weeks interval depending upon culture volume and growth of the microalgae strain.

### 3.4.6. Standardization and optimization of culture conditions

In order to select the efficient culture conditions to ascertain with superior growth and lipid content among the isolated microalgae, a multi-step cultivation strategy was designed. The experiments were conducted in batch mode and each batch experiment was performed in triplicates. Each step was designed specifically for the isolated microalgae strains using growth medium supplemented with alternate nitrogen sources, initial culture pH, light intensities, salinity, organic extracts (CD and PW) towards potential low-cost cultivation. The culture period was varied from 2-3 weeks according to the microalgae strains and growth performance was determined by regular growth

studies. Total lipid content was determined from oven dried biomass harvested at the end of culture period. Selection was made primarily on the basis of superior growth and lipid content. The following steps were followed sequentially in a multi-step cultivation approach.

**Step 1: Optimization of initial culture pH and light intensity**

Microalgae strains were screened for optimum initial culture pH and light intensity. Five levels of initial culture pH in increasing order from pH 5.0 to pH 9.0 and five levels of light intensity from 27, 33.7, 47, 67.5 and 81 $\mu$mol $m^{-2}s^{-1}$ (PAR) were studied for the selected microalgae strains.

**Step 2: Screening for suitable growth medium**

The isolated microalgae strains were initially grown in liquid BG11, which is widely used for green algae cultivation. The various nutrient compositions of BG11 are listed in Table 3.1. Algal strains well adapted in BG11 were selected for growth comparison in various autotrophic media (Table 3.1) at optimized growth conditions. This step was important for preliminary selection of suitable medium for optimum growth of specific microalgae strains, which may vary from species to species. A suitable medium is utmost necessary for a specific microalgae strain to maintain its optimum growth. Based on the growth performances measured in terms of cell count or optical density on regular basis, BG11 was chosen as basic media for cultivation and maintenance of the selected microalgae strains.

**Step 3: Medium supplemented with alternate sources of nitrogen**

Microalgae strains with optimized growth conditions (obtained from Step 2) were subjected to screening of growth in the medium supplemented with alternate nitrogen

(N) sources. For alternate N-sources, the sole N-source of BG11 medium was supplemented with equal molar concentration (17.6 mM) of selected alternate N-sources. For instance, $NaNO_3$ of BG11 medium was supplemented with $KNO_3$, $NH_4NO_3$ and Urea. Based on the results of growth performances, suitable N source was selected for further experimentation.

**Step 4: Salinity effect**

The effect of the salinity conditions on the cell growth and lipid production of selected microalgae strains was carried out by the addition of NaCl to the selected medium (BG11) at various concentrations ranging from 10.0-200 mM (Table 3.1). The NaCl concentrations were varied over the range such as 10, 30, 50, 70, 90, 110, 130, 150 and 200 mM respectively. Based on the preliminary growth study, NaCl concentration of 50 and 200 mM was selected for "two-stage stage cultivation. During the cultivation, 50 mM NaCl was added at the $1^{st}$ stage to collect the maximum biomass, in the $2^{nd}$ stage, as the species reaches its stationary phase, 200 mM NaCl concentration was added to the same culture. In this cultivation method, after 15 days of first stage cultivation, 50% of culture was harvested and the remaining culture batch was again fed with BG11+ 200 mM NaCl in order to induce stress condition at the stationary phase of the culture.

**Step 5: Screening of cow dung (CD) and piggery waste (PW) extract as low-cost medium**

This step was considered as an effective screening step for selecting the microalgae strains and to formulate the low-cost nutrient medium for mass cultivation. It was presumed that CD and PW extract could be potentially utilized for low-cost outdoor mass cultivation. Nevertheless, selection of a specific strain with faster and robust growth is an important factor in outdoor cultivation. In addition, strain must be able to

withstand the fluctuating light and temperature conditions. Hence, the selected microalgae strains were screened with nutrient medium supplemented with CD and PW extract at different concentration as stated in Section 3.2.2. The experiment was conducted both in batch mode and continuous culture under controlled growth conditions. Growth performance was determined by measuring cell density at regular time interval.

## 3.4. Identification of the selected microalgae strains

The microalgae strains, selected on the basis of their growth performance (see section 3.4.6) were cultivated for 2-3 weeks in batch incubation under the stated growth conditions in 250 mL Erlenmeyer flask containing 100 mL sterilized culture medium.

### 3.4. 1. Morphological identification

The preliminary identification of selected strains was carried out on the basis of characteristic morphological features. Morphological characteristics of the selected microalgae strains were evaluated from maintained unialgal cultures using a compound microscope (LABOMED, Model: ATC 2000) equipped with 8 x zoom digital camera (SONY, Model: DSC-W100, Japan) and Field Emission Scanning Electron Microscope (Zeiss, Model: Sigma). The observed characteristic features were compared with the available literature for preliminary identification of the strains, which incorporated with molecular taxonomic identification, confirmed the Phylogenetic position of the selected microalgae strains.

### 3.4.2. Field Emission Scanning Electron Microscopy (FESEM)

The FESEM analysis of sample was carried out by centrifuging an aliquot of each culture at 5000 rpm for 10 min. The pellet containing algal cells was kept for 4 h in

0.25% glutaraldehyde at 4ºC (primary fixation). The fixative was removed and pellet was washed in 0.1 M phosphate buffer. The secondary fixation was done with 1% osmium tetraoxide, followed by washing with 0.1 M phosphate buffer. The dehydration was performed in an acetone series such as 30%, 50%, 70%, 80%, 90%, 95% and 100% acetone for 3 min followed by air drying under vacuum. All the samples were sputter-coated with gold thin film and loaded for FESEM analysis (Zeiss, Model: Sigma).

### 3.4.3. Extraction of genomic DNA and 18S rRNA gene sequence analysis

The genomic DNA was extracted from lyophilized cell biomass of freshly harvested cultures according to the procedure described by Talukdar *et al*. (2013). For DNA extraction, freshly prepared extraction buffer containing Lithium Chloride (0.8 M), Sarkosyl (0.6%), EDTA (10 mM, pH 8.0), polyvinyl pyrrolidone (0.2%) and -mercaptoethanol (5%) was used.

The PCR products were then sent to Royal Life Sciences Pvt. Ltd., (Secunderabad, India) for DNA sequencing. The sequence was aligned using Clustal X and similarity analysis of 18S rRNA was completed with Basic Local Alignment Search Tool (BLAST) in GenBank database of the NCBI. The phylogenetic tree was constructed by MEGA 5.1 software and analyzed using Neighbor-Joining (NJ) algorithm, applying bootstrap analysis from 100 bootstrap replications.

## 3.5. Determination of algal growth

Growth characteristics of selected microalgae species was analyzed in a batch culture over a period of 7 - 28 days. The culture duration was depended on the microalgae strain. For a growth analysis definite volume of culture was withdrawn at specific intervals of time (culture age) for determination of growth by direct cell count. Equal volume of

fresh media was added after each withdrawal to maintain the total volume of the culture. For each studied parameter the data reported are the average of triplicate values.

### 3.5.1. Cell count and preparation of growth curve

The microalgae cell density was determined by counting the cell numbers with the help of a Neubour Haemocytometer. The cell numbers were counted randomly in some of the medium sized squares of haemocytometer at 200 × magnification of a compound microscope. The average number of cells per medium was calculated and multiplied with 25 to obtain the number of cells per large square. The calculated number was again multiplied with $10^4$ to obtain total number of cells per $cm^3$ (mL).

### 3.5.2. Determination of specific growth rate ($\mu$ $d^{-1}$), divisions per day (k) and doubling time ($T_2$)

The specific growth rate represents average growth rate of all cells present in a culture, which defines fractional increase in biomass over a unit time. In case of direct cell count method, the specific growth rate was calculated following the equation (1) (Levasseur *et al.*, 1993):

$$\mu = \frac{[Ln(N_2 / N_1]}{t_2 - t_1} \quad \textbf{(1)}$$

where, $N_1$ and $N_2$ = biomass at time ($t_1$) and time ($t_2$) respectively.

If "t" is expressed in days, the growth rate ($\mu$) can be converted to divisions or doublings per day (k) by dividing ($\mu$) by the natural log of 2 (= 0.6931), according to the Equation (2):

$$k = \frac{\mu}{0.6931} \quad \textbf{(2)}$$

The time required to achieve a doubling of the number of viable cells is termed as doubling time ($T_2$), which was calculated by using Equation (3):

$$T_2 = \frac{0.6931}{\mu} \quad \textbf{(3)}$$

### 3.6. Harvesting of cell biomass

Recovery of the algal biomass was initiated with the harvesting of cultures. The harvesting process adopted in this study was a combination of physical and chemical treatment process. The culture was first transferred to 20 L volume plastic container and chemical ($FeCl_3$) was added to facilitate sedimentation of the cell biomass.

Dewatering is the process in which supernatant *i.e.* biomass free medium is decanted and the biomass is collected separately. The biomass in agglomerate form was further centrifuged at 3000 to 5000 rpm for 8 - 10 min and the pellets were collected. The biomass in agglomerate form thus achieved was kept in a hot-air oven at 80ºC for 6 to 8 h for complete removal of water or drying. The dried algal flaks were stored at -20ºC for further processing and analysis.

### 3.7. Biomass determination

Biomass production was determined by measuring the dry cell weight gravimetrically. The dry weight of algal cells was measured by filtering an aliquot of culture suspension on pre-weighed Whatman filters (GF/C). The filters were rinsed with redistilled water and then dried in a hot-air oven at 80ºC for 6 to 8 h. The dried filters were cooled down to room temperature in desiccators over anhydrous silica gel until constant weight was obtained. Biomass productivity was expressed in gram per unit volume per day (g $L^{-1}d^{-1}$).

### 3.8. Biochemical composition analysis

The oven dried cell biomass of isolated microalgae strains were subjected for quantitative analysis of major biochemical contents such as, total lipids, carbohydrates and proteins.

To determine total lipid, carbohydrate and protein content, definite volume of respective microalgae species cultures were harvested either by filtration using pre-weighted and

pre-combusted GF/C filter or by centrifugation at specific interval of culture age. The algal cells were washed repeatedly first with 1% NaCl solution and then with double distilled water to remove the traces of media content and was oven dried before measuring the biomass weight.

### 3.8.1. Determination of total cellular protein content

Total protein determination was based on the adaptation of Lowry's method (Lowry *et al.*, 1951). According to this method a blue color develops in the reaction by reduction of phosphomolybdic-phosphotungstic component of folin-ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein. The protein standard stock solution was prepared using Bovine Serum Albumin (BSA) Fraction V from a stock solution of 500 mg BSA $L^{-1}$.

Protein estimation of the sample was carried out in a Phosphate buffer (pH 7.0). The sample to be tested was first weighed and homogenized with a mortar and pestle in 5 mL of buffer. The homogenate was centrifuged and supernatant was collected. From the supernatant 0.2 and 0.5 mL of the samples were taken for total protein estimation. Optical density of the colored solution produced during the reaction was measured at 660 nm. A calibration curve was prepared using Bovine Serum Albumin (BSA-fraction V) as standard ranging from 0, 20, 40, 60, 80, 100, 120 and 140 $\mu$g. The amount of protein present in the sample was estimated from the calibration curve using the following equation and expressed in mg of protein present per gm of the sample. The percentage (%) value was calculated from the final result using following equation:

$$\text{Protein yield (mg g}^{-1}) = \frac{\text{protein value from standard curve}}{\text{volume of test sample (mL)}} \times 1000 \qquad \textbf{(4)}$$

### 3.8.2. Determination of total cellular carbohydrate content

The carbohydrate content was estimated following the anthrone method (Hedge *et al*., 1962) with slight modification. According to the method carbohydrate was first hydrolyzed into simple sugars using HCl. In hot acidic medium the glucose components were dehydrated to hydroxymethyl furfural, which gives green colored product with anthrone.

100 mg of the test sample was weighed and then homogenized with 5.0 mL of 2.5 N HCl in a mortar and pestle. The homogenized sample was again added with 5.0 mL of 2.5 N HCl to adjust the volume and hydrolyzed in boiling water bath for 90 min. The sample was cooled down to room temperature (30 ± 2ºC) and neutralized with anhydrous sodium carbonate crystals. The solution volume was made up to 100 mL with distilled water and centrifuged at 1500 *g* for 10 min. The supernatant was collected and aliquot of 0.5 mL was used for analysis. Prior to analysis the volume was made up to 1 mL with distilled water. To this mixture 4 mL of anthrone reagent was added and placed in boiling water bath for approximately 8 min. After the heat treatment mixture was rapidly cooled to 4ºC. Optical density of the resultant dark green colored solution was measured at 630 nm. A calibration curve was prepared using glucose as standard at the concentration of 0.002 mg to 0.01 mg. The amount of carbohydrate present in the sample was calculated from the calibration curve using Equation (5) and expressed in mg $g^{-1}$. The percentage (%) value was calculated from the final result as represented below:

$$\text{Carbohydrate yield (\%)} = \frac{\text{Carbohydrate values from standard curve}}{\text{volume of test sample (mL)}} \times 100 \qquad \textbf{(5)}$$

### 3.8.3. Determination of total cellular lipid content

Microalgal lipid extraction was carried out by Bligh and Dyer, (1959) method. For extraction of lipid dry biomass of the sample was weighted and homogenized in a mortar

and pestle with 0.1 to 0.5 g of anhydrous $Na_2SO_4$ and 1 to 2 mL of 2% BHT (2.04 g of BHT in 100 mL $CHCl_3$). Total lipid was extracted from this homogenized powder with 5-10 mL of $CHCl_3$:MeOH (2:1) solvent mixture. The residue was extracted 2-3 times with chloroform or till it became colorless. The extracts were mixed together, filtered through Whatman No.1 filter paper and transferred to a separating funnel. To the separating funnel 0.9% NaCl solution (⅓ of the volume) and excess $CHCl_3$ was added, mixed thoroughly and kept undisturbed for overnight at room temperature to form a clear biphasic layer. The lower organic ($CHCl_3$) layer containing lipid components was collected in a clean pre-weighed ($W_1$) glass vial. Similarly, upper methanol-water layer was washed twice with chloroform and collected separately. All the collected chloroform layers were mixed together. The solvent was evaporated near to dryness in a water bath at 60ºC and dried in desiccators over anhydrous $Na_2SO_4$. The vial containing crude lipid extract was reweighed ($W_2$). The difference in weight ($W_2$-$W_1$) was recorded as total lipid content of the sample. The lipid content was determined gravimetrically and expressed as % dry weight.

### 3.9. Preparation of fatty acid methyl esters (FAME)

Approximately 1 g of dried algal biomass was used to prepare FAME. The in-situ transesterification of microalgae biomass was performed using a reaction mixture of 5% concentrated sulfuric acid in methanol (v/v). The biomass (1 g) was taken in a 50 mL sample tube glass and 3 mL of hexane was added to transfer the methyl esters in hexane phase. Freshly prepared above stated Me-$H_2SO_4$ reaction mixture (2 mL) was then added to the mixture. The vial was mixed well by vortex mixture at low speed followed by incubation at 90ºC for 4 h under constant stirring. After the reaction mixture was allowed to cool down to room temperature, then 1.2 mL of water was added to stop the reaction.

The mixture was centrifuged for 1 min and the upper hexane layer containing FAME was collected in a clean glass vial. The extracted FAME was dried by passing through anhydrous $Na_2SO_4$ and analyzed using $^1H$ NMR spectrometer.

### 3.10. Determination of fatty acid compositions

The $^1H$ NMR spectrum of FAME sample was obtained on (Advance III HD NMR spectrometer) 600 MHz proton frequency using a 5 mm broad band inverse probe head, equipped with shielded z-gradient accessories. Prior to the analysis extracted FAME sample was dissolved in 400 mL deuterated chloroform ($CdCl_3$). The deuterated chloroform chemical shift peak at 7.26 ppm was taken as the internal reference. The parameters followed in the analysis were maintained as described by Mazumdar *et al*., (2012).

The spectrum generated was analyzed to determine the proportion of linolenic (Ln), linoleic (L), oleic (O) and saturated (S) FA groups present in the FAME samples by using following equations (Yeung *et al*., 2008):

$$\text{Ln (\%)} = 100\left[\frac{B}{A+B}\right] \quad \textbf{(6)}$$

$$\text{L (\%)} = 100\left[\frac{E}{D} - 2\left[\frac{B}{A+B}\right]\right] \quad \textbf{(7)}$$

$$\text{O (\%)} = 100\left[\left(\frac{C}{2D}\right) - \left(\frac{E}{D}\right) + \left[\frac{B}{A+B}\right]\right] \quad \textbf{(8)}$$

$$\text{S (\%)} = 100\left[1 - \left(\frac{C}{2D}\right)\right] \quad \textbf{(9)}$$

where A (peak 1), B (peak 2), C (peak 5), D (peak 6) and E (peak 7) are areas of the peaks with chemical shift.

### 3.11. Determination of calorific value (CV)

The calorific value of microalgae oil/FAME was determined using an oxygen bomb calorimeter (1341 plain jacket calorimeter, Parr instruments USA). A crucible containing moisture-free (dry) algal oil (0.5 g) was ignited in the presence of oxygen (99.99% purity) at 3000 kPa. Calorific value was calculated using the following equation (10)

$$CV = \frac{\Delta T \times W\text{-}e1\text{-}e2\text{-}e3}{m} \quad \textbf{(10)}$$

Where, W = 2546.3415 cal $g^{-1}$ which is enthalpy of water

$\Delta T$ = difference in temperature

m = sample weight

And $e_1$, $e_2$ and $e_3$ represent correction in calories for heat of the formation of $HNO_3$, $H_2SO_4$ and fuse wire respectively.

### 3.12. Determination of cold flow properties

The oxidation induction temperature was determined using DSC instrument (model DSC1, stare system, Mettler Toledo) connected with a computer based controller. The test sample of approximately 5-10 mg was taken in a 40 $\mu$l sealed pan under a nitrogen flow rate of 40 mL $min^{-1}$. Four sequential active steps temperature program was used, in which the sample was heated from room temperature (RT) to 50ºC at a constant heating rate of 5ºC $min^{-1}$ and then it was hold under the isothermal condition for 10 min to homogenize the sample and dissolve a waxy material present in the sample. The sample was cooled using an external refrigeration system (Huber, TC-45, R290 refrigerant, USA) under the specified conditions as described previously (Borugadda *et al*., 2014).

### 3.13. Determination of Acid value and Iodine value

Acid value denotes free fatty acid (FFA) content of oil. It is defined as the amount of potassium hydroxide (mg) required for neutralizing 1 g of oil sample. Acid value was determined according to AOCS Official method (Te TA-64, 1997). The microalgae oil sample was titrated in neutral ethanol with 0.5 N KOH in association with Phenolphthalein solution as an indicator. Acid value was calculated using the following expression:

$$\text{Acid value (mg KOH g}^{-1}) = \frac{\text{Volume of the titrant (mL)} \times \text{N} \times 56.10}{\text{Mass of the sample (g)}} \quad \textbf{(11)}$$

where, N is the normality of accurately standardized sodium hydroxide solution.

Iodine value denotes the unsaturation (double bonds) contained in oil. The Wij's method was used to determine the iodine value and was calculated according to AOCS recommended practice Cd 1c-85.

0.1 g to 0.2 g of oil or FAME sample was weighed into a 250 mL Erlenmeyer flask, and added with 20 mL of carbon tetrachloride and 25 mL of Wijs reagent. Then the mixture was stored in a dark place at 25 ± 5ºC for 30 min. Thereafter sample was added with 10 mL of potassium iodide solution and 100 mL of distilled water followed by titration with 0.1 N sodium thiosulphate ($Na_2S_2O_3$). Titration was done in addition with 1 mL of starch indicator solution until the blue starch-iodine color disappears. The iodine value of the test sample was determined using the following equation:

$$\text{Iodine value} = \frac{N\left(V_3 - V_4\right) \times 12.6}{\text{m}} \quad \textbf{(12)}$$

where, N= 0.1 which is the normality of the $Na_2S_2O_3$ solution

m= mass of the oil sample in g

$V_3$= amount of $Na_2S_2O_3$ solution (mL) used for blank test

$V_4$= amount of $Na_2S_2O_3$ solution (mL) used for test sample

## 3.14. Thermogravimetric analysis (TGA)

Thermogravimetric is a method in which thermal properties of esters are measured as a function of temperature. The thermogravimetric behavior of microalgae oil and FAME sample was analyzed in pyrolytic conditions by means of a STA7200, Hitachi thermal analysis system instrument at inert (nitrogen) atmosphere. The non-isothermal analysis was performed at a heating rate of 10ºC $min^{-1}$ from room temperature to 800ºC. A high purity nitrogen gas (99.99%) was fed at a constant flow rate of 100 mL $min^{-1}$ as an inert purge gas to avoid the unwanted oxidation of sample. To perform the analysis, different amounts of samples were used and were measured in mg in an open silica crucible. The weight loss vs temperature data was recorded and obtained data was used to plot the TG and DTG curves. TG and derivative of TG (DTG) curves were used to determine the onset temperature of samples which indicate the thermal behavior of samples.

## 3.15. FTIR spectroscopy and data acquisition

FTIR attenuated total reflectance spectra were collected in Fourier transform infrared spectrophotometer (IR Affinity-1 Shimadzu). The test samples were scanned and acquired the spectra at a spectral resolution of 4 $cm^{-1}$ after 100 scans in the mid-IR region (4000 – 500 $cm^{-1}$). The baseline correction was carried out using inbuilt software to minimize differences between the recorded spectra due to baseline shift. The absorption spectra revealed clear signatures of fatty acids, which were assigned by their corresponding functional groups, based on available published information.

### 3.15.1. Chemometric analysis of FTIR fingerprints

FTIR spectra were preprocessed using the software Spekwin32-spectroscopy to obtain the smoothing points. Baseline corrected in the spectral region between 3000 and 1000 $cm^{-1}$ were used for multivariate data analysis. Principal Component Analysis (PCA),

Hierarchical Clustering Analysis (HCA), K-means clustering and Multidimensional scaling (MDS) were performed using the software SAS JMP, Version 11 and XLSTAT 2014 for windows.

# CHAPTER 4

# Results and discussions

# 4. RESULTS AND DISCUSSION

Investigations in this thesis are focused on the selection of potent microalgae species from existing 'microalgae resources' of the region and their potential towards the biodiesel production. The experimental findings are represented and categorized according to the defined objectives of the thesis. The respective tables and graphs of the study are placed after each objective of the thesis.

## 4.1. Isolation, screening, identification and establishment of unialgal culture of the selected freshwater microalgae

The unique diversity of microalgae and the spectrum of species available for biodiesel production have placed the microalgae in the priority list compared to other advanced biomass feedstocks. Microalgae are widespread in all over the existing earth ecosystems and around 30,000 species have been studied and analyzed (Mata *et al*., 2010). These microalgae species are classified into several groups, namely cyanobacteria (Cyanophyceae), green algae (Chlorophyceae), diatoms (Bacillariophyceae), yellow-green algae (Xanthophyceae), golden algae (Chrysophyceae), red algae (Rhodophyceae), brown algae (Phaeophyceae), dinoflagellates (Dinophyceae) and 'pico-plankton'(Prasinophyceae and Eustigmatophyceae) (Hu *et al.*, 2008). Among them, only few species such as *Botryococcus*, *Chlamydomonas*, *Chlorella*, *Dunaliella*, *Neochloris*, etc are known for biodiesel potential owing to their lipid content between 20% and 75% by the weight of dry biomass (Deng *et al*., 2009).

India has a very rich repository of natural resources. The northeastern states of India is acclaimed to be a genetic treasure land of plant and microbial resources, which forms a distinctive part of the Indo-Burma Hotspot that ranks 6$^{th}$ among the 34 mega biodiversity hotspots of the world with abundant natural resources available for

sustainable development (Conservation International, 2007). The microalgae species used in this study are isolated from a biodiversity rich fresh water body of Assam, India. This place is located south of the Eastern Himalayas (26.15ºN 91.77ºE) and bestowed with favorable climatic conditions for harboring a rich diversity of freshwater microalgae resources (Talukdar and Kalita, 2011). These diverse resources highly need to be explored for potential biotechnological applications. Particularly the region is well suited for the large-scale cultivation of freshwater microalgae owing to its unique landscape, geo-climatic conditions and other limnological and ecological features.

This objective deal with the isolation of potential native microalgae strains from the wetland of Assam, India. Isolation of the strain is followed by characterization and preliminary selection of potent species for study. The isolated strains are screened based on its dominancy, growth and biochemical compositions for potential utilization as renewable biomass feedstock for biodiesel production.

### 4.1.1. Sample collection, isolation and microscopic observation

For isolation, around 25-30 water samples were collected from the IIT Guwahati campus, Amingaon and Jalukbari area of Kamrup district, Assam, India (Figure 4.1). The preliminary ecological data of the sampling sites recorded onsite during the sample collection along with preliminary identification of significant microalgae species based on microscopic analysis are listed in Table 4.1. The collection site pH and temperature was in the range of 6.9-7.6 and 26-30ºC respectively. Microscopic observations of collected water samples from various locations revealed a rich diversity of freshwater microalgae strains. Prior to isolation of the desirable strains, each of the collected water samples was subjected to microscopic observation for preliminary morphological analysis.

Microalgae strains were isolated by a dilution method followed by repeated streaking on the agar-plate as described in section 3.4.3. Following the method as shown in Figure 4.2, microalgae strains were initially isolated and maintained their unialgal culture under controlled laboratory growth conditions as stated in Section 3.4.4. Therefore, to select the desirable strains of specific interest as specified in the objectives, four native strains were initially selected based on observed growth performances and available literature. The morphological features of the selected strains observed under a light microscope are represented in Table 4.1. Preliminary identifications of the species were done based on the comparison of data revealed by microscopic observations with the data available in the literature. Based on morphological features as shown in Figure 4.3, the selected microalgae isolates were initially identified as *Ankistrodesmus* sp (ADIITEC-I), *Scenedesmus* sp (ADIITEC-II), *Chloromonas* sp (ADIITEC-III) and *Navicula* sp (ADIITEC-V). Moreover, for standardization of various methodologies the identified *Scenedesmus* sp. (GUBIOTJT116) supplied by the Department of Biotechnology, Gauhati University, India was considered as a reference species.

### 4.1.2. Growth characteristics of the isolated microalgae species

The studied microalgae species grown in a normal BG11 medium under the growth conditions stated in Section 3.4.4 exhibited variations in their growth characteristics. Figure 4.4 depicts the growth curves of the isolated microalgae species in normal BG11 medium. Exponential growths of studied microalgae strains were observed in between 2-7 days after the day of inoculations. Among the isolated strains, *Navicula* sp. (ADIITEC-V) revealed a steady growth rate and a comparatively much slower specific growth (0.1 $d^{-1}$). Otherwise, *Scenedesmus* sp. (GUBIOTJT116) was found to be the most aggressive and fast growing species among all the studied microalgae isolates. In table 4.2,

*Scenedesmus* sp. (GUBIOTJT116) revealed the maximum specific growth rate of 0.26 ±0.02 $d^{-1}$ with the least doubling time of 2.66±0.17 days followed by *Chloromonas* sp. (ADIITEC-III) ($\mu$ = 0.21±0.03 $d^{-1}$ and $T_2$ = 3.3±0.48 days), *Ankistrodesmus* sp. (ADIITEC-I) ($\mu$ = 0.19±0.02 $d^{-1}$ and $T_2$ = 3.64±0.04 days) and *Scenedesmus* sp. (ADIITEC-II) ($\mu$ = 0.18±0.02 $d^{-1}$ and $T_2$= 3.85±0.53 days).

### 4.1.3. Biochemical compositions

Biochemical contents such as total cellular lipid, carbohydrate and protein of the selected microalgae strains were quantified from the oven dried cell biomass of cultures harvested during late exponential phase or an early stationary phase of growth. The percentage value of each component was determined from the mean values of three replicates and given in Table 4.2. The comparison of biochemical contents are represented in Figure 4.5.

#### 4.1.3.1. Total cellular carbohydrate and protein content

The cell dried biomass of isolated microalgae strains was analyzed for proximate values of the total cellular carbohydrate and protein content as shown in Figure 4.5. Among the microalgae strains, the dried biomass of *Navicula* sp (ADIITEC-V) showed significantly high yield of total carbohydrate content (26.5±0.65%), followed by *Scenedesmus* sp (ADIITEC-II) (24.5±0.6%), *Scenedesmus* sp (GUBIOTJT116) (23.4±0.75%) and *Chloromonas* sp (ADIITEC-III) (21±0.9%). *Ankistrodesmus* sp (ADIITEC-I) yielded the least amount (20.6±0.85%) of total cellular carbohydrate content. On the other hand, total protein contents from the biomass of studied microalgae strains were determined in the range between 9.5%-13% of dry cell weight (DCW). Among the microalgae strains, the yield of protein content by *Ankistrodesmus* sp (ADIITEC-I) and *Navicula* sp

(ADIITEC-V) (13±0.7% and 11.4±0.6% respectively) were found to be the highest, followed by *Chloromonas* sp (ADIITEC-III) (10.9±0.6%). Otherwise, the native strain of *Scenedesmus* sp (GUBIOTJT116) revealed the least amount (9.5±0.6%) of total protein (Table 4.2). The values may vary depending on the growth stage and cultivation conditions as described by Leonardos *et al*., (2000). It is also reported that stress conditions like nitrogen-starvation effectively drives the accumulation of carbohydrates in microalgae, but also considered as a strain dependent (Ho *et al*., 2013).

**4.1.3.2. Total lipid content**

The cell dried biomass of isolated microalgae strains was analyzed for the estimation of total lipid content and shown in Table 4.2. The maximum lipid content in case of *Chloromonas* sp. (ADIITEC-III) on a dry cell weight basis was found to 36.25±0.7%. Compared with other isolates total lipid content of *Scenedesmus* sp. (GUBIOTJT116) was around 34.14±0.8% followed by *Scenedesmus* sp. (ADIITEC-II) and *Ankistrodesmus* sp. (ADIITEC-I) 30±1.2% and 25.5±0.5% respectively. On the other hand, *Navicula* sp. (ADIITEC-V) yielded a comparatively least amount of total lipids, i.e. 18.2±0.75%. In this study, the data coincide with the fact that the nutrient limitations towards the stationary growth phase invariably cause a steady decline in the cell division rate, which in turn triggers the lipid metabolism (Sharma *et al*., 2012). In particular, nitrogen source of the nutrient played a crucial role in microalgae lipid metabolism. As reported in many species or strains of microalgae, a general trend towards the accumulation of lipids, particularly TAG, in response to nitrogen deficiency was also observed (Praveenkumar *et al*., 2012).

The microalgae strains grown in normal BG11 medium under similar state of controlled growth conditions, showed comparatively higher lipid content in *Scenedesmus*

sp. (GUBIOTJT116) and *Chloromonas* sp. (ADIITEC-III). The results from this preliminary screening thus revealed immense possibilities of these native microalgae strains for consideration of potential biofuel applications. Therefore, the native strains of microalgae *Scenedesmus* sp. (GUBIOTJT116) and *Chloromonas* sp. (ADIITEC-III) were selected for further studies.

### 4.1.4. Morphological characterization

The micro morphological features of *Chloromonas* sp. (ADIITEC-III) was observed under the light and FESE microscope (Figure 4.6. B). Under the light microscopy, the species showed motile nature. The motile cells were biflagellate, equal in length and sub-spherical in shape measuring 3 to 6 $\mu$m in diameter. The chloroplasts had a single eyespot and lacked pyrenoids, but the presence of anterior papilla was noticed in the cell. According to the traditional taxonomic system, the chloroplast of biflagellate cells devoid of pyrenoids indicates that they belong to the biflagellate volvocalean genus *Chloromonas* (Remias *et al*., 2010). Based on these preliminary observations on morphological characteristics the strain ADIITEC-III was initially identified as *Chloromonas* species. Nevertheless, further detail study on the taxonomic characterization need to be carried out, but was beyond the scope of this study.

### 4.1.5. Molecular characterization and phylogenetic placement

The initially identified as *Chloromonas* species of Chlorophyceae was further confirmed by 18S rRNA gene sequence based molecular phylogenetic analysis. The sequence of the isolated strain was submitted to NCBI GenBank under the accession number KF471125.1. In the reconstructed Phylogenetic tree, the molecular markers of internal transcribed spacers ITS1 and ITS2 of ADIITEC-III indicates a different clad. As shown

in Figure 4.7, when compared with the sequences retrieved from the NCBI database, provides the newly isolated microalgae sp. strain ADIITEC-III an independent status possibly of a species with close proximity with the *Chloromonas serbinowin* sp. bearing accession number AB624569.1.

Figure 4.1 Sample collection sites from a roadside pond

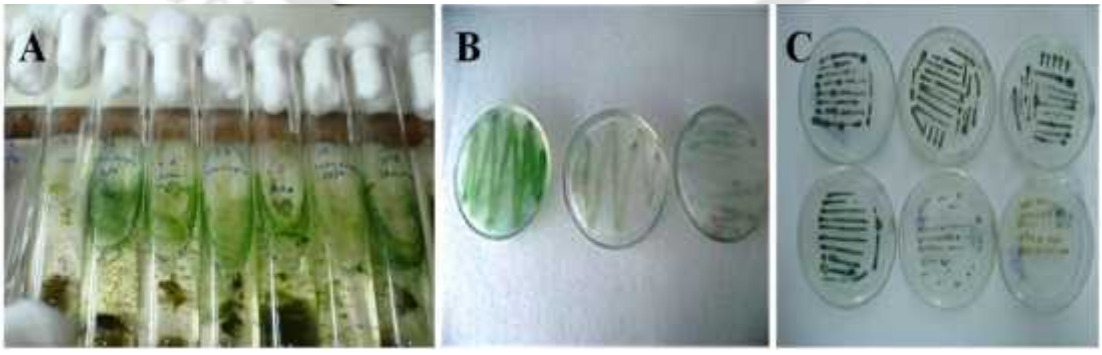


Figure 4.2 Processing of the collected water sample. A- Enrichment culture; B, C - solid culture for isolation

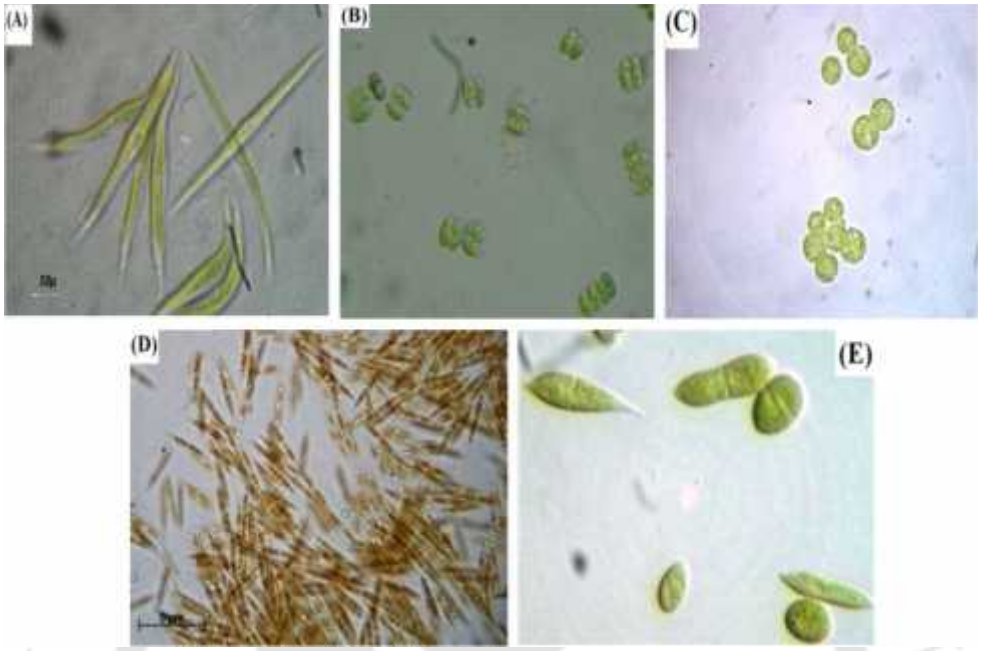


Figure 4.3 Micro photographs of the isolated microalgae strains. A- *Ankistrodesmus* sp (ADIITEC-I), B- *Scenedesmus* sp (ADIITEC-II), C- *Chloromonas* sp (ADIITEC-III), D- *Navicula* sp (ADIITEC-V), *Scenedesmus* sp (GUBIOTJT116)

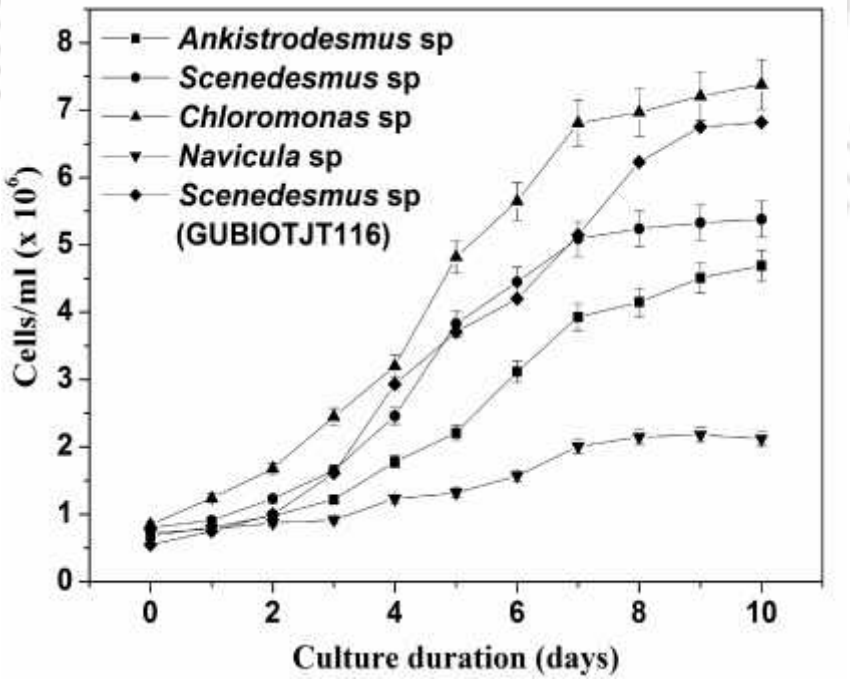

Figure 4.4 Comparative growth curves of isolated microalgae strains. Growth curves show average growth and doubling time of the studied microalgae strain in normal BG11 medium

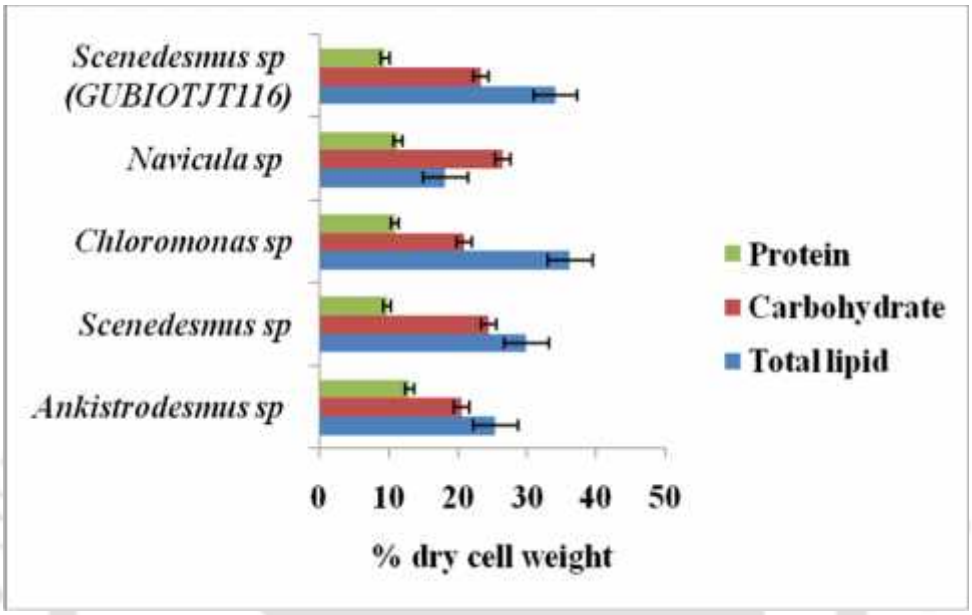


Figure 4.5 Comparison total lipid, carbohydrate and protein content of isolated microalgae strains grown in BG11 medium

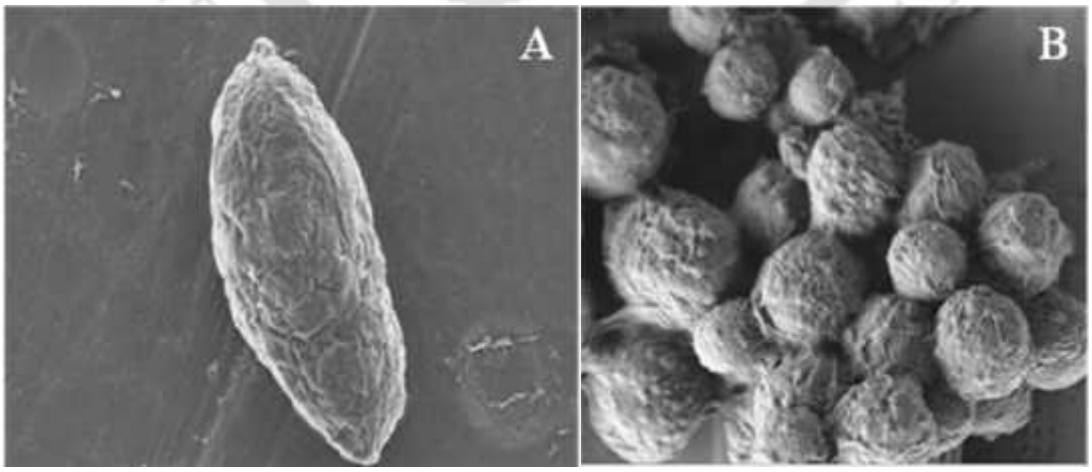


Figure 4.6 FESEM image taken under 9.43 KX magnifications. A- *Scenedesmus* sp. (GUBIOTJT116); B- *Chloromonas* sp. (ADIITEC-III)

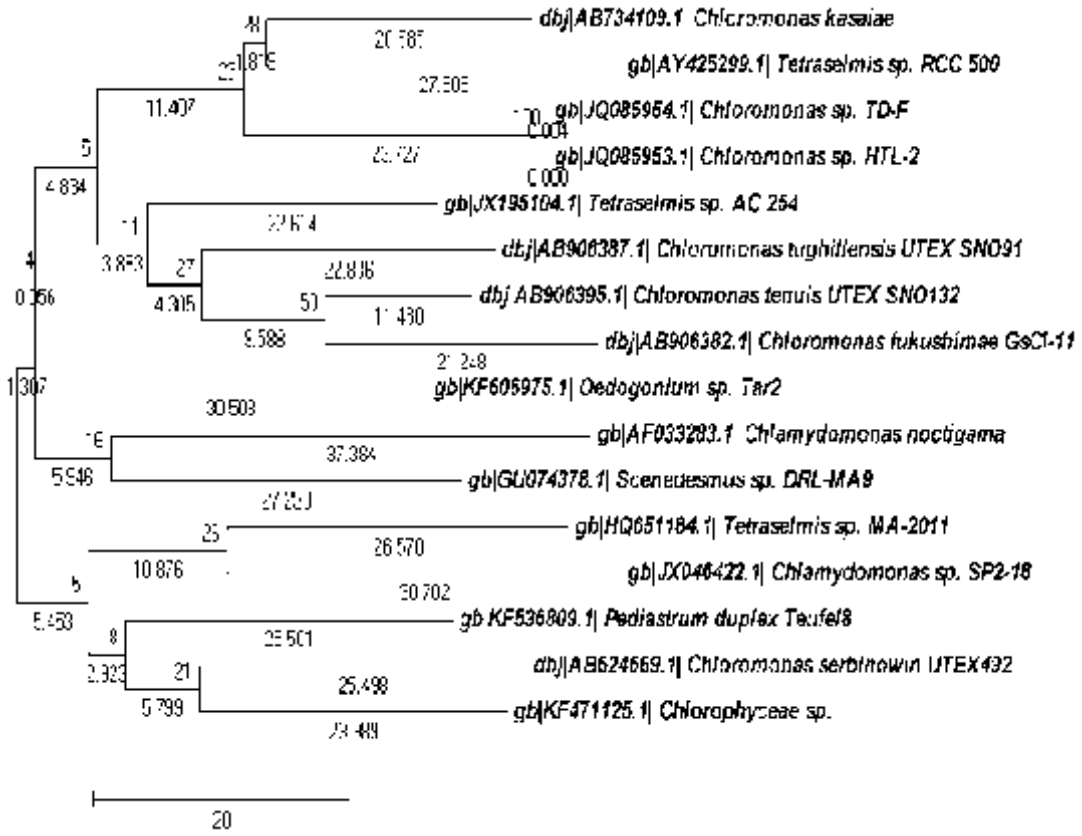


Figure 4.7 Phylogenetic tree of 18S rRNA sequences inferred with the neighbor – joining method. Sequences are identified by their corresponding GenBank accession numbers and organism name. Numbers shown at the branches are the credibility percentages using bootstrap test (100 replicates).The *Chloromonas* sp. ADIITEC-III (KF471125.1) is shown in the rectangular box

Table 4.1 Morphological characteristics of the selected microalgae strains

| Species /strains | Size ($\mu$m) | | Shape | Organization |
|---|---|---|---|---|
| | length | diameter | | |
| *Ankistrodesmus* sp. ADIITEC-I | 28.0 | 3.0-4.0 | Spindle shaped | Solitary or cluster |
| *Scenedesmus* sp. ADIITEC-II | 20.0 | 7.5 | Fusiformis, slightly acute | Solitary |
| *Chloromonas* sp. ADIITEC-III | 34.0 | 4.0 | Spindle shaped | Solitary or cluster |
| *Navicula* sp. ADIITEC-V | 4.5 - 5.0 | 4.5 | Spherical | Solitary |
| *Scenedesmus* sp. GUBIOTJT116 | 8.75 - 15.8 | 4.5-8.25 | Sub-spherical to pear shaped, embedded more or less within the matrix | Colonial |

Table 4.2 Comparison of growth and lipid yield of selected microalgae strains

| Strains | Growth media | Sp growth ($d^{-1}$) | Doubling time ($T_2$) | Total lipid (%) dry wt | Carbohydrate (%) | Protein (%) |
|---|---|---|---|---|---|---|
| *Ankistrodesmus* sp ADIITEC-I | BG 11 | 0.19±0.02 | 3.64±0.4 | 25.5 ±0.5 | 20.6 ±0.85 | 13±0.7 |
| *Scenedesmus* sp ADIITEC-II | BG 11 | 0.18±0.02 | 3.9±0.53 | 30±1.2 | 24.5±0.6 | 9.8±0.8 |
| *Chloromonas* ADIITEC-III | BG 11 | 0.21±0.03 | 3.3±0.48 | 36.25±0.7 | 21±0.9 | 10.9±0.6 |
| *Navicula* sp ADIITEC-V | BG 11 | 0.1±0.02 | 6.93±0.66 | 18.2±0.75 | 26.5±0.65 | 11.4±0.6 |
| *Scenedesmus* sp (GUBIOTJT116) | BG 11 | 0.26±0.02 | 2.66±0.17 | 34.14±0.8 | 23.4±0.75 | 9.5±0.6 |

## 4.2. Optimization and characterization of selected microalgae strains in terms of growth, biomass and lipid production

The physiological factors for microalgae cultivation are the preliminary footsteps to overcome the potential biomass demand. Optimization of microalgae cultivation is the basic requirement and always maintained with a combination of basic physiological factors like light intensity, pH and temperature for its optimum physicochemical and biological processes. Physiological factors including physical and chemical stimuli modulate the biochemical compositions of the microalgae (Richmond, 2004).The physical stimuli such as gravity and light invoke generalized cellular reactions (Phatarpekar *et al*., 2002). The chemical stimuli include alteration of media compositions which could modify and improve algal lipids (Richmond, 2004).

This objective focuses on the selection of appropriate physico-chemical cultivation conditions for growth, biomass and biochemical compositions of *Chloromonas* (Chlorophyceae) species ADIITEC-III and *Scenedesmus* species GUBIOTJT116. The study was conducted in a 250 mL culture flask in triplicate in a batch mode for the culture duration of 10 days. Isolates were studied in three media compositions, (BG11, BBM and Chu 13) and the optimum condition is taken forward for a range of pH conditions i.e. pH 5.0, 6.0, 7.0, 8.0 and 9.0 which is calibrated using acetate and phosphate buffer. The two isolates were cultivated at seven different light intensities (27, 40.5, 54, 67.5, 81 and 94.5 $\mu$mol $m^{-2}s^{-1}$). The lipid yield from the optimum condition is transesterified to FAME and their property is characterized.

### 4.2.1. Determination of growth and biomass production of *Chloromonas* (Chlorophyceae) species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 in different media compositions

As the *Chloromonas* (Chlorophyceae) sp. ADIITEC-III is reported for the first time, it is important to screen the optimum cultivation condition. Therefore, to choose the optimum media composition, three different types of synthetic media recipe were studied. Growth studies in terms of cell count in the selected medium was carried out in batch cultures for 10 days. As seen from the growth curve (Fig. 4.8), no obvious lag phase was observed after the day of inoculation and it continues steady growth till day 7 to 8. In fact, ADIITEC-III in BG11 medium showed a swift rise in cell number from day 2 and the maximum specific growth rate ($\mu_{max}$) was recorded to be 0.22 ± 0.02 $d^{-1}$ with a doubling time ($T_2$) of 3.1 ± 0.16 d. Among the studied media compositions, Chu 13 medium showed the least specific growth rate of 0.11 ± 0.01 $d^{-1}$ with a doubling time ($T_2$) of 6.33 ± 0.578 d followed by the BBM media. The cultures grown in BBM media, showed almost similar growth pattern as in BG11 medium with the specific growth rate of 0.21 ± 0.01 $d^{-1}$ and a doubling time ($T_2$) of 3.3 ± 0.157 d. However, in terms of biomass productivity, BG11 and BBM media showed maximal productivity compared to Chu 13 media. Although, no significant difference in biomass productivities between the media BG11 (0.03 ± 0.017 g $L^{-1}d^{-1}$) and BBM media (0.03 ± 0.015 g $L^{-1}d^{-1}$) was observed. Whereas Chu 13 media, with a minimum increase in net cell density indicates slow growth, leading the least biomass production (0.2 g $L^{-1}$) towards the $10^{th}$ day of the growth period. Thereby, the biomass productivity was recorded to be (0.02 ± 0.015 g $L^{-1}$ $d^{-1}$).

The *Scenedesmus* species GUBIOTJT116 grown under three different media compositions showed significant responses. During 10 days of cultivation, various

growth parameters, including maximum specific growth rate ($\mu_{max}$), and overall biomass productivity were monitored with respect to time. As illustrated from the growth curve (Fig. 4.9), the maximum specific growth rate (0.24 ± 0.02 $d^{-1}$) with a doubling time ($T_2$) of 2.89 ± 0.24 d was recorded in BG11 medium. This specific growth of *Scenedesmus* species GUBIOTJT116 is higher than the specific growth rate obtained from the BBM (0.23 ± 0.01 $d^{-1}$) and Chu 13 media (0.18 ± 0.02 $d^{-1}$) respectively. In Figure 4.9, it is clearly observed that the lag phase in all the three media compositions withstand up to 2 days and from where the growth continues till it reaches its stationary phase. From the data obtained, BG11 was found to be the most suitable media compositions in comparison to BBM and Chu 13 media. In addition, the biomass production of *Scenedesmus* species GUBIOTJT116 grown in BG11 medium (3.4 g $L^{-1}$) recovered from the 10$^{th}$ day of cultivation is much higher than the Chu 13 media (2.6 g $L^{-1}$). The similar response towards the biomass productivity was observed in BG11 (0.34 ± 0.02 g $L^{-1}d^{-1}$) and BBM medium (0.3 ± 0.015 g $L^{-1}d^{-1}$). The significant difference was observed in Chu 13 media with productivity (0.26 ± 0.015 g $L^{-1}d^{-1}$) which is much lower than the other two studied media compositions. Thereby, BG11 would be preferred over BBM and Chu 13 media to obtain the optimum specific growth rate ($\mu_{max}$) and overall biomass productivity.

### 4.2.2. Study on growth characteristics of ADIITEC-III and GUBIOTJT116 under different light intensity

The two isolates were cultivated at seven different light intensities (27, 40.5, 54, 67.5, 81 and 94.5 $\mu$mol $m^{-2}s^{-1}$). Each experiment was studied in triplicate in a batch mode for the culture duration of 10 days and the initial culture pH was adjusted to pH 7.5 prior to autoclaving. Growth study carried out using different light intensities showed a

significant effect on cell growth curve patterns of ADIITEC-III and GUBIOTJT116. The experiment at different light intensities on both the isolates as shown (Fig. 4.10 and 4.11) exhibits a slow growth rate and low biomass production under a low light intensity of 27 $\mu$mol $m^{-2}s^{-1}$, but the gradual increase in irradiance was associated with an increase in growth and biomass only up to a certain light intensity i.e. 81 $\mu$mol $m^{-2}s^{-1}$. Among the studied light regimes, ADIITEC-III exposed to 40.5 $\mu$mol $m^{-2}s^{-1}$ provides a continuous cell growth through an exponential phase till the $9^{th}$ day of cultivation with a maximum specific growth rate of 0.23 ± 0.017 $d^{-1}$ with doubling time ($T_2$) of 3.02 ± 0.23 days. Under the irradiance of 54, 67.5 and 81 $\mu$mol $m^{-2}s^{-1}$, no significant variation in growth rates were noticed as 0.2 ± 0.026, 0.19 ± 0.02 and 0.18 ± 0.02 $d^{-1}$ respectively. However, the further increased in light intensity to 94.5 $\mu$mol $m^{-2}s^{-1}$ showed a sudden drop in the growth rate (0.16 ± 0.02 $d^{-1}$) with a doubling time of 4.37 ± 0.55 days. As reported, the growth rate of *Dunaliella tertiolecta* and *Scenedesmus* sp. 11-1 in different light intensity was also studied (Tang *et al*., 2011; Liu *et al*., 2012). In their study, *Scenedesmus* sp. 11-1 showed high tolerance towards the light regimes and light intensity 400 $\mu$mol $m^{-2}s^{-1}$ indicates the most suitable condition for growth with the total biomass yield of 3.88 g $L^{-1}$ (Liu *et al*., 2012).

In the case of GUBIOTJT116, the growth rate is continuously supported under the irradiance of 81 $\mu$mol $m^{-2}s^{-1}$, at this light intensity cell density was 4.69 x $10^6$ cells $ml^{-1}$ towards the $10^{th}$ day of the experiment with maximum growth rate (0.25 ± 0.06 $d^{-1}$) and minimum doubling time (2.56 ± 0.13 days). The least increased in cell concentration (3.88 x $10^6$ cells $mL^{-1}$) was observed in the culture incubated under the light intensity of 94.5 $\mu$mol $m^{-2}s^{-1}$ with a minimum growth rate (0.1 ± 0.003 $d^{-1}$). However, culture grown under irradiance of 27 and 40.5 $\mu$mol $m^{-2}s^{-1}$ showed a growth rate of 0.16 ± 0.01 $d^{-1}$ and

0.17 ± 0.002 $d^{-1}$ respectively. The light intensity of 40.5 and 54 $\mu$mol $m^{-2}s^{-1}$ showed no significant variation in growth rate. A similar study conducted on *C. vulgaris* sp. has provided different aspects where a low light intensity range of 37.5 to 62.5 $\mu$mol $m^{-2}s^{-1}$ showed increased growth, biomass and exhibits a significant decrease in biomass at high light intensity of 100 $\mu$mol $m^{-2}s^{-1}$ (Khoeyi *et al*., 2012). It has also been observed that alteration in light intensity affects biomass and biochemical compositions and also exhibits their range of adaptations in different culture conditions accordingly (Khoeyi *et al*., 2012). Moreover, light intensity in nature goes to about 2,000 $\mu$mol $m^{-2}s^{-1}$ and which may limit growth as it is beyond the light saturation constants of some algae (Jeon *et al*., 2006; Liu *et al*., 2012). Indeed, depending on species to species, microalgae exhibits increased growth and biomass under the certain saturation point of light. But further increase in light intensity beyond the saturation point inhibits the growth and biomass causing photoinhibition (Xue *et al*., 2011). In this phenomenon, a photo oxidation reaction takes place inside the cell due to the inability of photosynthetic apparatus to absorb the excess light (Harrison *et al*., 1990; Ma *et al*., 1997; Phatarpekar *et al*., 2002; Richmond, 2004).

### 4.2.3. Determination of biomass and lipid production of ADIITEC-III and GUBIOTJT116 under different light intensity

Light is the major source of energy for the growth of microalgae cells. Therefore, light intensity played a significant effect on biomass production of the studied species towards the exponential growth phase. As shown in Figure 4.12 and 4.13, each microalga has a unique requirement of light intensity for biomass and lipid production. The growth characteristics, biomass and total lipid content of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 grown at different light intensities is tabulated in

Table 4.3 and 4.4 respectively. At a light intensity of 81 $\mu$mol $m^{-2}s^{-1}$, GUBIOTJT116 exhibited maximum growth and biomass production (0.32 ± 0.02 g $L^{-1}d^{-1}$) in comparison with ADIITEC-III (0.032 ± 0.01 g $L^{-1}d^{-1}$). But the subsequent increased in light intensity to 94.5 $\mu$mol $m^{-2}s^{-1}$ lowers the biomass production and in the contrary same light regime provide both ADIITEC-III and GUBIOTJT116 an effective increase in lipid content (% DCW) of 36.5 ± 0.75 and 38.8 ± 0.8 respectively. As observed, the GUBIOTJT 116 species at the series of light regimes (27, 40.5, 54, 67.5 $\mu$mol $m^{-2}s^{-1}$) follows the steady increase in biomass productivity. Therefore, in the present study on GUBIOTJT116, it was observed that a gradual increase in light intensities ranging from 27 $\mu$mol $m^{-2}s^{-1}$ to 81 $\mu$mol $m^{-2}s^{-1}$ favored the cell density increase which results in maximum biomass production as shown in Figure 4.13. However, the biomass productivity of GUBIOTJT116 was limited beyond the light regime 81 $\mu$mol $m^{-2}s^{-1}$ with minimum biomass production (0.14 ± 0.02 g $L^{-1}d^{-1}$) respectively at 94.5 $\mu$mol $m^{-2}s^{-1}$. In the contrary, ADIITEC-III under the light conditions (40.5, 54, 67.5 and 81 $\mu$mol $m^{-2}s^{-1}$), no significant variation in growth rates (0.23 ± 0.017, 0.2 ± 0.026, 0.19 ± 0.02 and 0.18 ± 0.02 $d^{-1}$) was observed.

From the obtained data it was clear that the particular species can be grown under the low light conditions ranging (40.5, 54, 67.5 $\mu$mol $m^{-2}s^{-1}$) for optimum growth and biomass production. On the other hand, our results on lipid content showed that an increase light intensity was associated with increased lipid yield (Figure 4.12 and 4.13). When compared with the low light conditions, the pattern of lipid content was almost similar for both the species. In case of ADIITEC-III, the low light conditions 27, 40.5 and 54 $\mu$mol $m^{-2}s^{-1}$ showed minimum lipid content (% DCW) i.e. 22.7 ± 1.61, 23.5 ± 0.95, and 28.4 ± 1.55 respectively. Likewise, minimum lipid accumulation was also

observed in case of species GUBIOTJT116 under the same light intensities and thereafter increased beyond the light intensity of 67.5 $\mu$mol $m^{-2}s^{-1}$. Thus, in the case of our studied species, light intensities ranging from 67.5 to 94.5 $\mu$mol $m^{-2}s^{-1}$ tends to promote the lipid yield. The total lipid content of the studied microalgae species was determined towards the late exponential phase of the culture period. As noted from the data obtained, the significant variation in lipid and biomass production was observed at different light intensities. The light regime carries an essential part in the production of triacylglycerides and its composition varies from species to species in respond to different light intensities (Wahidin *et al*., 2013). In regards, there had been many contradictory reports on the accumulation of lipid in relation to irradiance. Many have reported that increased light intensity supports enhancements in the lipid and hydrocarbon contents rather than biomass production (Metzger *et al*., 1999; Tansakul *et al*., 2005). However, on the other hand, high irradiance has been reported to be responsible for promoting increased growth but reduces the lipid content (Cheirsilp *et al*., 2012). It was reported that triacylglycerol is the main component of neutral lipid and during synthesis it requires a large amount of ATP and NADPH via photosynthesis. Therefore, on exposure to a large quantity of light energy, excess light energy may be directed towards the lipid accumulation on a unit biomass basis through the process of photosynthesis (Liu *et al*., 2012). Thus, the study describes modifications of light intensity in accordance to microalgae species may serve the required yield of biomass and lipid.

### 4.2.4 Study on growth and biomass production of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 in different pH conditions

The growth pattern of both the isolated strains was studied under controlled pH condition and pH was controlled using buffers. Microalgae growth is also sensitive to pH change, and showed an increased trend of pH towards the stationary stage of the growth phase due to their ability to metabolize the inorganic carbon $CO_2$ (Rocha *et al*., 2003). The effect of pH on growth of ADIITEC-III and GUBIOTJT116 are shown in Figure. 4.14 and 4.15. From the data obtained (Table 4.5), it was observed that pH of the media has a significant effect on the final cell number and growth rate of both isolates. Among the studied pH range, growth of both the species was enhanced at pH 7.0. Under this condition, the maximum specific growth rate of ADIITEC-III and GUBIOTJT116 was recorded to be 0.25 ± 0.03 $d^{-1}$ and 0.2 ± 0.0004 $d^{-1}$ towards the final day of the experiment. Results showed maximum biomass productivity of 0.15 ± 0.01 g $L^{-1}d^{-1}$ for ADIITEC-III and 0.23 ± 0.02 g $L^{-1}d^{-1}$ for GUBIOTJT116 at pH 7.0. In comparison, acidic condition (pH 5.0 and pH 6.0) does not enhance the cell density and shows minimum biomass productivity and therefore a linear decline in the cell density of both isolates was observed towards the acidic conditions. However, a steady cell growth with biomass productivity was observed in ADIITEC-III when grown at pH 8.0 and pH 9.0 with the yield of 0.11 ± 0.02 g $L^{-1}d^{-1}$ and 0.08 ± 0.015 g $L^{-1}d^{-1}$ respectively.

Whereas, pH 8.0 for GUBIOTJT116 was observed with specific growth 0.15 ± 0.001 $d^{-1}$ but further increase in pH to 9.0 revealed slower growth rates. Subsequently, biomass productivity was observed to be decreased slightly (i.e. 0.21 ± 0.02 and 0.2 ± 0.02 g $L^{-1}d^{-1}$) during pH 8.0 and pH 9.0 respectively. Similar response was also observed when the species was grown at lower pH, where the cell density becomes less resulting least biomass productivity among the studied pH conditions. At pH 5.0 and pH 6.0,

biomass productivity was recorded as 0.08 ± 0.02 g $L^{-1}d^{-1}$ and 0.1 ± 0.01 g $L^{-1}d^{-1}$ respectively. According to a report *Scenedesmus* species WC-1 showed the highest cell densities in buffered cultures pH 7.4 and pH 9.3 followed by pH 10.3 unbuffered media condition (Gardner *et al*., 2011).

However, *A. falcatus* sp. could grow in a wide range of culture pH (5.0 - 10.0) but favored the maximum growth between the pH range of 7.0 to 9.0 (Talukdar *et al*., 2012). The results agrees well with the reported literature (Gardner *et al*., 2011; Talukdar *et al*., 2012), where a similar pH trend ranging from 7.0 to 9.0 provided favorable condition for increased cell number and high growth rate. Usually, microalgae cultures provide a gradual elevation of pH condition towards the final stage of the growth. As reported in the literature, pH 8.2 condition found to be efficient in rapid nitrate uptake and almost the entire nitrate taken up was released as ammonia during this condition in the presence of $CO_2$, which might be the reason for an abrupt rise in medium alkalinity at the later stage of growth (Eisele *et al*., 1997). Hence, an intermediate key is required to control the fluctuating pH in order to support the continuous growth of the strain.

### 4.2.5. Characterization of oil and methyl esters (FAME) recovered from *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 in BG11 medium under optimized light and pH conditions

From the optimized condition, the oil recovered from the biomass of ADIITEC-III and GUBIOTJT116 is further transesterified into methyl esters (FAME). Since, fuel properties have been one of the known criterions in selecting the microalgae sp. for biodiesel production; therefore the retrieved oil and methyl esters (FAME) of isolates were further characterized.

**4.2.5.1. Fatty acid profile of methyl esters (FAME) of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116**

In microalgae, the most common fatty acid profiles consist mainly of five $C_{16}$ and $C_{18}$ fatty acids (Knothe, 2009). In pertinence to biodiesel, the carbon chain length and the degree of saturation and unsaturation of the fatty acid molecules amend the fuel property (Mazumdar *et al*., 2012). In this study, the fatty acid composition has been determined through $^{1}$H NMR spectrometer as shown in Figure 4.16 and 4.17. The results of this compositional analysis are summarized in Table 4.6. $^{1}$H-NMR spectrum of methyl esters showed the appearance of signal at δ 3.7 ppm refers the characteristics of oxymethylic hydrogen which attributed to methylic esters (biodiesel) (Rashid *et al*., 2010). Figure 4.16 and 4.17 depicts $^{1}$H NMR spectrum of methyl esters of ADIITEC-III and GUBIOTJT116 with a strong singlet peak at 3.65 ppm and 2.3 ppm of methoxy protons which confirms the presence of methyl esters and verifies the complete conversion of algal oil into biodiesel (Nautiyal *et al*., 2014). Therefore, the fatty acid composition of ADIITEC-III and GUBIOTJT116 retrieved from acid catalyzed transesterification reaction was largely occupied by oleic acid (32.54%) and (48.1%) of total fatty acid respectively. The methyl esters (FAME) of ADIITEC-III indicates total saturated fatty acid 39.79% and the most abundant unsaturated fatty acid was oleic acid (C18:0) followed by linolenic acid (C18:3). On the other hand in GUBIOTJT116, total saturated fatty acid and linolenic acid was recorded as 36.29% and 13.79% respectively. In reports, oils with high oleic acid content known to have reasonable ignition quality, combustion heat, and cold filter plugging point (CFPP), oxidative stability, viscosity, and lubricity (Abou-Shanab *et al*., 2011). According to the reports on *D. elegans* DRLMA13 species, linolenic acid (18:3) decreases towards the late stationary phase of cultivation and presumed to attain an increased amount of palmitic and oleic acid by prolonged

cultivation (Kaur *et al*., 2012). In regards, ADIITEC-III and GUBIOTJT116 methyl esters obtained after the cultivation for 30 days showed low yield of linolenic acid as 6.63% and 13.79% respectively. Generally, high energy yield, superior oxidative stability, and higher cetane numbers are directed by the presence of a high proportion of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), but again the oils dominated by these fatty acids are susceptive to low temperature and tends to solidify (Doan *et al*., 2011). Moreover, besides saturated fatty acids, many algal oils contain significant amounts of highly polyunsaturated fatty acids (HiPUFA; ≥ 4 double bonds) but have no information regarding their fuel properties (Knothe, 2012). However, the polyunsaturated fatty acids (PUFAs) imparts very good cold-flow properties, but susceptible to oxidation which causes deterioration in the quality of biodiesel upon storage (Doan *et al*., 2011). The findings of the study indicates that both the strains are largely occupied by saturated fatty acids which would amount to a negative impact on cold flow properties, but the presence of high oleic acid content might increase the oxidative stability of fuel, enabling longer storage (Abou-Shanab *et al*., 2011).

#### 4.2.5.2. Physico-chemical characteristics of oil and methyl esters (FAME) of *Chloromonas* (Chlorophyceae) species ADIITEC-III and *Scenedesmus* species GUBIOTJT116

The physico-chemical characteristics of oil and FAME of ADIITEC-III and GUBIOTJT116 were evaluated according to the American Standards for Testing Materials ASTM 6751 and represented in Table 4.6. In this study, the properties relevant to fuel use such as acid value, iodine value, calorific value and cold flow properties were determined. Evaluation of oil property is recorded to determine its efficiency in conversion to biodiesel fuel.

Acid value is one of the most critical quality parameters of biodiesel. As illustrated in Table 4.6, the initial acid value of ADIITEC-III and GUBIOTJT116 oil was found to be similar as 14.02 mg KOH $g^{-1}$ which was quite high compared to ASTM 6751 standards. This indicates the presence of free fatty acids in the sample contributing high FFA content of 7.04% which prefers acidic over alkaline catalysts for the biodiesel conversion process. Moreover, the tendency of soap formation is quite high with base catalyzed transesterification reaction which creates hindrance in separation of biodiesel from the glycerin fraction (Sahoo *et al*., 2007; Nautiyal *et al*., 2014). In fact, the FFA content (>3%) inhibits the alkali-catalyzed transesterification process due to the consumption of alkali catalyst by FFAs (Chen *et al.*, 2012). Therefore, both the sample was subjected to acid-catalyzed transesterification reaction and the acid value of ADIITEC-III and GUBIOTJT116 FAME after transesterification was found to be 0.48 mg KOH $g^{-1}$ and 0.52 mg KOH $g^{-1}$ respectively. As reported in the literature FFA content of algal oil can reach as high as 84% (oil weight) (Krohn *et al*., 2011). Although, the presence of such high levels of FFAs during the microalgae growth is quite dubious as it may cause cytotoxic effect on the cells (Chen *et al*., 2012). The reason behind the degradation of microalgae cellular lipid to FFAs is depicted due to long-term storage and via enzymatic action by internal enzymes (Alencar *et al*., 2010).

Biodiesel must also meet the parameters of iodine value (IV), which indicates the degree of unsaturation of fuel, expressing it's intended to oxidize (Kumar *et al*., 2012). The rate of autoxidation of unsaturated fatty acid compounds is known to depend on the number and position of double bonds (Kumar *et al*., 2012). The iodine value (IV) of ADIITEC-III oil and FAME obtained by the AOCS method was recorded as 114.21 and 112.3 g $I_2$ $100^{-1}$ g respectively. The similar iodine value was recorded for the prepared

methyl ester as the parameter typically dependent on the origin of oil (Mazumdar *et al*., 2012). Moreover, the iodine value (IV) of GUBIOTJT116 oil and FAME was also recorded to be 114 and 112 g $I_2$ $100^{-1}$ g respectively. The calculated iodine value was well below the European allowed biodiesel standards 120 g $I_2$ $100^{-1}$ g (Damiani *et al*., 2010). Pertaining the fact, in comparison to seed oil, microalgae oil contains a high amount of polyunsaturated fatty acids (PUFAs) which correspond to the less oxidation stability of algal-derived biodiesel (Kumar *et al*., 2012). The extensive unsaturation degree corresponds to the higher IV value denoting higher number of double bonds in the biodiesel.

Also, the calorific value produced from ADIITEC-III FAME (40 MJ $Kg^{-1}$) was comparatively higher than its oil (35 MJ $Kg^{-1}$). Even in the case of GUBIOTJT116, methyl esters (41 MJ $Kg^{-1}$) showed higher calorific value than oil (38 MJ $Kg^{-1}$). The calorific value determines the suitability of biodiesel as it measures the energy produced while burning the fuel completely (Arias-Peñarands *et al*., 2013). However, an increase in lipid yield of microalgae also suggests an increase in the energy value (Talukdar *et al*., 2013). In fact, the lipid content of microalgae can be initiated during the cultivation period to incorporate with the fuel suitability.

Biodiesel is often associated with the poor low temperature flow properties, causing a challenge in operating engine at cold climatic regions. Therefore, the study of a low temperature flow properties of the fuel i.e. cloud point (CP), pour point (PP) and cold filter plugging point (CFPP) is important. In this study pour point of oil and methyl esters of ADIITEC-III and GUBIOTJT116 were estimated according to the ASTM standards and comparative results are tabulated in Table 4.6. As shown in typical DSC thermogram (Figure 4.18, 4.19, 4.20 and 4.21), pour point of the samples showed a sharp

increase in energy flow due to the exothermic nature of increasing reaction. The length of fatty acids chain and its interaction between the molecules is responsible for the crystallization of esters. In our study, pour point of ADIITEC-III and GUBIOTJT116 oil sample was found to be -12ºC and -13ºC respectively, which is much higher than the pour point of methyl esters (-7ºC) and (-12ºC). According to the reports branched chain esters have a lower crystallization temperature than that of straight chain esters (Borugadda *et al*., 2014). At higher temperatures, saturated fatty acids tends to crystallize as it possess significantly higher melting points than unsaturated fatty acid compounds (Knothe, 2005). Therefore no heat change is observed as these fatty acids are miscible with each other at room temperature. Mono- alkyl esters with higher concentrations of saturated fatty acids and lower concentrations of low melting point unsaturated fatty acids exhibits poor cold flow properties (Knothe, 2007). In fact, more the unsaturated fatty acids content in the oil better are the cold flow properties (Borugadda *et al*., 2014).

#### 4.2.5.3. Thermogravimetric analysis of oil and methyl esters (FAME) of *Chloromonas* (Chlorophyceae) species ADIITEC-III and *Scenedesmus* species GUBIOTJT116

Figure 4.22 and 4.23 shows the thermogravimetric (TGA) and differential thermogravimetric (DTG) profile of ADIITEC-III oil and FAME sample under nitrogen atmosphere at a heating rate of 10ºC $min^{-1}$. From the available literature it is observed that the heating rate of 10ºC $min^{-1}$ yield better information about the thermal behavior, hence same has been selected in the present study (Grierson *et al*., 2009) (Shuping *et al*., 2010). From TGA and DTG plot it was observed that the thermal degradation of both the sample takes place in three consecutive steps and was in agreement with the reported literature (Shuping *et al*., 2010) (Marcilla *et al*., 2009). The thermal stability of the

sample depends on the chemical structure and fatty acid composition. While comparing the thermal decomposition profile at 10ºC $min^{-1}$ it was observed that the onset temperature was 166ºC and 161ºC for ADIITEC-III oil and FAME sample respectively. On the other hand, the onset temperature of GUBIOTJT116 oil and methyl esters was recorded as 176ºC and 174ºC respectively.

The obtained results could be better explained from the weight loss profiles (Table 4.7) of oil and methyl ester samples of both the species. The ADIITEC-III oil sample showed 10% weight loss at 177ºC, while in case of methyl esters it was at 174ºC which includes moisture content and decomposition of mono and polyunsaturated fatty acids (oleic, linoleic and linolenic) (Borugadda *et al*., 2014). For GUBIOTJT116 oil and methyl ester, 10% weight loss was observed at 217ºC and 235ºC respectively. In the case of ADIITEC-III, all the components of oil which accounts for almost 50% weight loss were decomposed at 332ºC, whereas in the case of methyl ester weight loss was observed at 249ºC, it includes the breakdown of saturated fatty acids. However, 50% of weight loss was observed at the temperature range of 359ºC for both oil and methyl esters sample of GUBIOTJT116. Moreover, the components of ADIITEC-III oil which accounted for almost 90% weight loss were decomposed at around 498ºC and in the case of methyl esters weight loss was observed at 477ºC. Similar observation was also recorded with GUBIOTJT116 oil and methyl ester sample where 90% weight loss was observed at 501ºC and 482ºC respectively. All the studied samples were completely burnt out after being heated up to 800ºC. From the above discussion, it was clear that initiation and complete thermal degradation of the ADIITEC-III and GUBIOTJT116 FAME sample was within a temperature range inferior to the oil sample, which may be due to certain compositional features of fatty acids.

According to the reports, certain compositional features of fatty acid such as chain length, degree of unsaturation and branching of the chain influences the thermo-oxidative properties of the fatty ester molecule (Borugadda *et al*., 2014). Hence, based on weight loss profile, it could be concluded that thermal stability of the oil sample of both the species was superior to the methyl esters. Hence, the data obtained shows that ADIITEC-III and GUBIOTJT116 oil samples were thermally more stable than the synthesized methyl esters. The thermal stability is indeed a very significant factor to determine the storage condition of biodiesel and its life span (Borugadda *et al*., 2013).

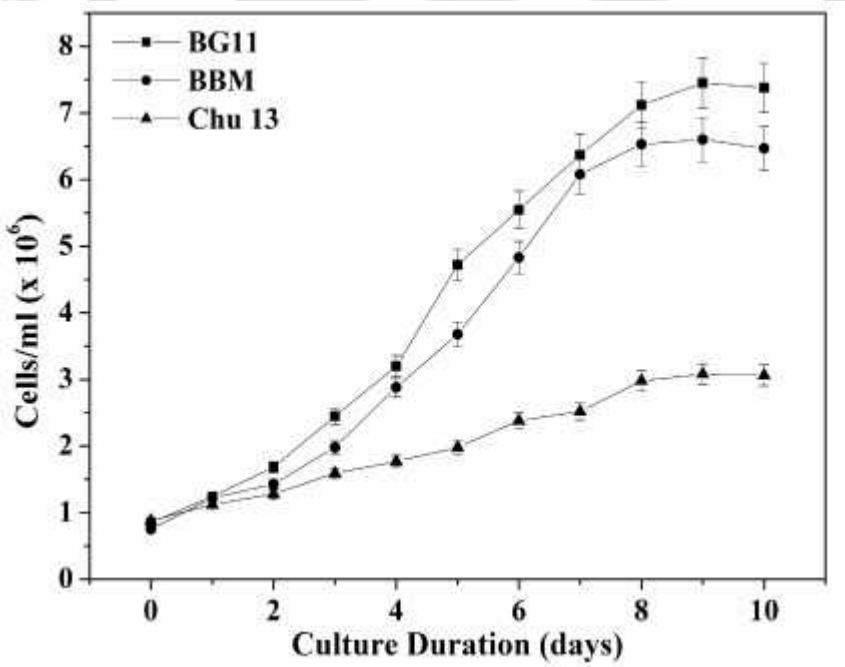


Figure 4.8 Growth curves of *Chloromonas* species ADIITEC-III in three media compositions (BG11, BBM and Chu 13)

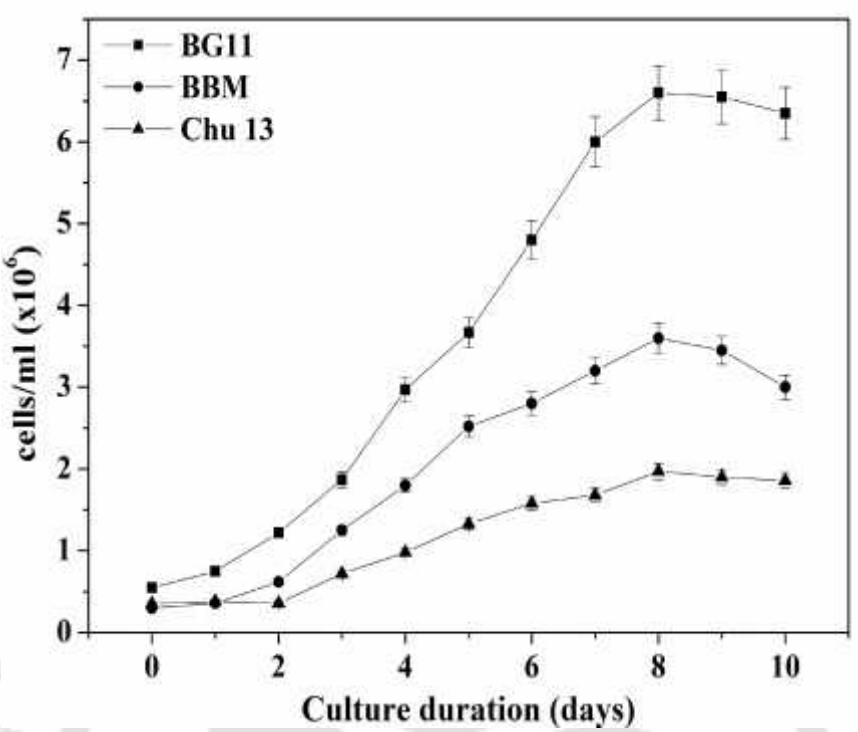


Figure 4.9 Growth curves of *Scenedesmus* species GUBIOTJT116 in three media compositions (BG11, BBM and Chu 13)

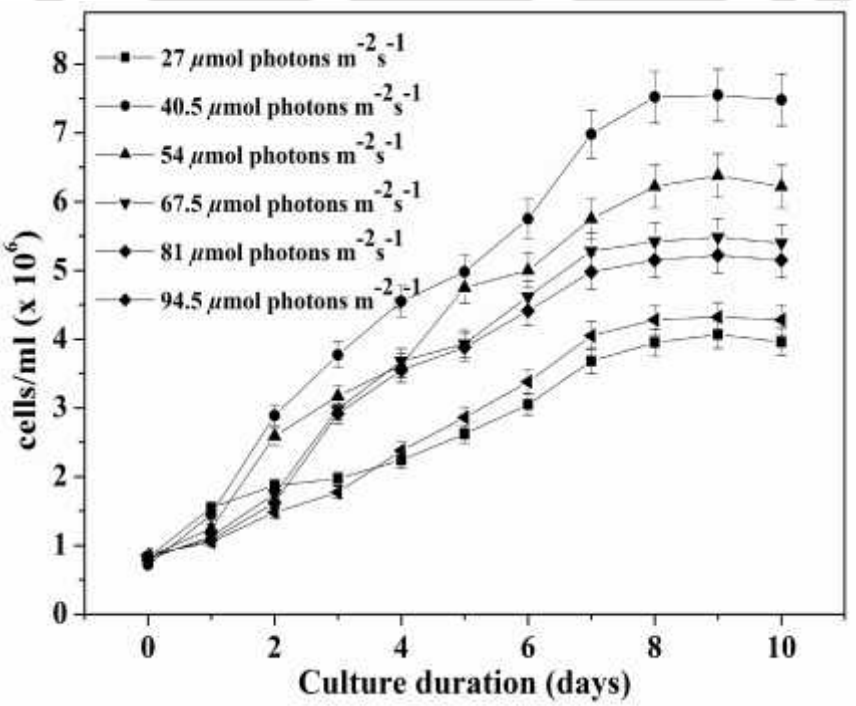


Figure 4.10 Growth curves of *Chloromonas* species ADIITEC-III in different light intensities (27, 40.5, 54, 67.5, 81 and 94.5 $\mu$mol $m^{-2}$ $s^{-1}$)

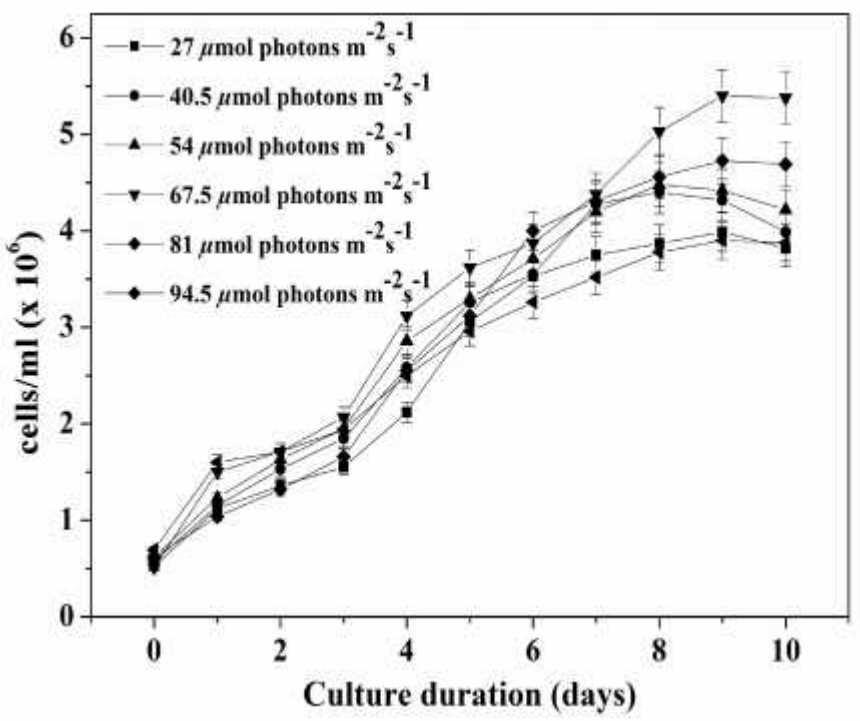


Figure 4.11 Growth curves of *Scenedesmus* species GUBIOTJT116 in different light intensities (27, 40.5, 54, 67.5, 81 and 94.5 $\mu$mol $m^{-2}$ $s^{-1}$)

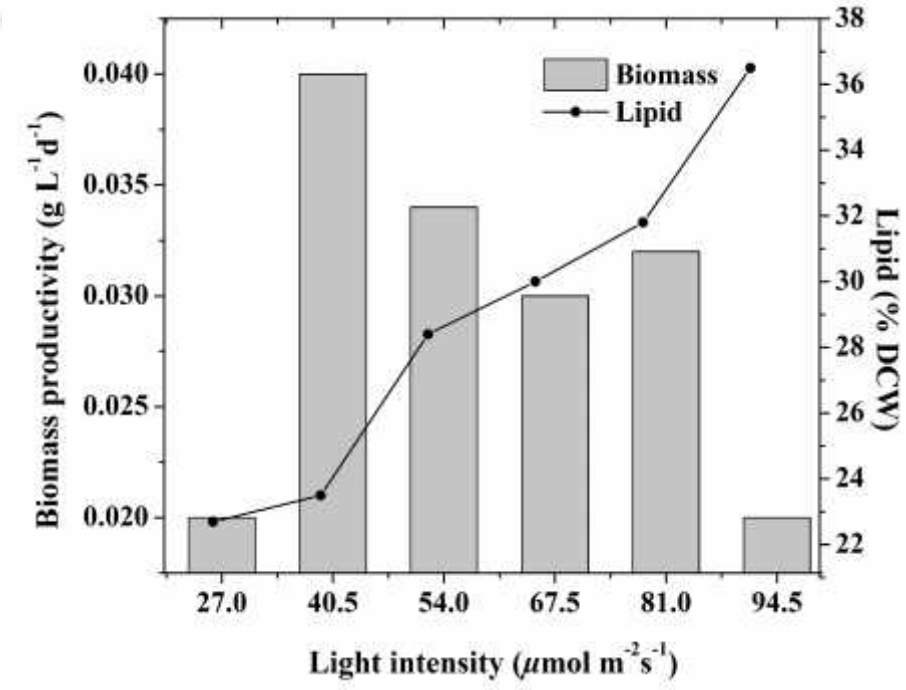


Figure 4.12 Relationship between biomass productivity and lipid content of *Chloromonas* species ADIITEC-III under the light intensity of 27, 40.5, 54, 67.5, 81, and 94.5 $\mu$mol $m^{-2}$ $s^{-1}$

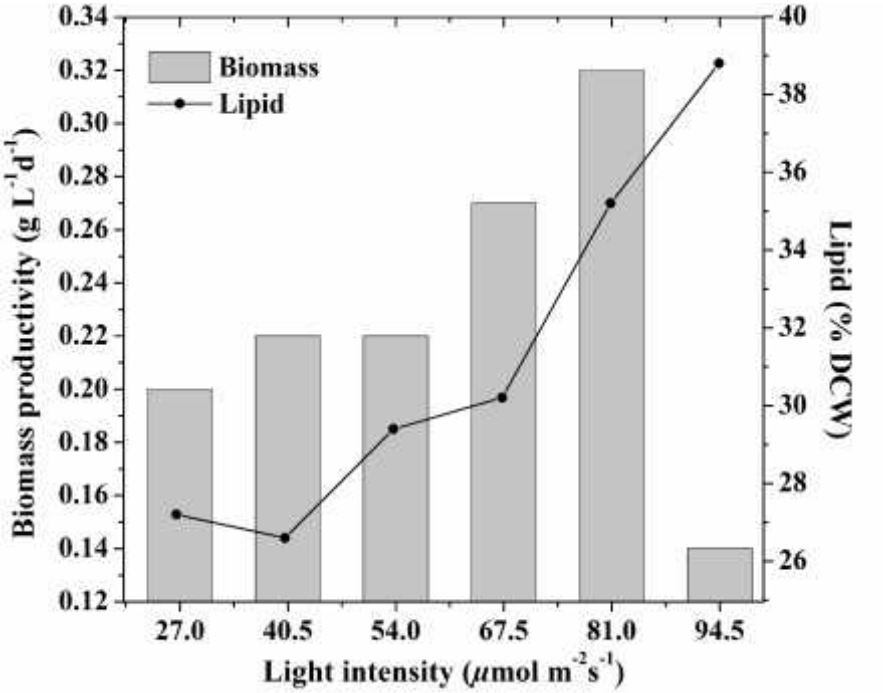


Figure 4.13 Relationship between biomass productivity and lipid content of *Scenedesmus* species GUBIOTJT116 under the light intensity of 27, 40.5, 54, 67.5, 81, and 94.5 μmol $m^{-2}$ $s^{-1}$

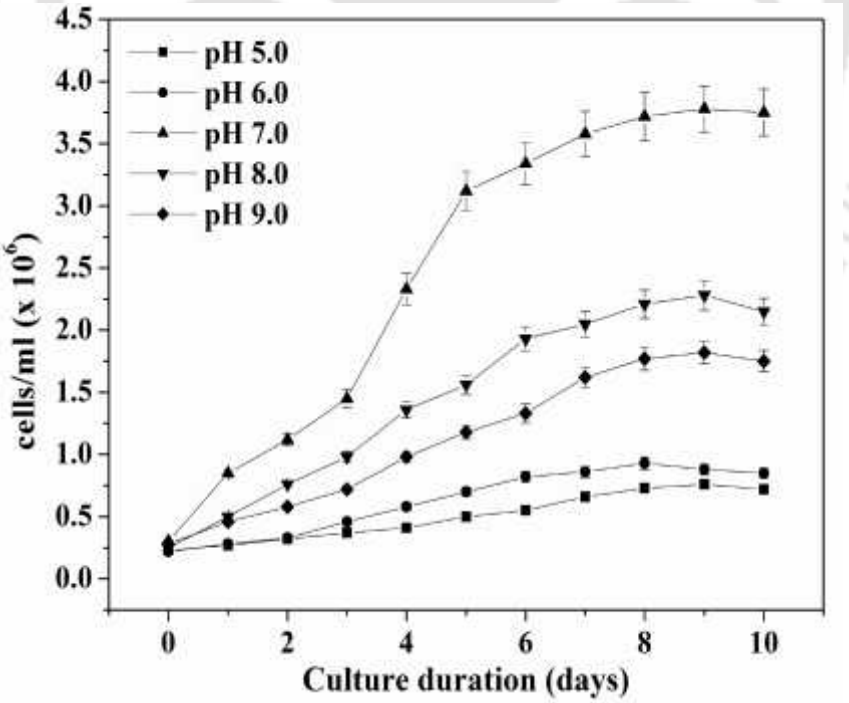


Figure 4.14 Growth curves of ADIITEC-III in different pH conditions (pH 5, 6, 7, 8 and 9)

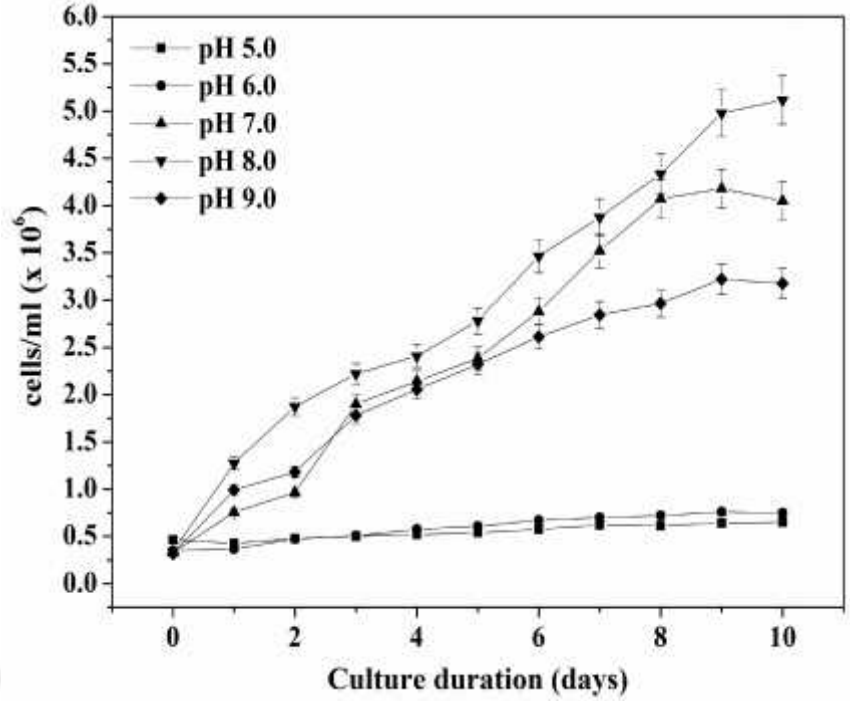


Figure 4.15 Growth curves of GUBIOTJT116 in different pH conditions (pH 5, 6, 7, 8 and 9)

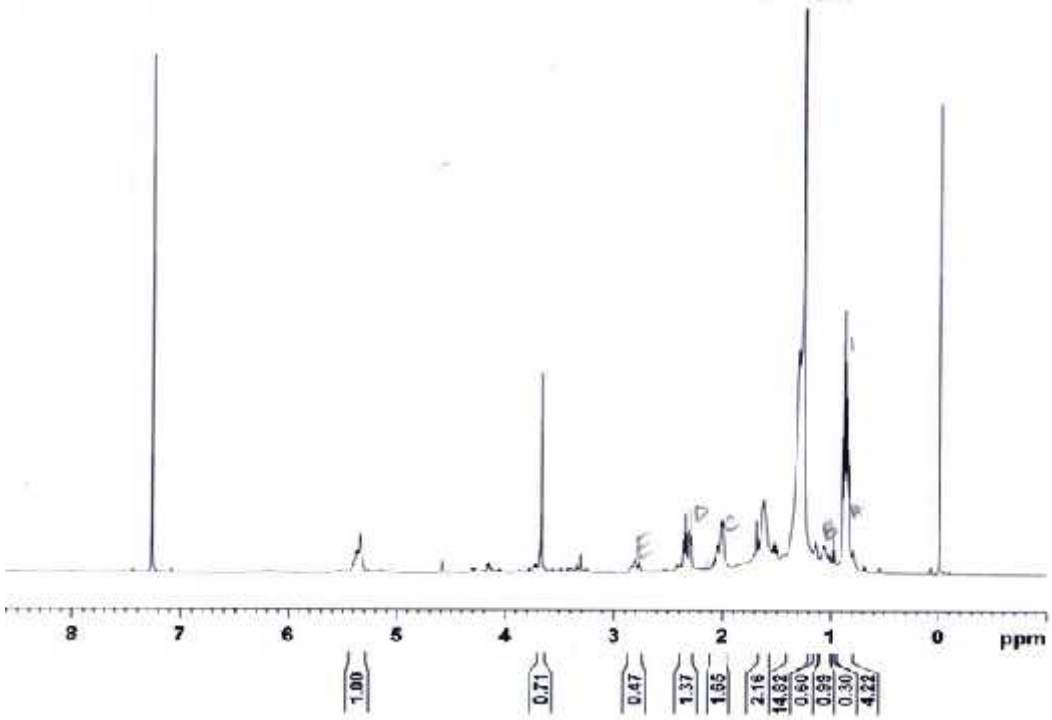


Figure 4.16 $^{1}$H NMR spectrum of methyl esters (FAME) of *Chloromonas* species ADIITEC-III, the strong singlet peak at 3.65 ppm is indicative of due to oxymethylic hydrogen referent to methyl esters

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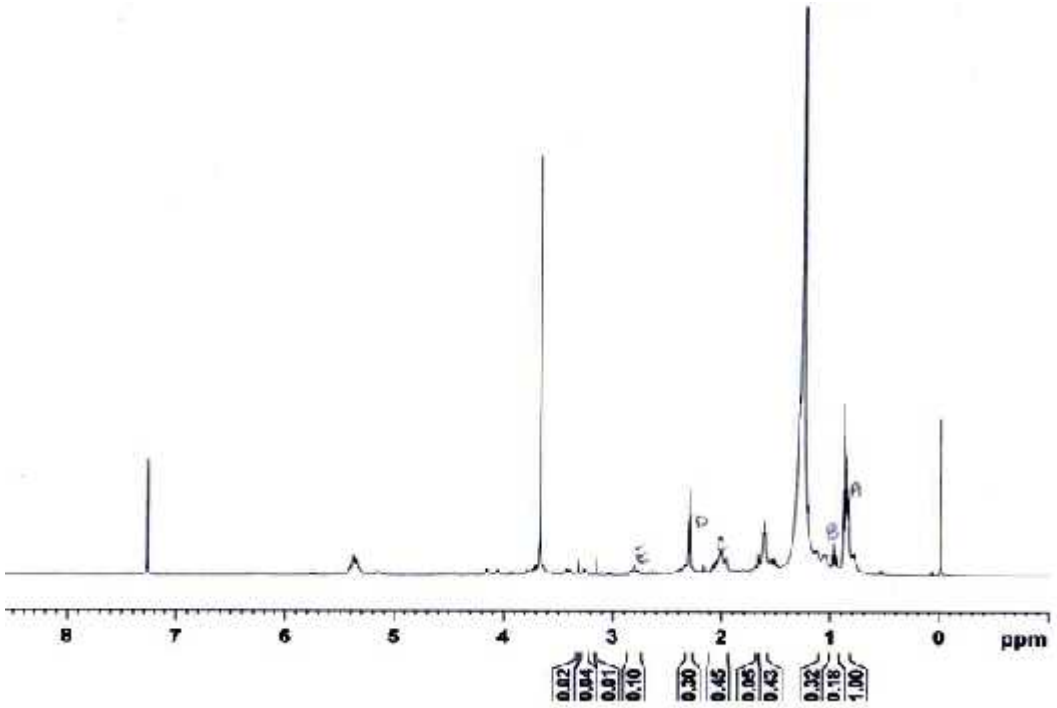


Figure 4.17 $^{1}H$ NMR spectrum of methyl esters (FAME) of *Scenedesmus* species GUBIOTJT116, the strong singlet peak at 3.65 ppm is indicative of due to oxymethylic hydrogen referent to methyl esters

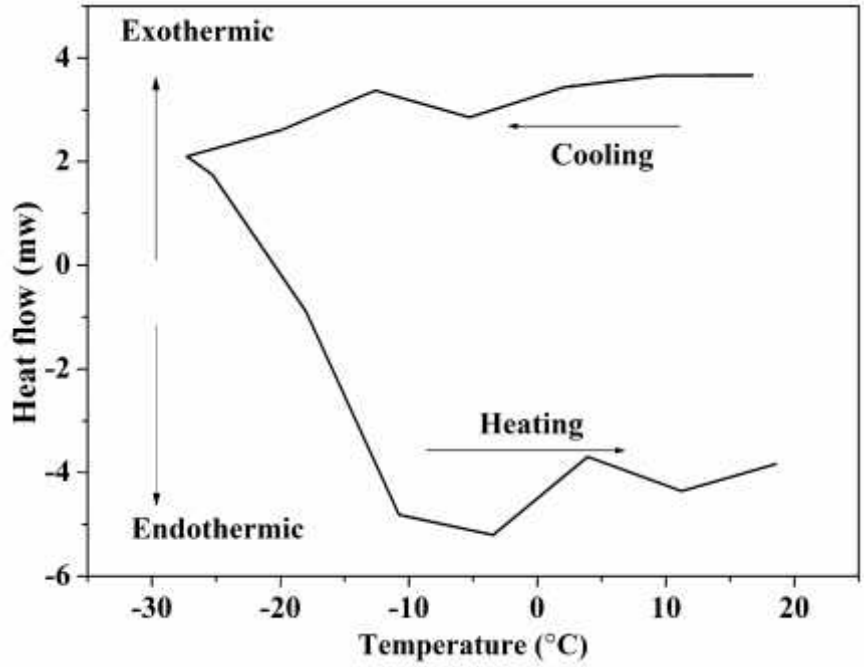


Figure 4.18 DSC thermogram of ADIITEC-III oil @ 5°C $min^{-1}$ under $N_2$ atmosphere

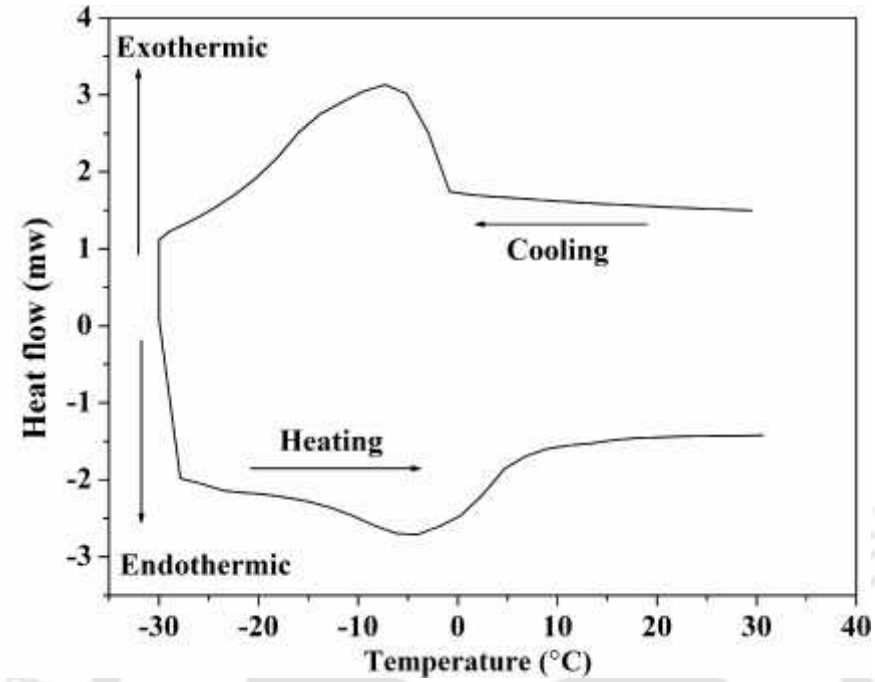


Figure 4.19 DSC thermogram of ADIITEC-III FAME @ 5°C $min^{-1}$ under $N_2$ atmosphere

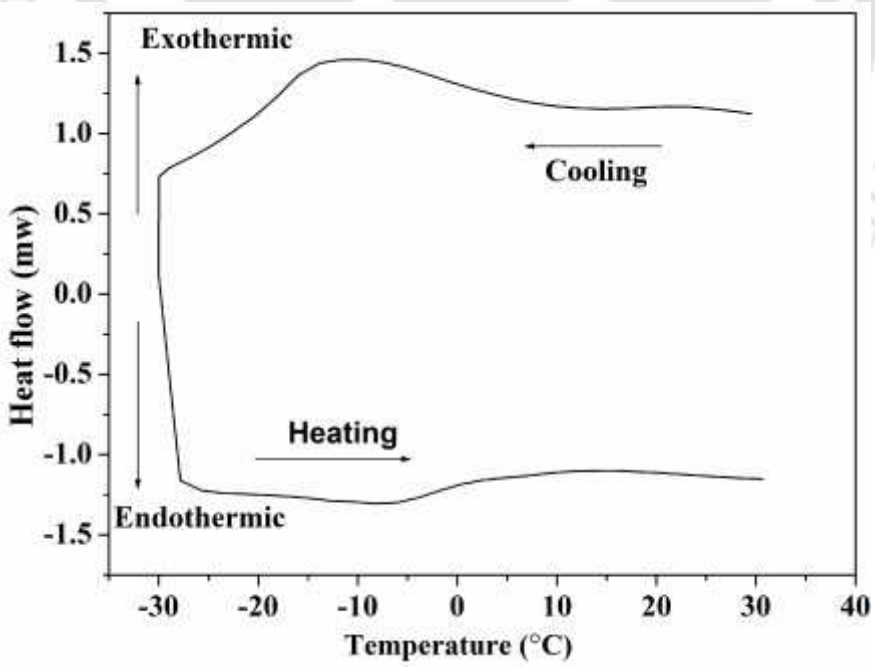


Figure 4.20 DSC thermogram of GUBIOTJT116 oil @ 5°C $min^{-1}$ under $N_2$ atmosphere

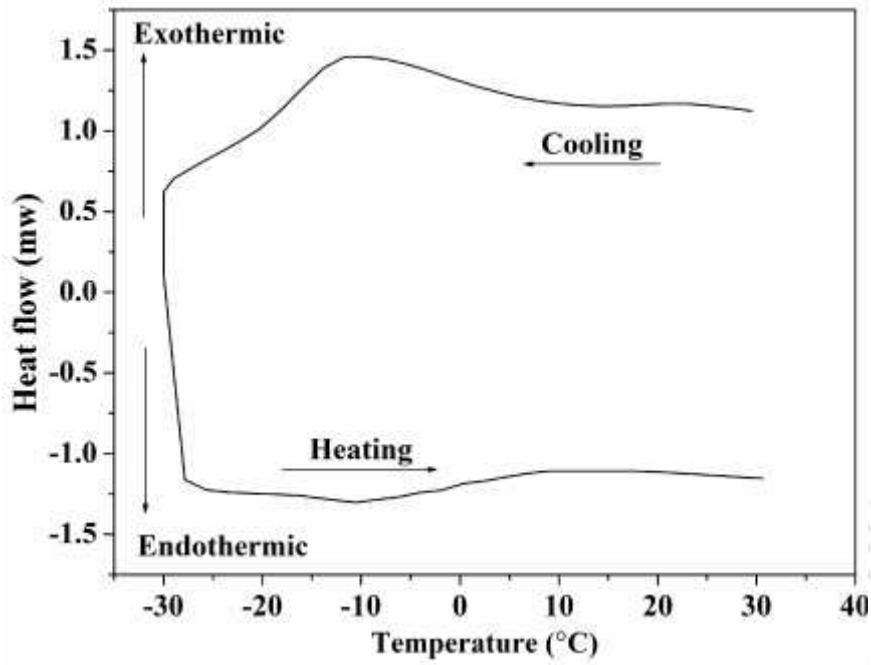


Figure 4.21 DSC thermogram of GUBIOTJT116 FAME @ 5°C $min^{-1}$ under $N_2$ atmosphere

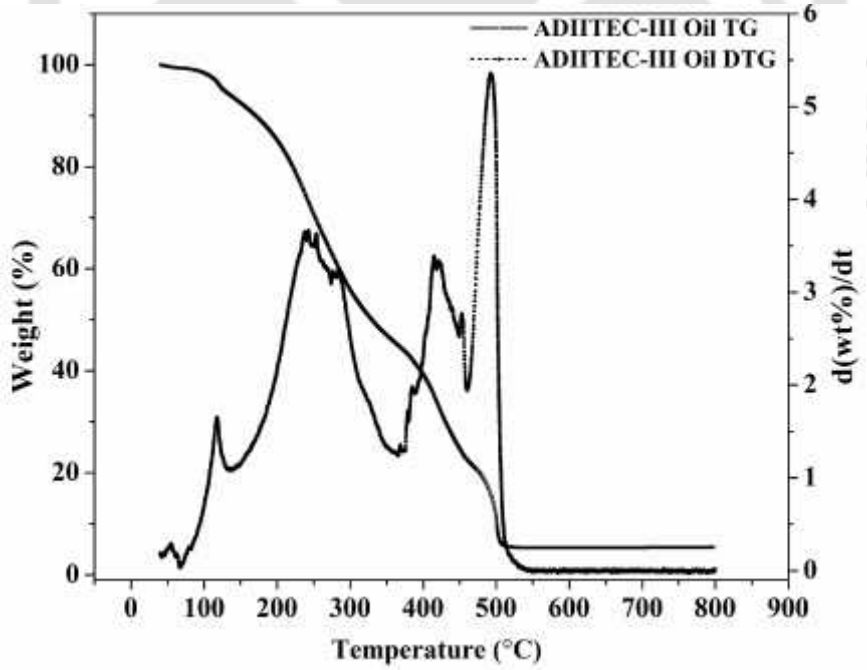


Figure 4.22 TG and DTG curves of ADIITEC-III oil @ 10°C $min^{-1}$ under $N_2$ atmosphere

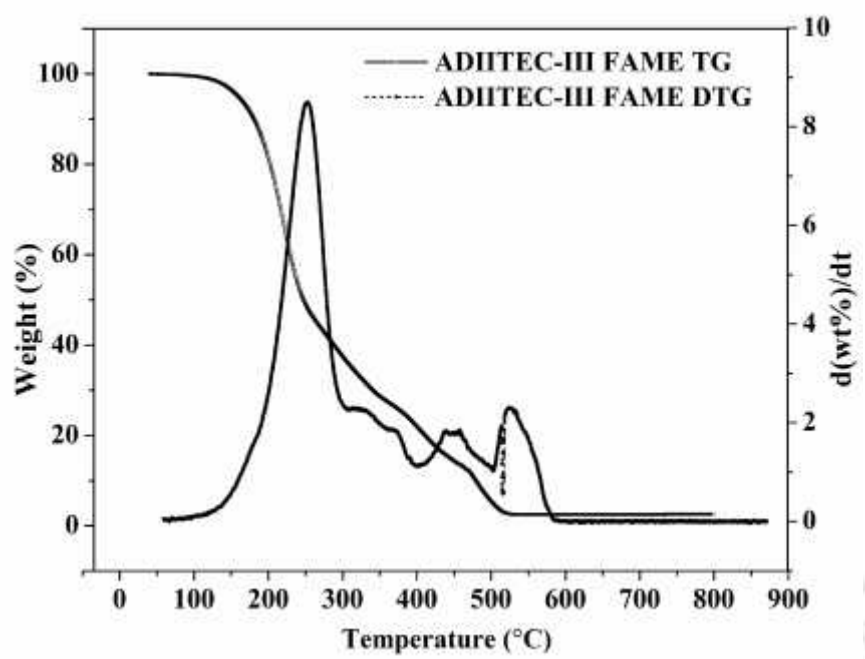


Figure 4.23 TG and DTG curves of ADIITEC-III FAME @ 10°C $min^{-1}$ under $N_2$ atmosphere

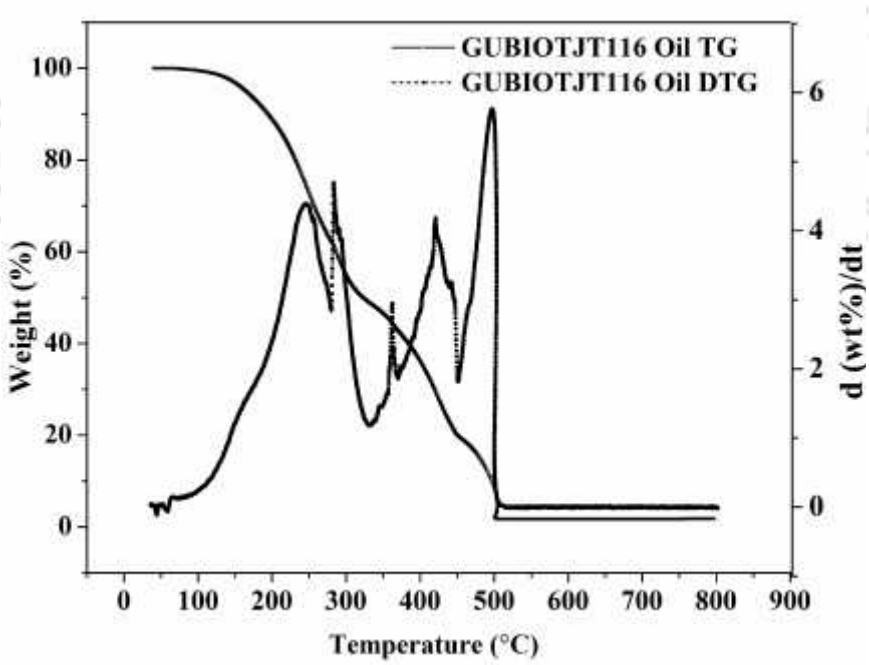


Figure 4.24 TG and DTG curves of GUBIOTJT116 oil @ 10°C $min^{-1}$ under $N_2$ atmosphere

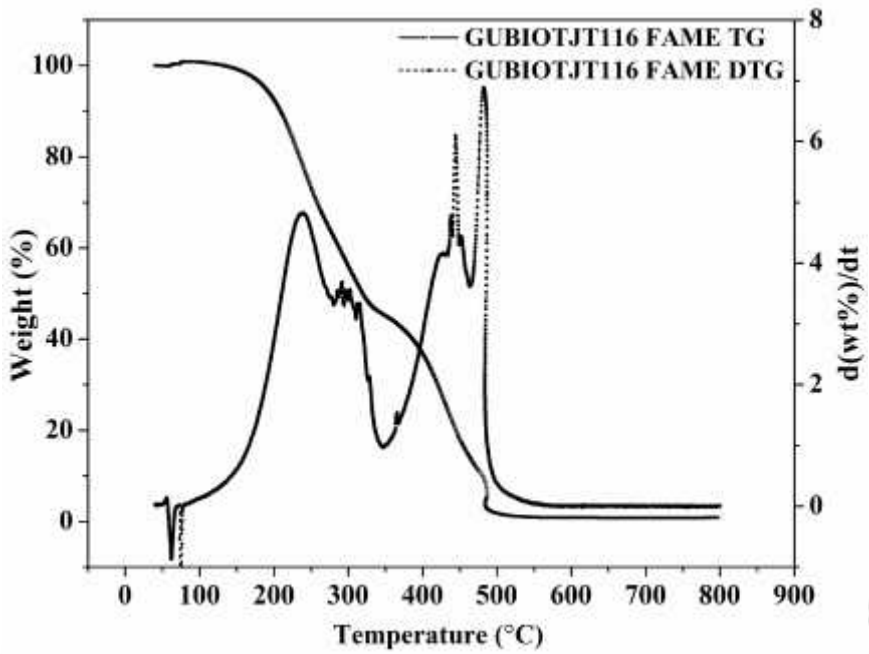


Figure 4.25 TG and DTG curves of GUBIOTJT116 FAME @ 10°C $min^{-1}$ under $N_2$ atmosphere

Table 4.3 Growth characteristics, biomass and total lipid content of *Chloromonas* species ADIITEC-III under different light intensities

| Light intensity ($\mu$mol $m^{-2}s^{-1}$) | Specific growth rate $\mu$, ($day^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity (g $L^{-1}d^{-1}$) | Total lipid content (% DCW) |
|---|---|---|---|---|
| 27 | 0.17 ± 0.02 | 4.11 ± 0.48 | 0.02 ± 0.011 | 22.7 ± 1.61 |
| 40.5 | 0.23 ± 0.017 | 3.02 ± 0.23 | 0.04 ± 0.015 | 23.5 ± 0.95 |
| 54 | 0.2 ± 0.026 | 3.5 ± 0.43 | 0.034 ± 0.01 | 28.4 ± 1.55 |
| 67.5 | 0.19 ± 0.02 | 3.81 ± 0.40 | 0.03 ± 0.01 | 30 ± 1.1 |
| 81 | 0.18 ± 0.02 | 3.88 ± 0.43 | 0.032 ± 0.01 | 31.8 ± 0.9 |
| 94.5 | 0.16 ± 0.02 | 4.37 ± 0.55 | 0.02 ± 0.015 | 36.5 ± 0.75 |

Table 4.4 Growth characteristics, biomass and total lipid content of *Scenedesmus* species (GUBIOTJT116) under different light intensities

| Light intensity ($\mu$mol $m^{-2}s^{-1}$) | Specific growth rate $\mu$, ($day^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity (g $L^{-1}d^{-1}$) | Total lipid content (% DCW) |
|---|---|---|---|---|
| 27 | 0.16 ± 0.01 | 3.8 ± 0.18 | 0.2 ± 0.01 | 27.2 ± 0.7 |
| 40.5 | 0.17 ± 0.002 | 3.8 ± 0.04 | 0.22 ± 0.02 | 26.6 ± 0.8 |
| 54 | 0.17 ± 0.002 | 3.8 ± 0.04 | 0.22 ± 0.02 | 29.4 ± 0.8 |
| 67.5 | 0.2 ± 0.02 | 3.0 ± 0.37 | 0.27 ± 0.02 | 30.2 ± 0.72 |
| 81 | 0.25 ± 0.01 | 2.56 ± 0.13 | 0.32 ± 0.02 | 35.2 ± 0.72 |
| 94.5 | 0.1 ± 0.003 | 5.77 ± 0.13 | 0.14 ± 0.02 | 38.8 ± 0.8 |

Table 4.5 Growth characteristics and biomass production of *Chloromonas* species ADIITEC-III and *Scenedesmus* species (GUBIOTJT116) under different pH conditions

| pH | *Chloromonas* species ADIITEC-III | | | *Scenedesmus* species (GUBIOTJT116) | | |
|---|---|---|---|---|---|---|
| | Specific growth rate $\mu$, ($d^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity (g $L^{-1}$ $d^{-1}$) | Specific growth rate $\mu$, ($d^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity (g $L^{-1}$ $d^{-1}$) |
| 5 | 0.08 ± 0.01 | 8.02 ± 0.55 | 0.02 ± 0.015 | 0.04 ±0.003 | 13.8 ± 0.64 | 0.08 ± 0.02 |
| 6 | 0.12 ± 0.03 | 5.8 ± 0.48 | 0.04 ± 0.011 | 0.05 ±0.004 | 7.7 ± 0.25 | 0.1 ± 0.01 |
| 7 | 0.25 ± 0.03 | 2.7 ± 0.16 | 0.15 ± 0.01 | 0.2 ±0.001 | 2.8 ± 0.005 | 0.23 ± 0.02 |
| 8 | 0.21 ± 0.02 | 3.36 ± 0.25 | 0.11 ± 0.02 | 0.15 ±0.001 | 4.07 ± 0.03 | 0.21 ± 0.02 |
| 9 | 0.18 ± 0.03 | 3.72 ± 0.31 | 0.08 ± 0.015 | 0.13 ±0.001 | 4.6 ± 0.04 | 0.2 ± 0.02 |

Table 4.6 Physico chemical properties of oil and methyl ester compositions of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116

| Properties | *Chloromonas* species ADIITEC-III | | *Scenedesmus* species GUBIOTJT116 | | ASTM D-6751 limits |
|---|---|---|---|---|---|
| | Oil | FAME | Oil | FAME | |
| Acid value mg KOH $g^{-1}$ | 14.02 | 0.48 | 14 | 0.52 | 0.50 max |
| Iodine value g $I_2100^{-1}$g oil | 114.21 | 112.3 | 114 | 112g | 120 max |
| DSC (pour point) | -12ºC | -7ºC | -13ºC | -12ºC | -15 to 10 |
| Calorific value MJ $Kg^{-1}$ | 35 | 40 | 38 | 41 | - |
| Fatty acid compositions of ADIITEC-III and GUBIOTJT116 | | | | | |
| Fatty acid profile | ADIITEC-III FAME | | GUBIOTJT116 FAME | | ASTM D-6751 limits |
| Linolenic acid (18:3) | 6.63 % | | 13.79 % | | - |
| Linoleic acid (18:2) | 21.04 % | | 1.83 % | | - |
| Oleic acid (18:1) | 32.54 % | | 48.1 % | | - |
| Total saturates | 39.79 % | | 36.29 % | | - |

Table 4.7 Temperature characteristics for the thermogravimetric analysis of oil and methyl esters of *Chloromonas* sp and *Scenedesmus* species grown in ammonium nitrate and urea

| Microalgae | Onset temperature (ºC) | Mass loss (%) | | |
|---|---|---|---|---|
| | | 10% | 50% | 90% |
| ADIITEC-III oil | 166 | 177ºC | 332ºC | 498ºC |
| ADIITEC-III FAME | 161 | 174ºC | 249ºC | 477ºC |
| GUBIOTJT116 oil | 176 | 217ºC | 359ºC | 501ºC |
| GUBIOTJT116 FAME | 174 | 235ºC | 359ºC | 482ºC |

## 4.3. Determination of growth, biomass and the lipid yield of selected microalgae strains under different nitrogen sources and characterization of derived methyl esters

The mass cultivation of microalgae culture in terms of biofuel production depends mainly on the biomass and lipid productivity. These two factors are the key parameters affecting the economic feasibility of the biodiesel production (Wu *et al*., 2013). Studies conducted in the previous chapter have demonstrated that biomass and lipid content could be increased by different cultivation conditions such as light intensity and pH condition. Moreover, the reports also suggested enhanced lipid yield via other cultivation conditions such as nitrogen deprivation, low temperature, high salt concentration and high iron concentration (Hsieh *et al*., 2009). Among these factors, nitrogen plays an important role in microalgae lipids and fatty acid metabolism. In addition, nitrogen is considered to be easy to manipulate and are cheaper in compare to other factors (Takagi *et al.*, 2000). Microalgae lipid can be enhanced during the cultivation by incorporating

various nitrogen sources such as ammonia, nitrate, nitrite and urea (Becker, 1994). Also, the reports suggested, the use of urea is more feasible than the other nitrogen sources in terms of cost for large scale microalgae cultivation (Danesi *et al*., 2002; Matsudo *et al*., 2009).

In this study, the effect of variation of nitrogen sources on the cultivation of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 to enhance the lipid content have been summarized. Four different nitrogen sources (i.e ammonium nitrate ($NH_4NO_3$), potassium nitrate ($KNO_3$), urea (U) and sodium nitrate ($NaNO_3$) were employed for cell growth and lipid accumulation in batch mode. Based on the data obtained from the previous chapter, BG11 medium was selected for these experiments. The sole nitrogen source in BG11 medium is sodium nitrate which is considered as a control condition to compare the other nitrogen sources used in the study. Section 3.4.6; demonstrate the detailed methodology for culture preparation with alternate nitrogen sources. Thereafter, the FTIR spectrum of the oil sample of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 obtained under different nitrogen sources were investigated in detailed using the chemometric techniques of discriminate analysis and multivariate calibrations. The technique was basically used to comprehend the large and complex data set of vibrational frequencies (wave numbers) in a simplified manner. It helps to short-list the optimum nitrogen source showing a significant effect on maximum lipid yield. Subsequently, the prepared methyl ester was also further characterized for their physico-chemical properties.

### 4.3.1. Effect of alternate nitrogen sources on growth, biomass and lipid production of ADIITEC-III and GUBIOTJT116

Under the stated growth conditions, *Chloromonas* species ADIITEC-III and GUBIOTJT116 was cultivated over 10 days to determine the appropriate nitrogen source in terms of growth, biomass and lipid production. The growth curve as shown in figure 4.26 and 4.27 for four different nitrogen sources, revealed an initial lag phase from the day of inoculation. Both the species, supplemented with $KNO_3$ and $NaNO_3$ showed (Table 4.8 and 4.9) almost similar trend of growth with the maximum specific growth rate ($\mu$max) and biomass production under the similar growth condition. The biomass production of ADIITEC-III and GUBIOTJT116 was significantly higher in $NaNO_3$ than the other nitrogen sources i.e. 0.04 ± 0.01 and 0.27 ± 0.015 g $L^{-1}$ respectively at the terminal day (10 days). In contrast, ADIITEC-III and GUBIOTJT116 supplemented with $NH_4NO_3$ were not much responsive for algal growth showing specific growth rates of 0.17 ± 0.015 $d^{-1}$ and 0.15 ± 0.02 $d^{-1}$ respectively. And these growth rates were invariably similar to that of culture cultivated in urea (U). Beside their slow growth in $NH_4NO_3$, *Chloromonas* species ADIITEC-III showed a high accumulation of lipid yield i.e. 35.86 ± 2.3% in comparison to other nitrogen sources (Figure 4.26). But, GUBIOTJT116 cultured with urea showed better lipid yield (37.2 ± 0.36%) followed by ammonium nitrate 35.5 ± 0.7% (Figure 4.27). The findings clearly indicated that the nitrogen in the nitrate forms ($KNO_3$ and $NaNO_3$) favored the algal growth over $NH_4NO_3$ and urea. The result obtained in this study with GUBIOTJT116 agrees well with the reported data on *Neochloris oleoabundans* (Li *et al*., 2008). As per their study, $NaNO_3$ favors cell growth and accumulates higher lipid than urea and does not support the growth in medium with ammonium as the nitrogen source. However, our study on both the species showed contradictory results in terms of lipid recovery from urea. Infact, the culture fed with

urea accumulates more lipid yield than $NaNO_3$. Moreover, it is also reported that the nitrate and ammonium uptake interaction inhibits the microalgae cells in nitrate uptake, as the product obtained through ammonium assimilation causes a rapid and reversible inactivation of nitrate transport (Flynn, 1991). Several studies on nitrogen supplementation for algal growth, biomass and lipid production has been reported in many species such as *Porphyridium purpureum* (Becker, 1994), *Scenedesmus dimorphous* (Benider *et al*., 2001), *Tetraselmis suecia*, *Skeletonema costatum* and *Thalassiosira pseudonana* (Arumugam *et al*., 2003). The study also supports that the nitrogen source supplied in any form promotes the growth and lipid accumulation in microalgae.

### 4.3.2. Characterization and evaluation of synthesized methyl esters

From the results it was observed that both the isolates grown in potassium nitrate ($KNO_3$) and sodium nitrate ($NaNO_3$) showed better growth response. Among the studied nitrogen sources, urea (U) enhances the lipid yield of GUBIOTJT116. Whereas, the lipid content of ADIITEC-III was increased when supplemented with ammonium nitrate ($NH_4NO_3$). Therefore, based on high lipid yield, the oil recovered from the isolates was transesterified into methyl esters (FAME). Subsequently, the synthesized methyl esters are characterized to understand the effect of nitrogen sources on fuel properties.

#### 4.3.2.1. Fatty acid profile of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116

The fatty acid composition retrieved from both the species grown in nitrogen source which enhances the lipid yield is listed in Table 4.10. Among the different nitrogen sources, urea (U) and ammonium nitrate ($NH_4NO_3$) showed enhancement in the lipid

yield of GUBIOTJT116 and ADIITEC-III respectively. The derived methyl esters showed abundance of monounsaturated oleic (C18:1), linolenic acid (C18:3) and linoleic (C18:2) and saturated fatty acids. In this study, the fatty acid composition of the derived methyl esters was estimated by using $^{1}H$ NMR spectrometer technique (Figure 4.28 and 4.29). The results of this compositional analysis are summarized in Table 4.10. From the fatty acid profile it was observed that oleic acid was the predominant fatty acid in *Scenedesmus* species (56.92%) followed by saturated fatty acid (25%), linolenic acid (15.25%) and linoleic acid (2.83%). Similarly, oleic acid was predominant in *Chloromonas* species (47.03%) followed by saturated fatty acid (36.9%), linoleic acid (8.84%) and linolenic acid (7.23%). Altogether 63.1% of the fatty acids in *Chloromonas* species were unsaturated fatty acids, while in case of *Scenedesmus* species unsaturated fatty acid percentage was recorded as 75%. Therefore, the content of C18 fatty acids in both the microalgae species was significantly higher than that of many other reported algae, such as *S. rubescens* (Lin *et al*., 2011), *Monodus subterraneus* (Khozin-Goldberg and Cohen, 2006), *Haematococcus pluvialis* (Damiani *et al*., 2010) and *Chlorella vulgaris* (Converti *et al*., 2009). It has been reported that, fatty acids composition of some micro-algae could be manipulated by altering the cultivation conditions (Damiani *et al*., 2010). In this study, comparison of the methyl esters of both the microalgae species cultured in BG11 ($NaNO_3$) media with their respective high lipid yielding nitrogen sources, showed a significant change in their composition of saturated fatty acid and C18 series content (as % of total FAME). In *Chloromonas* species, the culture grown in ammonium nitrate ($NH_4NO_3$) showed high increment of 14.49% in oleic acid and a slight increase in linolenic acid (0.6%) compared to BG11 ($NaNO_3$) media. However, the content (%) of linoleic and saturated fatty acid was found to decrease in culture with

ammonium nitrate ($NH_4NO_3$). Similarly, in case of *Scenedesmus* species significant rise in oleic (8.82%) and linolenic acid (1.46%) content was recorded. The total saturated fatty acid was observed to decrease up to (11.29%) and increment of 1% in linoleic acid was recorded in *Chloromonas* species. Therefore it can be said that the use of different nitrogen sources significantly influences the total content of saturated fatty acid and C18 series of *Chloromonas* and *Scenedesmus* species which is the main fatty acids composition in biodiesel.

#### 4.3.2.2. Physico-chemical characteristics of methyl esters (FAME) produced from *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 grown under optimized nitrogen source

The biodiesel produced from the oil obtained from *Chloromonas* and *Scenedesmus* species under optimized nitrogen source was characterized for the fuel properties such as acid value, iodine value, calorific value and cold flow property. Predicted properties are summarized in Table 4.10, and compared with American Standards for Testing Materials ASTM 6751.

Acid value is considered as important characteristics of biodiesel. It determines the amount of free fatty acids present in a sample. As per the ASTM standard the maximum acid value suggested for the biodiesel is 0.50 mg KOH $g^{-1}$. The acid value of FAME sample of *Chloromonas* species at optimized nitrogen source (ammonium nitrate) was found to be 0.48 mg KOH $g^{-1}$ which agrees well with the ASTM standard. Similarly, the acid value of *Scenedesmus* species grown in urea and sodium nitrate ($NaNO_3$) was also observed in the same range i.e. 0.50 mg KOH $g^{-1}$. The iodine value (IV) determines the degree of unsaturation which influences fuel oxidation (Lang *et al*., 2001). According

to ASTM D-6751 limits, biodiesel fuel should have an iodine value less than 120 g $I_2$ $100^{-1}$ g oil.

The iodine value of the methyl ester of *Chloromonas* species grown in ammonium nitrate and sodium nitrate was found to be similar i.e. 112.3 g $I_2$ $100^{-1}$ g. However, *Scenedesmus* species grown under urea showed slightly higher iodine value 114 g $I_2$ $100^{-1}$ g than in the BG11 medium ($NaNO_3$). The rate of oxidation of unsaturated fatty acid compounds depends on the number of double bond in the fatty acid chain (Kumar *et al*., 2012). Therefore, while heating the unsaturated fatty acid, the polymerization of triglycerides might lead to the gum formation (Lang *et al*., 2001). Also, the calorific value is an important property of biodiesel that determines its suitability. The methyl ester of *Chloromonas* species grown in ammonium nitrate showed a calorific value of 40 MJ $Kg^{-1}$. On the other hand, *Scenedesmus* species grown in urea showed a higher calorific value of 42 MJ $Kg^{-1}$ than sodium nitrate (40 MJ $Kg^{-1}$).

Cold flow properties are determined to specify the usability of fuel oil in cold temperature (Lang *et al*., 2001). In this study, pour point of methyl esters of ADIITEC-III and GUBIOTJT116 in ammonium nitrate and urea respectively were estimated according to ASTM standards and comparative results are tabulated in Table 4.10. Pour point values were recorded according to their respective DSC thermogram (Figure 4.30 and 4.31). Biodiesel is a mixture of fatty acid esters (usually FAMEs) with a different carbon chain length and degree of saturation (Borugadda *et al*., 2014). The carbon chain length of fatty acid and their interaction between the molecules are responsible for crystallization of esters (Rodrigues *et al*., 2006). The crystallization in a saturated fatty acid ester takes place at higher temperature due to their high melting points. Also it has been stated that, lower crystallization temperature was observed in branched chain esters

than that of straight chain esters (Knothe, 2005). Therefore, more the unsaturated fatty acids content better the cold flow properties of sample (Borugadda *et al*., 2014). In our study, the pour point value of methyl esters of *Chloromonas* in ammonium nitrate was same as that of sodium nitrate (-7°C). However, *Scenedesmus* species in urea showed better pour point values (-13°C) than sodium nitrate (-12°C). This pour point values could be well explained from the fatty acid compositions of both the species as tabulated in Table 4.10. The *Scenedesmus* species in urea showed better pour point values that may be because of higher unsaturation content (75%) as compared to sodium nitrate (63.72%). Whereas, pour point values for *Chloromonas* species in both ammonium nitrate and sodium nitrate are similar as there is no much variation in their fatty acid composition, mainly unsaturation content.

#### 4.3.2.3. Thermogravimetric analysis of methyl esters (FAME) recovered from *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 grown under optimized nitrogen source

The thermal stability of prepared methyl ester samples of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 grown under optimized nitrogen source were determined by Thermogravimetric (TG) analysis. Based on available literature, thermal behavior is best understood with a heating rate of 10ºC $min^{-1}$, hence same has been selected in the present study (Grierson *et al*., 2009; Shuping *et al*., 2010). The onset temperature of decomposition under nitrogen atmosphere determines the thermal stability of the sample (Borugadda *et al*., 2014). The TGA and DTG plot at a constant heating rate of 10ºC $min^{-1}$ under nitrogen atmosphere are shown in Figure 4.32 ad 4.33. The plot shows that the thermal degradation of the sample takes place in three consecutive steps, and was in agreement with the reported literature (Marcilla *et al*.,

2009; Shuping *et al*., 2010). These degradation steps occur because the thermal stability of esters is dependent on their chemical structure and fatty acid composition. In particular, chain length, a degree of unsaturation and branching of the chain influences the thermo-oxidative properties of fatty acid ester. Thereby, the samples with high saturated fatty acid content are thermally more stable than the unsaturated ones (Borugadda *et al*., 2014). During the thermal decomposition, the oxygenated hydrocarbons of methyl esters break down into a volatile lower molecular hydrocarbon, carbon dioxide and carbon monoxide.

The onset temperature recorded for methyl esters of *Chloromonas* species in ammonium nitrate and *Scenedesmus* species in urea are 112ºC and 163ºC respectively. The weight loss profiles of both the species are summarized in Table 4.11. The FAME sample of *Chloromonas* species in ammonium nitrate showed 10% weight loss at 153ºC, while in case of *Scenedesmus* species it was at around 195ºC, which includes moisture content and decomposition of mono and polyunsaturated fatty acids (Borugadda *et al*., 2014). Almost 50% weight loss of *Chloromonas* species was recorded at 324ºC, whereas in case of *Scenedesmus* species weight loss was observed at 345ºC; it includes the breakdown of saturated fatty acids. Moreover, the methyl esters of *Chloromonas* species which accounted for almost 90% weight loss at around 475ºC and in case of *Scenedesmus* species the weight loss was observed at 480ºC.

The result obtained with the methyl esters of both the strains in sodium nitrate condition was compared in Table 4.11. The data revealed that initiation and completion of the thermal degradation of both the species in their optimized nitrogen sources was within a temperature range inferior to the sodium nitrate condition. This may be due to higher polyunsaturated fatty acids (Table 4.10) content in both the species in their

optimized nitrogen sources (ammonium nitrate and urea) compared to sodium nitrate. The high polyunsaturation content in the fatty acid composition of the sample lowers its thermal stability. This is due to the lower boiling point of unsaturated fatty acids compared to the saturated fatty acid (Park *et al*., 2008). Hence, it could be concluded that the *Chloromonas* species and *Scenedesmus* species grown in sodium nitrate were thermally more stable than their optimized nitrogen condition in terms of lipid yield.

#### 4.3.3.1. FTIR analysis of oil sample of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 grown under optimized nitrogen source

Fourier transform infrared (FTIR) spectroscopy is considered as an effective tool for characterization of algal biomass and lipid screening due to its non-destructive and rapid nature (Talukdar *et al*., 2014). Figure 4.34 and 4.35 exhibits FTIR spectrum of the lipid extracted from biomass of *Chloromonas* species and *Scenedesmus* species grown under different nitrogen sources (i.e ammonium nitrate ($NH_4NO_3$), potassium nitrate ($KNO_3$), urea (U) and sodium nitrate ($NaNO_3$)). The peaks or bands attributed in the FTIR spectrum are due to the functional groups present in the lipid samples. Since, biodiesel is mainly composed of mono-alkyl ester, the ester C=O stretching band appears at around 1740 $cm^{-1}$ for the studied lipid samples. The mid- infrared region 3500-1000 $cm^{-1}$ of spectra illustrates the distinct absorption bands which was assigned and characterized based on biochemical standards and published literature (Stehfest *et al*., 2005; Dean *et al*., 2010). The band region 3000−2800 $cm^{-1}$ was attributed from the aliphatic C−H stretching vibration and C−H bending region (1500−1300 $cm^{-1}$); whereas, the intense absorption bands (1746−1654 $cm^{-1}$) corresponds to the C=O ester band (Talukdar *et al*., 2014). The appearance of FTIR spectrum of treated lipid extracts illustrated the distinct IR bands in the aforementioned regions which suggest the distinct nature of lipid

existence. The near compositional similarities and dissimilarities of the FTIR spectrum was served as frequency region selection for classification and quantification of lipid recovered from the treatment. Herein, to evaluate the possible spectral variations among the FTIR spectrum of the lipid extracts, the key absorption bands throughout two spectral regions were chosen: the hydrocarbon region (3000−2800 $cm^{-1}$) and the bimolecular region (1800−1000 $cm^{-1}$). Therefore, apparent distinctions among the studied nitrogen sources in the selected regions are further explained by multivariate analysis.

#### 4.3.3.2. Principal component analysis (PCA) of oil sample of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 grown under different nitrogen sources

The FTIR spectrum of lipid extracted from the biomass grown under different nitrogen sources showed significant variations in their IR response. This may be attributed to the chemical heterogeneity in the samples. Hence to understand such variations IR spectra's was then subjected to PCA for comparative analysis. The PCA was performed in the selected spectral regions which help in reproducing the most prominent variation pattern in the data. The clear absorption band obtained at 3000-2800 $cm^{-1}$ and 1800-1000 $cm^{-1}$ was selected for this study, since these spectral ranges are dominated by the lipid acyl chain absorption and biomolecular fingerprint respectively.

At the beginning, analysis was performed in the region between 3000-2800 $cm^{-1}$; PCA score plots obtained by analyzing the measured IR spectrum represent the good discrimination of the data. The PCA score plots for *Chloromonas* species in different nitrogen sources are represented in Figure 4.36 and 4.37. From the data obtained, it can be seen that PC1 and PC2 contributed to a majority of the total variations which was equivalent to 98.79% (PC1 and PC2 accounted for 92.3 and 6.49%, respectively) and are

expected to be useful for disclosing the correlations of data points. Whereas, PC1 and PC2 for *Scenedesmus* species under different nitrogen sources (Figure 4.38 and 4.39) contributed 93.31% and 5.61% respectively. As it has been observed that the variables are clearly resolved by the two PCs which explain the correlations near a periphery of the circle. In *Chloromonas* species, the differences between the variables are represented by ammonium nitrate and urea, which are above the horizontal axis, whereas sodium nitrate and potassium nitrate are below the horizontal axis. On the other hand, *Scenedesmus* species in their loading plot potassium and ammonium nitrate present pole apart from the horizontal axis. However, urea and sodium nitrate was observed at the horizontal axis. The scatter plot of *Chloromonas* and *Scenedesmus* species shown in Figure 4.36 and 4.38 indicates a distinct group of spectral data, which might be due to similar chemical structure of lipids extracted from different variables. Nevertheless, the dissimilar chemical nature contributing to the spectral profiles creates the spread of variation along the two principal axis. Moreover, the first principal component (PC1) accounted the largest contributions which may be due to the $CH_2$ stretching modes at around 2,850 $cm^{-1}$ and 2,922 $cm^{-1}$, followed by the $CH_3$ component at around 2,960 $cm^{-1}$. In *Chloromonas* species, among the variables, maximum variance of 26.88% was contributed by urea in PC1 and ammonium nitrate contributed maximum variance of 60.19% in PC2 (Table 4.12). Similarly in *Scenedesmus* species, maximum variance was contributed by urea (26.46%) in PC1 and potassium nitrate in PC2 (51.66%) (Table 4.13). Therefore, the data obtained well explains to confirm the above result which is depicted by the correlations of the original variables and the cumulated percentage of variance of each variable explained by PC1 and PC2.

To better evaluate the lipid changes in the different treatments, PCA has been extended to the spectral range between 1800 and 1000 $cm^{-1}$, since this spectral range is considered as the biomolecular fingerprint region. In addition, the multidimensional scaling of the region 1800 $cm^{-1}$ and 1000 $cm^{-1}$ also suggested that the calculated distances were much larger than the analysis performed at higher wave numbers (Table 4.14 and 4.15). The complexities of IR responses in this region are due to absorption of lipids and biomolecules which were better explained in the score plots obtained from PC analysis. The score plot of the region (i.e. 1800-1000 $cm^{-1}$) of *Chloromonas* species (Figure 4.40 and 4.41) showed that potassium nitrate and urea are separated by the first PC factor from sodium nitrate and ammonium nitrate. From the obtained data, PC1 contributed 87.24% of the total variations, whereas PC2 contribution was 7.62%. The score plot of *Scenedesmus* species (Figure 4.42 and 4.43) depicted that sodium nitrate is separated by the first PC factor from urea, sodium nitrate and ammonium nitrate. Also the loading plot showed total variation of 72.21% in PC1 and 20.63% in PC2. From Figure 4.40 and 4.42, it was noticed that the two-dimensional score plots (PCA) obtained by analyzing the IR spectra of different treatments indicates that PC1 is mainly governed by C=O ester band and C=C stretching. Furthermore, multidimensional scaling (MDS) for both the regions, i.e. 3000-2800 and 1800-1000 $cm^{-1}$ showed significant variation in the studied nitrogen sources. In *Chloromonas* and *Scenedesmus* species, Shephard diagram (Figure 4.44 and 4.45) of a region 1800-1000 $cm^{-1}$ with the stress value (Kruskal's stress) 0.011 and 0.012 respectively showed a good fit of the model. Whereas, in *Chloromonas* and *Scenedesmus* species, Shepard diagram (Figure 4.46 and 4.47) of a region 3000-2800 $cm^{-1}$ showed the stress value (Kruskal's stress) of 8.251 E-4 and 0.001 respectively. The data obtained from MDS analysis showed minimum dissimilarity of

107.101 between urea and sodium nitrate in the region 3000-2800 $cm^{-1}$ for *Chloromonas* species (Table 4.16). Similarly, minimum dissimilarity of 132.134 was observed between urea, and sodium nitrate at the spectral region 1800-1000 $cm^{-1}$ (Table 4.17). Overall, MDS analysis showed close proximity between urea and sodium nitrate and discriminates potassium nitrate in both the spectral regions. On the other hand, *Scenedesmus* species showed close proximity between potassium and sodium nitrate variation with minimum dissimilarity of 77.954 in the region 3000-2800 $cm^{-1}$ (Table 4.18). While, minimum dissimilarity of 209.697 was observed between ammonium, and potassium nitrate at the spectral region 1800-1000 $cm^{-1}$ (Table 4.19). Altogether MDS analysis in this case discriminates urea in both the spectral regions.

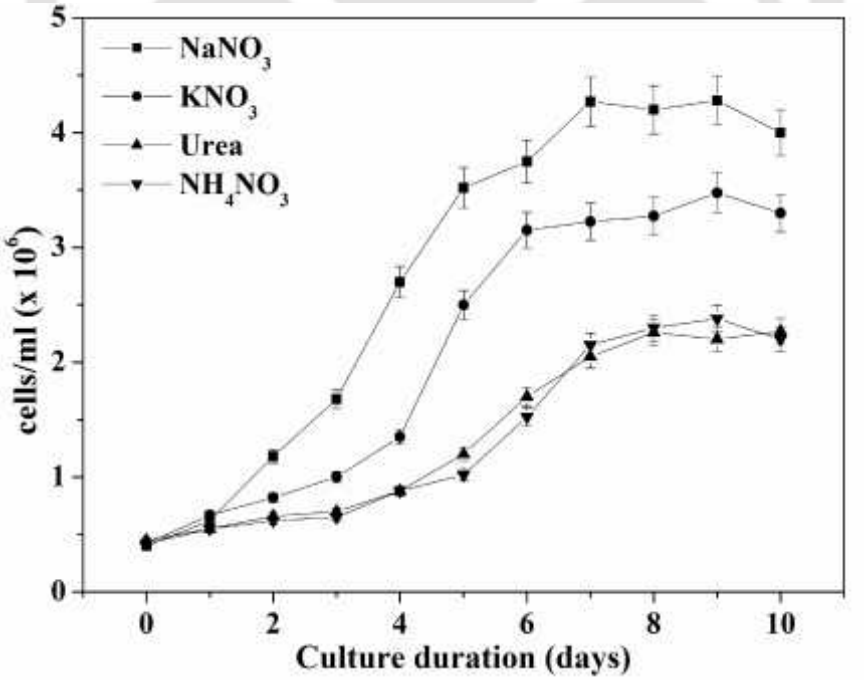


Figure 4.26 Growth curves of *Chloromonas* species cultivated in different nitrogen sources

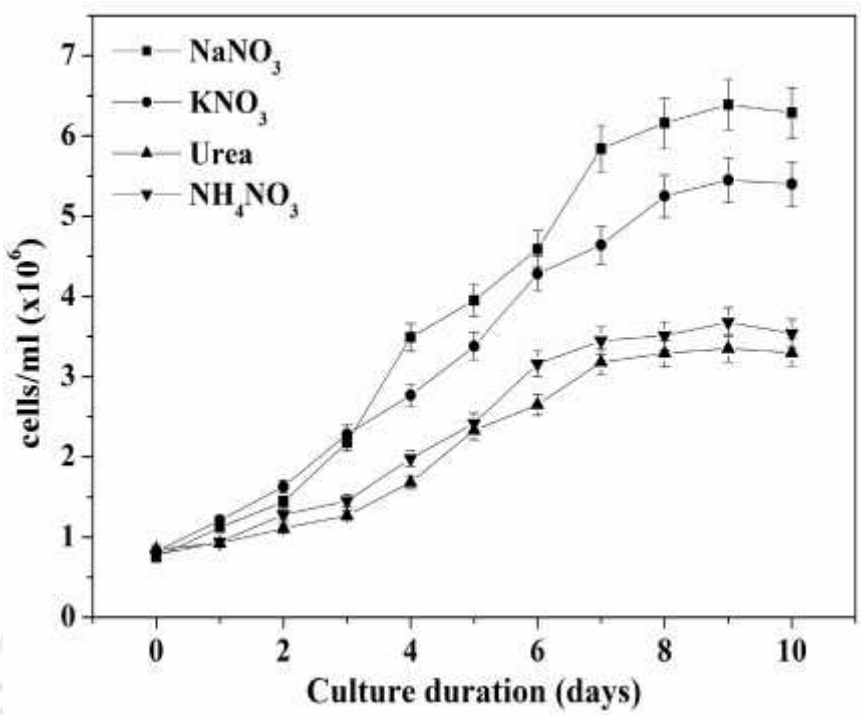


Figure 4.27 Growth curves of *Scenedesmus* species cultivated in different nitrogen sources

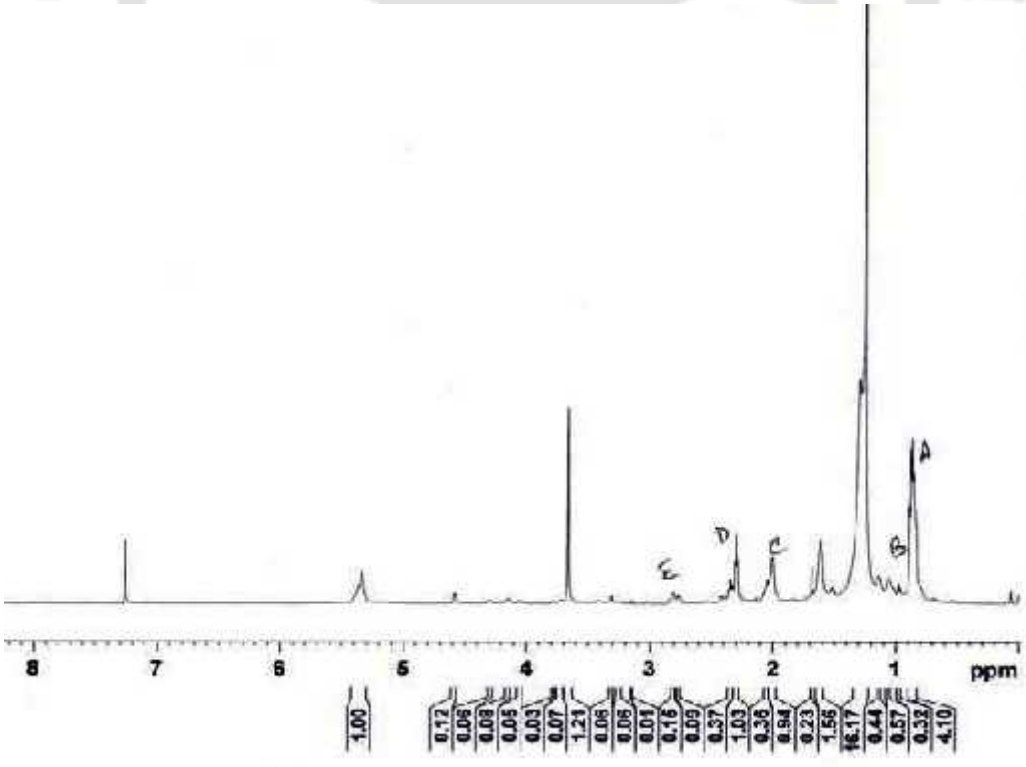


Figure 4.28 $^{1}$H NMR spectrum of methyl esters (FAME) of *Chloromonas* sp ADIITEC-III grown in ammonium nitrate

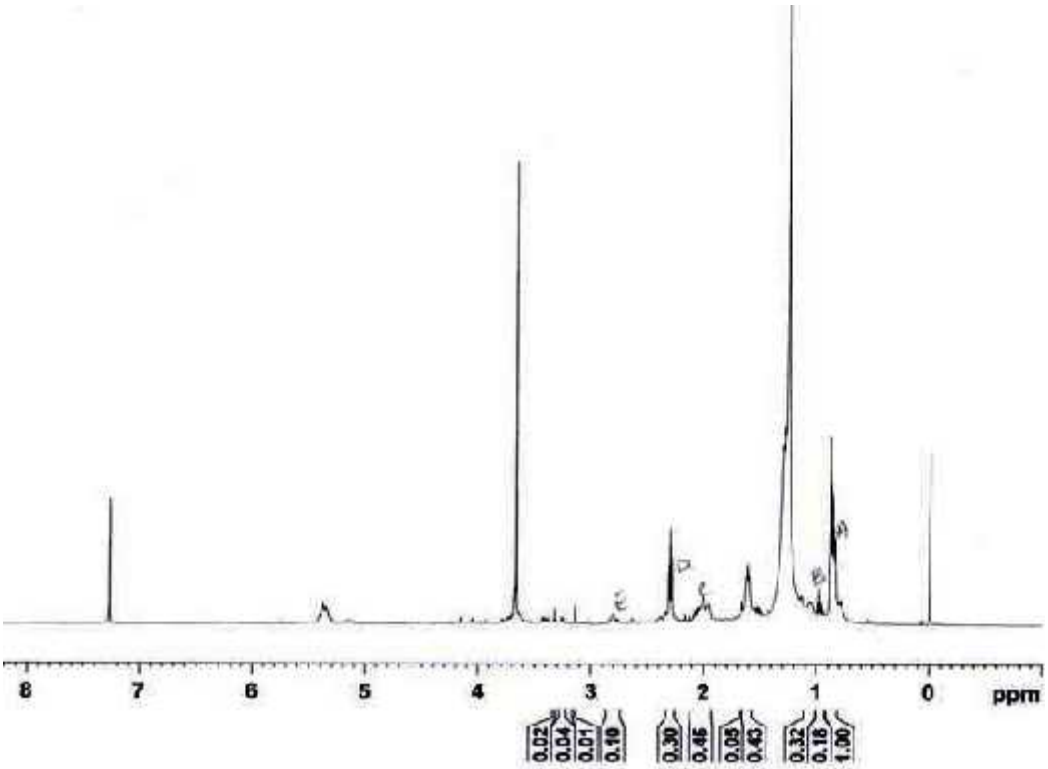


Figure 4.29 $^{1}$H NMR spectrum of methyl esters (FAME) of *Scenedesmus* sp grown in urea

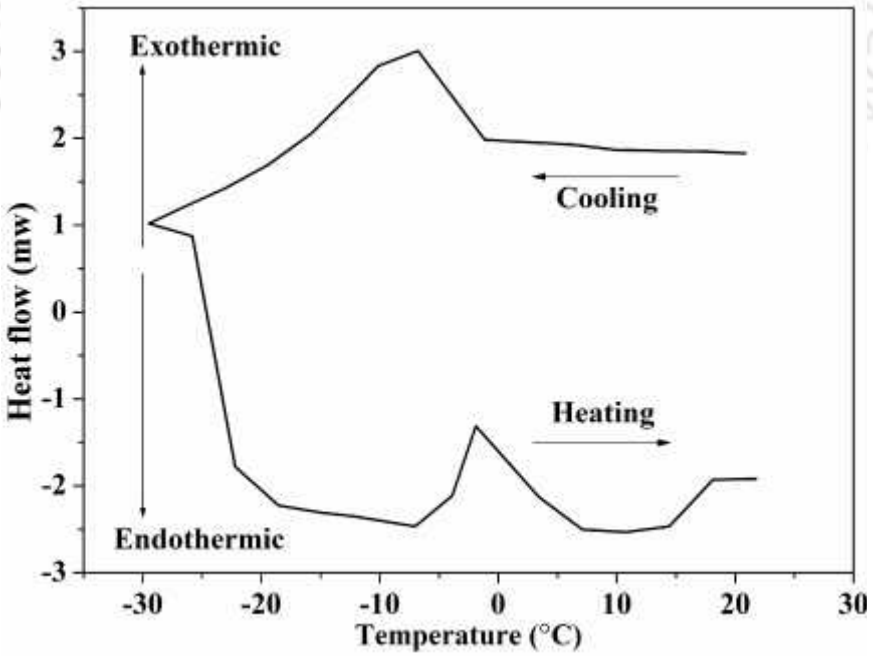


Figure 4.30 DSC thermogram of *Chloromonas* sp FAME @ 5°C min$^{-1}$ under $N_2$ atmosphere

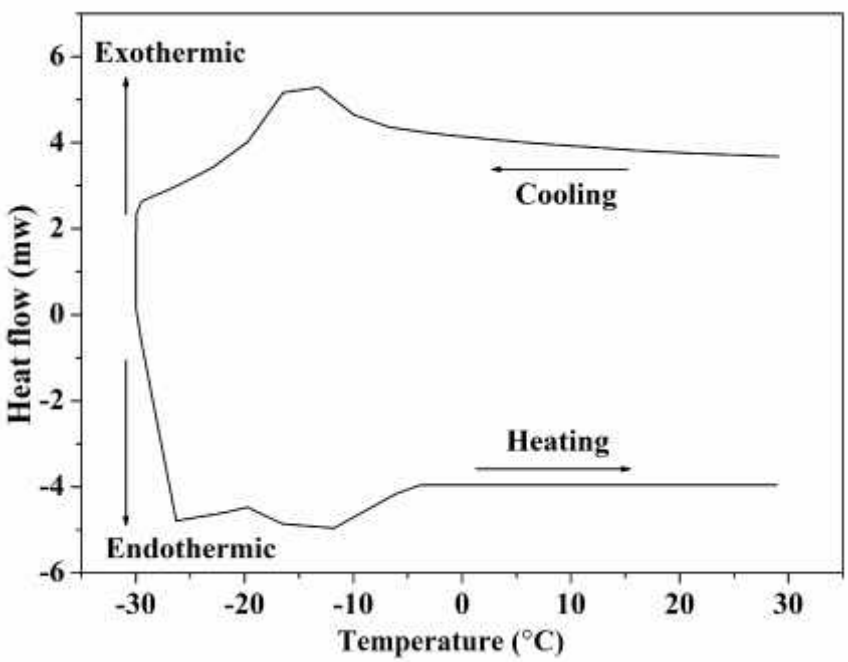


Figure 4.31 DSC thermogram of *Scenedesmus* sp FAME @ 5°C $min^{-1}$ under $N_2$ atmosphere

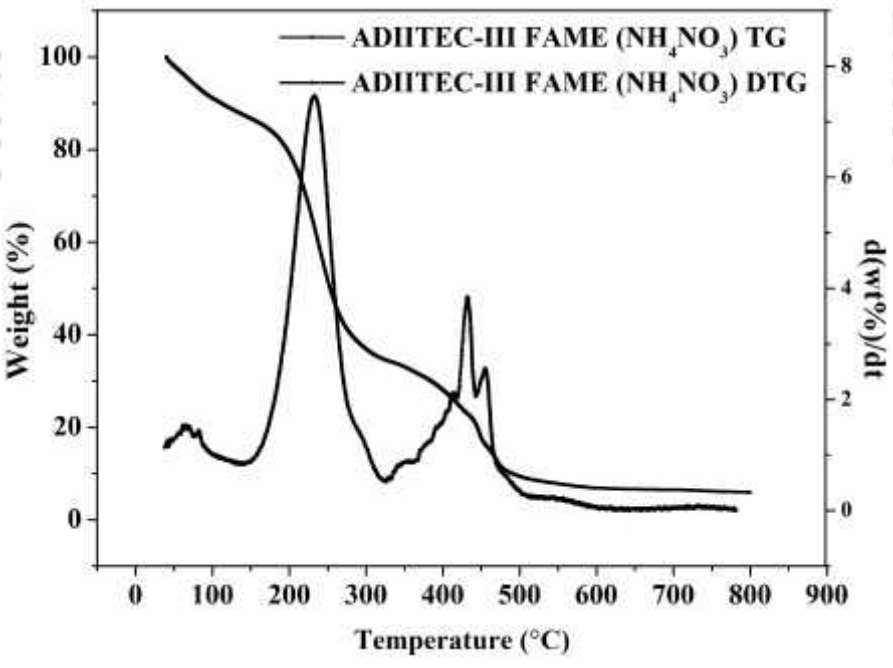


Figure 4.32 TG and DTG curves of *Chloromonas* sp FAME 10°C $min^{-1}$ under $N_2$ atmosphere

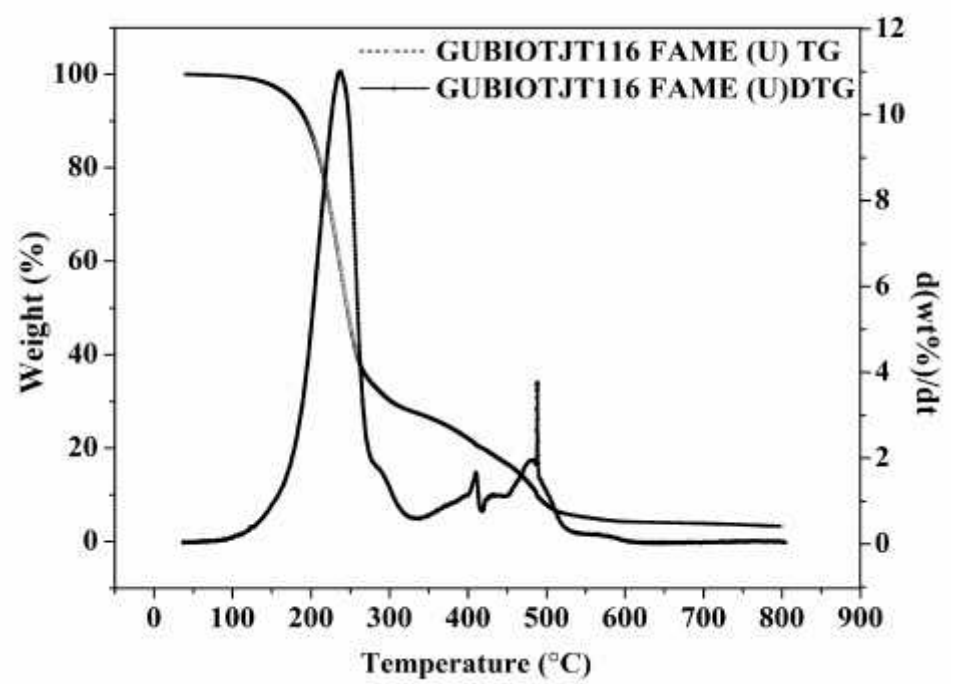


Figure 4.33 TG and DTG curves of *Scenedesmus* sp FAME 10°C $min^{-1}$ under $N_2$ atmosphere

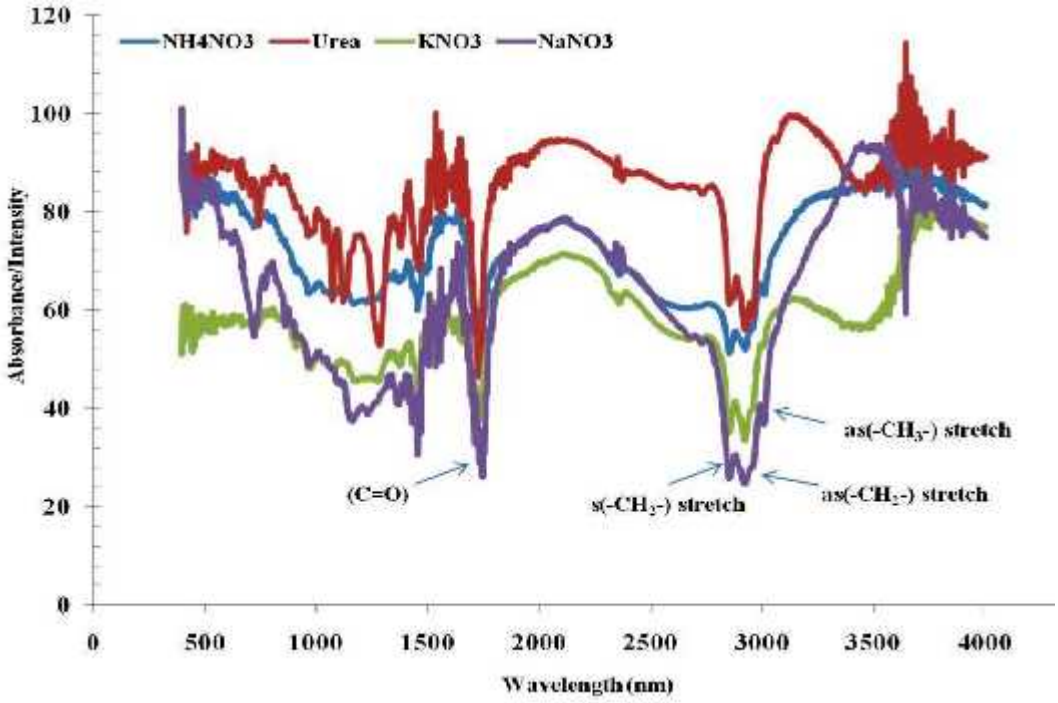


Figure 4.34 FTIR spectra of methyl ester of *Chloromonas* sp scanned at the region (4000−399 $cm^{-1}$). FTIR spectrum showing the characteristic lipid hydrocarbon bands apparent in the spectra of different nitrogen sources

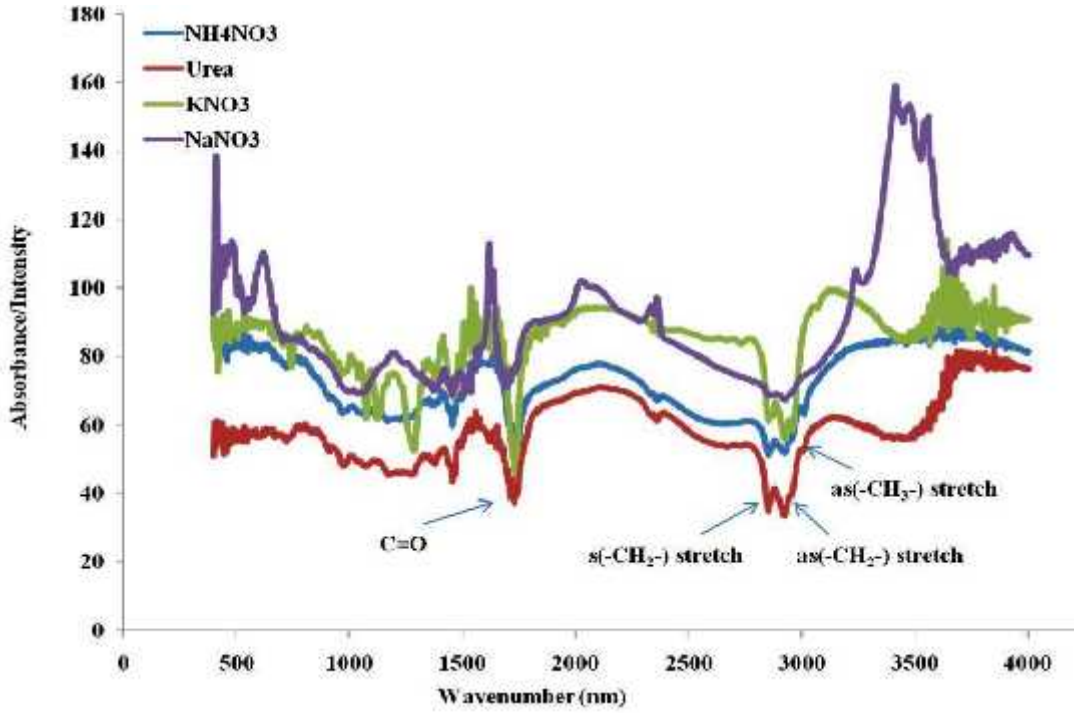


Figure 4.35 FTIR spectra of methyl ester of *Scenedesmus* sp scanned at the region (4000−399 $cm^{-1}$). FTIR spectrum showing the characteristic lipid hydrocarbon bands apparent in the spectra of different nitrogen sources

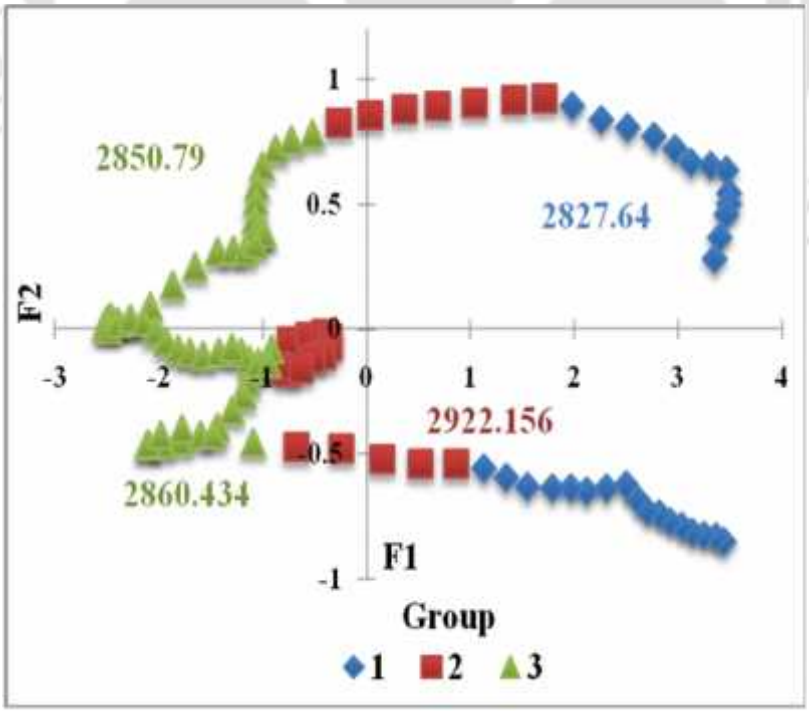


Figure 4.36 Principal component analysis of *Chloromonas* sp at the region (3000−2800 $cm^{-1}$). 2D scatter plot of PC1 × PC2 showing variance contribution

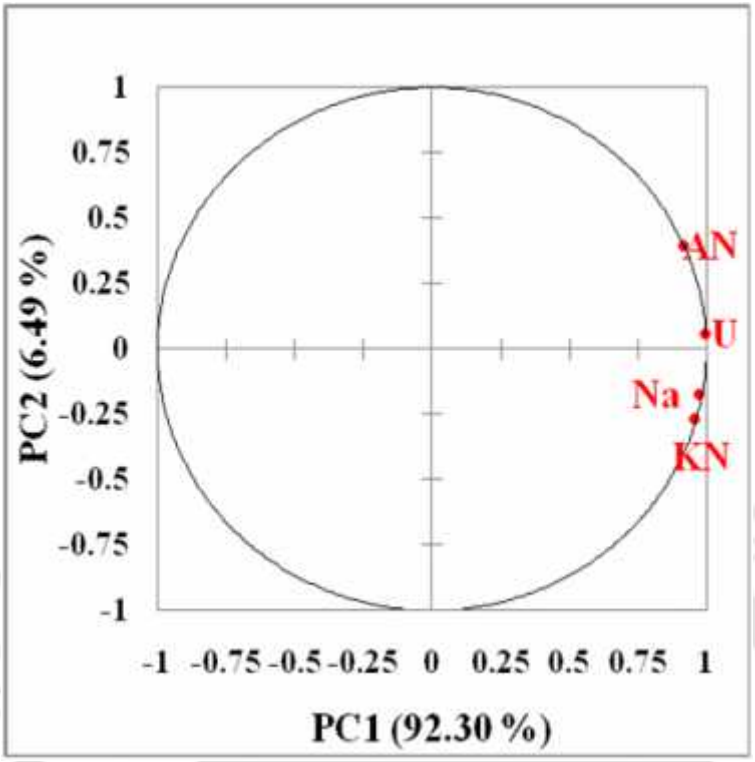


Figure 4.37 Principal component analysis of *Chloromonas* sp at the region (3000−2800 $cm^{-1}$). Loading plot showing the linear coefficients

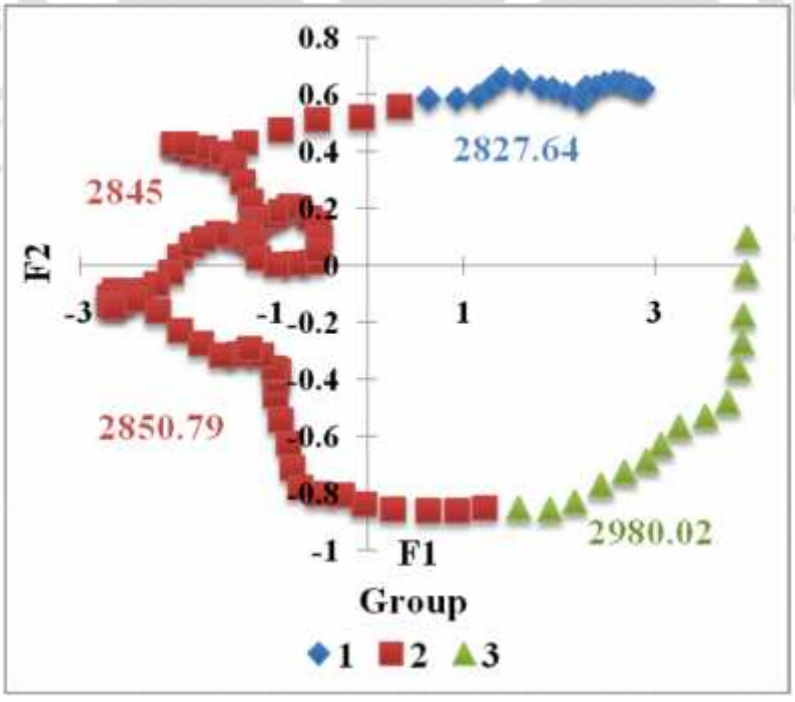


Figure 4.38 Principal component analysis of *Scenedesmus* sp at the region (3000−2800 $cm^{-1}$). 2D scatter plot of PC1 × PC2 showing variance contribution

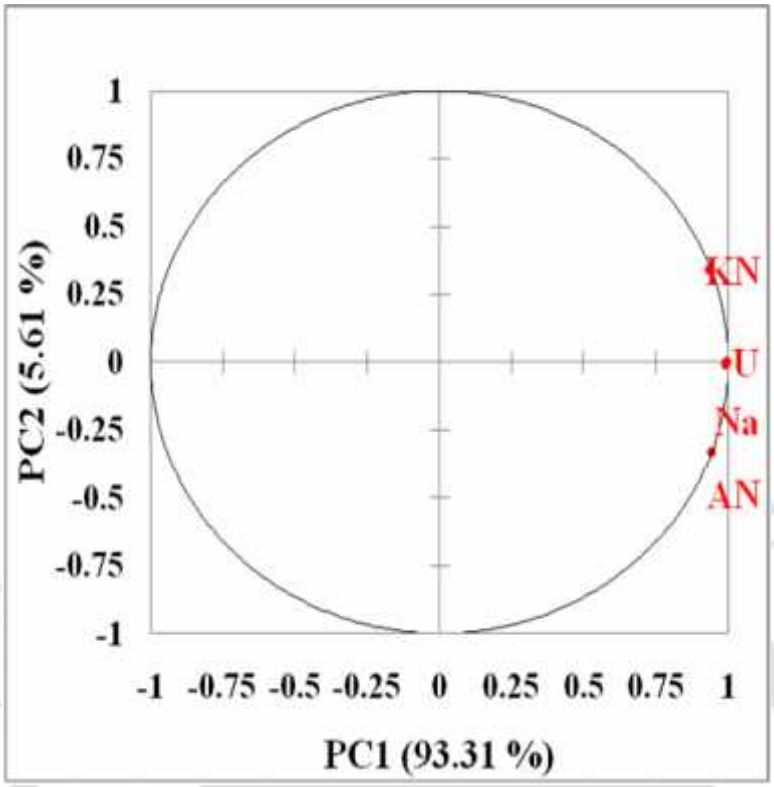


Figure 4.39 Principal component analysis of *Scenedesmus* sp at the region (3000−2800 $cm^{-1}$). Loading plot showing the linear coefficients

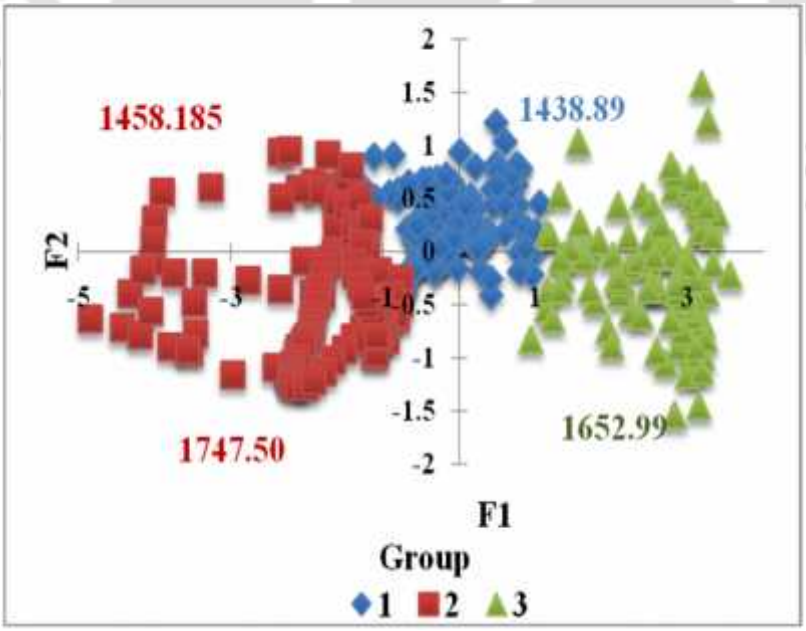


Figure 4.40 Principal component analysis of *Chloromonas* sp at the region (1800−1000 $cm^{-1}$). 2D scatter plot of PC1 × PC2 showing variance contribution

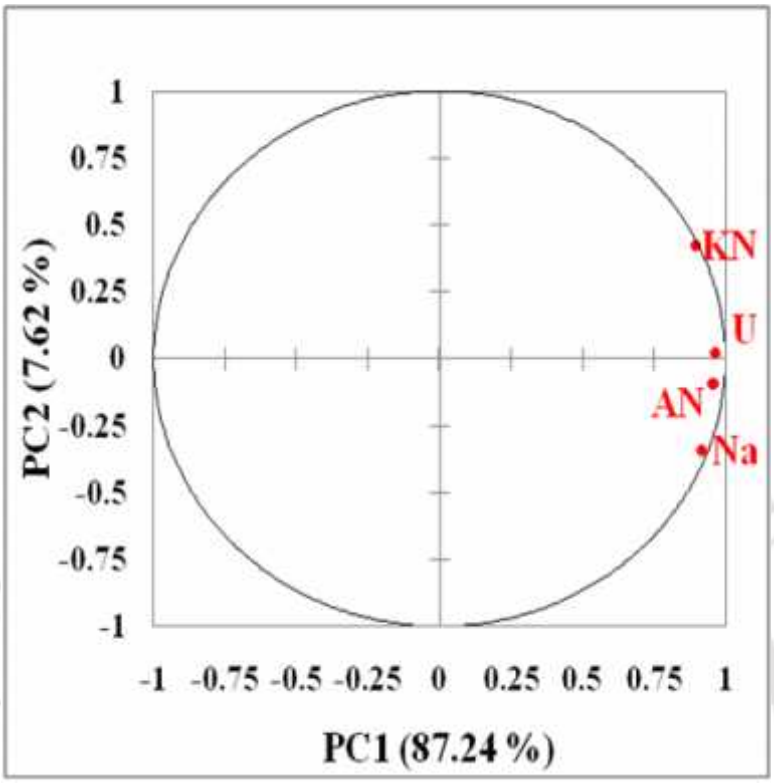


Figure 4.41 Principal component analysis of *Chloromonas* sp at the region (1800−1000 $cm^{-1}$). Loading plot showing the linear coefficients

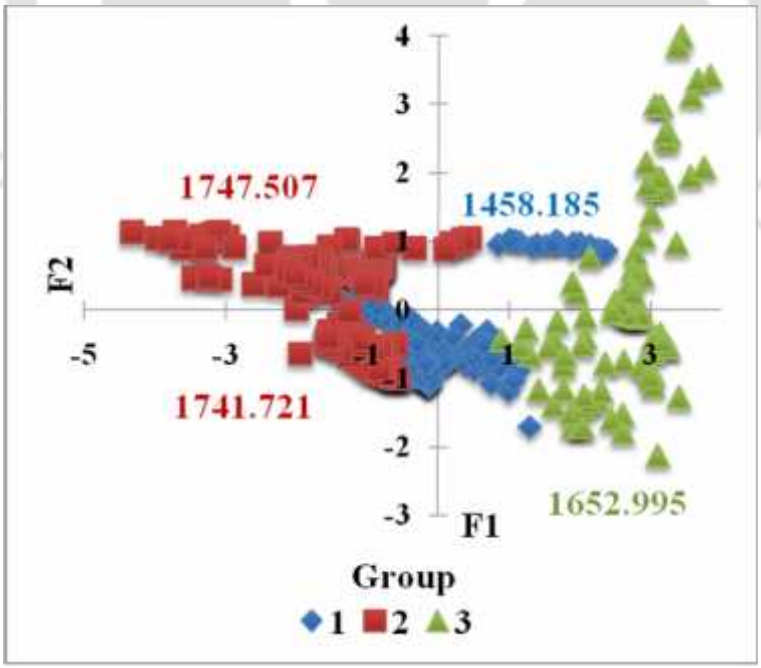


Figure 4.42 Principal component analysis of *Scenedesmus* sp at the region (1800−1000 $cm^{-1}$). 2D scatter plot of PC1 × PC2 showing variance contribution

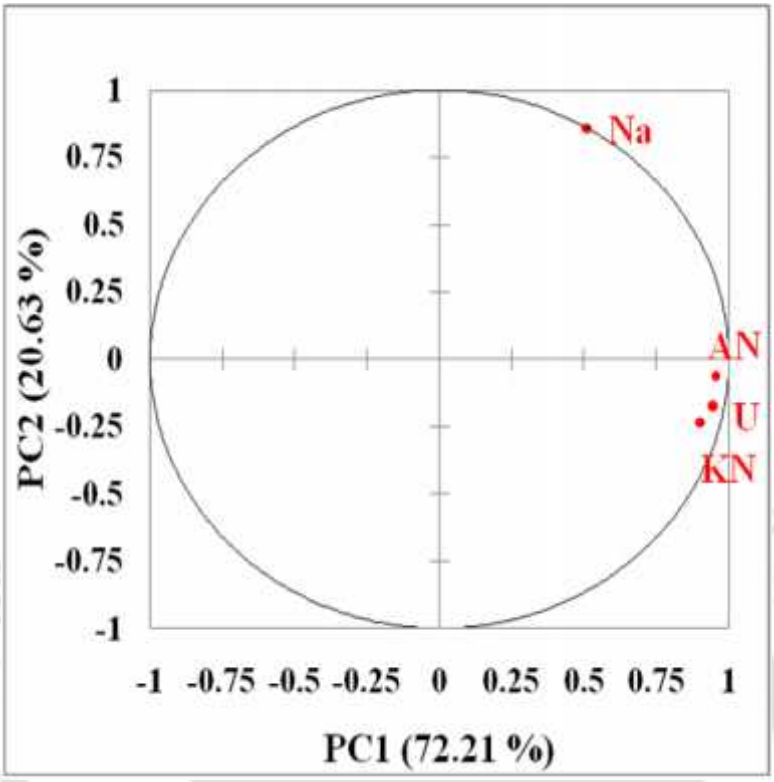


Figure 4.43 Principal component analysis of *Scenedesmus* sp at the region (3000−2800 $cm^{-1}$). Loading plot showing the linear coefficients

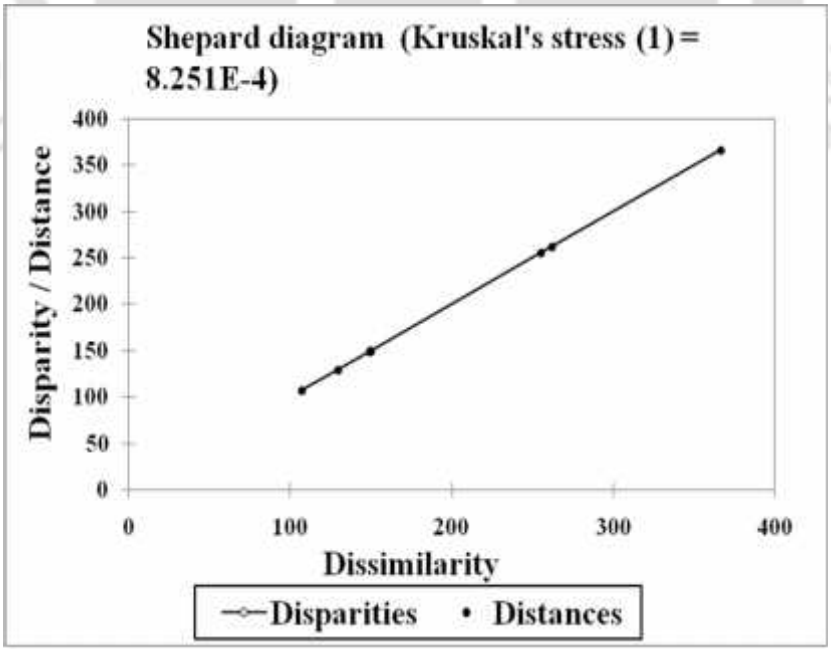


Figure 4.44 Shepard diagram of *Chloromonas* sp at the region (1800−1000 $cm^{-1}$) showing Kruskal's stress value (1) = 0.011)

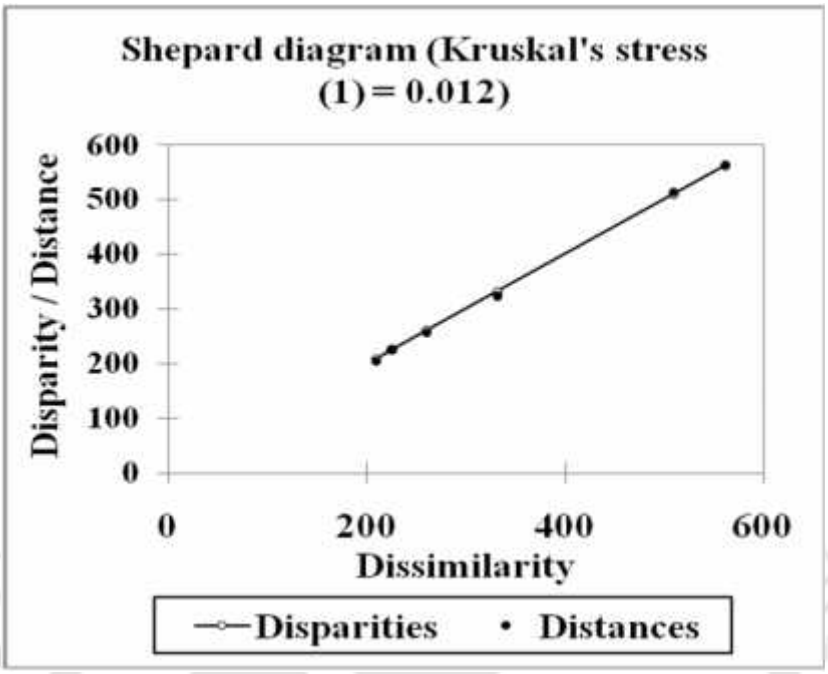


Figure 4.45 Shepard diagram of *Scenedesmus* sp at the region (1800−1000 $cm^{-1}$) showing Kruskal's stress value (1) = 0.012)

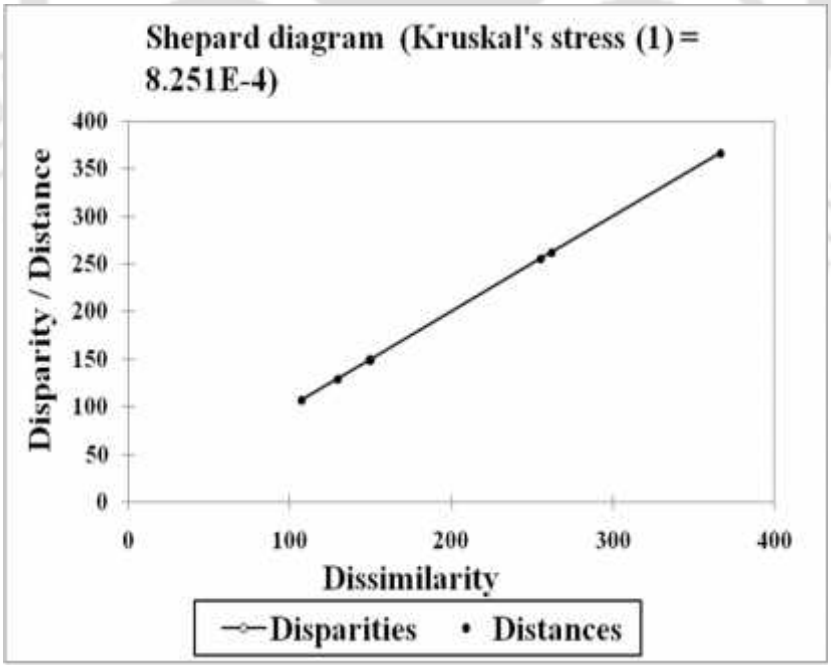


Figure 4.46 Shepard diagram of of *Chloromonas* sp at the region (3000−2800 $cm^{-1}$) showing Kruskal's stress value (1) = 8.251 E-4)

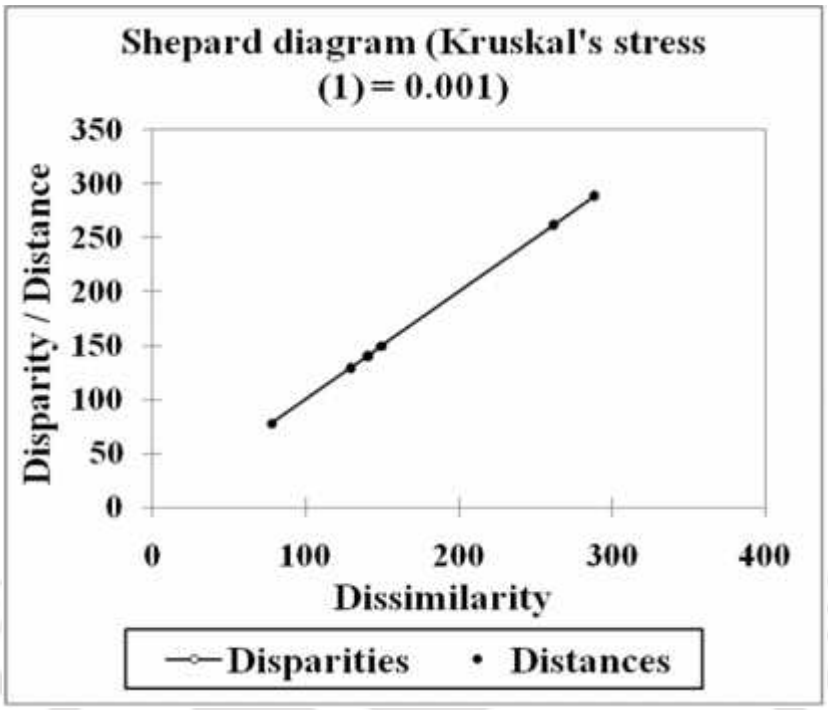


Figure 4.47 Shepard diagram of *Scenedesmus* sp at the region (3000−2800 $cm^{-1}$) showing Kruskal's stress value (1) = 0.012

Table 4.8 Growth characteristics, biomass and lipid production of *Chloromonas* species in different nitrogen sources

| $N_2$ source | *Chloromonas* species (ADIITEC-III) | | | |
|---|---|---|---|---|
| | Specific growth rate $\mu$, ($d^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity (g $L^{-1}$ $d^{-1}$) | Total lipid content (% DCW) |
| $NaNO_3$ | 0.23 ± 0.015 | 3.01 ± 0.21 | 0.04 ± 0.01 | 28.40 ± 2.85 |
| $KNO_3$ | 0.20 ± 0.02 | 3.46 ± 0.35 | 0.03 ± 0.01 | 34.35 ± 0.32 |
| Urea | 0.16 ± 0.015 | 4.33 ± 0.38 | 0.02 ± 0.02 | 30.2 ± 0.87 |
| $NH_4NO_3$ | 0.17 ± 0.015 | 4.07 ± 0.35 | 0.02 ± 0.02 | 35.86 ± 2.3 |

Table 4.9 Growth characteristics, biomass and lipid production of *Scenedesmus* species in different nitrogen sources

| $N_2$ source | *Scenedesmus* species (GUBIOTJT116) | | | |
|---|---|---|---|---|
| | Specific growth rate $\mu$, ($d^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity ($g\ L^{-1} d^{-1}$) | Total lipid content (% DCW) |
| $NaNO_3$ | 0.21 ± 0.02 | 3.3 ± 0.31 | 0.27 ± 0.015 | 30 ± 0.4 |
| $KNO_3$ | 0.19 ± 0.01 | 3.64 ± 0.2 | 0.24 ± 0.02 | 32.8 ± 0.66 |
| Urea | 0.14 ± 0.02 | 4.95 ± 0.72 | 0.18 ± 0.015 | 37.2 ± 0.36 |
| $NH_4NO_3$ | 0.15 ± 0.02 | 4.62 ± 0.44 | 0.2 ± 0.02 | 35.5 ± 0.3 |

Table 4.10 Physico chemical properties of methyl ester of *Chloromonas* species ADIITEC-III in ammonium nitrate and *Scenedesmus* species GUBIOTJT116 in urea

| Properties | *Chloromonas* species ADIITEC-III | | *Scenedesmus* species GUBIOTJT116 | | ASTM D-6751 limits |
|---|---|---|---|---|---|
| | FAME (BG11) | FAME ($NH_4NO_3$) | FAME (BG11) | FAME (Urea) | |
| Acid value mg $KOHg^{-1}$ | 0.48 | 0.5 | 0.52 | 0.52 | 0.50 max |
| Iodine value g $I_2$ $100^{-1}$g oil | 112.3 | 112 | 112 | 114 | 120 max |
| DSC (pour point) | -7ºC | -7ºC | -12ºC | -13ºC | -15 to 10 |
| Calorific value MJ $Kg^{-1}$ | 40 | 40 | 41 | 42 | - |
| Fatty acid compositions of ADIITEC-III and GUBIOTJT116 | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| Linolenic acid (18:3) | 6.63% | 7.23% | 13.79 % | 15.25% | - |
| Linoleic acid (18:2) | 21.04% | 8.84% | 1.83 % | 2.83% | - |
| Oleic acid (18:1) | 32.54% | 47.04% | 48.1 % | 56.92% | |
| Total saturates | 39.79% | 36.9% | 36.29 % | 25% | - |

Table 4.11 Temperature characteristics for the thermogravimetric analysis of methyl esters of *Chloromonas* sp and *Scenedesmus* species grown in ammonium nitrate and urea respectively

| Microalgae | Onset temperature (ºC) | Mass loss (%) | | |
|---|---|---|---|---|
| | | 10% | 50% | 90% |
| ADIITEC-III FAME ($NH_4NO_3$) | 112 | 153ºC | 324ºC | 480ºC |
| ADIITEC-III FAME ($NaNO_3$) | 161 | 174ºC | 249ºC | 477ºC |
| GUBIOTJT116 FAME (Urea) | 163 | 195ºC | 345ºC | 488ºC |
| GUBIOTJT116 FAME ($NaNO_3$) | 174 | 235ºC | 359ºC | 482ºC |

Table 4.12 PCA of *Chloromonas* species. Correlations between variables and first two Principal Components with contribution of the variables to the total variance explained by each of the PCs (3000-2800 $cm^{-1}$)

| Variables | PC1 | | PC2 | |
|---|---|---|---|---|
| | Correlations | Variance (%) | Correlations | Variance (%) |
| AN | 0.918 | 22.808 | 0.395 | 60.197 |
| KN | 0.954 | 24.659 | -0.264 | 26.903 |
| U | 0.996 | 26.882 | 0.058 | 1.314 |
| Na | 0.973 | 25.651 | -0.173 | 11.587 |

Table 4.13 PCA of *Scenedesmus* species. Correlations between variables and first two Principal Components with contribution of the variables to the total variance explained by each of the PCs (1800-1000 $cm^{-1}$)

| Variables | PC1 | | PC2 | |
|---|---|---|---|---|
| | Correlations | Variance (%) | Correlations | Variance (%) |
| AN | 0.941 | 23.778 | -0.329 | 48.278 |
| U | 0.994 | 26.462 | 0.002 | 0.001 |
| KN | 0.938 | 23.593 | 0.340 | 51.664 |
| Na | 0.988 | 25.167 | -0.011 | 0.056 |

Table 4.14 PCA of *Chloromonas* species. Correlations between variables and first two Principal Components with contribution of the variables to the total variance explained by each of the PCs (1800-1000 $cm^{-1}$)

| Variables | PC1 | | PC2 | |
|---|---|---|---|---|
| | Correlations | Variance (%) | Correlations | Variance (%) |
| AN | 0.961 | 26.472 | -0.091 | 2.716 |
| KN | 0.894 | 22.921 | 0.424 | 58.860 |
| U | 0.962 | 26.506 | 0.023 | 0.169 |
| Na | 0.917 | 24.102 | -0.341 | 38.255 |

Table 4.15 PCA of *Scenedesmus* species. Correlations between variables and first two Principal Components with contribution of the variables to the total variance explained by each of the PCs (1800-1000 $cm^{-1}$)

| Variables | PC1 | | PC2 | |
|---|---|---|---|---|
| | Correlations | Variance (%) | Correlations | Variance (%) |
| AN | 0.954 | 31.514 | -0.060 | 0.441 |
| U | 0.948 | 31.110 | 0.177 | 3.805 |
| KN | 0.904 | 28.293 | 0.236 | 6.753 |
| Na | 0.512 | 9.083 | -0.857 | 89.000 |

Table 4.16 Multidimensional Scaling of *Chloromonas* species. Comparative distance table showing the results of MDS analysis (3000-2800)

| Pair | Dissimilarity | Disparity | Distance | Rank (Dissimilarity) | Rank (Disparity) | Rank (Distance) |
|---|---|---|---|---|---|---|
| U - Na | 107.101 | 107.101 | 106.900 | 1 | 1 | 1 |
| AN - KN | 129.317 | 129.317 | 129.310 | 2 | 2 | 2 |
| AN - U | 149.401 | 149.401 | 148.850 | 3 | 3 | 3 |
| AN - Na | 255.185 | 255.185 | 255.735 | 4 | 4 | 4 |
| KN - U | 261.875 | 261.875 | 262.231 | 5 | 5 | 5 |
| KN - Na | 366.211 | 366.211 | 365.857 | 6 | 6 | 6 |

Table 4.17 Multidimensional Scaling of *Chloromonas* species. Comparative distance table showing the results of MDS analysis (1800-1000)

| Pair | Dissimilarity | Disparity | Distance | Rank (Dissimilarity) | Rank (Disparity) | Rank (Distance) |
|---|---|---|---|---|---|---|
| U - Na | 132.134 | 132.134 | 130.632 | 1 | 1 | 1 |
| AN - KN | 209.697 | 209.697 | 207.926 | 2 | 2 | 2 |
| AN - U | 331.809 | 331.809 | 325.422 | 3 | 3 | 3 |
| AN- Na | 406.873 | 406.873 | 411.985 | 4 | 4 | 4 |
| KN - U | 509.646 | 509.646 | 515.322 | 5 | 5 | 5 |
| KN- Na | 580.314 | 580.314 | 576.169 | 6 | 6 | 6 |

Table 4.18 Multidimensional Scaling of *Scenedesmus* species. Comparative distance table showing the results of MDS analysis (3000-2800)

| Pair | Dissimilarity | Disparity | Distance | Rank (Dissimilarity) | Rank (Disparity) | Rank (Distance) |
|---|---|---|---|---|---|---|
| KN - Na | 77.954 | 77.954 | 77.943 | 1 | 1 | 1 |
| AN - KN | 129.317 | 129.317 | 129.365 | 2 | 2 | 2 |
| AN - Na | 140.584 | 140.584 | 140.641 | 3 | 3 | 3 |
| AN - U | 149.401 | 149.401 | 149.504 | 4 | 4 | 4 |
| U - KN | 261.875 | 261.875 | 261.819 | 5 | 5 | 5 |
| U - Na | 288.601 | 288.601 | 288.553 | 6 | 6 | 6 |

Table 4.19 Multidimensional Scaling of *Scenedesmus* species. Comparative distance table showing the results of MDS analysis (1800-1000)

| Pair | Dissimilarity | Disparity | Distance | Rank (Dissimilarity) | Rank (Disparity) | Rank (Distance) |
|---|---|---|---|---|---|---|
| AN - KN | 209.697 | 209.697 | 209.791 | 1 | 1 | 1 |
| KN - Na | 225.320 | 225.320 | 225.292 | 2 | 2 | 2 |
| AN - Na | 260.915 | 260.915 | 260.986 | 3 | 3 | 3 |
| AN - U | 331.809 | 331.809 | 331.958 | 4 | 4 | 4 |
| U - KN | 509.646 | 509.646 | 509.546 | 5 | 5 | 5 |
| U - Na | 561.594 | 561.594 | 561.538 | 6 | 6 | 6 |

## 4.4. The effect of saline (NaCl) condition and a two-stage cultivation of selected microalgae strain on growth, biomass and lipid yield

To enhance the lipid yield of microalgae two different culture conditions such as saline condition and two stage cultivation strategy was carried out. As per the reported literature few culture condition such as nitrogen starvation (Pancha *et al*., 2014), light intensity (George *et al*., 2014) and mixotrophic growth condition (Pancha *et al*., 2015) play a significant role in enhancing biomass, lipid and carbohydrate contents in various microalgal species. But despite their potential role, nitrogen starvation (i.e. nitrate and phosphate limitations) and mixotrophic growth conditions are associated with certain drawbacks. During nitrogen starvation, biomass production is quite low, which leads to lower lipid yield. Whereas, the high cost of organic carbon during mixotrophic cultivation make it a cost intensive process (Pancha *et al*., 2015). To overcome such problems, many researchers have introduced the idea of two stage cultivation, where microalgae is grown initially in nutrient sufficient condition and after recovering sufficient biomass stress phase is applied (Sun *et al*., 2014). Similar to nutritional starvation, study on salt stress to enhance lipid accumulation has also been reported in various microalgae like *Chlamydomonas* sp. JSC4 (Ho *et al*., 2014), *Desmodesmus abundans* (Xia *et al*., 2014), *Nannochloropsis sp*. (Pal *et al*., 2011) etc. Studies on salinity effect have been mainly carried out in marine microalgae and very few reports are available on freshwater microalgae for lipid and carbohydrate production (Pancha *et al*., 2015). The two stage cultivation with salt stress (NaCl) is assumed to be practically more convenient than the two stage TAG accumulation methods in large scale open ponds (Yang *et al*., 2014).

The present study was aimed to understand the effect of saline (NaCl) stress on growth, biomass and biochemical composition of *Chloromonas* species (ADIITEC-III)

and *Scenedesmus* species GUBIOTJT116 under single and two stage cultivation regimes. For the salt stress study NaCl concentration was varied over the range such as 10, 30, 50, 70, 90, 110, 130, 150 and 200 mM respectively. The changes in methyl ester induced during the two stage cultivation was characterized and compared with methyl ester recovered from the microalgae grown in culture media without NaCl.

### 4.4.1. Effect of salt stress (NaCl) on growth, biomass and lipid production of *Chloromonas* and *Scenedesmus* species

During the study it was observed that addition of NaCl to the growth medium significantly affected the biomass and lipid production of *Chloromonas* and *Scenedesmus* species. The growth rate of both the microalgae species was improved as the concentration of NaCl increased from 0 to 50 mM (Figure 4.48 and 4.49). From the study, specific growth, biomass and lipid production obtained for *Chloromonas* and *Scenedesmus* species are tabulated in Table 4.20 and 4.21 respectively. Among the studied concentrations, 90 mM favored the specific growth (0.25 ± 0.02) and biomass productivity (0.2 ± 0.02 g $L^{-1}d^{-1}$) in *Chloromonas* species. Whereas increased specific growth (0.26 ± 0.02) and biomass productivity (0.37 ± 0.02 g $L^{-1}d^{-1}$) of *Scenedesmus* species was observed at 50 mM of NaCl. Both the concentrations showed better growth of the strains than that of in control BG11 media. However, the growth of *Chloromonas* and *Scenedesmus* species was found to be inhibited at NaCl concentrations above 90 mM and 50 mM respectively. The results of the study revealed that the salt concentration between 10 to 50 mM NaCl were appropriate for the promotion of the growth rate of *Scenedesmus* species GUBIOTJT116. These findings are in accordance with reported studies on *S. obliquus (*Kaewkannetra *et al*., 2012). The high oil content of 38.8 ± 0.5% (dry biomass weight) was observed in the culture of *Chloromonas* species which was

supplemented with 200 mM NaCl. In the absence of salt, i.e. control condition (BG11 media), the lipid yield over the 15 day period was observed to be approximately 30.2 ± 0.72% (dry biomass weight). It is evident that cultivation of *Chloromonas* species at higher NaCl concentrations leads to higher lipid yield, but results in decreased biomass production. At high salinity conditions, lipid yield of algae increases due to the accumulation of glycerol molecule in reponse to osmotic pressure of the environment (Salama *et al*., 2013). For *Scenedesmus* species, the highest lipid yield was found to be 37.8 ± 0.5% at 90 mM NaCl concentration in the culture media. However, decrease in biomass production was observed with further increase in concentration beyond 130 mM. The decrease in cell biomass may be attributed to the non-adaptability of the organism at high salinity conditions (0.1–0.75 M) (Vazquez-Duhalt and Arredondo-Vega, 1991; Ben-Amotz *et al*., 1985). Whereas, *Dunaliella tertiolecta* ATCC 30929 known as halo tolerant species, can grow under salt stress of up to 1.0 M NaCl (Takagi *et al*., 2006). The purpose of two stage cultivation was to obtain maximum biomass with high yield of lipid. Based on the data obtained, ADIITEC-III was considered for further study as GUBIOTJT116 was not able to sustain at high salinity condition and resulted in slow growth and biomass productivity.

### 4.4.2. Effect of two stage salt stress (NaCl) cultivation on biomass and lipid production of *Chloromonas* species ADIITEC-III

The effect of two stage salinity stress condition on biomass and lipid content was investigated in *Chloromonas* species (ADIITEC-III). The microalgae sp. was first grown in BG-11 medium with low salt stress of 50 mM and after 15 days of cultivation high salt stress was applied by addition of 200 mM of NaCl. The reason for addition of 50 mM of NaCl to the 1$^{st}$ stage was to collect the maximum biomass. In the 2$^{nd}$ stage, as the species

reaches its stationary phase, 200 mM of NaCl was added to the same culture. The salts were added into the cultures when cells achieved a biomass concentration of 1.5 ± 0.3 g $L^{-1}$ after 15 days of cultivation i.e. at the late exponential phase. The addition of salt concentration was selected based on the preliminary screening of salts (10 mM to 200 mM) on growth and lipid yield. Figure 4.50 shows the effect of two stage salinity stress on lipid yield and biomass productivity of microalgae *Chloromonas* species. For comparisons, the species grown in BG-11 medium without any salt additions are considered as the control culture. Therefore, the results obtained from the 1$^{st}$ stage cultivation shown that biomass production increased to 1.5 ± 0.3 g $L^{-1}$, whereas in control it was 0.45 ± 0.03 g $L^{-1}$. In the 2$^{nd}$ stage addition of 200 mM of NaCl increased the lipid content up to 40.8 ± 0.2%. The lipid content obtained in this study was higher than that obtained with *D. abundans* (i.e. 34.70%) at 20 g $L^{-1}$ NaCl (Xia *et al*., 2014). The addition of NaCl might help in accumulating neutral and polar lipids, or triacylglycerols and glycerol, which support the organisms to tolerate high salt stress (Xia *et al*., 2013). The results emphasize the importance of salt stress as stimuli for lipid induction in freshwater green algae and further confirm the feasibility of two-phase cultivation mode for microalgae biodiesel production.

### 4.4.3. Comparisons of fatty acid profile of culture grown in two-phase mode and BG11 media without NaCl

Besides lipid content, FAME profile is also a significant factor in terms of biodiesel quality to determine the feasibility of this two-stage cultivation process using salt addition. In this study fatty acid composition of lipid sample was determined through $^{1}$H NMR spectrometer as shown in Figure 4.51.

The derived methyl esters showed abundance of monounsaturated oleic (C18:1), linolenic acid (C18:3) and linoleic (C18:2) and saturated fatty acids (Table 4.22). In two-stage cultivation, most abundant fatty acids observed in *Chloromonas* species were oleic acid (18:1) and linoleic acid (18:3). A remarkable increase in monounsaturated fatty acid (40.49%) content was observed under high salinity compared to control condition (32.54 %) (i.e. without NaCl). Whereas, substantial decreased in linolenic acid (1.51%) was recorded compared to control condition (6.63%). Also, total saturated fatty acid content of the sample was found to be 35.04%. These findings are consistent with the earlier reported literature on freshwater microalgae *Chlamydomonas mexicana*, *S. obtusus* XJ-15 (Salama *et al*., 2013; Xia *et al*., 2013), and *D. abundans* (Xia *et al*., 2014) under high salinity stress condition. Xia et al. (2014) in their study also observed increase in oleic acid (36.94%) content under NaCl concentration of 20 g $L^{-1}$ compared to control condition (18.39%). The variations in fatty acid composition under high salinity stress condition are inevitable to maintain the membrane fluid and prevent it from destruction (Xia *et al*., 2014). However, stress conditions alter fatty acid synthesis to produce more of the monounsaturated FAs (MUFA) that mainly make up neutral lipids which in turn favors biodiesel production (Hu *et al*., 2008). The results of the study revealed that *Chloromonas* species can also accumulate lipids under varying environments like high salt concentrations which in turn make this microalgae sp. a very versatile and promising species for large-scale production for lipid-rich biomass.

### 4.4.4. Comparison of the physico-chemical properties of FAME produced under two different conditions (two - phase mode and BG11 media without NaCl)

The important characteristics of the biodiesel produced from *Chloromonas* species under high salinity stress condition are summarized in Table 4.22. The FAME of *Chloromonas*

species grown in two – phase mode showed a similar range of acid value than that of the control condition (0.48 mg KOH $g^{-1}$). The iodine value of biodiesel produced from *Chloromonas* sp. grown in high salt media was found to be 114 g $I_2$ $100^{-1}$ g and meet the ASTM standards for biodiesel showing better biodiesel characteristics than control condition (112.3 g $I_2$ $100^{-1}$). The calorific value of FAME sample obtained from two – phase mode cultivation was around 42 MJ $Kg^{-1}$ and was found to be higher than culture without salt (40 MJ $Kg^{-1}$). The cold flow property (CFPP) is also an important characteristic of biodiesel and used to determine the flow performance of biodiesel at low temperatures. Earlier studies on CFPP suggested that fatty acid with more unsaturation content correspond to better cold flow properties (Borugadda *et al*., 2014). In this study, total unsaturated fatty acid content of the sample was recorded to be 64.94% in two-phase mode, whereas in control condition it was 60.21%. Therefore, two-phase mode cultivation showed better clod flow property (-8ºC) compared to control condition (-7ºC) (Figure 4.52 and Figure 4.19.).

Similarly, thermal stability of the FAME sample was determined using thermogravimetric analysis (TGA) technique. The onset temperature of decomposition under nitrogen atmosphere determines the thermal stability of the sample (Borugadda *et al*., 2014). The TGA and DTG plot at constant heating rate of 10ºC $min^{-1}$ under nitrogen atmosphere are shown in Figure 4.53. The onset temperature for methyl esters of *Chloromonas* species in two-phase mode and control culture condition was recorded to be 171ºC and 161ºC respectively. The weight loss profiles obtained in two- phase cultivation are summarized and compared with control culture in Table 4.23. The FAME sample of *Chloromonas* species in two-phase mode showed 10% weight loss at 195ºC, 50% weight loss at 311ºC and almost 90% weight loss were observed at around 498ºC.

The weight loss includes moisture content and decomposition of mono and polyunsaturated fatty acids (oleic, linoleic and linolenic) (Borugadda *et al*., 2014). Hence, thermal stability could be better explained based on fatty acid compositions. As represented in Table 4.22, polyunsaturated fatty acid content was high in control condition than the NaCl supplemented culture. These high levels of polyunsaturation content in the sample are responsible for the lower thermal stability (Park *et al*., 2008). Therefore, methyl ester sample of two-phase cultivation was thermally more stable than culture grown in control condition.

The results of the study revealed that *Chloromonas* species grown in high salinity stress condition (NaCl) showed better biodiesel properties than control condition. Moreover, low cost of NaCl suggests its usage as a best stimulus for lipid induction in terms of enhanced lipid productivity and improved biodiesel quality (Xia *et al*., 2014). Our results showed that *Chloromonas* species can also accumulate lipids under high salt concentrations which make this microalgae species a very versatile and promising sp. for large-scale cultivation. Nevertheless, this two-stage cultivation strategy was not only feasible, but also effective in terms of biodiesel quality.

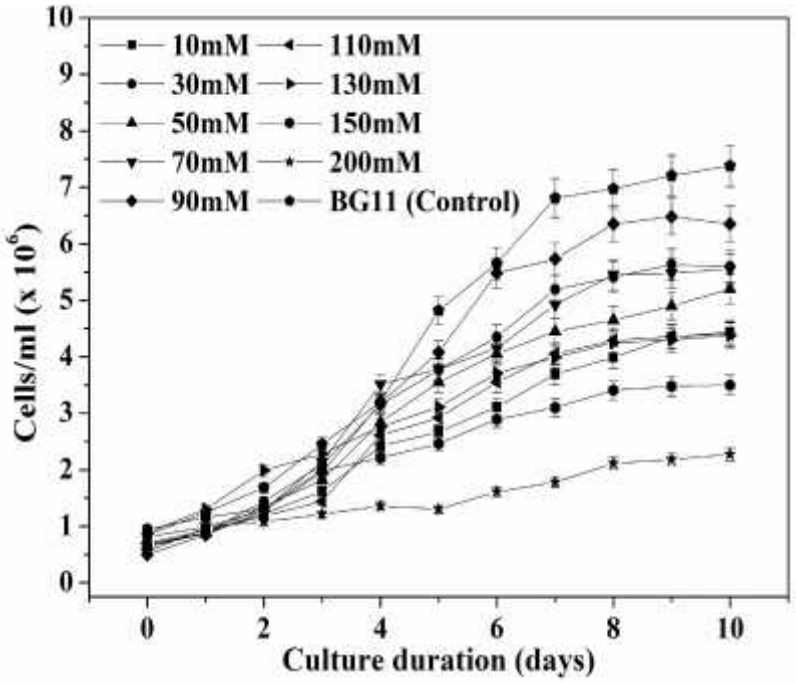


Figure 4.48 Growth curves of *Chloromonas* species cultivated in different NaCl concentration

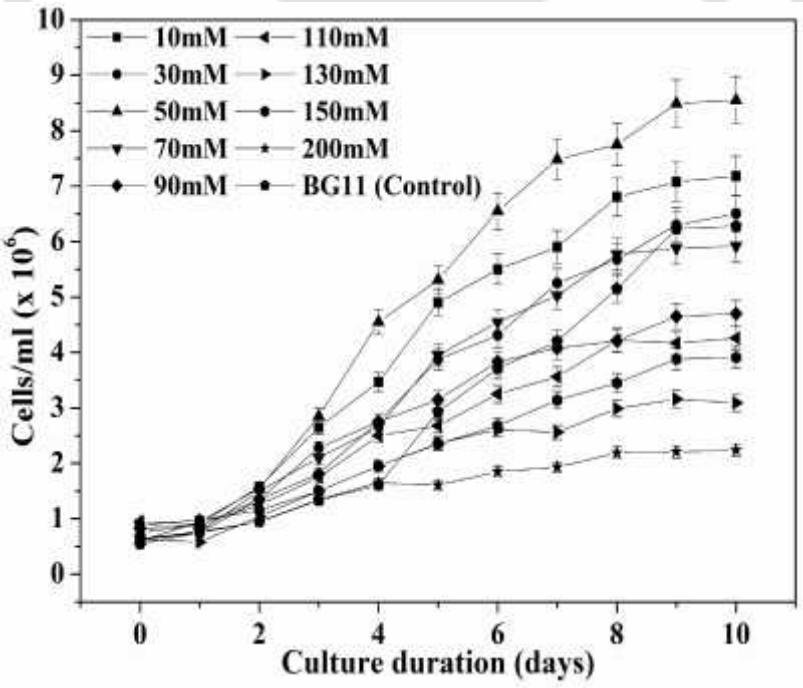


Figure 4.49 Growth curves of *Scenedesmus* species cultivated in different NaCl concentration

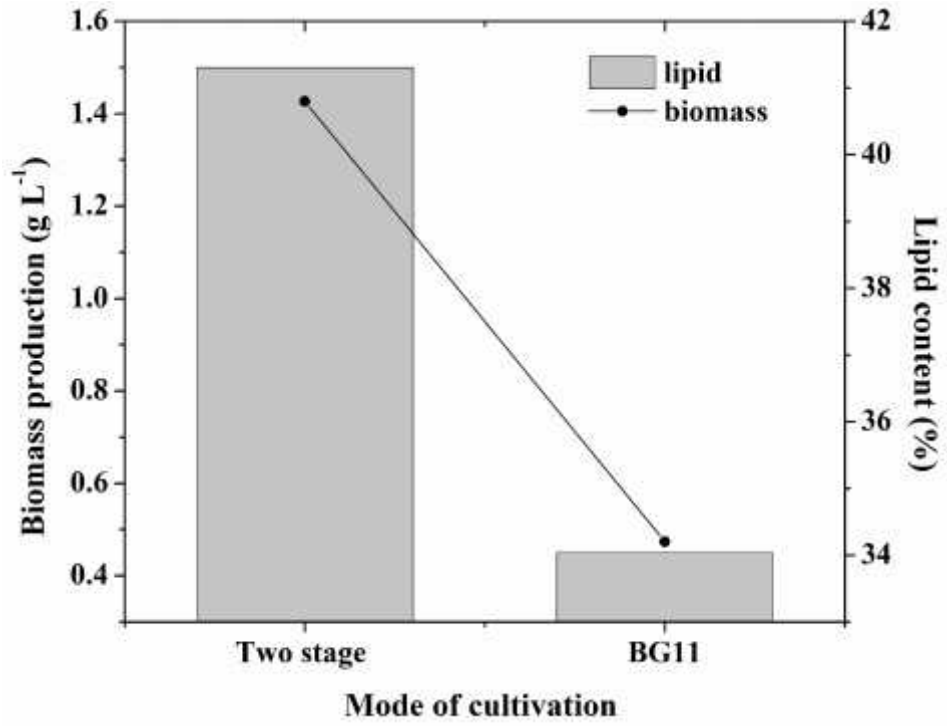


Figure 4.50 Biomass and lipid production of *Chloromonas* species cultivated in two-phase mode

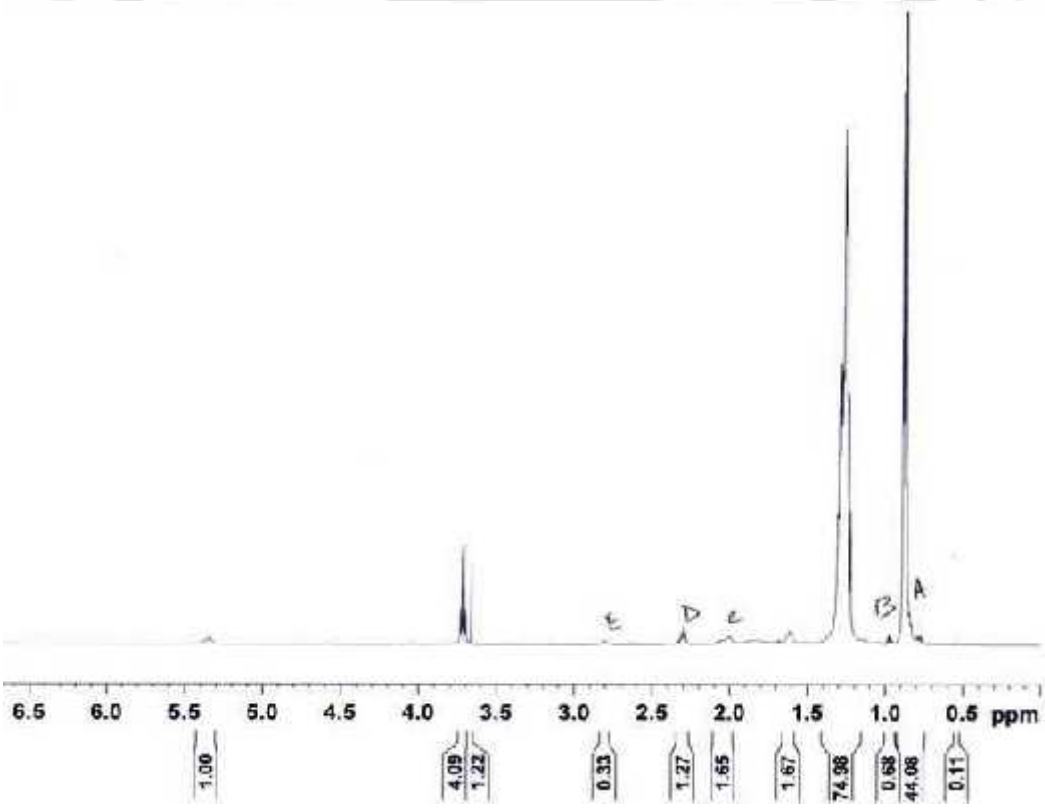


Figure 4.51 $^{1}$H NMR spectrum of methyl esters (FAME) of *Chloromonas* species recovered from two stage cultivation

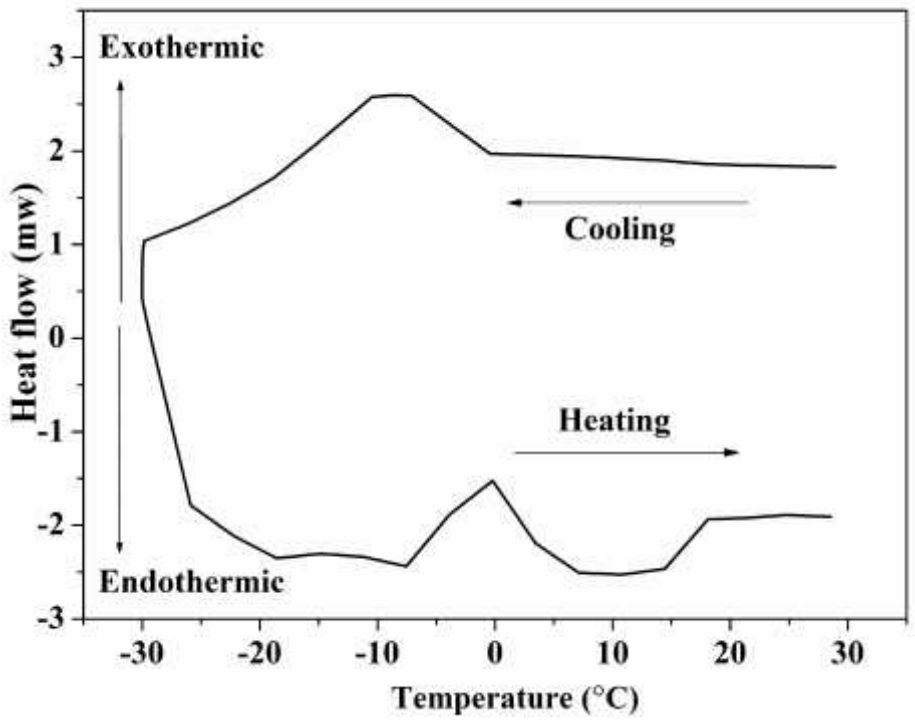


Figure 4.52 DSC thermogram of *Chloromonas* species FAME grown in two stage cultivation

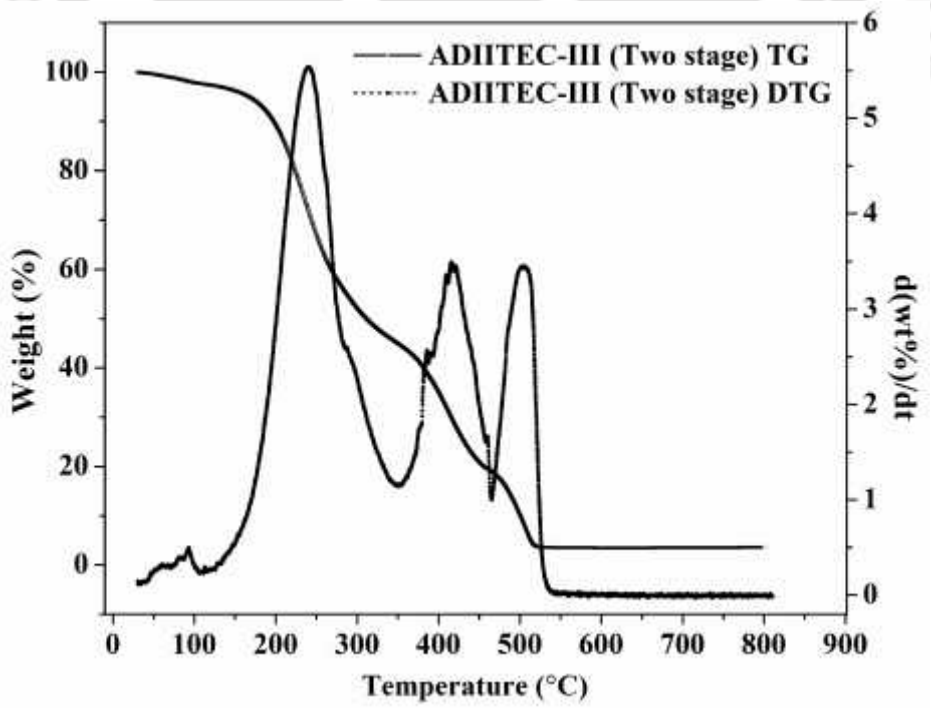


Figure 4.53 TG and DTG curves of *Chloromonas* sp FAME recovered from two stage cultivation

Table 4.20 Growth characteristics, biomass and lipid production of *Scenedesmus* species in different NaCl concentration

| NaCl Conc. | Specific growth rate $\mu$, ($day^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity ($g\ L^{-1}d^{-1}$) | Total lipid content (% DCW) |
|---|---|---|---|---|
| 10 mM | 0.22 ± 0.02 | 3.15 ± 0.27 | 0.3 ± 0.04 | 29.8 ± 0.2 |
| 30 mM | 0.23 ± 0.02 | 3.01 ± 0.32 | 0.34 ± 0.02 | 30 ± 0.5 |
| 50 mM | 0.26 ± 0.02 | 2.66 ± 0.2 | 0.37 ± 0.02 | 32.2 ± 0.25 |
| 70 mM | 0.22 ± 0.01 | 3.15 ± 0.14 | 0.28 ± 0.015 | 34.8 ± 0.2 |
| 90 mM | 0.17 ± 0.02 | 4.07 ± 0.57 | 0.21 ± 0.015 | 37.8 ± 0.5 |
| 110 mM | 0.15 ± 0.03 | 4.62 ± 0.96 | 0.2 ± 0.015 | 30.8 ± 0.2 |
| 130 mM | 0.13 ± 0.015 | 5.33 ± 0.58 | 0.14 ± 0.02 | 26.5 ± 0.4 |
| 150 mM | 0.12 ± 0.01 | 5.77 ± 0.48 | 0.11 ± 0.01 | - |
| 200 mM | 0.1 ± 0.015 | 6.93 ± 0.8 | 0.06 ± 0.015 | - |
| Control | 0.24 ± 0.02 | 2.89 ± 0.24 | 0.34 ± 0.02 | 34 ± 0.35 |

Table 4.21 Growth characteristics, biomass and lipid production of *Chloromonas* species in different NaCl concentration

| NaCl Conc. | Specific growth rate $\mu$, ($day^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity ($g\ L^{-1} d^{-1}$) | Total lipid content (% DCW) |
|---|---|---|---|---|
| 10 mM | 0.18 ± 0.02 | 3.88 ± 0.43 | 0.03 ± 0.01 | 28 ± 0.55 |
| 30 mM | 0.21 ± 0.03 | 3.34 ± 0.48 | 0.05 ± 0.01 | 29.8 ± 0.4 |
| 50 mM | 0.2 ± 0.002 | 3.48 ± 0.35 | 0.08 ± 0.02 | 31.5 ± 0.65 |
| 70 mM | 0.23 ± 0.02 | 3.02 ± 0.26 | 0.15 ± 0.02 | 34.8 ± 0.35 |
| 90 mM | 0.25 ± 0.02 | 2.75 ± 0.27 | 0.2 ± 0.02 | 35 ± 0.3 |
| 110 mM | 0.18 ± 0.03 | 3.92 ± 0.66 | 0.16 ± 0.02 | 35.8 ± 0.2 |
| 130 mM | 0.16 ± 0.02 | 4.37 ± 0.55 | 0.12 ± 0.03 | 36 ± 0.3 |
| 150 mM | 0.13 ± 0.015 | 5.24 ± 0.58 | 0.1 ± 0.04 | 37 ± 0.3 |
| 200 mM | 0.1 ± 0.01 | 6.37 ± 0.8 | 0.05 ± 0.025 | 38.8 ± 0.5 |
| Control | 0.21 ± 0.03 | 3.3 ± 0.48 | 0.03 ± 0.017 | 36.2 ± 0.4 |

Table 4.22 Fatty acid composition of methyl esters of *Chloromonas* species recovered from two stage cultivation

| Fatty acid profile | *Chloromonas* species FAME (two Stage) | *Chloromonas* species FAME (BG11) |
|---|---|---|
| Acid value | 0.48 | 0.48 |
| Iodine value | 114 g $I_2$ $100^{-1}$g oil | 112 g $I_2$ $100^{-1}$g oil |
| Calorific value | 42 MJ $Kg^{-1}$ | 40 MJ $Kg^{-1}$ |
| DSC (pour point) | -8ºC | -7ºC |
| Fatty acid compositions | | |
| Linolenic acid (18:3) | 1.519 % | 6.63 % |
| Linoleic acid (18:2) | 22.94 % | 21.04 % |
| Oleic acid (18:1) | 40.49 % | 32.54 % |
| Total saturates | 35.04 % | 39.79 % |

Table 4.23 Comparison of temperature characteristics for the thermo gravimetric analysis of methyl esters of *Chloromonas* sp recovered from BG11 media and two stage cultivation

| Microalgae | Onset temperature (ºC) | Mass loss (%) | | |
|---|---|---|---|---|
| | | 10% | 50% | 90% |
| ADIITEC-III FAME | 161 | 174 ºC | 249 ºC | 477 ºC |
| ADIITEC-III FAME (two stage ) | 171 | 195 ºC | 311 ºC | 498 ºC |

## 4.5. Cultivation of Chloromonas species ADIITEC-III and Scenedesmus species GUBIOTJT116 in cow dung and piggery waste as a cheap media composition to produce biodiesel and its property assessment

The large scale cultivation of microalgae generally requires addition of nutrients (e.g., N and P) in large quantities, which is one of the expensive steps in processing algae to biofuel (Levine *et al.*, 2011). According to the life-cycle assessment (LCA), utilization of synthetic fertilizers for algal feedstock production could account for approximately 50% of the energy and greenhouse gas (GHG) emissions (Clarens *et al.*, 2010). Instead agricultural and domestic wastes can be employed as a promising source of nutrients for algal cultivation (Cantrell *et al.*, 2008). As a result, to achieve cost-effective microalgae biomass production, the domestic wastes can be effectively replaced by the synthetic nutrient inputs.

This chapter focuses on the characterization and utilization of animal waste for growth and biomass production of two indigenous strains i.e. *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116. The cow dung and piggery waste are used as synthetic alternative media which are known to be rich in nutrients especially the much needed nitrogen. In support, elemental analysis of the wastes was carried out and based on the compositions a range of concentrations i.e. 5%, 10% and 15% were considered for the experimentation. The lipid obtained at optimized condition was transesterified to produce biodiesel and the produced biodiesel was further characterized for various properties, and was compared with ASTM biodiesel standards.

### 4.5.1. Elemental analysis of cow dung and piggery waste

The cow dung and piggery waste are characterized for their elemental composition, and the results obtained are summarized in Table 4.24. The waste used in this study showed

the presence of major element, i.e. nitrogen and phosphorous. As shown in Table 4.25, the total nitrogen content was found to be a maximum (5400 mg $kg^{-1}$) in piggery waste compared to cow dung waste. Findings of the study showed that in both the waste, most of the nitrogen was present in the form of ammonium-N. The tolerance of microalgae towards the presence of excess ammonium-N may vary from species to species. It has been reported that the non-filamentous green algae such as *Chlorella* and *Scenedesmus* species could tolerate high concentration of nitrates or ammonium solutions (Chen *et al*., 2012). The total phosphorous content was more in piggery waste i.e. 258.75 mg $kg^{-1}$ than cow dung waste. Though phosphorous is a necessary entity for the microalgae cultivation but it has much less effect on algal growth compared to excessive nitrogen (Chen *et al*., 2012). Some traces of heavy metals have also been observed during the study and the micronutrient needed for microalgae growth was also present in the form of metals like Cu, Zn, Ni, Fe etc in both the waste sample. All the elements required for the optimum cultivation of microalgae was obtained in both the organic compositions and therefore it could be further utilize as a cheap alternate media source for mass cultivation of microalgae species.

### 4.5.2. Effect of different concentration of cow dung and piggery waste on growth, biomass and lipid content of ADIITEC-III and GUBIOTJT116

The effect of cow dung waste concentrations on algal growth and composition was studied by varying the concentration in the range 5%, 10% and 15%. The influence of cow dung waste on microalgae cultivation is also compared with BG11 medium. The growth curves of *Chloromonas* and *Scenedesmus* sp. grown in cow dung and piggery waste are represented in Figure 4.54, 4.55, 4.56, 4.57 respectively. The growth curves showed lag phase of two days, where both the strain ADIITEC-III and GUBIOTJT116

demonstrated fast growth for 15% cow dung waste with maximum specific growth of 0.21 ± 0.011 and 0.25 ± 0.011 $d^{-1}$ respectively. The concentration appears to support exponential growth phase of both the species till 7$^{th}$ day, after which declining phase was observed. The growth behavior of strains was typically compared with optimized synthetic medium i.e. BG11, which represent a similar growth patterns as depicted in Table 4.26, 4.27, 4.28 and 4.29. In case of GUBIOTJT116 15% concentration of cow dung waste showed higher biomass yield (3.2 g $L^{-1}$) compared to ADIITEC-III (0.6 g $L^{-1}$). On the other hand, lower concentration (5% and 10%) doesn't support robust growth but enhance the lipid production. According to a study conducted by Levine *et al*., (2011), algal cells achieve highest cell density at the nitrogen (in the form of nitrate) concentration range 70 to 140 mg $L^{-1}$; but again, it may vary from species to species (Levine *et al*., 2011). Similarly, in the present study 15% cow dung waste concentration found to contain 157.8 mg $L^{-1}$ of nitrate (Table 4.25) which might be one of the reasons to enhance the growth of ADIITEC-III and GUBIOTJT116. Although, the nitrate content in 15% cow dung concentration was quite high, but still increase in the growth rates were not significant. This may be due to the soluble colored organic compounds of the cow dung, which hinders the clarity of water and interferes with light penetration (Wang *et al*., 2012). In addition, ADIITEC-III and GUBIOTJT116 grown at lower concentrations of cow dung (5% and 10%) showed slow growth rates, but attributes increase lipid yield.

Likewise, for piggery extract study was carried out using an identical range of concentrations. The higher concentrations of piggery extract (i.e. 10% and 15%) revealed better biomass and growth for GUBIOTJT116 (Table 4.29). Whereas, concentration beyond 10% showed insignificant effect on ADIITEC-III growth Figure 4.55 and 4.57, growth curves of both the species depicted the lag phase of two days at all the

concentrations of piggery waste. However, the species continue to extend its exponential growth till 7 to 8 days. As summarized in Table 4.29, both maximum biomass production and growth of GUBIOTJT116 reached the highest value at 15% concentrations of piggery waste with productivity of 0.25 ± 0.02 g $L^{-1}d^{-1}$ and a specific growth rate of 0.22 ± 0.015 $d^{-1}$. However, cell growth was negatively impacted with a specific growth rate of 0.14 ± 0.015 $d^{-1}$ at lower concentration (i.e. 5%). The growth and biomass production of GUBIOTJT116 in piggery waste responded better than the ADIITEC-III. This attributed to the high nitrate content (5400 mg $kg^{-1}$), in which ammonical nitrate was in a major quantity (4780 mg $kg^{-1}$) as shown in Table 4.24. It has been reported that non-filamentous green algae such as *Chlorella* and *Scenedesmus* could tolerate increased amount of nitrates or ammonium solutions (Chen *et al*., 2012). ADIITEC-III appeared to be intolerant to increased piggery waste concentration (15%) in which the nitrate content was recorded to be 400 mg $L^{-1}$. The species exhibited poor algal biomass production, which may be due to deleterious effect of nitrogen at higher concentrations (Arumugam *et al*., 2013). The minimum biomass productivity of 0.042 ± 0.007 g $L^{-1}d^{-1}$ was observed in ADIITEC-III at 15% piggery waste concentration. Similar pattern of lipid yield was exhibited in varied concentrations of piggery waste as well as cow dung waste. ADIITEC-III culture grown at lower piggery waste concentrations (5% and 10%) enhances the lipid yield to 35 ± 0.91 and 28.6 ± 0.52% respectively.

### 4.5.3. Characterization and evaluation of synthesized methyl esters obtained at optimized condition

Generally, percentage of triacylglycerides (TAG) of total lipid varies according to the species and physiological state of the culture **(**Wang *et al*., 2010). The biomass obtained at optimized media condition (cow dung and piggery waste) based on the high lipid yield

was further processed into methyl esters (FAME). This FAME content was compared with the methyl esters obtained from culture grown in BG11 media. The physico-chemical characteristics of synthesized methyl esters (FAME) of ADIITEC-III and GUBIOTJT116 were evaluated according to the American Standards for Testing Materials ASTM 6751 and represented in Table 4.30. In this study, the properties relevant to fuel use such as acid value, iodine value, calorific value and cold flow property were determined and compared with ASTM standards.

#### 4.5.3.1. Fatty acid profile of methyl esters (FAME) of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116

$^{1}$H NMR spectra of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 methyl esters (FAMEs) are presented in Figure 4.59, 4.60, 4.61 and 4.62 respectively. The fatty acid composition is determined as the methyl esters of fatty acids by $^{1}$H NMR spectroscopy. The obtained lipid was transesterified to produce fatty acid methyl esters (FAMEs) by methylation (Brennan and Owende, 2010; Chisti, 2007; Hu *et al*., 2008). However, all fatty acids are not suitable for biodiesel production. In fact, triacylglycerides are more responsible than phospholipids and glycolipids as they show higher fatty acid content (Miao and Wu, 2006; Ördög *et al*., 2013). In plant cells, the most abundant TAGs are palmitic acid, stearic acid, unsaturated oleic acid, linoleic and linolenic acid (Gurr and James, 1980; Wang *et al*., 2010). Table 4.30 shows the fatty acid profiles of the species cultivated in cow dung and piggery waste at optimized concentrations. In both the species, oleic acid was more prevalent and found to be in higher amount than the saturated fatty acids. In particular, results of the study revealed a dramatic increase of linoleic acid in piggery waste. Namely, the proportion of linoleic and linolenic acid content was high in the piggery waste while the saturated fatty acids

declined significantly. On the other hand, FAMEs derived from cells in cow dung showed opposite trend. Both the species showed a variation in their fatty acid profile. The fatty acid profile of *Chloromonas* species ADIITEC-III in cow dung revealed decreased in linolenic acid content from 19% to 2.75%. Decreased was also observed in GUBIOTJT116 (linolenic acid) from 13.79% to 9.06%. The oleic acid accumulation trend was similar in both the species; in fact it was more in cow dung culture than piggery waste. The fatty acid profile trend of ADIITEC-III grown in BG11 media was oleic acid > saturated fatty acid > linoleic acid > linolenic acid. But the trend was different when grown in piggery waste with main fatty acids being linoleic acid > oleic acid > linolenic acid > saturated fatty acid. Whereas, fatty acid profile observed in GUBIOTJT116 grown in BG11 media was similar to that of ADIITEC-III. The study also observed sudden variation in fatty acid profile of both the species with respect to linoleic, linolenic acid and saturated fatty acid content when grown in piggery waste.

It should be noted that the data obtained are the fatty acid composition generated from the total lipid extracts instead of individual lipid classes. Therefore, slight deviations in the data are expected (Hu *et al*., 2008). In addition, the variation observed in fatty acid profile was much obvious as the microalgae's are known to vary quantitatively and qualitatively depending on their growth period and physiological status such as temperature, light intensity and nutrients respectively (Hu *et al*., 2008). However, fatty acid compositions of all the parameters showed satisfied proportion of oleic acid which is an ideal component of biodiesel. According to the literature high oleic acid content provides a reasonable balance of fuel properties (Abou-Shanab *et al*., 2013).

**4.5.3.2. Characterization and evaluation of synthesized methyl esters**

The synthesized methyl esters of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 was evaluated with the American Standards for Testing Materials ASTM 6751 for biodiesel fuel.

The main biodiesel properties of both the species were estimated and tabulated in Table 4.30. The *Chloromonas* species ADIITEC-III grown in 5% cow dung and piggery waste showed the highest lipid yield and at this media condition, acid value was recorded to be 0.48 mg KOH $g^{-1}$ (Section 4.5.2). On the other hand, initial acid value of GUBIOTJT116 oil derived from BG11 medium was recorded as 14 mg KOH $g^{-1}$. However, after acid catalyzed transesterification acid value was reduced to 0.52 mg KOH $g^{-1}$. As depicted in Table 4.28, insignificant variation in the acid value (0.52 mg KOH $g^{-1}$) was observed for the methyl esters synthesized from microalgae grown in 5% of cow dung and piggery waste. According to ASTM standard, acid value of methyl esters should be lower than 0.50 mg KOH $g^{-1}$. In case of GUBIOTJT116 acid value of methyl esters at all the culture parameters was recorded to be higher than ASTM standard. The sample also showed high calorific value which measures the energy produced while burning the fuel completely (Arias-Peñarands *et al*., 2013). Similarly, calorific value (41 MJ $Kg^{-1}$) was also high for ADIITEC-III FAME obtained from piggery and cow dung waste. The calorific values obtained from waste samples were slightly higher than that of methyl esters derived from BG11 medium (40 MJ $Kg^{-1}$). Whereas, in case of GUBIOTJT116, calorific value of the methyl esters derived from BG11 medium and cow dung waste was in a similar range, i.e. 41 MJ $Kg^{-1}$, but the value was slightly low in piggery waste (i.e. 38.8 MJ $Kg^{-1}$).

The iodine value is the measure of degree of unsaturation of the methyl esters. In fact, more the double bonds in a fatty acid chain, higher the iodine value of the sample

(Knothe, 2012). The methyl esters of ADIITEC-III obtained from cow dung and piggery waste showed insignificant variation in iodine values, i.e. 114 g $I_2$ 100 $g^{-1}$ respectively. However, slight variation in the iodine value was observed for methyl esters of GUBIOTJT116 (i.e. 114 and 116 g $I_2$ 100 $g^{-1}$), the values obtained are well within the limits of ASTM D6751 biodiesel standards. Higher iodine values of the sample may result in the polymerization of glycerides and deposition of lubricant in the engine (Francisco *et al*., 2010).

Low temperature performance is one of the most important properties for users of biodiesel, and it is mainly indicated by cloud point and pour point. In this study pour point of the sample was estimated by DSC technique. Typical DSC thermogram of ADIITEC-III and GUBIOTJT116 for cow dung and piggery waste is shown in Figure 4.63, 4.64, 4.65 and 4.66. The difference in the pour point of studied methyl esters sample can be explained from the fatty acid profile (Table 4.30). Higher the unsaturated fatty acids content in the oil better the cold flow properties (Borugadda *et al*., 2014). The unsaturated ester due to different three-dimensional conformations crystallizes at lower temperatures than the saturated ester. The intermolecular interactions in unsaturated ester molecules are weaker than the saturated esters which allow the unsaturates to crystallize at the lower temperatures (Zuleta *et al*., 2012). Hence, the thermogram of methyl esters predicts better during cooling than the data obtained during heating (Dunn *et al*., 1999).

As can be seen from the Table 4.30, total saturated ester content is responsible for the variation in pour point. Therefore, from the comparative evaluation of fatty acid composition, ADIITEC-III grown in cow dung and piggery waste was expected to show favorable cold flow properties than GUBIOTJT116 and same was noticed in the present study. This may be because of higher unsaturation content (81.84%, 86.7%) in the

methyl esters of ADIITEC-III grown in cow dung and piggery waste respectively. As a result, ADIITEC-III grown in cow dung and piggery waste showed higher pour point of -13ºC and -14ºC respectively. In comparison, these pour point values are higher than the pour point values of GUBIOTJT116 -7ºC and -14ºC respectively.

**4.5.3.3. Thermogravimetric analysis methyl esters (FAMEs) of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 in cow dung and piggery waste**

The weight loss and derivative of TGA curves (DTG) of ADIITEC-III and GUBIOTJT116 methyl esters sample is shown in Figure 4.67, 4.68, 4.69, 4.70 respectively. The TGA analysis was performed under nitrogen atmosphere at a heating rate of 10 ºC $min^{-1}$. The DTG curves of microalgae sample revealed that the thermal degradation of the methyl esters occurred in three stages. Thermal stability of methyl esters sample was estimated by onset temperature and is defined as the temperature at which decomposition of the sample starts (Borugadda *et al*., 2014; Peng *et al*., 2001). DTG curves of methyl esters sample showed three steps of mass loss attributed to the decomposition and/or volatilization in various temperature ranges. Similar decomposition pattern was noticed by Shuping *et al*., (2010) and Marcilla *et al*., (2009) during their study on TGA analysis of methyl esters sample and found to be in agreement with the present study. The onset temperature of fatty acid methyl esters obtained for ADIITEC-III grown in cow dung and piggery waste was found to be 147ºC and 145ºC respectively (Table 4.31). On the other hand, onset temperature values for GUBIOTJT116 were 182ºC and 174ºC respectively.

The results obtained are comparable with the methyl esters of respective species grown in BG11 medium as discussed earlier section 4.2.5.1. The variations in the results

were mainly due to the chemical structure of esters and fatty acid compositions. It has been reported that esters with high unsaturated fatty acids content are thermally less stable than saturated ones (Borugadda *et al*., 2013). The results obtained could be better explained from the weight loss profiles (Table 4.31) of methyl ester samples of both the species. The methyl ester of ADIITEC-III in cow dung and piggery waste showed 10% weight loss at 170ºC and 168ºC, while for GUBIOTJT116 weight loss was observed at same temperature i.e. 198ºC. The weight loss includes moisture content and decomposition of mono and polyunsaturated fatty acids (i.e. oleic, linoleic and linolenic acid) (Borugadda *et al*., 2014).

All the components of ADIITEC-III methyl esters (i.e. cow dung and piggery waste) which accounts for almost 50% weight loss were decomposed at 247ºC and 241ºC respectively. In the case of GUBIOTJT116 FAME, 50% weight loss was observed at 386ºC and 337ºC respectively. This weight loss includes the breakdown of saturated fatty acids such as palmitic and stearic acid. Similarly, the components of ADIITEC-III FAME in cow dung and piggery waste accounted for almost 90% weight loss at around 468ºC and 448ºC respectively. Similar observation was also recorded with GUBIOTJT116 methyl ester sample in cow dung and piggery waste, where 90% weight loss was observed at 488ºC and 477ºC respectively. All the studied samples were completely burnt out after being heated up to 800ºC. From the above discussion, it was clear that initiation and complete thermal degradation of FAME sample of ADIITEC-III and GUBIOTJT116 in piggery waste were within a temperature range inferior to cow dung sample, which may be due to certain compositional features of fatty acids. According to the reports, certain compositional features of fatty acid such as chain length, degree of unsaturation and branching of the chain influence the thermo-oxidative

properties of fatty ester molecule (Borugadda *et al*., 2014). Hence, the data obtained shows that the FAME samples of ADIITEC-III and GUBIOTJT116 in cow dung are thermally more stable than piggery waste sample. Interestingly, both the species in cow dung showed better thermal stability than the culture grown in BG11 medium. The thermal stability is indeed a very significant factor to determine the storage condition of biodiesel and its life span (Borugadda *et al*., 2013).

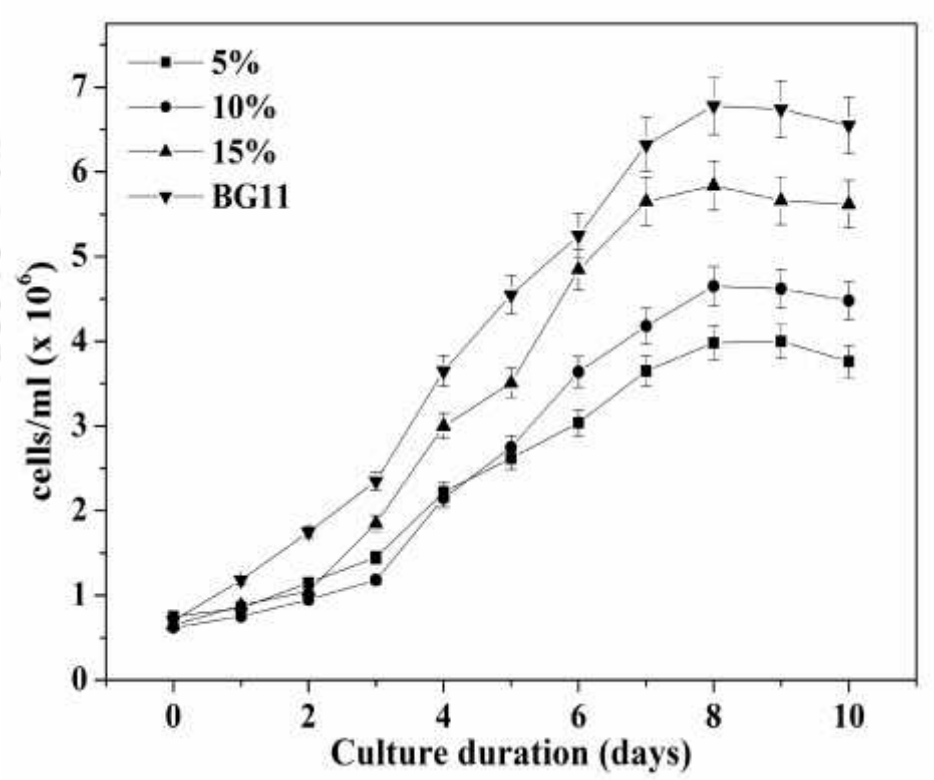


Figure 4.54 Growth curves of *Chloromonas* species in different concentrations of cow dung waste i.e. 5%, 10% and 15%

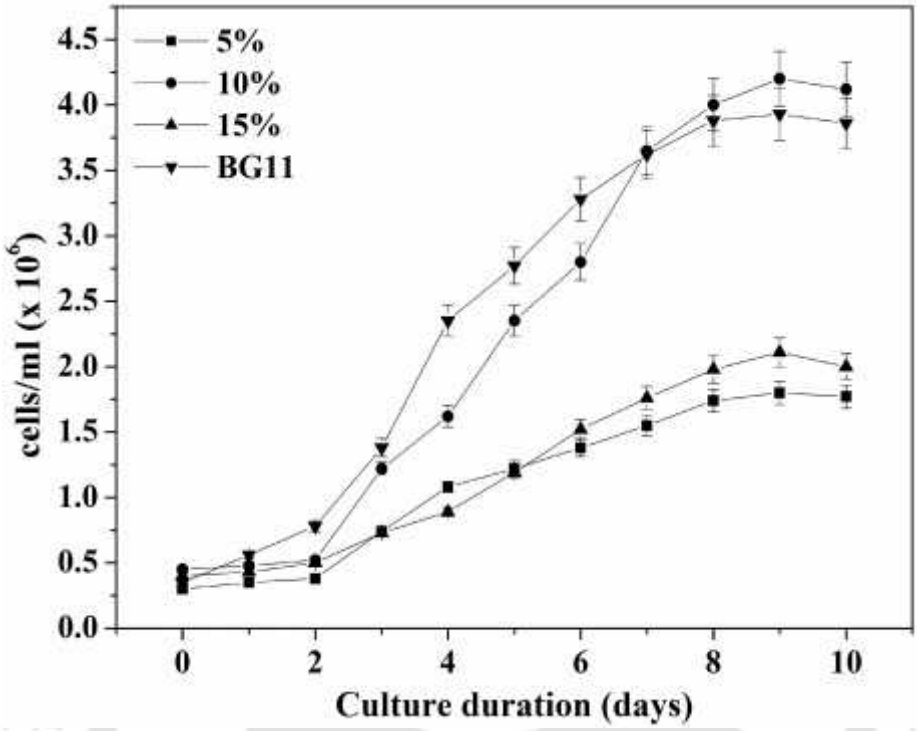


Figure 4.55 Growth curves of *Chloromonas* species in different concentrations of piggery waste i.e. 5%, 10% and 15%

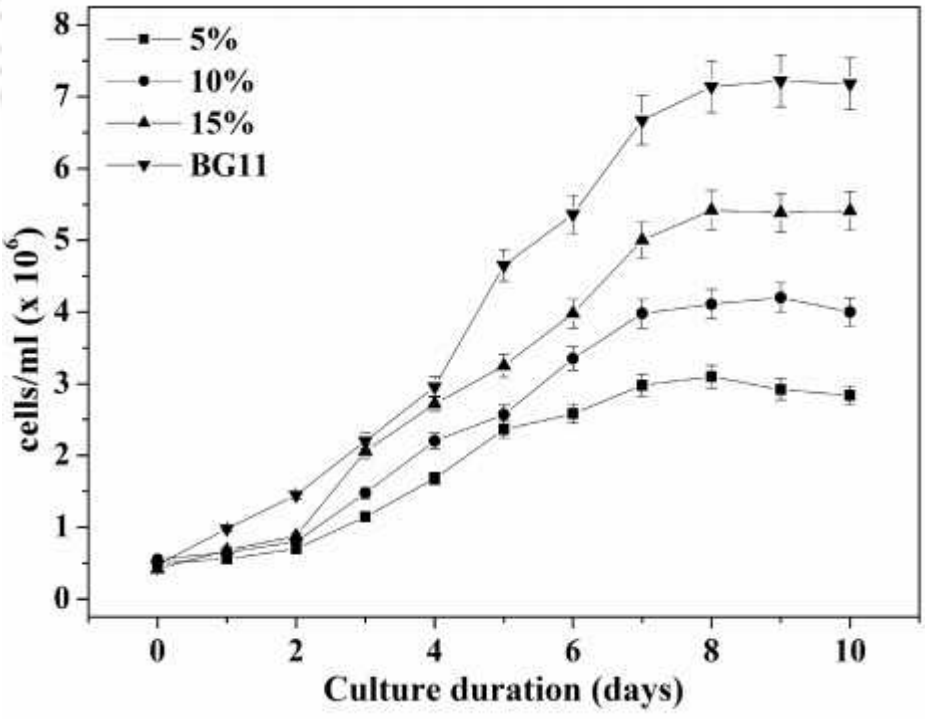


Figure 4.56 Growth curves of *Scenedesmus* species in different concentrations of cow dung waste i.e. 5%, 10% and 15%

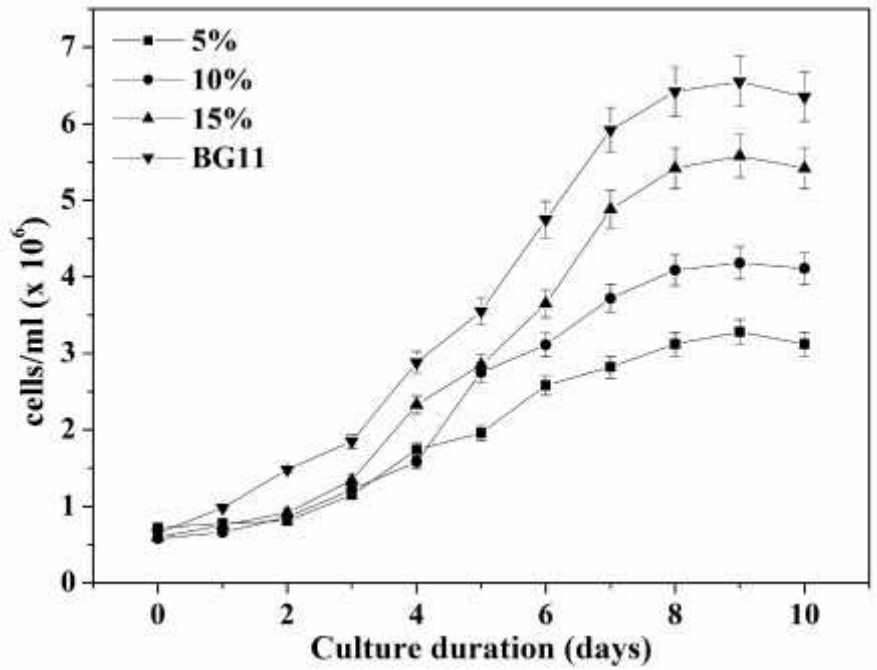


Figure 4.57 Growth curves of *Scenedesmus* species in different concentrations of piggery waste i.e. 5%, 10% and 15%

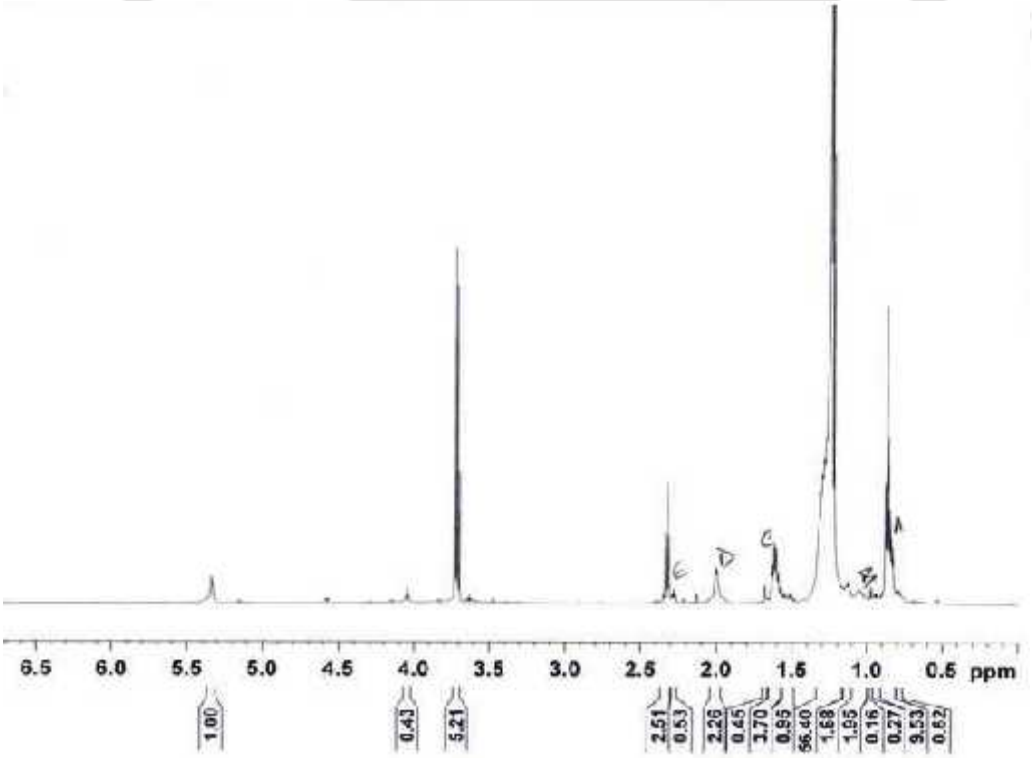


Figure 4.58 $^{1}$H NMR spectrum of methyl esters (FAME) of *Chloromonas* species ADIITEC-III grown in 5% of cow dung compositions, the strong singlet peak at 3.65 ppm is indicative of due to oxymethylic hydrogen referent to methyl esters

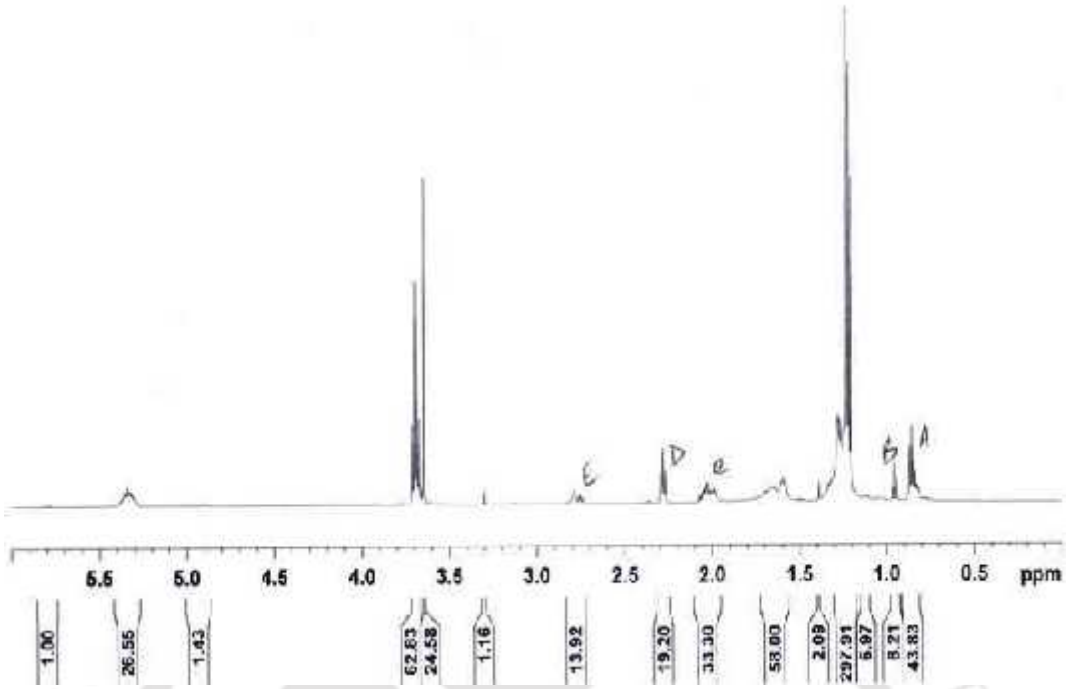


Figure 4.59 $^{1}$H NMR spectrum of methyl esters (FAME) of *Chloromonas* species ADIITEC-III grown in 5% of piggery waste, the strong singlet peak at 3.65 ppm is indicative of due to oxymethylic hydrogen referent to methyl esters

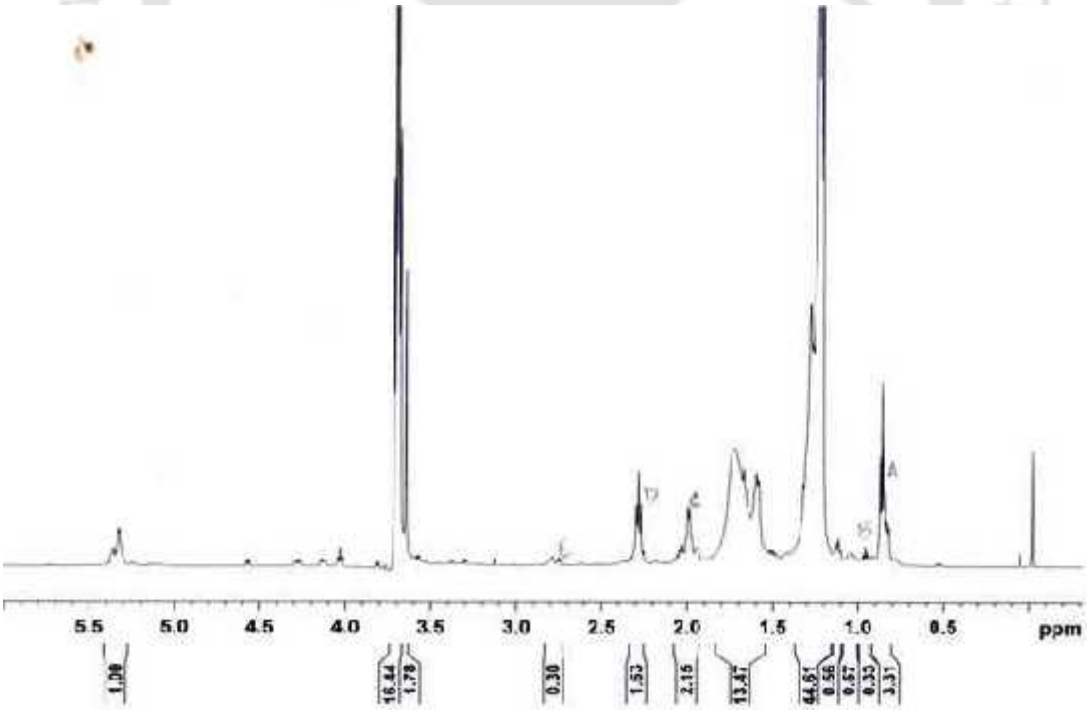


Figure 4.60 $^{1}$H NMR spectrum of methyl esters (FAME) of *Scenedesmus* species grown in 5% of cow dung waste, the strong singlet peak at 3.65 ppm is indicative of due to oxymethylic hydrogen referent to methyl esters

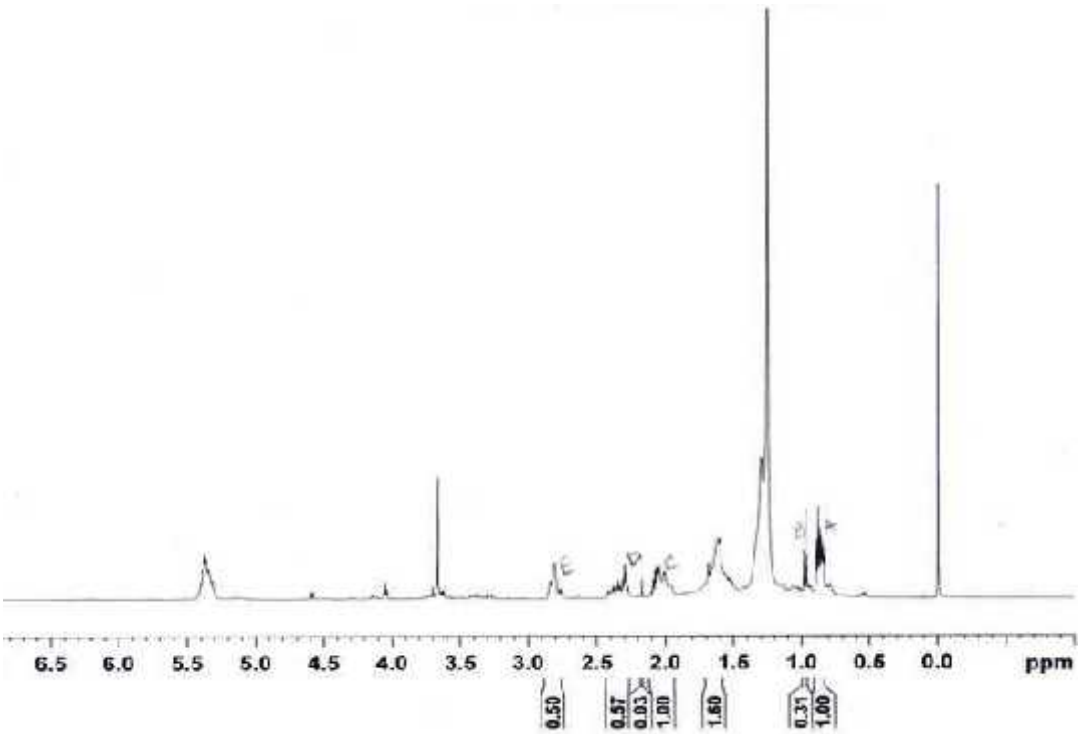


Figure 4.61 $^{1}$H NMR spectrum of methyl esters (FAME) of *Scenedesmus* species grown in 5% of piggery waste, the strong singlet peak at 3.65 ppm is indicative of due to oxymethylic hydrogen referent to methyl esters

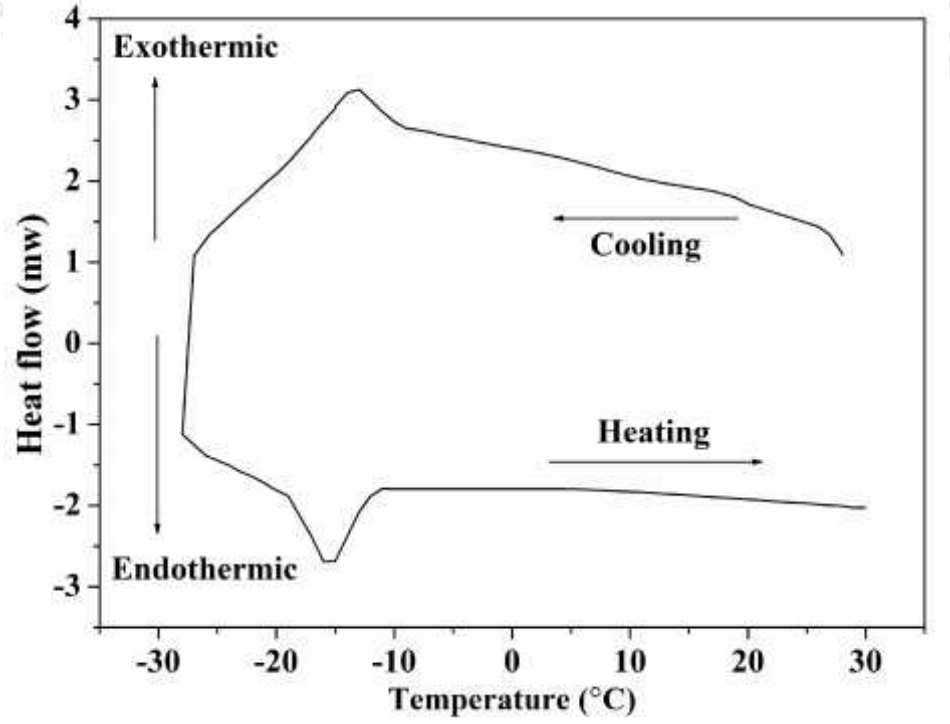


Figure 4.62 DSC thermogram of *Chloromonas* species FAME derived from 5% cow dung @ 5°C min$^{-1}$ under $N_2$ atmosphere

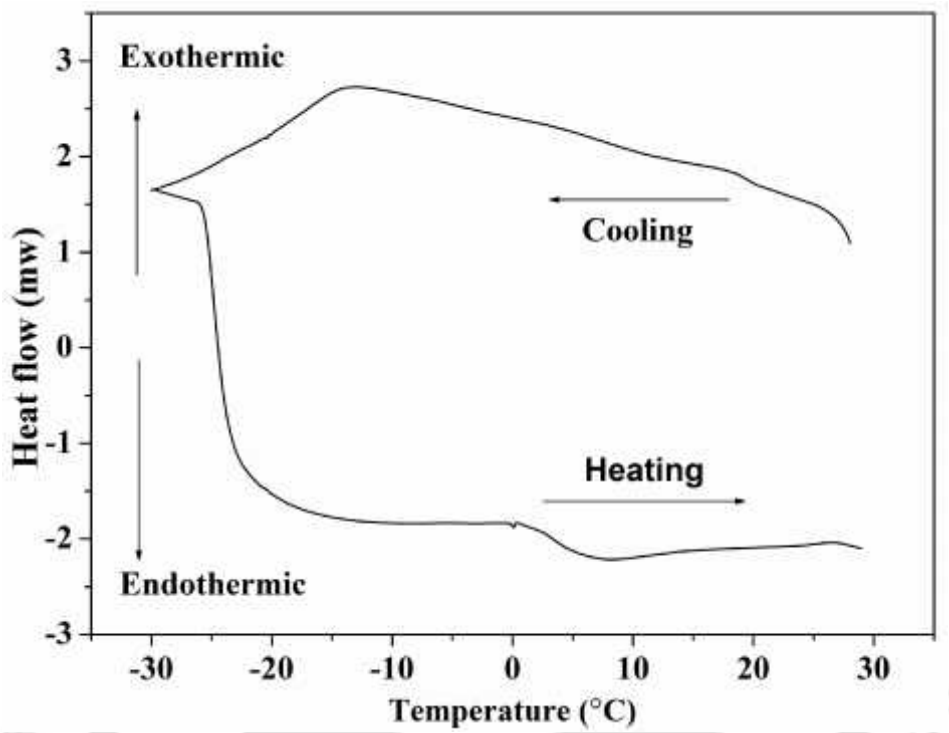


Figure 4.63 DSC thermogram of *Chloromonas* species FAME derived from 5% piggery waste @ 5°C $min^{-1}$ under $N_2$ atmosphere

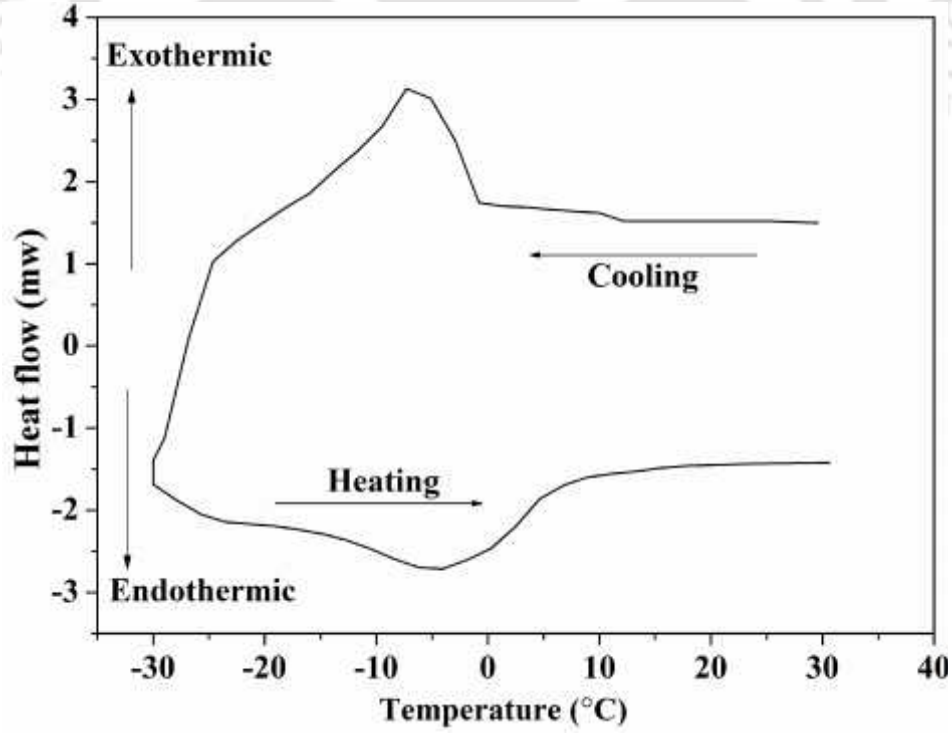


Figure 4.64 DSC thermogram of *Scenedesmus* species GUBIOTJT116 FAME derived from 5% cow dung @ 5°C $min^{-1}$ under $N_2$ atmosphere

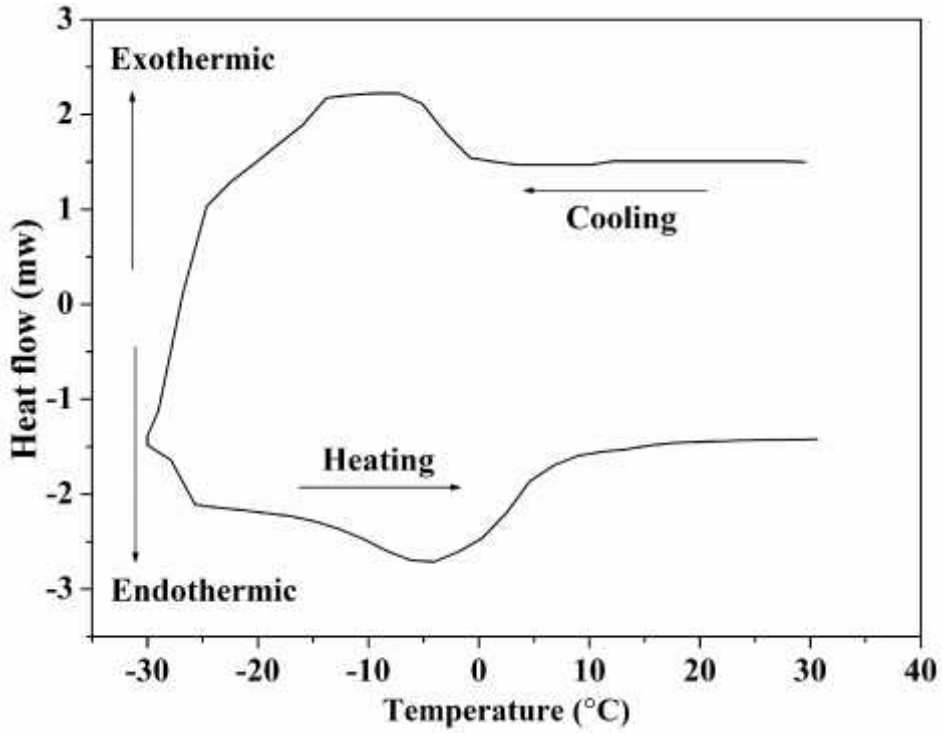


Figure 4.65 DSC thermogram of *Scenedesmus* species GUBIOTJT116 FAME derived from 5% piggery waste @ 5°C $min^{-1}$ under $N_2$ atmosphere

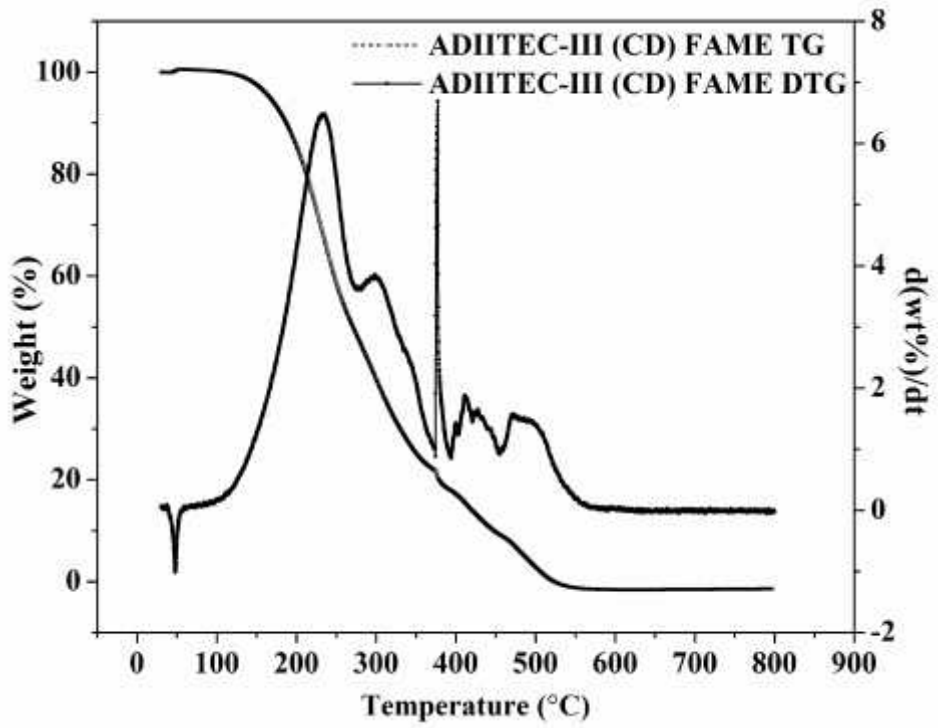


Figure 4.66 TG and DTG curves of ADIITEC-III FAME in cow dung @ 10°C $min^{-1}$ under $N_2$ atmosphere

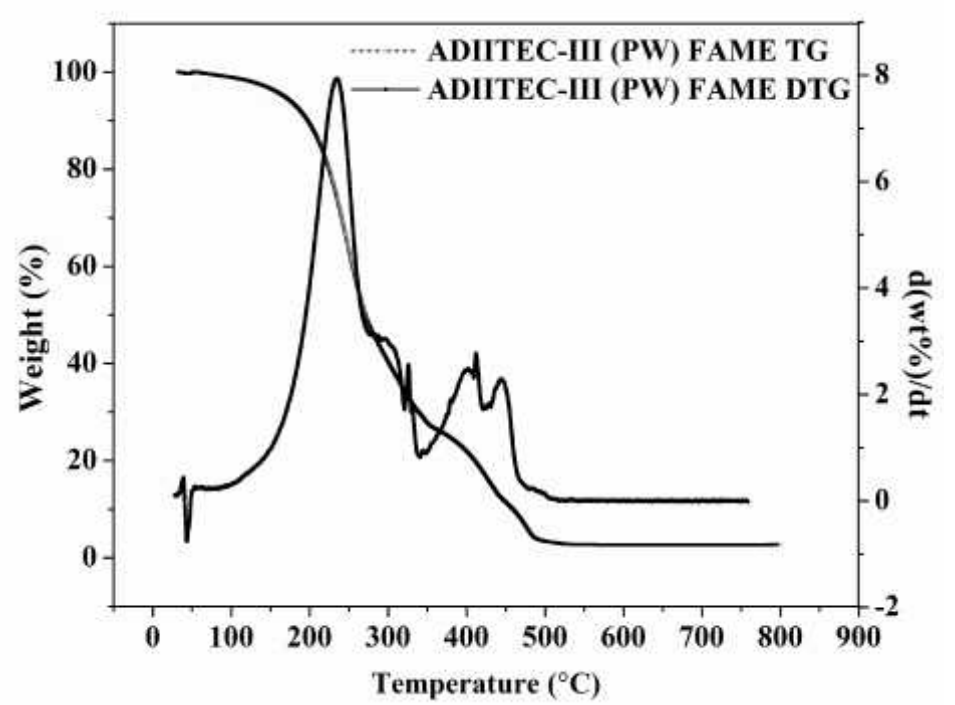


Figure 4.67 TG and DTG curves of ADIITEC-III FAME in piggery waste @ 10°C $min^{-1}$ under $N_2$ atmosphere

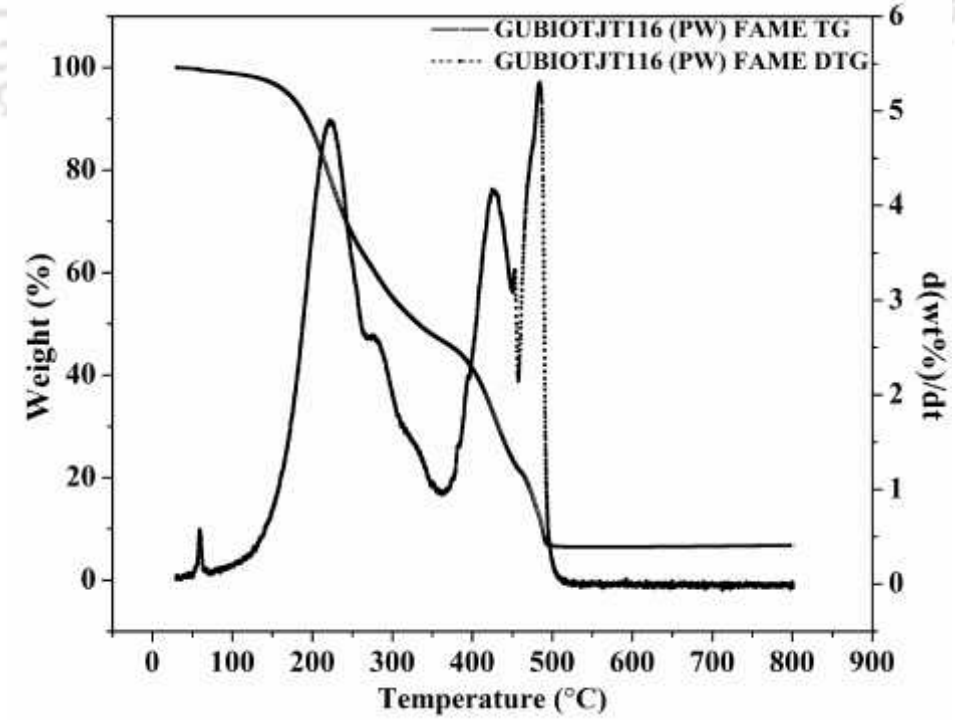


Figure 4.68 TG and DTG curves of *Scenedesmus* species FAME in piggery waste @ 10°C $min^{-1}$ under $N_2$ atmosphere

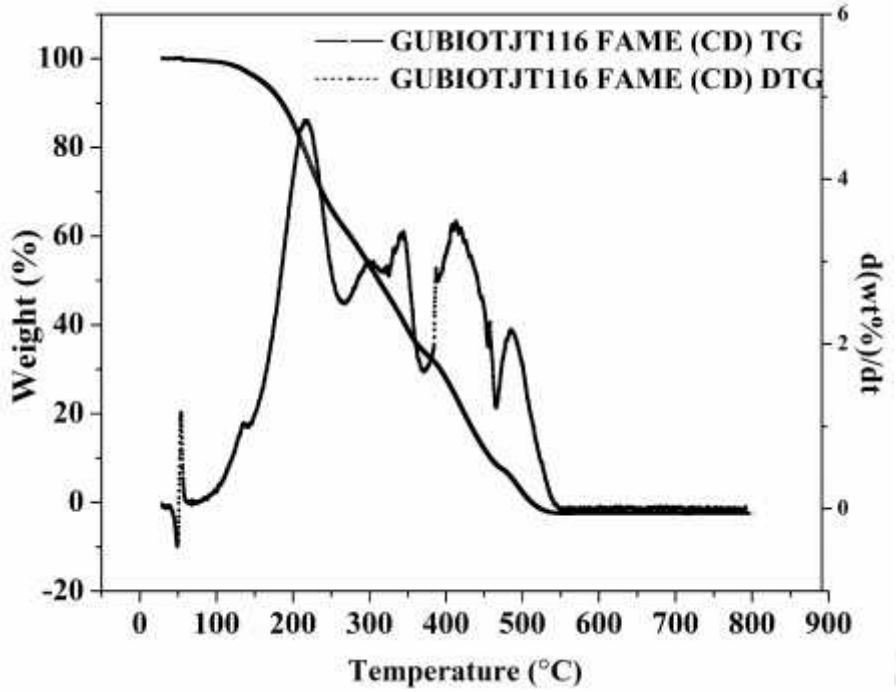


Figure 4.69 TG and DTG curves of *Scenedesmus* species FAME in cow dung waste @ 10°C $min^{-1}$ under $N_2$ atmosphere

Table 4.24 Elemental characterization of cow dung and piggery waste

| Characteristics of animal waste | Cow dung waste (mg $Kg^{-1}$) | Piggery waste (mg $Kg^{-1}$) |
|---|---|---|
| Zinc(Zn) | 171 | 191.25 |
| Copper (Cu) | 55.75 | 62.25 |
| Manganese (Mn) | 395.75 | 422.25 |
| Iron (Fe) | 9493.75 | 24000 |
| Nickel (Ni) | 232.5 | 270.75 |
| Lead (Pb) | 870 | 972.5 |
| Cadmium (Cd) | 53.5 | 61.5 |
| Chromium (Cr) | 63 | 90.25 |
| Sodium (Na) | 610 | 755 |
| Potassium (k) | 10585 | 4610 |
| Calcium (Ca) | 3485 | 2235 |
| Total phosphorous | 197.45 | 258.75 |
| Available phosphorous | 119.1 | 106.1 |
| Total nitrate | 2105 | 5400 |
| Ammonical nitrate | 1645 | 4780 |

Table 4.25 Concentration of total nitrate and ammonical nitrate present in 5%, 10% and 15% of cow dung and piggery waste

| Concentration | Cow dung waste | | Piggery waste | |
|---|---|---|---|---|
| | Total nitrate (mg $L^{-1}$) | Ammonical nitrate (mg $L^{-1}$) | Total nitrate (mg $L^{-1}$) | Ammonical nitrate (mg $L^{-1}$) |
| 5% | 52.62 | 41.12 | 135 | 119.5 |
| 10% | 105.25 | 82.25 | 270 | 239 |
| 15% | 157.8 | 123.37 | 400 | 458.5 |

Table 4.26 Growth characteristics, biomass and total lipid content of *Chloromonas* species in different concentrations of cow dung extract i.e. 5%, 10% and 15%

| Cow dung extract (%) | Specific growth rate $\mu$, ($day^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity (g $L^{-1}d^{-1}$) | Total lipid content (% DCW) |
|---|---|---|---|---|
| 5% | 0.16 ± 0.015 | 4.26 ± 0.38 | 0.03 ± 0.011 | 34.8 ± 0.91 |
| 10% | 0.19 ± 0.015 | 3.53 ± 0.28 | 0.05 ± 0.015 | 30.6 ± 1.41 |
| 15% | 0.21 ± 0.011 | 3.25 ± 0.18 | 0.06 ± 0.015 | 28.8 ± 1.11 |
| BG11 | 0.22 ± 0.02 | 3.16 ± 0.29 | 0.08 ± 0.02 | 32.5 ± 0.97 |

Table 4.27 Growth characteristics, biomass and total lipid content of *Chloromonas* species in different concentrations of piggery waste i.e. 5%, 10% and 15%

| Piggery extract (%) | Specific growth rate $\mu$, ($day^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity ($g\ L^{-1}d^{-1}$) | Total lipid content (% DCW) |
|---|---|---|---|---|
| 5% | 0.17 ± 0.01 | 4.07 ± 0.24 | 0.048 ± 0.006 | 35 ± 0.91 |
| 10% | 0.22 ± 0.02 | 3.16 ± 0.29 | 0.08 ± 0.02 | 28.6 ± 0.5 |
| 15% | 0.16 ± 0.02 | 4.37 ± 0.55 | 0.042 ± 0.007 | 26.8 ± 0.52 |
| BG11 | 0.23 ± 0.026 | 3.03 ± 0.37 | 0.09 ± 0.035 | 33.6 ± 0.87 |

Table 4.28 Growth characteristics, biomass and total lipid content of *Scenedesmus* species in different concentrations of cow dung waste i.e. 5%, 10% and 15%

| Cow dung extract (%) | Specific growth rate $\mu$, ($day^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity ($g\ L^{-1}d^{-1}$) | Total lipid content (% DCW) |
|---|---|---|---|---|
| 5% | 0.16 ± 0.02 | 4.37 ± 0.55 | 0.23 ± 0.026 | 35 ± 0.91 |
| 10% | 0.19 ± 0.01 | 3.65 ± 0.19 | 0.27 ± 0.015 | 29.6 ± 1.24 |
| 15% | 0.25 ± 0.015 | 2.73 ± 0.15 | 0.32 ± 0.026 | 25.8 ± 1.4 |
| BG11 | 0.27 ± 0.011 | 2.53 ± 0.1 | 0.35 ± 0.015 | 33.6 ± 1.6 |

Table 4.29 Growth characteristics, biomass and total lipid content of *Scenedesmus* species in different concentrations of piggery waste i.e. 5%, 10% and 15%

| Piggery extract (%) | Specific growth rate $\mu$, ($day^{-1}$) | Doubling time $T_2$, (days ) | Biomass productivity ($g\ L^{-1}d^{-1}$) | Total lipid content (% DCW) |
|---|---|---|---|---|
| 5% | 0.14 ± 0.015 | 4.87 ± 0.5 | 0.19 ± 0.015 | 33.5 ± 1.45 |
| 10% | 0.19 ± 0.02 | 3.67 ± 0.38 | 0.21 ± 0.02 | 31.8 ± 0.41 |
| 15% | 0.22 ± 0.015 | 3.11 ± 0.21 | 0.25 ± 0.02 | 29.8 ± 0.9 |
| BG11 | 0.23 ± 0.01 | 3.01 ± 0.13 | 0.27 ± 0.01 | 35.4 ± 0.808 |

Table 4.30 Physico chemical properties of methyl ester of *Chloromonas* species ADIITEC-III and *Scenedesmus* species GUBIOTJT116 in cow dung and piggery waste

| Properties | ADIITEC-III FAME | | | GUBIOTJT116 FAME | | | ASTM D-6751 limits |
|---|---|---|---|---|---|---|---|
| | Cow dung (5%) | Piggery waste (5%) | BG11 | Cow dung (5%) | Piggery waste (5%) | BG11 | |
| Acid value mg ($KOHg^{-1}$) | 0.48 | 0.48 | 0.48 | 0.52 | 0.52 | 0.62 | 0.50 max |
| Iodine value ($I_2$/100g oil) | 114 g | 114 g | 112.3 g | 114 g | 116 g | 114 g | 120 max |
| Pour point | -13ºC | -14ºC | -7ºC | -7ºC | -14ºC | -12ºC | -15- 10 |
| Calorific value | 41 | 41 | 40 | 41 | 38 | 41 | - |
| Fatty acid compositions of ADIITEC-III and GUBIOTJT116 | | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Linolenic acid (18:3) | 2.75 % | 15.77 % | 6.63 % | 9.06 % | 23.66 % | 13.79 % |
| Linoleic acid (18:2) | 17.94 % | 40.96 % | 21.04% | 0.28 % | 40.38 % | 1.83 % |
| Oleic acid (18:1) | 61.15 % | 29.97 % | 32.54% | 56.5 % | 23.66 % | 48.1 % |
| Total saturates | 18.15 % | 13.3 % | 39.79% | 34.1 % | 12.3 % | 36.29 % |

Table 4.31 Comparison of temperature characteristics for the thermogravimetric analysis of methyl esters of *Chloromonas* and *Scenedesmus* species grown in cow dung and piggery waste

| Microalgae | Onset temperature (ºC) | Mass loss (%) | | |
|---|---|---|---|---|
| | | 10% | 50% | 90% |
| ADIITEC-III FAME | 161 | 174 ºC | 249 ºC | 477 ºC |
| ADIITEC-III cow dung (CD) | 147 | 170 ºC | 247 ºC | 468 ºC |
| ADIITEC-III piggery waste (PW) | 145 | 168 ºC | 241 ºC | 448 ºC |
| GUBIOTJT116 FAME | 174 | 211 ºC | 362 ºC | 480 ºC |
| GUBIOTJT116 cow dung (CD) | 182 | 198 ºC | 386 ºC | 488 ºC |
| GUBIOTJT116 piggery waste (PW) | 174 | 195 ºC | 337 ºC | 477 ºC |

# CHAPTER 5

# IMPORTANT FINDINGS AND CONCLUSION

# 5. IMPORTANT FINDINGS AND CONCLUSION

## 5.1. Important findings

This entire study was focused on isolation of native freshwater microalgae species from relatively unexplored microalgae resources of the NE region, identification and characterization of suitable oleaginous microalgae species and testing various physico-chemical parameters for biodiesel production. Therefore, based on the study conducted, the important findings of the entire thesis are listed below:

1. A newly isolated strain was identified as *Chloromonas* species (ADIITEC-III) through 18S rRNA sequence analysis.
2. Under the optimized light and pH condition, total lipid yield of *Chloromonas* species (ADIITEC-III) and *Scenedesmus* species (GUBIOTJT116), the reference algal strain was recorded to be 31.8 ± 0.9% and 35.2 ± 0.72% respectively.
3. The fatty acid composition of ADIITEC-III and GUBIOTJT116 was estimated and found to contain mainly oleic acid, linoleic acid, linolenic acid and total saturated fatty acid. The fatty acid profile showed higher percentage of oleic acid in their composition i.e. 32.54% and 48.1% respectively.
4. The use of ammonium nitrate as a nitrogen source for ADIITEC-III resulted in higher lipid yield (i.e. 35.86 ± 2.3%), whereas, in case of GUBIOTJT116 urea enhanced the lipid yield to 37.2 ± 0.36%.
5. Increase in oleic acid content of ADIITEC-III and GUBIOTJT116 with the variation of nitrogen source was found to be 47.04% and 56.92% respectively.
6. The *Chloromonas* species (ADIITEC-III) can grow up to a salt stress of 200 mM NaCl with a maximum lipid yield of 38.8 ± 0.5%.

7. Increased biomass production (1.5 ± 0.3 g $L^{-1}$) and lipid content (40.8 ± 0.2%) was observed in the two-stage cultivation strategy compared to control culture (0.45 ± 0.03 g $L^{-1}$).
8. The use of cow dung and piggery waste as alternate low-cost media supported the growth of ADIITEC-III and GUBIOTJT116 and increased the lipid yield at 5% waste concentration compared to culture grown in BG11 medium.

## 5.2. Overall conclusion

In the present study, four freshwater microalgae strains were isolated from IIT Guwahati campus, Amingaon and Jalukbari area of Kamrup district, Assam, India. The isolation of microalgae strains was carried out by dilution method followed by repeated streaking on the agar-plate. Among the four native isolated strains, ADIITEC-III exhibited higher growth rates and lipid yield; therefore, it was selected for further identification and characterization. Apart from that, *Scenedesmus* species GUBIOTJT116 was used as a reference species for the thesis work. The isolate was identified as *Chloromonas* species ADIITEC-III on the basis of 18S rRNA gene sequence (GenBank accession number KF471125.1) analysis. The potential of this strain to produce higher amount of lipid and biomass with appropriate biodiesel production was evaluated by altering various physiological conditions. The species ADIITEC-III and GUBIOTJT116 displayed significant lipid yield of 36.5 ± 0.75% and 38.8 ± 0.8% respectively under high light regime 94.5 $\mu$mol $m^{-2}s^{-1}$. In comparison, low light intensities (i.e. 24 and 40.5 $\mu$mol $m^{-2}s^{-1}$) favored the growth rate of ADIITEC-III, whereas the growth rate of GUBIOTJT116 was continuously supported up to irradiance of 81 $\mu$mol $m^{-2}s^{-1}$. The characterization of oil and methyl ester of ADIITEC-III and GUBIOTJT116 revealed significant properties of biodiesel. Initially oil samples with high acid values were subjected to acid catalyzed

transesterification reaction which resulted in values within ASTM D-6751 standards. The pour point of oil samples was comparatively higher than the methyl esters. The pour point values of ADIITEC-III and GUBIOTJT116 oil sample was found to be -12°C and -13°C respectively, which is much higher than the pour point of methyl esters (-7°C) and (-12°C). The fatty acid profile of isolates showed abundance of oleic acid followed by total saturated fatty acid. Moreover, oil sample of GUBIOTJT116 was found to be thermally more stable than ADIITEC-III. Therefore, both isolates were found to be a potential candidate for biodiesel production.

For microalgae cultivation, nitrogen plays an important role in micro algal growth, lipids and fatty acid metabolism. Among the four different nitrogen sources, ammonium nitrate and urea favored the lipid yield in ADIITEC-III and GUBIOTJT116 respectively. In contrary, this two nitrogen source showed unfavorable condition for the growth of isolated strains. The study on FTIR spectrum of lipid extracts of both the species grown under studied nitrogen sources was further coupled with principal component analysis to reproduce the most prominent variation pattern in the data. In case of ADIITEC-III, ammonium nitrate exhibits maximum variation of 60.19% in the spectral region 3000−2800 $cm^{-1}$. Similarly, in case of GUBIOTJT116, maximum variation of 51.66% was contributed by potassium nitrate. However, in the spectral region (i.e. 1800-1000 $cm^{-1}$) of ADIITEC-III, the maximum variation was observed in potassium nitrate (58.86%), whereas sodium nitrate contributed maximum variation of 89% in the spectral region of GUBIOTJT116. Moreover, multidimensional scaling analysis of FTIR spectrum of ADIITEC-III showed close proximity between urea and sodium nitrate, and discriminates potassium nitrate in both spectral regions. On the other hand, MDS analysis of GUBIOTJT116 showed discrimination of urea in both spectral regions. The physico-chemical characterization of methyl ester samples of ADIITEC-III and GUBIOTJT116

grown in ammonium nitrate and urea were determined and compared with ASTM D-6751 standards. The oleic acid yield of both the species grown at optimized nitrogen source was found to be high compared to normal BG11 medium ($NaNO_3$). The methyl esters of ADIITEC-III grown in ammonium nitrate showed high content of oleic acid (47.04%) which was quite higher than the FAME retrieved from control condition (32.54%). On the other hand, GUBIOTJT116 showed higher yield in oleic acid (56.92%) when grown in urea than the controlled condition (48.1%). However, isolates grown in sodium nitrate proved to be thermally more stable than other optimized nitrogen condition due to high saturated fatty acid content in their composition. Hence, for large scale cultivation, ammonium nitrate and urea could be used as a potential nitrogen source for increased lipid yield. Nevertheless, nitrogen source may vary from species to species. The results of chemometric analysis provided detailed information of FTIR spectrum and its response on various nitrogen sources.

The two-stage cultivation study was used to produce maximum biomass and lipid at the same cultivation period. The species ADIITEC-III and GUBIOTJT116 was screened based on their tolerance towards high NaCl concentration. From the study, it was found that ADIITEC-III was able to sustain at extreme NaCl salt stress. In case of ADIITEC-III, 90 mM salinity favored the specific growth (0.25 ± 0.02) and biomass productivity (0.2 ± 0.02 g $L^{-1}d^{-1}$) whereas increased specific growth (0.26 ± 0.02) and biomass productivity (0.37±0.02 g $L^{-1}d^{-1}$) of GUBIOTJT116 was observed in 50 mM of NaCl. The NaCl concentration beyond 130 mM in GUBIOTJT116 showed significant decreased in biomass production, thus leading to difficulty in lipid extraction.

The two-stage cultivation of ADIITEC-III showed increased biomass production (1.5 ± 0.3 g $L^{-1}$) and lipid content (40.8 ± 0.2%) compared to control culture (0.45 ± 0.03 g $L^{-1}$). The derived methyl ester sample showed abundance of oleic acid (40.49%) followed

by saturated fatty acid (35.04%) in their compositions. Accordingly, pour point value and thermal stability of the samples were found to be superior compared to the culture grown in normal BG11 medium. The data obtained from this study provides important inputs for further investigations in the cultivation process to employ suitable stress phase according to the species in order to obtain high biomass and lipid.

Moreover, characterized cow dung and piggery waste compositions demonstrated itself as a potent alternate low-cost culture medium for mass cultivation of both the species. The 5% cow dung concentration favors the strains with increased lipid yield, whereas 15% cow dung supports the growth and biomass production. The derived methyl esters of isolated strains revealed high polyunsaturated fatty acids content compared to the culture grown in normal BG11 media. Similarly, pour point values were significantly higher than the culture grown in BG11 media, but lower pour point was observed for GUBIOTJT116 grown in cow dung waste. Likewise, GUBIOTJT116 grown in cow dung was thermally more stable than the other culture compositions. Hence, animal waste adopted in this work can form a framework to investigate large scale microalgae cultivation for biomass and lipid production.

Overall, the physico-chemical characteristics of derived methyl esters revealed that both the strains i.e. ADIITEC-III and GUBIOTJT116 could act as a prospective feedstock for biodiesel production. However, for large scale implementation, GUBIOTJT116 would be the better strain as the species is very robust and dominant in nature. Nevertheless, newly isolated strain *Chloromonas* species ADIITEC-III showed its ability to withstand high saline condition which makes it suitable to grow at extreme conditions.

### 5.3. Future work

Following are the scopes for future work:

- ✓ As the growth rate is concern, *Chloromonas* species (ADIITEC-III) needed to be improvised prior to consider for outdoor open mass cultivation. Future studies on this area could lead promising result in this regard.
- ✓ The optimization of outdoor culture conditions is imperative for maximum biomass and lipid output. The optimizations necessitate special emphasis on nutrient input, light and temperature limitations and operating parameters. Thus, future research in this area would endeavor to determine the factors which could improve growth and lipid production for outdoor cultivations.
- ✓ Finally, while the outcomes of this study indicate a high potential for large-scale cultivation of selected microalgae strains, the cost effective measures must be considered for economic sustainability, which would require a complete life cycle analysis (LCA) of the system.

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# ANNEXURE

# Annexure A

## A.a. Forward and reverse sequences of *Chloromonas* species ADIITEC-III

### ADIITEC-III_Forward

GGGTAACAGCTTCGAGCCACACCGAGAACCACTCTTGTGCGGTCCCCTGCAACT
GTGTGGGCCCTAGCGGCTGCCTCTCTTCCGTGCTGTCTGGAGGATAGTGGCGAT
CCCCCTGCACACGAGTAGTAGCGACCCTTGTCTGCCCGGGGCGCGAGCCCCTCT
GACCTCTTAACTGAAGGCATGGAAGAGGGCTGGCCACCCCTTAACCAAATAACC
CAGCACCAAACAAAGCCTAACCATACATACCAAGATCTGGGTCTCGGCCTGGCC
AAAGCATCCTAACGGAGAGTACTCTCAACAGCCGATATGCTTGGATCTCGCAAC
GATGAATAACGCACACGAAATGCTGATACGCCGTGCGCATTGCTGAATTTCGTG
AGTCTTCGCGTCTTTGAACGCATATCGCGCTCGAGGCTTCTGTCGTCAGCCTGTC
TGCCTCAGCGTCGGGCTAACATCGCCATCCCTATAGCTAAGAATAAAGGCCTGG
CGGACCTGGCTGTTCCAGCTCTCAAGCGTCCTGCACCCTTCCGCGTACAGGCTCG
CGCCGAGCATCTGGATCAGCTGAAGACCACACGTTAACCTCTGGACCCGCTAAG
GGCCGCAGATGGCTGGGCTGACAAAGGCTATATTCTTAGTTGATGGTCTGGGAC
AGGACTTGTAGACCCTCGAACATGACACTTAAGCCTTCATTATCTCGACCAGAG
CTCAAGCAAGATTATCCGCTGAACTTAAGCATATCAATACCCCGGACGAATGAG
CTCTATACGGAAACACAAACAATAAACAAGACCTGGGTCGAACTTGTGAATCGC
TTACCAATCGTCGGATCATCATGCTATCAAGGGGAAGAACGTTTCCCCGGGGCC
TTTGTGCGCCACCCACCCTTTCCCACCGCCCCGGAGTTATGTAAAACCCACAAAT
CCCCTTGACGGCAAACCCTTCTTCCGACCCCCAATCCCCCCCCAAAGGGGGGACA
GAGGATAAGGNGGGAGCAGGATAGG

### ADIITEC-III Reverse

TAAGGGGGCCCTTCGGAAGGTGACTGGGAAGATCATTAATCTATCAATCCAACC
CGAGACCTCTATGTTCGGTCCCCTGAACAGCGTGCCCTTAGTGCTTCAGTTGCCG
TGTGCTGTAGGATAGAGAGCGAGCACTGCAAAGGGGTAGTAGCTCCTTGTCTGC
GCAGGGTCTACCCCGTCTGCGTGTTACTGAGGGCATGGGCGAGGGCTGGCCACC
CCTTAAACAAACACCCCAACATCAAACACAACCTTACAATACATACCAAGATAT
GCATCTGGCCTGGCCAATGCATCCTAACTGAGACAACTCTCAACACCGGATATC
TTGGCTCTCGCAACGATGAAGAACGCAGTGAAATGCGATACGTAGTGTGAATTG
CAGAATTCCGTGAATCATGGAATCTTTGAACGCATATTGCAGCTCGAGGCTTCG
GCCAAGAGCATGTCTGCCTCAGCGTCGGGTTAATATCGCCATCCCTATACCTAG
GTATAGAGTCCTGGCGGACCTGGCTGTTCCAGCTCTCAAGCGTCGTGCACCCTT
GGGGGTACAGGCTCTCGCCAAGCATATGGATCAGCTGAAGAGCAGAGGTTAAC
CAAGGACCCGCTAAGGGCCGCAACTGGGTGGGCTGTCAAAGGCTATATTCTTAG
TTGTTGGTCTGGGACTTTCCTTGTTGACCCTCAAACAGGAAACTTAACCCTTCAT
ATCTCGACCTGAGCTCAGTCAGATGCCGATCCGC

## A.b. Calibration curve of biochemical compositions

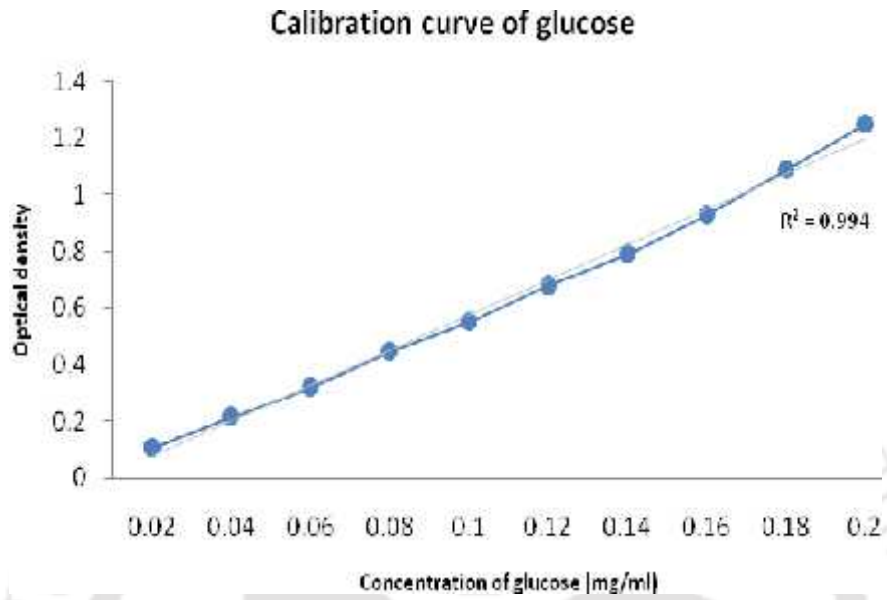


Figure A.1 Calibration curve of glucose for estimation of carbohydrate

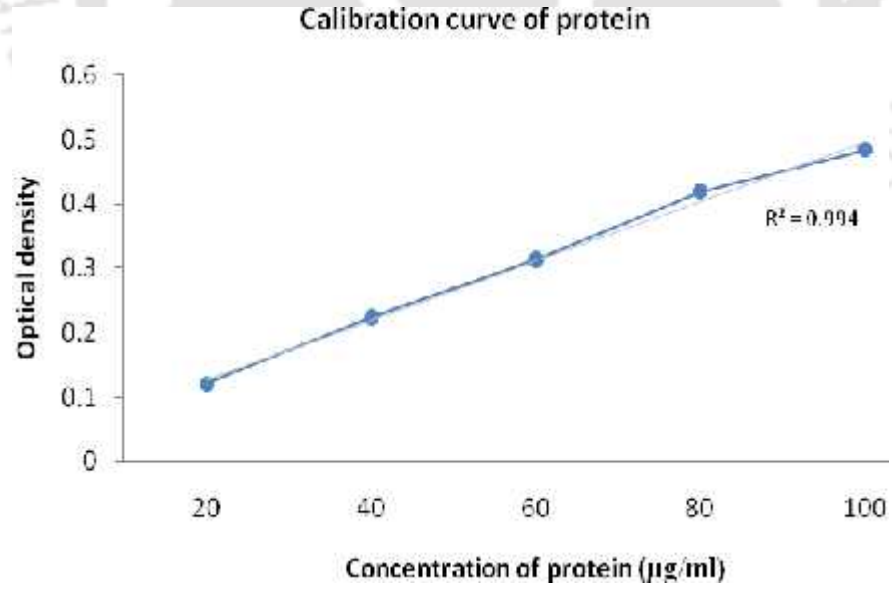


Figure A.2 Calibration curve of BSA for protein estimation

# Research output

**Book Chapter**

- A. Difusa, K. Mohanty, V.V. Goud, Advancement and challenges in harvesting techniques for recovery of microalgae biomass, in **Environmental Sustainability-Role of Green Technologies** (Editors: P. Thangavel and G. Sridevi), Springer, 2015, 159-169.

**Peer-Reviewed Research Papers**

- **A. Difusa**, J. Talukdar, M. C. Kalita, K. Mohanty, Vaibhav V. Goud (2015) Effect of light intensity and pH condition on the growth, biomass and lipid content of microalgae *Scenedesmus* species. **Biofuels**, pp 37-44.
- **A. Difusa**, K. Mohanty, V.V. Goud. The chemometrics approach applied to FTIR spectral data for the analysis of lipid content in microalgae cultivated in different nitrogen sources. **Biomass Conversion and Biorefinery**, DOI 10.1007/s13399-016-0198-6.
- **A. Difusa**, K. Mohanty, Vaibhav V. Goud. Physico - chemical characteristics of a newly isolated strain of the microalgae Chloromonas (Chlorophyceae) species ADIITEC-III from the Wetland of Assam for biodiesel production, Submitted to **Algal Research**.

**Conferences**

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