Exploiting local algal diversity for bio-diesel production

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Exploiting local algal diversity for bio-diesel production

Dissertation submitted in partial fulfillment

of the requirements of the degree of

Doctor of Philosophy

in

Chemical Engineering

by

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based on research carried out

under the supervision of

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September, 2017

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Acknowledgment

I would like to express my appreciation to all those people who have contributed to make this work possible through their help and support along the way. My deepest gratitude goes to my thesis supervisor Prof. (Dr.) R. K. Singh for giving me the opportunity to work on this interesting project and for their valuable guidance, inspiration, constant encouragement, heartfelt good wishes and support through all the phases of the project. I take this opportunity to express my deep sense of gratitude to the members of my Doctoral Scrutiny Committee Prof. Sujit Sen of Chemical Engineering Department, Prof. Japesh Bera of Ceramic Engineering Department and Prof. B. G. Mishra of Chemistry Department for thoughtful advice during discussion sessions.

I express my immense gratitude to Prof. Mukesh Kumar Gupta of the Biotechnology and Medical Engineering for his valuable suggestions and encouragement. I wish to extend my special thanks to Prof. (Mrs.) Kakoli Karar (Paul) for allowing me to carry many repeated experiments in her lab. Special thanks goes to the following professors during various stages of the work Prof. P. Rath, Prof. (Ms.) Madhushree Kundu, Prof. Santanu Paria of the Department of Chemical Engineering, Prof. Preetam Sarkar, Prof. Sabyasachi Mishra of Department of Food Process Engineering.

This work also would not have been possible without the help of all the research group members seniors and friends. I would like to express my gratitude to Ms Debalakshmi, Mr Suresh Chaluvadi, Mr Sowhm Swain Mohapatra, Ms Bageshwari Sanga, Mr. A.V.S.L. Sai Bharadwaj, Dr Sachin Kumar, and Dr Achyut Kumar Panda for their support and good wishes.

Finally, I express my humble regards to my parents and other family members, for their immense support, sacrifice and their unfettered encouragement at all stages. Last but not the least I am grateful to Manish, my love for his trust, tolerance, support, understanding and eternal love.

September 15, 2017
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Abstract

Algae is proven potential resources for eco-friendly bio-diesel production. But research efforts are still required for worthwhile results. The present study aims to utilize locally available algae biomass resources for bio-diesel production. The samples were collected from the air, water depositories and soil within the Institute campus. Further purification, culture, morphological identification, preservation, lipid extraction, transesterification and bio-diesel properties estimation were also conducted.

In the first set of experiment, water strains were collected, isolated up to the molecular level and utilized for vitrification protocol development. The isolated *Oocystis sp*. and *Anabaena sp*. confirm an improvement in survival percentage over conventional encapsulation-vitrification method. The viability concentration was also enhanced further by the addition of 2-mercaptoethanol and glutathione. The developed methodology further used for the preservation of collected and isolated cells during the study.

Further, the study compared the pre-treatment strategies for improvement in overall lipid extraction rate. Comparison were done for dry vs. wet, with cell disruption vs. without cell disruption and conventional Soxhlet method vs. Folch's methods. Results show that the combination of dried algal biomass with the Folch's method yields more than 27% lipid which was comparatively higher than the traditional Soxhlet methodology i.e. 15%. The mixed population includes *Chlorella, Anabaena, Euglena, Oocystis and Sphaerocystis* species. Fatty acids present in lipid consist majorly of the C-18 molecule i.e. linolenic acid (C18:3), linoleic acid (C18:2) and oleic acid (C18:1). The other varieties of comparatively short carbon chain fatty acids were also observed which were considered to give the best fuel properties. Therefore, this local algal mixed diversity was found to be suitable for biofuel as well as various other fatty acids production.

Similarly, dominant airborne species throughout the year in this locality were found to be *Scenedesmus sp., Chlorella sp., Pteromonas, Sphaerocystis sp., Oocystis sp., Oedogonium sp., Anabaena, Pseudanabaena sp., Gloeocapsa sp., Microcystis sp., Naviculoid Diatoms, Mastogloia, Striatella sp., Euglena sp., Phacus sp.* and two unidentified species. Availability of algae was found to be maximum during post monsoon and minimum in rainy season. Lipid

estimation and FAME analysis were conducted by spectrofluorometry, CHNS, FTIR, GC-MS. Basic bio-diesel properties were also performed to know the suitability of extracted oil as raw material for bio-diesel production. Further, probable sugars, acids and alcohol were also estimated from methanol layer after lipid extraction. As varieties of algae were found in the month of October, therefore, overall lipid content and other functional groups were also found higher in the analysis. Summarizing, the obtained airborne algal oil fraction was suitable to use as bio-diesel. As the airborne algae were also oleaginous, hence these could not be considered as contamination during large scale open culture system.

In the further experiments, bio-diesel and other co-products were produced using soil algal biomass. All the three layers during lipid extraction i.e. chloroform:methanol:residual layers were considered for this study. The dominant species include *Chlorella, Euglena, Oocystis, Anabaena, Pseudomonas* and one unreported species. Bottom chloroform layer consists of lipid which was transesterified and analyzed by GC-MS for the presence of FAME and phytol. Phytol is hydrolyzed component of chlorophyll molecule and precursor of vitamin E, and K. HPLC of methanol layer shows the presence of various carbohydrates, acids and other commercially valuable components. Therefore, methanol layer could be further purified and utilize as the source of carbohydrates and other useful chemicals. The cell debris was physically activated to use it as bio-char. Comparative characterization of raw algae, residual algae and algae biochar by proximate, elemental, TGA, FTIR, XRD, SEM-EDX were done. The results show that volatile matter was depleted after lipid extraction but fixed carbon increases. Peaks of FTIR study identified many chloroalkanes repeat in all the three states which were consistent with EDX analysis. EDX shows the presence of high amount of carbon, oxygen along with few inorganic substitutes like chlorine, calcium, etc. SEM and XRD pattern reveals the surface morphology of raw, residue and bio-char of algae. The residual algae are much crystalline in comparison to other two states which may be due to the extraction of intracellular components. Hence raw and residual algal biomass could not be utilized directly as the adsorbent. The further physical treatment creates the pores in crystalline surface hence could be used as the adsorbent. Organic and inorganic materials present in algal biomass shows that it can also be utilized as fertilizer for agricultural purpose. Further, methylene blue dye adsorption study was also conducted to know the suitability of biochar as the adsorbent. More than 90% of the dye was absorbed after the interval of one hour. In summary, the algae are very valuable biomass, and wise utilization could provide various value added products for human benefit.

Keywords: local algae, bio-diesel, pre-treatment, airborne, co-product, cryopreservation

Contents

Certificate of Examination .... *ii*

Supervisors' Certificate .... **iii**

Dedication .... **iv**

Declaration of Originality .... **v**

Acknowledgment .... **vi**

Abstract .... **vii**

Abbreviation .... **xv**

Introduction **1**

1.1 Problem Statement *2*

1.2 Solution strategies *3*

1.3 Motivation of Work *5*

1.3.1 Indigenous algae 5
1.3.2 Lipid extraction methodology 5
1.3.3 Preservation scenario 6

1.4 Scope of this study *6*

1.5 Organization of thesis *8*

Literature Review **9**

2.1 Biofuel as alternative *9*

2.2 Status of Biofuel from microorganism *10*

2.2.1 An account on Microalgae 12

2.3 Improved strategies for algae biofuel production *12*

2.3.1 Algae contents 13
2.3.2 Harvesting 14

2.4 Present market scenario *14*

2.4.1 Status in India 14
2.4.2 Worldwide scenario 16

2.5 Strategies required for commercialization of algae biofuels *18*

2.5.1 Cryopreservation 19
2.5.2 Lipid extraction pre-treatment 22
2.5.3 Selection of strain 23
2.5.4 Bio-diesel co-product production 26

2.6 Objectives of Present work *27*

Materials and Methods **28**

3.1 Summary *28*

3.2 Materials *28*

3.3 Methods *29*

3.3.1 Study Site 29
3.3.2 How to collect? 30
3.3.2.1 Water 30
3.3.2.2 Soil 30
3.3.2.3 Air 31
3.3.3 Measurement of environment physiochemical parameters 31
3.3.3.1 Water Analyses 31
3.3.3.2 Soil Analyses 32
3.3.3.3 Air Analysis 32
3.3.4 Growth condition 32
3.3.4.1 Scale-up of algae culture 34
3.3.4.2 Dewatering 35
3.3.4.3 Monitoring cell growth 35
3.3.5 Morphology study by microscopy 36
3.3.6 Maintaining Microalgae Stocks 37
3.3.7 Lipid Estimation 37
3.3.7.1 Lipid staining 37
3.3.7.2 Lipid Extraction 38
3.3.7.3 Transesterification 38
3.3.7.4 Bio-diesel analysis 38

Open encapsulation-vitrification for cryopreservation of algae 40

4.1 Introduction 40

4.2 Materials and Methods 40
4.2.1 Isolation and culture of algae samples 40
4.2.2 Molecular Identification 40
4.2.3 Vitrification 41
4.2.4 Warming and rehydration 42
4.2.5 Assessment of viability 42
4.2.6 Statistical analyses 42

4.3 Result and Discussion 44
4.3.1 Isolation of algae 44
4.3.2 Comparison of open and closed systems of vitrification 45
4.3.3 Influence of bead size 48
4.3.4 Effect of antioxidants on post-warming viability 49

4.4 Conclusion 55

Pre-treatment and Bio-diesel production from fresh water algal culture 56

5.1 Introduction 56

5.2 Materials and Methods 56
5.2.1 Algae culture 56
5.2.2 Water analysis 57
5.2.3 Extraction pre-treatment 57
5.2.4 Lipid Extraction 57

5.3 Results and Discussion 58
5.3.1 Water analysis 58
5.3.2 Growth and Identification of algae 59
5.3.3 Selection of lipid extraction methodology 62
5.3.4 FAME profile analysis 63

5.4 Conclusion 66

Bio-diesel production from airborne algae 67

6.1 Introduction 67

6.2 Materials and methods 67

6.2.1 Sample collection ... 67
6.2.2 Lipid extraction ... 68
6.2.3 Analytical methods ... 68

6.3 Results and Discussion ... 69
6.3.1 Effect of climate ... 69
6.3.2 Identified Algae ... 71
6.3.3 Lipid estimation ... 76
6.3.4 Analysis of FAME ... 77
6.3.4.1 CHNS analysis ... 77
6.3.4.2 FTIR ... 78
6.3.4.3 GC-MS ... 81
6.3.4.4 Fuel Properties ... 84
6.3.4.5 HPLC ... 85

6.4 Conclusion ... 87

Bio-diesel and co-product production from soil algal biomass ... 88

7.1 Introduction ... 88

7.2 Matertials and Method ... 88
7.2.1 Algae Strain ... 88
7.2.2 Lipid and other Co- Product Extractions ... 89
7.2.3 Bio-char Preparation and characterization ... 89

7.3 Results and discussion ... 90
7.3.1 Soil Analysis ... 90
7.3.2 Algae Identification ... 92
7.3.3 Nutrient profile of raw algae ... 94

7.4 FAME analysis ... 95
7.4.1 GC-MS ... 95
7.4.2 FTIR ... 97
7.4.3 Detection from MeOH layer ... 97
7.4.3.1 Chlorophyll ... 97
7.4.3.2 FTIR ... 98
7.4.3.3 HPLC ... 99
7.4.4 Dectection from Solid Fraction ... 100
7.4.4.1 TGA ... 101
7.4.4.2 FTIR ... 103
7.4.4.3 XRD ... 106
7.4.4.4 SEM-EDX ... 108
7.4.5 Effect of time as adsorbent ... 109

7.5 Conclusion ... 110

Conclusion and Recommendation for Future ... 112

8.1 Summary and Conclusions ... 112

8.2 Recommendations for future ... 118

Reference ... 119

Dissemination ... 132

Biography ... 133

List of Figures

Figure 1. Citations by year from a web of science search related to “algae” and “biofuels (Miodrag Belosevic 2014).” 17
Figure 2. Geographical locations of sampling site downloaded through Google earth. “W”, “S” marks indicates the algae collection site from water ans soil respectively. Logitude and latitude location of S is 22°15'22.4"N 84°54'18.5"E; W is 22°15′19.5″N; 84°54′14.5″E and W is 22°15'03.7"N 84°54'38.6"E. 29
Figure 3. Sample collection site. 30
Figure 4. Experimental set-up for large scale algal growth 35
Figure 5. *Scenedesmus* cells stained by Nile red have characteristic red fluorescence 38
Figure 6. Brief outline of steps followed during experiment 43
Figure 7. Morphological appearance of *Oocystis sp.* (A) and *Anabaena sp.* (B) isolated in the present study. Panel 1: Light Microscopy. Magnification: 1000x. Panel 2: FESEM. Magnification: 10,000x. A, Autospore. AK, Akinete. H, Heterocyte. Red line indicates the mucilage layer. 45
Figure 8. Post-warming viability of Oocystis sp. (Panel 1) and Anabaena sp. (Panel 2) vitrified by open (A1, B1) or closed (A2, B2) system of vitrification. Cells were stained with FDA after warming and were visualized under UV-illumination. A3, B3: Appearance of cells after 7 days of post-warming culture. Inset box shows a magnified dead cell. Magnification, 4000 x; A, Autospores. 48
Figure 9. Growth index of vitrified-warmed *Oocystis sp.* in the presence of A: GSH (0, 10, 50, 100 μM) or B: βME (0, 10, 50, 100 μM). Panel 1: OEV; Panel 2: CEV. 52
Figure 10. Growth index of vitrified-warmed *Anabaena sp.* in the presence of A: GSH (0, 10, 50, 100 μM) or B: βME (0, 10, 50, 100 μM). Panel 1: OEV; Panel 2: CEV. 54
Figure 11. Growth curve of algae culture used in experiment. 59
Figure 12. Microscopic view of predominant species present in the study. Panel 1 and Pannel 2 represents the image of before and after addition of lugol’s reagent. (A1, A2) *Chlorella sp.* (B1, B2) *Anabaena sp.* (C1, C2) *Euglena sp.* (D1, D2) *Oocystis sp.* (E1, E2) *Sphaerocystis sp.* 62
Figure 13. Lipid yield obtained by different techniques of extraction. Grey bar indicates wet biomass and black bar indicates dried biomass. F (Folch’s method of lipid extraction), CD (Cell disruption), Pet Eth (lipid extraction using Petroleum ether as solvent) 63
Figure 14. Chromatograms of FAMEs obtained during experiment. 64
Figure 17. Micrograph of sample under 100x resolution. Chlorophyta: *Scenedesmus dimorphus* (A), *Scenedesmus ovalternus* (B), *Scenedesmus bijugatus* (C), *Chlorella sp.* (D), *Pteromonas* (E), *Sphaerocystis sp.* (F), *Oocystis sp.* (G), *Oedogonium sp.* (H), Cyanobacteria: *Anabaena sp.* (I), *Pseudanabaena sp.* (J), *Gloeocapsa sp.* (K), *Microcystis sp.*(L), Bacillariophyceae: *Naviculoid diatoms* (M), *Mastogloia elliptica* (N), *Striatella sp.* (O), Euglenozoa: *Euglena sp.* (P), *Phacus sp.* (Q), Unidentified species (R,S) 76
Figure 18. Monthwise comparision of FTIR spectra of transesterified product. 80
Figure 20. Monthwise GC–MS chromatogram (A) January (B) December (C) August (D) September (E) November and (F) October 84
Figure 21. Algae identification by optical microscopy (100x) magnification (left) and SEM analysis (> 3000x) magnification (right). (A) Chlorella, (B) Euglena, (C) Oocystis, (D) Unknown, (E) Anabaena, (F) Pseudoanabaena species. 94

Figure 22. Schematic diagram of expected products by utilizing all the layers during lipid extraction ... 95
Figure 23. GC–MS chromatogram of transesterified product ... 96
Figure 24. FTIR analysis of transesterified product ... 97
Figure 25. FTIR analysis of compounds soluble in MeOH layer ... 99
Figure 26. Comparative TGA analysis of raw, residual and bio-char of algae biomass ... 102
Figure 27. FTIR analysis of raw, residual and bio-char of algae biomass ... 105
Figure 28. XRD pattern of raw, residual and bio-char of algae biomass ... 107
Figure 29. SEM-EDX analysis of raw, residual and bio-char of algae biomass. ... 108
Figure 30. Effect of contact time on the amount of methylene blue adsorption. ... 109

List of Tables

Table 1. Examples of current achievements by Algae biofuel Industries............................ 18
Table 2. Enlist of viability % and loading solution in algae encapsulation vitrification till date.. 21
Table 3. Examples of pre-treatment along with extraction methods 23
Table 4. Nutrient ingredients of used culture media.. 34
Table 5. Post-warming viability (Mean±SE) of cryopreserved cells by open or closed system of vitrification. 47
Table 6. Influence of bead size on Post-warming viability (Mean±SE) of cryopreserved cells. 49
Table 7. Influence of antioxidant treatment on post-warming viability (Mean±SE) of cryopreserved cells.. 50
Table 8. Physico-chemical characteristics of water sample from algae collection area 58
Table 9. Fatty acid profile detected at various nutrient conditions..................................... 64
Table 10. Comparative analysis of Bio-diesel properties. (Atabani et al, 2012; Sivaramakrishnan et al, 2012; Jain et al, 2010; Knothe, 2002; Islam et al, 2013)................ 65
Table 14. Common metal contents present in algae of different months. 78
Table 15. Monthwise comparision of functional groups present in transesterified product. 79
Table 16. Properties of Bio-diesel 85
Table 17. HPLC data for probable sugar, acids and other water soluble compound analysis. 86
Table 18. Physicochemical properties of collected soil.. 91
Table 19. Nutritional profile (wt%) of raw algae biomass 95
Table 20. FTIR analysis of transesterified product.. 97
Table 21. FTIR analysis for compounds present in MeOH layer 98
Table 22. HPLC analysis of MeOH layer for detection of sugar, acids and other water soluble products 99
Table 23. Proximate and Elemental analysis of raw, residual and bio-char of algae biomass 101
Table 24. Comparative FTIR analysis of raw, residual and bio-char of algae biomass 103

Abbreviation

AAS- Atomic absorption spectroscopy
BBM- Bold's Basal Medium
βME- 2-mercaptoethanol
BOD- biochemical oxygen demand
BET- Brunauer-Emmett-Teller
CEV- closed encapsulation vitrification
CFPP- Cold Filter Plugin point
CHNS- Carbon Hydrogen Nitrogen Sulphur
CN- Cetane Number
COD- Chemical Oxygen Demand
CP- Cloud Point
DMSO- Dimethyl sulfoxide
DO- dissolved oxygen
DU- Degree of Unsaturation
EC- electrical conductivity
EDX- Energy-dispersive X-ray spectroscopy
FAME- Fatty acid methyl ester
FDA- 3'6'diacetyl fluorescein diacetate
FESEM- field emission scanning electron microscope
FTIR- Fourier transform infrared spectroscopy
GC-MS- Gas chromatography–mass spectrometry
GSH- glutathione
HHV- Higher Heating Value
HPLC- High-performance liquid chromatography
IV- Iodine Value
LCSF- Long-Chain Saturated Factor
LN_2- liquid nitrogen
MeOH- methanol
MUFA- Monounsaturated Fatty Acids
NTU- Nephelometric Turbidity Unit
OD- Optical Density
OEV- open encapsulation vitrification
OS- Oxidation Stability
PUFA- Polyunsaturated Fatty Acids
PVS2- Plant vitrification solurion 2
SEM- Scanning electron microscope
SFA- Saturated Fatty Acids
SV- Saponification Value
TDS- total dissolved solids
TGA- triacylglycerides
TSS- total suspended solids
XRD- X-ray diffractometer

Chapter 1

Introduction

In the present industrialised era, the need for fuel is growing day by day and fossil fuel reservoirs are limited in the earth crust. Even though International agencies claim to discover new depositories but may not be sufficient to fulfil the enormous demand of the population. Gross annual demand increment of oil, gas and coal are 1.4 million barrels, 4.5 billion cubic feet and 3.1 million tonnes respectively(Abas, Kalair, and Khan 2015). Overall, the energy is utilised to make electricity, power automobiles, and industries by investing 4.4 billion tonnes of petroleum per year worldwide (Roddy 2013). It has also been estimated that fossil fuel growth worldwide will follow trapezoidal graph i.e. till 2025 fuel production will grow, but after that, it will remain in the steady state and finally will decline. According to present scenario, coal may rapidly be consumed and natural gases may remain longer in near future (Mohr et al. 2015) .

Underground mining of fossil fuels is proven dangerous occupation. Thousands of fatality and accidental hazardous directly or indirectly occurs during fuel digging operations. Sixty to seventy thousand occupational accidents or death reports have been recorded in Chinese and Spanish mining sectors [2003-2012] (Geng and Saleh 2015; Sanmiquel, Rossell, and Vintro 2015). It has been observed that traffic accidents were associated with shale gas drilling that might be due to geographical disturbances in Pennsylvania (Graham et al. 2015). During an analysis of 134 oil well blowout operations, it was concluded that chances of the accident are higher during the drilling operation. Offshore Blowout cause much more damage in onshore by awful environmental pollution (Statistics and Analysis on Causes for Well Blowout Accident 2015).

The ecological imbalance caused by fossil fuel is also another major impact. Burning petroleum products cause the release of carbon dioxide and other greenhouse gases. Excess of these gases results in anthropogenic changes globally. For example acidification and warming of oceans has affected the development of marine life (Padilla-Gamiño et al. 2013). Change in entire ecosystems has also started changing the seasonal climates and overall global temperature (Yinzhan Liu, Peter B. Reich, Guoyong Li 2011). Greenhouse gases

accumulation, hole in ozone layer, acid rain, climate change, aquatic life trauma by oil spills has also severely increased nowadays by consumption of conventional fuel(Tingzhen et al. 2014) . Environmental research committees worldwide are also worried for increased death and diseases hike caused by pollution alone. A survey in Mexico City states that exposure to fossil fuel particulate matter enhances the risk of obesity, diabetes, premature cardiovascular problems, cognitive impairment and Alzheimer's disease (Calderon-Garciduenas et al. 2015).

Fossil fuel dependency in 2008 was approximately 88% and was estimated to increase due to industrial and economic progress day by day (Brennan and Owende 2010). Sustainable carbon energy resources like bioethanol or bio-diesel from agriculture waste or food have potential to reduce the foreign outsourcing of oil and the GHG levels. The leading nations around the world have already started to reduce the dependency on petroleum resources. The renewable sources were derived from biological sources and were therefore called biofuels.

1.1 Problem Statement

The other considered energy renewable alternative is biofuel derived from organic resources and presently facing many issues for large scale industrialisation. There are three major concerns that limit the viability of terrestrial biofuels

1) Reduction in biodiversity

2) Destruction of arable land, and

3) Negative environmental impacts.

Terrestrial plants acquire the arable fertile land which could be utilised elsewhere for the production of food or other commercial crops. It not only effects the current tradition of agriculture but will also loss natural ecosystems as well as the biodiversity associated with the area. Clearing of rainforests, peatlands, savannas, as well as grassland in Brazil, Southeast Asia, and the United States (the current leaders in biofuel production), would actually result in a carbon debt (Smith et al. 2010). It has been estimated that this conversion of land could potentially release approximately 3.8 billion megatons of carbon dioxide into our atmosphere (Koh and Ghazoul 2008). The release of the carbon dioxide is due to the soil and plant matter actually store carbon within their mass and act as a carbon sink. A popular practice in the tropical areas in the world is through slash and burn in which the natural vegetation is cut down and burnt for monoculture plantations.

Terrestrial biofuels need high yield for feedstock availability, and that may refer to negative environmental impacts. The practice to maintain the high yield would utilise fertilisers, pesticides, leads to acidification, eutrophication as excess nitrates and phosphates enter receiving waters. From 1850 to 1940 due to similar practice the great lakes in Canada and the United States have the problem of nutrient enrichment (R. J. Radmer 2009). These excess nutrient loads promote algal blooms into water systems. Additionally, the dominating species mostly reported are cyanobacteria in such conditions. These have the ability to produce cyanotoxins, and that could even inhibit protein synthesis resulting in genetic damage to cells (Dow and Swoboda 2002). This would pose a threat to the organisms occurring within the affected ecosystem either by reducing their fitness or by killing the species.

Similar practice results in the use of the excess of pesticides like organophosphates and organochlorines. These chemicals have the ability to bio amplify, bioaccumulate, and induce acute toxic effects (Karami-Mohajeri and Abdollahi 2011). Pesticides like organophosphates are also harmful to non-target organisms as it lacks specificity. For example, during the 1980s there were a large number of bee deaths were reported after aerial spraying with organophosphorus compounds (Fletcher and Barnett 2003). Additionally, there is the gap in research about the environmental effect by using such pesticides. Approximately over 2000-3000 chemicals on the market have deficient data related to the environmental release and toxicity level (Ragnarsdottir 2000). Lastly, biofuels, when compared to their fossil fuel counterparts, do possess drawbacks. Although the environmental impacts of biofuels like ethanol, hydrogen or bio-diesel are much lower than their fossil fuel counterparts. They are often more expensive, require engine modifications to use and generally do not contain as much energy content thus requiring more fuel to attain the same level of performance.

1.2 Solution strategies

The advantages of algae as a feedstock for bioenergy have been push into center since the mid-twentieth century. Meier (1955), Oswald and Golueke (1960) in the late 1950s suggested that carbohydrate fraction of algal cells could be utilize for the production of methane gas and hence could be another mean of energy resources (Research 1995; Oswald and Golueke 1960).

During early 1970s the population was facing difficulties of energy price hikes and was pressure under DOE (Department of Energy). The concept of conversion from lipid to energy, however, was gained serious attention at that decade that ultimately turns the step towards DOE's Aquatic Species Program (1978-1996). The Aquatic Species Program also funded approximately $25 million research at many academic institutions through subcontracts (Nrel 1998). Previous research focused on hydrogen production from algae biomass, but the target shifted towards liquid fuel in the early 1980s.

Research advancement were made through algal strain isolation, species collection from geographical distinct environment, characterization, studies of algal physiology, biochemistry, genetic engineering, process development, and mass scale algal culture. Collection of more than 3,000 strains of microalgae was narrowed to the 300 most assured quality strains. The isolates were selected for their basis of tolerance to fluctuations in salinity, pH, temperature, and lipid accumulation. After screening and identification of microalgae, further studies were conducted to examine the ability of lipid accumulation in strains. The various stress conditions like the nutrient limitation, heavy metal accumulation were conducted, but it overall reduces the growth rate of strain in culture.

Finally, it was conformed that some species in the collection accumulated about 60% lipid primarily triacylglycerides (TAGs) of their dry weight under inducing conditions (Chisti 2008). A study conducted in Cyclotella cryptica found that insufficient silicon triggers oil accumulation by changing biochemical pathway. A key enzyme acetyl-CoA carboxylase (ACCase) belongs to fatty acid biosynthesis was found to increase under the nutrient stress conditions and was used to manipulate the TAG synthesis (Roessler 1988). This was the first successful transformation of microalgae for lipid production (Dunahay, Jaruis, and Roessler 1995). Other studies were also conducted on carbohydrate synthesis as these compounds could also be further used for lipid formation. Finally, Aquatic Species Program was successful in manifesting the feasibility of algae as a source of bio-diesel and explained the keynote findings for improvement or advances in the technology. However, the work highlighted the need to understand and maximise the research requirements of algal lipid production.

1.3 Motivation of Work

1.3.1 Indigenous algae

Nature is the most prolific habitat for survival of diversity of organism that provide tremendous variety of useful compounds for mankind. Approximately 70% of the Earth's surface is covered by water bodies and containing a largely unexplored algal biodiversity that offers an enormous resource of novel compounds. According to the Marine Literature database, varieties of metabolites exceeded 27,000 only from marine macro- and microorganisms. Therefore, the finding pave the path for future isolation and development of new products in the pharmaceutical, food, cosmeceutical, chemical, and agrochemical sectors. Algae from the euphotic zone was probably the first marine organisms utilised for investigation as sources of food, nutritional supplements, agricultural fertilisers and other valuable secondary metabolites. Varieties of species have the capability to produce varieties of compounds, and these diverse ranges of species survive in the diverse geographical environment. Therefore, current research also works in continuous discoveries of new indigenous species. Accordingly, these local species grow and survive best in the same belonged ecological niche. Therefore, workings in the local algal population have better survival and performance chance in outdoor culture plant. Reduced risk of contamination by other organisms could also observe. This is also one of the economical ways for large scale culture set up. As it manage the extra cost of procuring algae from outside, genetic modification for suitable growth or survival, photobioreactor maintenance cost, etc.

1.3.2 Lipid extraction methodology

Various protocols have been known today for lipid extraction and conversion into bio-diesel. Every protocol has its advantages and disadvantages. Extraction methods reported in algal cells were pyrolysis, supercritical fluid extraction, direct or stepwise transesterification, hydrothermal liquification, conventional soxhlet apparatus, Folch's method, etc. Further, pre-extraction techniques also improves oil fraction. Wet or dry algae biomass, with or without cell disruption, single or binary solvent extraction system, extraction time or temperature, acid or base pre-treatment are few simple but result determining step. These pre-extraction or extraction downstream processes also need to be improved by research.

Leftover algae residues have also used for various purposes like fertiliser, biochar, animal feed, aquafeed, and poultry feed, etc. However, study includes preparation, characterization and discussion of possible utility of obtained bio-char. The extensive literature reported that, the biochar could restore degraded soil, increase crop productivity, fix CO_2 and absorb contaminants. In general, the biochar is more stable than other organic matter on the ground, and thus it can persist in the soil for many years (Lehmann 2007). It was found that, the biochar has the potential to reduce CH_4 and N_2O emission from soil, especially in rice paddy soil, which is considered as a major source of greenhouse gas in China (Knoblauch et al. 2011).

1.3.3 Preservation scenario

According to literature, lipid and other value-added products were obtained by several algae species. Several commercial agencies or cell banks are putting research effort for developing new technologies for better cryopreservation results. Almost every phycological collection sustentation by routine sub-culturing. However, sub-culture on a regular basis is hectic and wastage of infrastructure. *Cryptococcus neoformans* strains were evaluated in Fungus Collection of the Tropical Medicine Institute, Brazil and revealed that phenotypic as well as genotypic alterations induced by a long period of subculture practice (Cavalcante et al. 2007). Consequently, low-temperature freezing is preferred unexceptionally. Nevertheless, the requirement of expensive programmable freezer/controlled-freezing system leads to search for other cheaper solutions. Vitrification has the advantage of the rapid cooling rate, practically applicable, economically suited and has shown high viability percentage in the diversity of cells (Gupta, Uhm, and Lee 2007; Gupta, Uhm, and Lee 2010). However, the large number and large size of the explant are difficult to handle at the same time, as the duration between successive steps are short and fixed. The recently developed encapsulation-dehydration methodology has an advantage over vitrification protocol of manipulation in precise duration. However, the technique consists of lengthy and time taking procedure. However, encapsulation-vitrification method has combined the benefit of these both techniques.

1.4 Scope of this study

Energy need is key principle to maintain socio-economic affairs which should be considerable particularly in terms of minimisation of environmental impact and natural resource

management. It is quite known that continuous consumption of fossil fuel could not be longer sustainable for increasing demand and it also increases potential greenhouse gas emission. Therefore, searching alternatives are highly required. Although several alternatives are in progress but every client have its own advantage and disadvantage. Biofuel is one of them which targets carbon as a source of energy by utilizing renewable natural resources. However, the first generation biofuels were derived from derived from the starch, sugar, animal fats, and vegetable oil which ultimately affects food resources. The second generation biofuels were derived from lignocellulosic biomass which are agricultural or forest residues. Whereas, it has disadvantages of requiring several steps for conversion to refined fuel and feedstock also competes with resources utilized for food crops. Microalgae derived biofuel is now considered as third generation resources as it devoid of the major drawbacks associated with first and second generation biofuels feedstocks. But as stated earlier, research still needs to overcome for the practice utilization of algae as biofuel. Therefore, the present study aims to use indigenous algae for bio-diesel production and described in different sections.

Research still discovers many unknown algal species, but on an approximation, the figure could be in between one to ten million (Metting 1996). Therefore, in the present study, we selected this geographical climate to know the algal diversity and possible ways to cultivate and extract bio-diesel. NIT, Rourkela premises has been chosen as the location of study which includes different physiological conditions. Based on previous literature, geographically distinct vicinity effect the variation in algal species and growth rate. Solar availability, ambient temperature, relative humidity, precipitation, presence of suitable land and water quality, availability of beneficial nutrients throughout the year are major deciding factors. Large culture system also faces several difficulties such as low mass culture, interspecific species contamination, non-uniform distribution of nutrient throughout the culture system, etc. The dominant species collected during the experiment were cultivated and preserved. The new methodology of preservation was also developed for comparatively higher viability percentage. Isolated cells were cultured in fabricated bioreactor set-up. Further, bio-diesel was extracted and characterized. Leftover algae residue were also utilised for bio-char production. Possible utilisation by biochar characterization was also included in the study.

1.5 Organization of thesis

The present work has been divided into several sections and discussed in different chapters which include introduction, literature, experimental methodology, bio-diesel production from mixed algae culture, airborne culture, biochar characterization from leftover algae residue developed cryopreservation protocol and conclusion of the study.

- Chapter 1: The present chapter is an introductory chapter that was focused on the need of research by briefly explaining in different subheadings.
- Chapter 2: contains literature review for biofuel as alternative sources, biofuel from microorganisms, current algae biofuel market scenario, future strategies for commercialization of algae biofuels and objective of present work.
- Chapter 3: Presents the materials and experiments selection for the collection site; purification, identification of samples; lipid estimation, extraction; FAME conversion and analytical characterization.
- Chapter 4: Includes the study of algae collected from water, identification, pre-treatment, lipid extraction, FAME conversion and characterization.
- Chapter 5: The chapter describes the collection of airborne algae throughout the year, purification, identification, climate changing effect, lipid extraction, FAME conversion and analytical studies.
- Chapter 6: The chapters discuss the bio-diesel and other possible co-product production from soil algae. Mainly three layers of chloroform: MeOH were used for possible utilisation as the source of saccharides, acids, chlorophyll pigment, bio-diesel, phytol and bio-char. Detail characterization of raw algae, residual algae and algae biochar was accomplished for compassion changes during every downstream step.
- Chapter 7: The study was regarding the development of cryopreservation protocol called open encapsulation-vitrification that enhanced the viability in comparison to the conventional method. The protocol was established on isolated and characterized water algae i.e. *Oocystis* and *Anabaena* as the model organism.
- Chapter 8: Presents the summary of the work and some suggestions for further study and finally the references used in this work.

Chapter 2

Literature Review

2.1 Biofuel as alternative

Biofuels could be the excellent alternative for both energy crisis and global warming. These renewable resources could be generated from the wide range of organic materials or even waste products. Leftovers could be efficiently recycled, could be exposed to open environment as these are biodegradable. Biofuels are thought to decline the troubles with conventional fossil fuels as they are capable of sequestering atmospheric carbon di-oxide gases. This is achieved by the photosynthesis in which light energy was absorbed by the chlorophyll for oxidation-reduction reaction.

Exhaust gases from bio-diesel has the less harmful impact on human health than petroleum diesel fuel as it contents comparatively lower nitric oxide, carbon and sulphur compounds (Volli, Singh, and Murugan 2014; Prakash, Singh, and Murugan 2013; A. Sharma and Murugan 2013). Biofuel based manufacture and refining industries are safer than underground mining and drilling impermeable rocks. These fuels lubrication could run most of the engines without major modification (Volli, Singh, and Murugan 2014). Apart from that the dependency from foreign petroleum outsourcing may decrease and will also provide employment within the country. Biofuels could be stored chemically in the form of liquid and has this advantage over other renewable sources of energy (solar, tidal, wind) since this fuel type can be easily integrated into current machinery (Amaro, Guedes, and Malcata 2011). Global production of bio-diesel begun in 1991 and reached to the production of approximately 2 billion gallons in 2006 (Birur, Hertel, and Tyner 2009).

Our world is dependent upon non-renewable resources today, knowing the fact that petroleum sources are already been depleting and may extinguish. Thinking for the alternative is the only option. According to renewables 2012 Global status Report, 80.6% of global final energy consumption depends upon fossil fuel, 16.7% on renewable resources whereas 8.5% on traditional biomass, 3.3% on biomass/solar/geothermal hot water/hydropower and 0.9% on

biomass/wind/solar/geothermal power generation respectively. In conclusion biomass is undoubtedly preeminent choice. But there are some conflict in biomass resources as it has other human consumable usages too. Therefore, current research also focuses on the alternatives of biomass resources.

2.2 Status of Biofuel from microorganism

An alternative fuel needs to be eco-friendly, economically competitive and overall net energy gain over the energy sources used to produce it. In recent years, a large number of hydrophobic lipid accumulating microorganisms have been studied especially used in the production of bio-diesel. Numerous oleaginous microorganism have been reported to grow and accumulate significant amounts of lipids similar to vegetable oil, methyl esters and soaps (Meng et al. 2009). Lipid accumulation and composition varies as per different species of microorganism and culture conditions (such as temperature, pH, culture time, etc.).

Among all other microbes, bacteria is known for higher growth rate and easy culture methods. It could accumulate oil content on average about 20–40% of dry biomass such as *Arthrobacter sp.* (40%) and *Acinetobacter calcoaceticus* (38%). Lipid accumulating bacteria, particularly of the *Actinomycete* group synthesizes fatty acids up to 70% of the cellular dry weight (Alvarez and Steinbüchel 2003). But literature suggests that generally bacteria accumulate lower lipid content than microalgae and very few species are oleaginous in nature. Complex lipoids are mostly concentrated in outer membrane which is difficult to extract. On the other hand, bacteria also requires simple carbon sources such as glucose under controlled conditions for intracellular TAGs accumulation. Therefore, currently these species are not considered as competitive client for bio-diesel production (Meng, Yang et al. 2009, Ratledge 1993).

Yeasts and fungi were also reported as oleaginous in nature since 1980s (Khambhaty et al. 2013). Some yeast strains, such as *Rhodosporidium sp., Rhodotorula sp.* and *Lipomyces sp.* could accumulate maximum intracellular lipids up to 70% of their biomass dry weight. *Crptococcus curvatus* was reported to accumulate storage lipid up to >60% on a dry weight basis and under N-limiting experimental condition it might also increase up to 90% TAG having about 44% of SFA similar to many vegetable oils (Meng et al. 2009). Mostly, oleic

(18:1), linoleic (18:2), palmitic (16:0) or palmitoleic acid (C16:1) are frequently found fatty acids in these organisms. But as yeasts are heterotrophic, they need to be grown under carbohydrate supplement. The choice of feedstock for growth of yeast also needs optimization and usually reported to grow on glucose or glucose syrups which is economically not cheaper. These organisms could only be maintained in designed series of fermenters. Maintenance of fermenter plants, controlling pH or aeration, sterilization of plant and media makes it economically non-feasible. Yeasts are also sensitive and easily contaminated by other bacteria or organisms. If were not maintained carefully, final culture easily dominated by other species. The situation even could run worse by development of pathogens in fermenter tank [Ratledge et al, 2008].

Among natural resources, algae have raised an enormous interest as these are photosynthetic organisms and exist in enormous form, from unicellular microscopic (microalgae) to multicellular (macroalgae or aquatic plants). Microalgae are known for higher photosynthetically efficient than plants for the production of biomass and oil. In fact depending on species, microalgae oil production can be 10–100 times higher than terrestrial plants. Algae as a resource for biofuel production not only will compete with the current demand but will not be contender candidate for food. Similar to other freshwater and marine organisms, algae can adapt to environmentally adverse conditions by the production of the variety of secondary metabolites, which are rarely found in other organisms. Algae have also been used for the multipurpose activity like wastewater purification, extraction of high value-added foods, pharmaceutical, nutraceuticals and for aquaculture (Guedes, Amaro, and Malcata 2011).

Algal biomass cultivated in the wastewater and seawater has the extra advantage of metal removal, nutrient uptake and decrease pressure on freshwater resources. Dependency on wastewater, however, could not be reliable as the quality varies from changing source and may fluctuates over time. Accordingly, diversity of algal blooms also fluctuates. A survey with 85–90% carpet industry effluents along with 10–15% municipal sewage evaluate that both the freshwater and marine algae could efficiently thrive and produce biomass and bio-diesel (Chinnasamy et al. 2010). Economics of producing microalgal bio-diesel need to improve considerably for market competition. Producing low- budget microalgal bio-diesel demands main improvements to algal biology, species selection, genetic or metabolic engineering,

improvement in the use of the biorefinery, photobioreactor design and downstream process optimization, etc. (Chisti 2008).

2.2.1 An account on Microalgae

The term microalgae most often considered as groups of diatoms, green algae, golden brown, *Prymnesiophytes, Eustigmatophytes* and *Cyanobacteria* (Metting 1996). However, group of *Cyanobacteria* are in conflict of being not algae but a class of photosynthetic bacteria. As per literature, these all members have been investigated as potential fuel production strains. Microalgae species also survive in a wide range of environments, freshwater, saturated saline, rocks, soil, humid air and many more. Algae contributes more than 40% of the global carbon fixation which are majorly efficient due to marine microalgae (Aishvarya et al. 2012). Microalgae can produce biomass very rapidly in comparison to other macro algae and even some species reports to have doubling time of 6 hour (Collet et al. 2011). Almost all algae have the capacity to accumulate energy-rich lipid whereas many microalgae species have been found to naturally accumulate high oil. Such as some *Botryococcus sp*. were reported to have up to 50% long chain hydrocarbons of their dry mass stored as (Metzger and Largeau 2005). Studies also says that these microalgae species have broader ancestral relationships than any known potential biofuel crops (Deschamps and Moreira 2009).

2.3 Improved strategies for algae biofuel production

Every algal species have their own constant lipid profile if grown under the same conditions. However, a number of studies were reported regarding increased lipid accumulation in algae under certain conditions due to variation in physio-chemical parameters like nutrients, metal deficiency and sun light, etc. Similar study was conducted by providing high iron concentration in nutrient media which leads to lipid accumulation in *C. vulgaris* probably due to modification in some metabolic pathways related to the lipid (Z. Y. Liu, Wang, and Zhou 2008). Another strategy includes the changes in downstream processing which may change the quality or quantity of accumulated overall lipid within the cell. It has been demonstrated that the overall light conversion efficiency of bioreactors can be noticeably improve lipid quantity as it reduces the number of the chlorophyll-binding LHC proteins in each cell (Mussgnug et al. 2007). Therefore, it is important to utilize species that have a suitable lipid

profile by maintaining suitable environment for bio-diesel production (Schenk et al. 2008).

Further, the high lipid yield microalgae strain usually obtained by screening of a wide range of naturally available isolates and the efficiency of those can be enhanced by selection, adaptation and genetic engineering. Fatty acid enzyme pathway had the great impact on quality and quantity of bio-diesel production. Therefore with the help of recombinant DNA technology and genetic engineering the fatty acid key enzymes or lipid synthesis pathways are targeted. For example, by up-regulation or down-regulation of fatty acid biosynthesis and b-oxidation respectively. By gene knock out or modifying polyunsaturated lipids synthesis enzymes could increase the proportion of monounsaturated lipids. At present and may be of future algal strain improvement will be screened by lipidomics, genomics, proteomics, and metabolomics that exhibit high growth rate, lipid accumulation, environmental adaptation and high value-add by-products (Schenk et al. 2008).

2.3.1 Algae contents

Natural water resources vary in different geographical conditions due to the presence of different organic and inorganic substances, but the origin of most of them is unknown (H. W. Kores 1972). The presence of algal blooms might be one of the possibilities among several reasons. Either by algae cells or whole thalli disintegration or by the liberation of extracellular compounds. Five groups of extracellular substances were found during extraction process from *Chlorococcum* and *Chlamydomonas*. These includes steam-volatile acids; yellow, water-soluble phenolic compounds; lipophilic substances; proteins and polysaccharides (H. W. Kores 1972).

As residual co-product, the aqueous layer after hydrothermal liquefaction of *Nannochloropsis oculata* was used as growth nutrient media for other microorganisms such as *Escherichia coli, Pseudomonas putida*, and *Saccharomyces cerevisiae*. Growth rates, yields, and carbon/nitrogen/phosphorus uptake were measured and found suitable for microbial culture simultaneously. These key finding could pave the easy pathways for biorefinery industries (Nelson et al. 2013). However, it is not yet clearly known that weather the liberation of substances follows a general pattern in different species, or the species differ more or less in the products they give off. Algal biomass is primarily made up of lipids, carbohydrates and

protein, but the percentage constitution varies widely from species to species and its culture conditions. The uniqueness with this biomass is the absence of lignin, the presence of phospholipids, high-quality carbohydrate fractions, antioxidants as well as the variation in quantity or quality. However, the collaboration of green chemistry and green engineering improves the prospects for our energy and material economy.

2.3.2 Harvesting

As microalgae cells are tiny microscopic organism therefore harvesting is tedious task which needs research attention. Present technologies used for harvesting of algae biomass includes flocculation, filtration, centrifugation and ultrasonic aggregation. Every mentioned techniques are having its own advantage and disadvantages. Such as, biological or chemical flocculation have advantage of cheap functioning budget but disadvantage of long duration and product decomposition. Whereas other techniques such as filtration, centrifuge, ultrasonic flocculation are more efficient but not suitable for large scale production and are not economically feasible too (Li et al, 2008).

2.4 Present market scenario

2.4.1 Status in India

Optimization of biofuel from renewable biomass resources and eco-friendly wastes are now not new in Indian scenario (Nayan, Kumar, and Singh 2013). Several researches also confirm the better engine performance from biofuel than diesel (Prakash, Singh, and Murugan 2013). Limitation of terrestrial biomass resources over algae biomass makes it the superior choice. In 1973 freshwater algal mass cultivation on pilot plant scale started at Central Food Technological Research Institute (CFTRI), Mysore (Becker and Venkataraman 1984). Twelve National Laboratories/Institute/University all over India are now under an obligation to work on algae biofuel, especially focusing on the area of collection, characterization, storage & improvement of oleaginous algae strains, design and development of the low-cost system of cultivation as well as oil recovery (Hemaiswarya et al. 2012). Group of researchers in CSMCRI, Gujarat has contributed in this field by utilised the marine yeast (Candida sp.) for converting *Kappaphycus alvarezii* (red algae) into ethanol under highly saline condition (Khambhaty et al. 2013). Further, they found buffered solvent system the most appropriate for

lipid research in the comparative evaluation of lipid extraction methods like Bligh and Dyer, Folch, Cequier-Sánchez methods, sonication and buffer extraction in green (*Ulva fasciata*), red (*Gracilaria corticata*) and brown algae (*Sargassum tenerrimum*) (Kumari, Reddy, and Jha 2011). Similarly, Bioengineering and Environmental Sciences team of IICT, Hyderabad discloses the effect of CO_2 sparging rate, duration and interval in growth and lipid accumulation of mixotrophic algae (Prathima Devi and Venkata Mohan 2012). Further, the team also produced a unique integrative process by combining Acid-rich effluent generated from acidogenic biohydrogen as a substrate for algae growth and lipid accumulation, and data supports the conformation as well (Venkata Mohan and Prathima Devi 2012). Other CSIR laboratories like IIMT, Bhubaneswar also optimized the alkali (NaOH) concentration for enhancement of CO2 sequestration in *Chlorella sp*. IMMTCC-2 (Aishvarya et al. 2012).

Apart from the effort of Indian Government research lab and universities, now few industries are also deciding to assemble. As of 19 July 2013, the Indian Oil listed on its website that Indian oil and Department of Biotechnology jointly set up a Centre for Advanced Bio-Energy Research in the campus of Indian Oil's R&D Centre at Faridabad. They also signed the agreement with Lanza Tech, USA for further development of Microalgae Technology. As per update of 15 July 2013, the live mint and wall street journal, Reliance Industries Ltd launched a pilot "algae-to-oil" project at a 100-acre facility in Jamnagar, Gujarat known to be the world's largest single-location refining complex. At present they are able to produce 10 barrels a day but aim is to increase up to 100 barrels. Some current research facts enforces for further investment in this domain. In a study, it was found that, Indian climate is tropical (37°C) which is very suitable for microalgae cultivation in raceway pond especially in plain and coastal areas, without the need of photobioreactors (Khan et al. 2009). A survey report confirms the four following locations to yield algae at satisfactory growth rate (Chanakya et al. 2013).

A. In paddy fields as a multi-tier crop (20 Million Hectare)
B. In saline, brackish region of Kachch (Gujarat; 3 Million Hectare)
C. Urban domestic waste water (40 billion Litre/day)
D. On fishery deficient seashores

The Indian researchers in this field are just on the verge of starting and many challenges still need to be focused.

2.4.2 Worldwide scenario

Algae market is yet to be senesce. It has all the capabilities to accomplish the expected energy demand. According to International Energy Agency (IEA) 2012 report, the energy demand of more than one-third will increase by 2035, especially in China, India and the Middle East. According to DBT, India is 5th larger energy consumer. In the year 2003-04 about 90 million tonnes of crude oil has been imported, and the demand graph is still in logarithmic phase. IEA 2012 report also claims increment of 3.6°C global warming. Research could make the versatile profit from Algae culture i.e. environment safety and balance economy of the country.

Presently, US rank first with 78%, followed by Europe 13% in algae biofuel production. More than 30 million £ by Europe (UK Carbon Trust), 6 million € by Scotland (BioMara), 100 million $ by US (Sapphire Energy), 92 million $ by collaboration of Aurantia (Spanish company) and green Fuel technology (Massachusetts, US) has been already invested before 2011 (J. Singh and Gu 2010). Leverage also has successful stories of the establishment of the industry as well as methodologies. Many more companies are in this success list which includes Aurora Algae, Synthetic Genomics, Petro Algae, Bioalgene, Phycal, Live fuels, Aquaflow, Bionavitas, Bodega Algae, General Atomics and much more. Altogether more than hundreds of companies worldwide are involved, less than 25 are in pilot phase, few have invested 10 million $ to optimization of fuel production (Amaro, Guedes, and Malcata 2011). Table 1 summarizes the claims by respective companies in their website for commercialization.

Interest increment of biofuel and algae can be estimated by observation of yearly citation data (Fig 1). Patent application filing competitions in the field of algae biofuel are increasing day by day. Few important (optimization of cultivation, separation and oil extraction) recent patents discussed here shows the tough contest of present era. Strain improvement for desired product yield was accomplished and patented (US20120329099 A1). Matthew C. et al. discloses the discovery of genetically modified algae which accumulates the much greater amount of starch. The Modified mutant was previously starchless phenotype than has been complemented with one or more copies of wild-type STA7 isoamylase gene construct [76]. Algae cultivation cost reduction was targeted by US20120282651 A1 and US20120202242 A1. The patent US20120282651 A1 claims to invent a co-cultivation system of together

microalgae & filamentous fungi for low cost separation from the medium, to achieve high biomass and lipid yield. Whereas David A et al in US20120202242 A1 stated the fabrication of a non-sterile heterotrophic cultivation system which includes a continuous stirred tank reactor (CSTR) followed by two Plug Flow Reactor (PFR). First CSTR tank will be loaded with nutrient and algae cell than travel through first nutrient depleted PFR to decrease other competent & will finally be transferred to another PFR having organic carbon.

Similarly, patent WO2011159547 A1 discloses a unique system to utilise pollutant contaminated fluid. In a chamber, waste fluid will be collected which will receive scrubber solution after treatment fluid will enter to the bioreactor for algae growth. Different recycle device separates algae after biofuel processing, scrubber solution and extra fluid from the system. The downstream system for separation and collection of lipid as well as oil was simplified by US20120045800 A1 and US20130115147 A1. The former claims to form a modified, advance separator system which separates nutrient and algae from cultivation chamber, lysed algae residue from oil after oil recovery and biofuel, glycerine after processing. Similarly, the later patent claims to comprise a microspike board. The array of spikes inclined in certain angles punctures the algae cells which release bio oil content within. Other than bio-diesel algae also have the large industrial application such as nutraceuticals, fertilisers, animal or fish feed, pharmaceuticals, hydrocolloids, substitutes for synthetics, cosmetics, chemicals and pigments (Tabernero, Martin Del Valle, and Galan 2013).

Figure 1. Citations by year from a web of science search related to “algae” and “biofuels (Miodrag Belosevic 2014).”

Table 1. Examples of current achievements by Algae biofuel Industries

Name of Company	Location	Current Achievements
Algenol	Florida	Producing 9000 gallon ethanol per acre per year in 4 acre land with help of 160 member team
Solix	Colorado, US	Production of more than 3000 gallons of algae oil per acre per year in its own designed Lumian AGS4000 successfully in 2010.
Sapphire Energy	San Diago, California	Developed successful technology to produce 91 octane gasoline, 89 cetane diesel and jet fuel.
Solazyme	South San Francisco, California	In 2010 delivered 80,000 liters of algae diesel and jet fuel to US Navy. Next target of company is to provide 5, 50,000 additional liters. In a test drive, vehicle ran thousands mile with unblended Solazyme Algae fuel without modification in engine.
Seambiotic	Israel	Grows microalgae culture using flue gas collected from nearby coal plant as feed stocks. It is producing biofuel as well as omega-3 food additives in pilot scale.

2.5 Strategies required for commercialization of algae biofuels

Fossil fuel reservoir beneath the earth surface is fixed and non-renewable. According to IEA (International Energy Agency) and BP (British Petroleum) reports techniques are in progress to discover new depositories but one day will not be sufficient to satisfy the demand of exponentially growing population (Khan et al. 2009). Increased consumption of conventional fuel majorly for industrialization and transportation is immersing our ecological balance day by day. Greenhouse gases accumulation, hole in ozone layer, acid rain, climate change, aquatic life trauma by oil spills are severe infrastructural damage to nature (Tingzhen et al. 2014). The present era demands new technologies to overcome these situations, and biofuels are the excellent alternative. These renewable energy feedstocks could be derived from the wide range

of organic materials or even waste products. Leftovers could be efficiently recycled, could be exposed to open environment as these are biodegradable. Bio-diesel exhaust has the less harmful impact on human health than petroleum diesel fuel as it contents comparatively lower nitric oxide, carbon and sulphur compounds (Prakash, Singh, and Murugan 2013). Dependency from foreign petroleum outsourcing may decrease and will also provide employment within the country.

2.5.1 Cryopreservation

A large number of algae have been isolated, characterized, selected, mutated and genetically modified worldwide to address the shortage of fossil fuels and to understand the ecological response patterns to climate change. Algae are also considered to be useful as agro foods, human supplements, the source of preservatives, and bio-fertilizers (R. J. Radmer 2009; Teimouri, Amirkolaie, and Yeganeh 2013; Vidyashankar et al. 2015). Several products have been known to obtain from microalgae yet such as biofuel (A. K. Singh and Singh 2014), fatty acids (K. Sharma and Schenk 2015), alcohols (Ellis et al. 2012), carbohydrates (Chia et al. 2015), therapeutic proteins (Gimpel et al. 2015), vaccines (Gimpel et al. 2015), etc. and many more could be possible in the near future as thousands of Algal strains known having "unique" characteristics of their own, such as *Oocystis sp*. Fatty acids for biofuel, bioconversion to obtain hydrocortisone, monoterpenes and bio-adsorption of heavy metals are current on-going research using this species (Ghasemi et al. 2009; Chinnasamy et al. 2010).

Several research communities and various industries, therefore, maintain a rich genetic diversity of different algal forms including microalgae and cyanobacteria. Unfortunately, the study on higher viability after cryopreservation of these valuable species are limited. Axenic cultures isolation is the tedious task and hence is necessary to preserve for the long duration. Therefore optimization to maximize viability using cryopreservation is a research requisite.
Most of the microalgae species were successfully cryopreserved, but due to unknown reasons still few species among them either fails to survive or shows low viability percentage (Brand and Diller 2004; Day and Fleck 2015). Similarly, macroalgae diversity like filamentous or seaweeds and geographically distinct algal species were yet not satisfactorily attempted (Day 2007). For other alternatives, algal culture banks are practicing non-axenic culture storage or density dependent preservation protocols, etc. (Amaral et al. 2013; Piasecki, Diller, and Brand

2009).

Long-term maintenance of algal collections on liquid or solid media does not assure their long-term maintenance and availability as these processes are labor intensive, costly and subject to contamination and genetic drift (Cavalcante et al. 2007). However, cryopreservation offers a cost-effective solution for long-term maintenance and transport of stable stocks of the wide variety of microorganisms including bacteria, fungi and algae. Among different methods of cryopreservation, vitrification offers an advantage of being simple, cost-effective and does not require any expensive device such as programmable freezer (Schiewe et al. 2015). However, its success is limited in most species of algae (Buhmann, Day, and Kroth 2013). Moreover, procedural steps such as the concentration and timing of loading and unloading of cryoprotective agents (CPA), cell density, etc. can influence the success of the algal cryopreservation (Tzovenis et al. 2004).

Encapsulation-vitrification technique recently developed and has simple and easy protocols (Tanaka et al. 2004). Encapsulation-vitrification has been successfully accomplished in various plant materials like axillary buds of potato (Dai Hirai and Sakai 1999), mint (D. Hirai and Sakai 1999), axillary shoot tips of apple (Paul, Daigny, and Sangwan-Norreel 2000), shoot tips of raspberry (Q. Wang et al. 2005), embryogenic cell grapevine rootstocks (Q. Wang et al. 2004), strawberry germplasm (Dai Hirai et al. 1998), somatic embryos of Olive and many more. It has recently been adapted for algae preservation. Optimisation parameters for the encapsulation-vitrification technique of vitrification duration and effect of vitrification solutions in each step for algal species namely, *Porphyra yezoensis* (seaweed); *Nitzschia closterium, Chaetoceros muelleri* (marine diatom); *Undaria pinnatifida* (sea vegetable) was identified (H. Liu et al. 2004; Zhang et al. 2009; B. Wang et al. 2011). (Table 2) Research still needs excellence in protocols like the rapid cooling rate. Recent studies have also shown that CPA- exposure and cooling can generate intracellular free radicals which can cause oxidative stress and induce apoptosis and cell death (Day, Fleck, and Benson 2000). The free radicals may also cause genetic alterations. Accordingly, use of antioxidants during vitrification was proposed to contribute to improving cryotolerant and viability of vitrified-warmed algae (Fleck et al. 2003). However, studies on possible beneficial effects of antioxidants on the viability of vitrified-warmed algae are scarce.

Studies in mammalian oocytes and embryos have shown that direct contact of the biological samples with liquid nitrogen (LN_2) during vitrification can significantly improve their post-warming viability (Vajta, Rienzi, and Ubaldi 2015). Such system, called open system of vitrification, allows the rapid rate of cooling due to minimal vitrification solution surrounding the biological sample and absence of insulating wall of the cryo-containers. Consequently, the open system of vitrification has become a popular method for vitrification of animal oocytes and embryos. In contrast, in the closed system of vitrification, samples are loaded and sealed in a cryo-container before vitrification to eliminate the direct contact of the biological sample with the LN_2. The latter system is advantageous for human samples to avoid possible cross-contamination with bacteria and viruses such as human immunodeficiency virus and hepatitis B and C viruses which are known to survive in LN_2 (Jensen et al. 2015).

Table 2. Enlist of viability % and loading solution in algae encapsulation vitrification till date.

Algae description		**Viability %**	**Loading condition**	**Dehydrating condition**	**reference**
Porphyra yezoensis (Seaweed)	protoplasts	66.5%	25% VS6 for 5 min at 0 °C	ice-cold concentrated VS6 for 3 min	(H. Liu et al. 2004)
Undaria pinnatifida (Seaweed)	Male gametophyte	26%	2 M glycerol and 0.6 M sucrose for 120 and 90 min at 25 °C	PVS2 for 50 and 40 min at 0 °C	(B. Wang et al. 2011)
	female gametophyte	31.2%			
marine diatom	*N. closterium*	73.8%	50% PVS2 for 60 min	100% PVS2 for 60 min at 0°C	(Zhang et al. 2009)
	C. muelleri	48.2%	50% PVS2 for 40 min	100% PVS2 for 60 min at 0°C	
Green Algae	*Oocystis sp.*	~54.20%	50% PVS2 (30 min)	100% PVS2(15 min)	Present
Cynobacteria	*Anabaena sp.*	~45.78 %	50% PVS2 (30 min)	100% PVS2(15 min)	Present

2.5.2 Lipid extraction pre-treatment

The current supply of bio-diesel in comparison to other conventional fuel is very less as raw products usages fertile land, water, competitive with food or other consumables and seasonal based supply of raw material. One of the capable substitutes is algae that overcome these limitations (Clarens et al. 2010). Selective algae stains have also benefited from higher growth rate, potential to produce higher triglycerides per area than terrestrial plants, can be cultivated in the useless land, polluted water, uptakes greenhouse gases, heavy metals from the environment and can be the source of various commercial products (R. Radmer 1996).

Algae biomass having catalytic, physical, chemical pre-treatment studies are limited but have overall importance as other downstream processing. Pretreatment of a substrate before extraction allows improving its biodegradability while acting on its physicochemical properties significantly. Used physical and chemical pretreatment processes include acids or bases treatment, thermal treatment and ultrasonic lysis, high-pressure homogenization. The effect of temperature, duration of the treatment, pH, and substrate concentration also affects the overall quality or quantity of final obtained yield (Table 3).

Increased pressure or temperature disrupts the cell wall, and this increases the extraction efficiency. This pre-treatment practice increases the solvent contact with the cellular materials via diffusion (Geciova, Bury, and Jelen 2002). Comparative to conventional extraction the lipid extraction followed by FAME conversion increases in microwave or bead beating treatments. It has also been noticed that freezing samples are also considered in pre-treatment but yet not much difference has been reported. Lipid yield also depends on final extraction procedure in combination with pre-treatment. For example, pre-treatment in the case of supercritical carbon dioxide extraction enhance both the yield and purity. The yield of sonicated cells do not differ much in gravity measurement but has high FAME conversion than conventional due to much solvent interaction in ruptured cells. Pre-treatment using pressure also includes autoclaving at 125 ˚C under pressure of 1.5 MPa and was conducted using *Botryococcus sp., Chlorella vulgaris*, and *Scenedesmus sp*. However, the study concluded that this treatment was not suitable and overall extraction was just 13% (J. Y. Lee et al. 2010). Similarly, Dote et al. treated the *B. braunii* suspended in water at 200–300 ˚C under high

pressure using an autoclave and found that liquid fuel could be obtained at a yield greater than the original content of hydrocarbons (Dote et al. 1994). Even the thermal pretreatment using wet algae also recovers more extracts (Kita et al. 2010).

Table 3. Examples of pre-treatment along with extraction methods.

Algae/seaweed/aquatic plants	**Pre-treatment**	**Extraction method**	**Refs.**
D. tertiolecta, P. tricornutum	Lyophilized (24–48 h)	Chloroform: MeOH (2:1), rotary wheel for 4 h, vacuum concentrator (60C). lyophilized, extracted with hot water for 1 h, combined with the MeOH and water. Soluble phase was dried again and analysed.	(Foster, Thomson, and Maher 2008)
P. australis C. officinalis, M. fragilis, L. obtuse, L. sp., D. pulchra, U. rigida, C. flexilis, C. herpestica, C. cactoides, R. implexium, C. subsimplex	freeze dried and homogenized	Chloroform: MeOH (2:1), vortexed on rotatory wheel (4 h), vacuum drying, addition of hot water to residue, stored frozen for analysis.	(Foster, Thomson, and Maher 2008)
Laminaria Digitata	Air dried, homogenized, freeze dried and powdered	Chloroform: MeOH (2:1) under sonication, enzyme hydrolysis (phospholipase D)	(Devalla and Feldmann 2003)
D. splendida	Air drying	chloroform–MeOH (2:1)	(El-Moneim M. R. Afify, Shalaby, and Shanab 2010)
Mixed cultures	Air drying	Soxhlet extraction (120 min, hexane)	(Vijayaraghavan and Hemanathan 2009)
S. bacillaris	Freeze-drying	chloroform–MeOH (2:1)	(Olivieri et al. 2011)
D. tertiolecta	Glass beads cell disruption	chloroform–MeOH (2:1)	(Tang et al. 2011)
C. pyrenoidosa	Drying	chloroform–MeOH (2:1)	(D'oca et al. 2011)

2.5.3 Selection of strain

World's rising population lacks livelihood needs of the energy to run the eminent industrialization by maintaining the sanctified atmosphere. Energy is essential requirements

for economic development of all developed as well as developing countries. Till date world's transportation is dependent on non-renewable fossil fuels. The phase of limited supplies is raising the oil prices day by day. Many nations are facing the similar present challenge and are searching for the attractive alternative to meet the future demand. Countries also have different propaganda like reduce the dependency of fossil fuel import, reducing greenhouse gas emission, etc. Currently, biomass feedstock of forestry, crops, waste biomass are still contributing to world's primary energy supplies and are expected to actively participate towards present problematic scenario. However, it still needs some research solutions. The pitfall areas are production management, pressure on natural resources may lead to the shortage of food, economic loss of high pricing crops, land or water management and may reduce quality of life.

Current research also suggests that biofuel or ethanol could be derived from microorganisms. Bacteria, fungus and algae have the potential to accumulate oil, and that could be derived for future fuel purpose. These alternatives also could overcome the drawbacks of other biomass resources. Algae is having certain advantages like rapid growth rate, estimated to yield more biofuel per hectare than other agricultural sources, can sequester significantly excess carbon dioxide, low toxic gas emission as bio-diesel, highly biodegradable, does not compete with food, fiber or other agricultural valuable sources, will not disturb natural habitats. Microalgae stores lipids in their membrane components, storage vacuoles and as metabolites. Under stress conditions, microalgae accumulate lipids in comparatively higher percentage. It can even grow by up taking nutrient from wastewater ponds, animal waste and other liquid wastes. Open large scale culture having limitations of climate, as defined by temperature and sunlight. According to geographical distribution southern parts of the United States and Asia, most of South America, Africa and Australia are many suitable areas for algae cultivation. Other places like Australia, South-east Asia and the Pacific area are having the advantage of comparably favorable winter and night temperature fall (Brennan and Owende 2010). Additionally, resources of saline or waste water, GHG abatement, sewage farms, industrial wastes or animal feed lots having sufficient nutrient content.

Biofuels from algae biomass have several advantages over terrestrial crops but have entered the spotlight lately. However, many significant obstacles still need to be the deal for achieving

economically competitive with energy from basic petroleum fuels. Associated people from oil companies or entrepreneurs and investors were looking options from decades for conversion of photosynthetic powers into available energy resources, and now it is possible to convert green slime, algae, into energy. Industry giants such as Dow Chemical Co., ExxonMobil Corp., BP p.l.c., and Chevron Corp. are planning to develop renewable fuels from this prolific group of algae. The planning includes selection and collection of algae strains for varying ratios of oils, proteins, starch reside within their cells. Briefly, industries are exploring to achieve vast options for exploiting energy from these microbes. Algae strain belongs either from indigenous strains or possibly genetically engineered are continuously screened. Few varieties have been reported for high lipid, carbohydrate and protein percentage in same species (Miodrag Belosevic 2014).

Along with pros, the idea has few cons that needs to be resolved. Research is in progress to troubleshoot these difficulties. Recent studies report the finding of airborne alga in the environment. Fungal or bacterial air contamination is familiar but airborne algae reports, and its related consequences are seldom described. The first finding was mentioned in 1910 at Germany. After that several literatures of different places were reported like in the metropolitan area of Mexico City by Sokoloff in 1931, algae from house dust were detected in the United States (D 1931; Burge and Rogers 2000). Similarly, viable green algae such as *Chlorococcum, Chlorella*, *Stichococcus*; *Cyanobacteria* such as *Nostoc, Phormidium* and *Anabaena* were detected from a healthcare center, a recreational center and several nursery schools in Sweden (Marshall and Chalmers 1997). Reports from Malaysia, Spain, Antarctica, and Delhi, India were also previously reported (Ng, Chu, and Ambu 2011; Tormo et al. 2001; Marshall and Chalmers 1997; Mittal A, M K Agarwal 1979). However, the diversity and abundance of these airborne algae species vary by changing climate, topography, geography, diurnal and seasonal effects. It has also been known that diversity and abundance of airborne algae are higher in tropical regions (Schlichting 1969).

The atmosphere is considered to harbour a wide mixture of microorganisms in all possible locality, such as air, water and soil. On literature survey, it is quite clear that very limited research has been carried out on airborne algae in comparison to water as well as soil algae. As for other microorganisms, air acts as a medium of transport for airborne algae also. As a

matter of fact, life cycles of such species are interlinked to the seasonal changes, meteorological conditions and also the morphological features of entire units of scattering are an adaptive response to transport phenomena. Health issue due to air pollution is now one of the challenging problem worldwide (Annesi-Maesano et al. 2007). The atmosphere has been recognized as a “spora” of microorganisms, including viruses, bacteria and microalgae, which can induce allergies and airborne diseases. However, relatively few studies have focused on bioaerosol associations with adverse human health effects (Douwes et al. 2003). Even scarcer are the data on airborne algae and cyanobacteria and related health effects, although their ecological and economic importance has long been recognized.

2.5.4 Bio-diesel co-product production

World’s rising population lacks livelihood needs of the healthy diet, sanctified atmosphere and energy. FAO reports, 925 million or 13.1% of world’s population or every 1 in 7 people do not have enough food to survive (Fao 2013). Air pollution alone in China comprises loss of 25 million life in 2010 (International Energy Agency 2003). Fossil fuel depositories are in evasive status to fulfil the demand of eminent industrialization. Convergent efforts to resolve the problem is in progress. Algae alone might be a versatile solution! The limiting factor to achieving the marketable algae biofuel is economical and efficient extraction technology. The applied solvents do not ideally separate cellular lipids from other intracellular materials like proteins, polysaccharides, sugars, pigments, metals, etc. As the biological materials differ in structure, morphology, texture, therefore, the same methodology may not give most efficient extraction to all. The Folch method or similar Bligh and Dyer method use the binary solvent combination of chloroform–MeOH (2:1) for endogenous cells lipid extraction (Folch, Lees, and Stanley 1957). The above method with some modification is majority utilised for the estimation of algal lipids.

The obtained residues from the process were considered as processed wastes that represent a cost liability for its further disposal and treatment. As the algae residues are protein rich, therefore it could be used as a nutrient additive in livestock feeds (Tabernero, Martin Del Valle, and Galan 2013). But for that, the process for bio-diesel production should be non-toxic, or the residue should be treated further. Hence, this downstream processing is the extra burden to industries. The extensive literature has been supported by few experiments for the

production of CH_4 using post transesterified residual algae biomass (Ehimen et al. 2011). The concept of recycling the nutrients are possible, but somewhere there is a sacrifice of high-value products i.e. bio-diesel. However, no experiments yet are available to prove that these nutrients recycling could exist in the dry route process. However, as it contains the high value of ‘‘N’’ and ‘‘P’’ amount, therefore, residue could be used as a fertiliser for terrestrial crops (Mullen et al. 2010). But residual algae obtained after transesterification may lose some nutritional value. However, research also discusses for obtaining ethanol or methane via fermentation but experiments regarding is scare for residual biomass.

2.6 Objectives of Present work

Based on current scenario and literature survey the overall objective of this work was to study the local diversity of algae ingathering, environmental variation during collection, morphological identification, extraction, accumulation of lipid, conversion into FAME and characterization.

The specific objectives of this study are:

- Developments of the new method of cryopreservation i.e. open encapsulation-vitrification for the preservation of these valuable algae biomass.
- Production and characterization of FAME from water algae and effect of pre-treatment in lipid extraction.
- Production and characterization of FAME from airborne algae and seasonal availability obtained throughout the year.
- Production of FAME and co-product obtained from all layers of MeOH: chloroform extraction and detailed comparison of raw, residue and bio-char from soil algae.

Chapter 3

Materials and Methods

3.1 Summary

This chapter describes the details regarding materials, chemicals and steps followed during experiments conducted for this study. Methodology mainly includes details regarding sample collection site, collection methods, analysis of site, algae purification or isolation, growth conditions, preservation, lipid extraction, transesterification, analytical technique details and fuel property analysis. As every experiment was not similar in all the chapters, therefore few particular details of protocols were mentioned in individual chapters as well.

3.2 Materials

All reagents of analytical reagent grade were used. Double deionized water (Milli-Q Millipore) was used for all required experiments. Chemicals and reagents for Algae culture growth media i.e. Algae culture agar, Algae culture broth, Nutrient agar, BBM reagents were purchased from Himedia Pvt Ltd, Mumbai. Reagents for water and soil analysis such as potassium dichromate, potassium chloride, sulfuric acid, ferrous ammonium sulphate, silver nitrate, ortho-phosphoric acid, sodium hydroxide, dickman-bray reagent, stannous chloride, ferrion indicator, Erichrome Black T indicator, methyl orange indicator, diphenylamine indicator, bromothymol blue indicator etc were purchased from Thermo scientific, sigma, Merck and Himedia chemicals.

Similarly, chemicals used during algae morphology study potassium iodide, iodine, glutaraldehyde, osmium tetroxide were also of analytical grade from sigma-aldrich. Organic solvents for lipid extraction such as petroleum ether, chloroform and MeOH, DMSO were procured from Merck, Mumbai, and Nile red for lipid estimation was of Himedia Pvt. Ltd. All the plastic and glassware used in the experiment were at first soaked in dilute HNO_3 (1/9, v/v), autoclaved followed by rinsing with distilled water prior to use.

3.3 Methods

3.3.1 Study Site

Rourkela is situated on the northern border of Odisha, Sundergarh district. This area is rich in iron ore and therefore many industrial plants are located here. Geographically, Rourkela is located at 84.54E longitude and 22.12 N latitude at about 219 meters above sea level. The city is located in the hilly area and surrounded by rivers as well. The NIT campus is located at the eastern end of Rourkela city and is also bordered by small mountains on the south. Lushed green campus occupied by more than 6000 people. The overall campus area is about 1024 acres (2nd largest campus in India, after IIT Kharagpur).Therefore this undulating topography was selected for the site of sample collection. The algae samples were collected from different locations within the NIT premises. Algae samples were collected from soil, water and air that include stressed conditions and nutrient enrichment area too. Therefore, water and sand were also collected for further analysis. Fig 2 and 3 demonstrates the geographical location of water and soil sampling site.

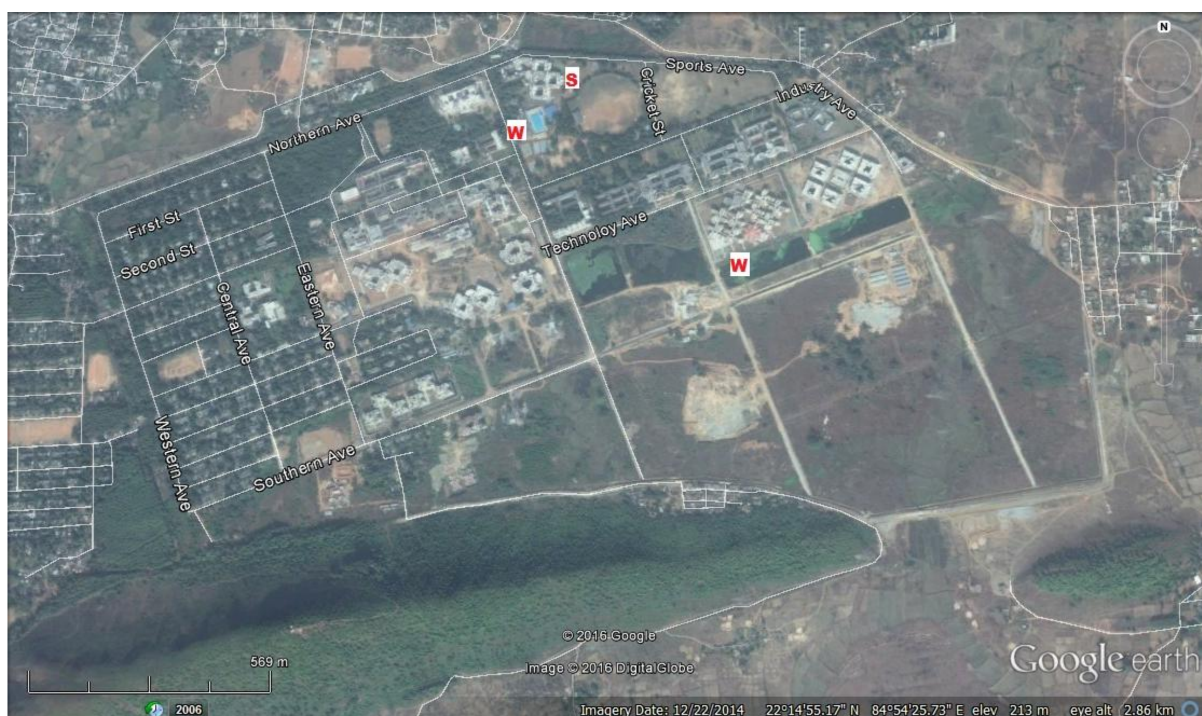

Figure 2. Geographical locations of sampling site downloaded through Google earth. "W", "S" marks indicates the algae collection site from water and soil respectively. Longitude and latitude location of S is 22°15'22.4"N 84°54'18.5"E; W is 22°15′19.5″N; 84°54′14.5″E and W is 22°15'03.7"N 84°54'38.6"E.

Figure 3. Sample collection site.

3.3.2 How to collect?

3.3.2.1 Water

Algal samples from water bodies were collected in a 2-litre polyethene carboy. Plastic bottles of 100 ml for algae sample collection and another 1-litre water was also collected for water analysis and keeping cells alive in that source water for few more days. The bottles were rinsed thrice with water collected during samples collection. Samples were collected only two different times throughout the study and used for study proceeded in chapter 4 and 5. Geographical location was already mentioned in text. During sample collection temperature of water was about 25-27 ℃ and were collected early morning time. Samples were not collected during rain event. The collected samples were brought to the lab and immediately dewatered and serially cleaned with tap water followed by distilled water. Samples were dewatered by sieving the biomass through mesh nylon netting. Finally, both the water and algae samples were labelled, sealed and stored in a refrigerator at 4 ℃.

3.3.2.2 Soil

Soil having algae accumulation were collected using 1.5 cm diameter sterile stainless steel cylinders with removable steel caps. In the field, the cores were pushed into the ground to remove a relatively undisturbed core of soil and left for few minutes to loosen the crust. Soil moisture of site was determined during collecting soil samples. Samples were collected once throughout the study and used for study proceeded in chapter 7. Geographical location was

already mentioned in text. Samples were collected early morning time but not during rain event. After collection, the well-labelled sample holders were brought to the lab. The top few millimetres of soil were removed with an alcohol-sterilized razor blade and placed in sterile flasks. An attempt was made to remove maximum soil by this process or by serial dilution or by placing the flask in the shaker for proper separation of soil and algae.

3.3.2.3 Air

The collection of air samples was done by two random ways. Firstly, the air stream containing the microbial particles was directed onto the surface of BBM agar in a sterile petri dish (90 mm in diameter) exposed above the ground. Secondly, wide mouth plastic bottles containing liquid BBM left open on the sample collection area. Further, the Petri dishes were removed and sealed immediately with parafilm. The Petri dishes were labelled and kept in an incubator for the growth of algae. Similar procedures were followed for plastic liquid media too. Visible algal growth was usually observed after 20 to 30 days or till the examination of the algae and cyanobacteria.

3.3.3 Measurement of environment physiochemical parameters

3.3.3.1 Water Analyses

The water samples from the site were analyzed for various physicochemical parameters includes temperature, pH, turbidity, dissolved oxygen (DO), total dissolved solids (TDS), total suspended solids (TSS), biochemical oxygen demand (BOD), Chemical Oxygen Demand (COD), hardness, electrical conductivity (EC), total alkalinity, chloride and Methodology for measurement of metal concentration by AAS. Calibrated multiprobes were used for estimation of temperature, pH, Turbidity, DO and EC.

Other analyses includes TSS were conducted by filtering collected sample water through Whatmann 41 filter paper. Biological Oxygen Demand (BOD) was analysed by measurement of oxygen depletion of water sample after three days of incubation period at 27 °C. This depletion majorly depends upon aerobic microbes present in water sample. Similarly, COD was calculated by sample oxidation with excess acidified potassium dichromate solution followed by titration against standard ferrous ammonium sulphate solution using ferrion indicator. The hardness of sample water was determined by NH_4Cl, NH_4OH buffer at pH 8 -

10 and titrated against standard EDTA using Erichrome Black T as the indicator. Chloride content was calculated by titrating against N/50 solution of silver nitrate using KCr as the indicator. The concentration of few metals like Fe, Cu, Ca, Mg were measured by AAS. Experiments were repeated minimum thrice and mean results were represented.

3.3.3.2 Soil Analyses

Physiochemical analysis of soil was determined in laboratory such as pH, moisture content, organic matter content and percentage of calcium carbonate. The alkalinity, moisture and DO content were determined as per Indian standards i.e. IS: 2720(Part-26) IS: 2720 (Part II)-1973 and Winkler method. Similarly, bulk density and specific gravity were determined gravimetrically as per known standard protocols. Organic matter and calcium carbonate portion were determined by various reagents and indicators like potassium dichromate, ortho-phosphoric acid and diphenylamine indicator in case of organic matter percentage whereas bromothymol blue indicator and sodium hydroxide solution was used for final titration to know calcium carbonate percentage in soil. The air dried soil was mixed with distilled water and potassium chromate indicator. Further silver nitrate titration was added to determine final chloride content. Available soil phosphorus was determined by mixing with the equal volume of soil and Dickman-bray reagent followed by addition of stannous chloride solution. Flame photometry was also used to determine potassium content.

3.3.3.3 Air Analysis

The light intensity, temperature and relative humidity were measured at randomly selected sampling sites in the campus during the study period. The measurements were taken during the early morning at the time of sample collection. Light intensity was measured with a photometer, while the temperature and relative humidity were measured simultaneously with a hygrometer. The physical parameters were measured at five randomly selected sites during each day visit, and the average was considered as the final result for a month.

3.3.4 Growth condition

Basic microbiological aseptic techniques like dilution, serial dilutions, colony streaking, spread plates and several times sub-culturing were used for day-to-day experiments for isolation or purification of algae colonies. Obtained strains or even mixed cultures were

initially maintained in modified BBM, nutrient agar, algae culture agar or algae culture broth (Table 4). Final pH was maintained by required sodium hydroxide or sulfuric acid. The process was repeated as per need and calibrated to 7.0±0.5. Growth was compared in different culture media and based on obtained results, BBM was selected for further large scale culture (Figure 4). In present work, Gentamicin, vancomycin, penicillin and nystatin were used in combination as per prescribed dose in earlier literature (A. K. Jones, Rhodes, and Evans 1973). Cultures were maintained in an incubator shaker with the constant shaking of 120 rpm, at 25 ± 2 °C under the light intensity of 50 ± 5 µmol m^{-2} sec^{-1} and 18:6 (light: dark) hours photoperiod. Solid agar plates were also maintained in the parameters as mentioned earlier without shaking. 0.1% of dextrose was also supplied in few selected culture at the particular stage to enhance the growth rate.

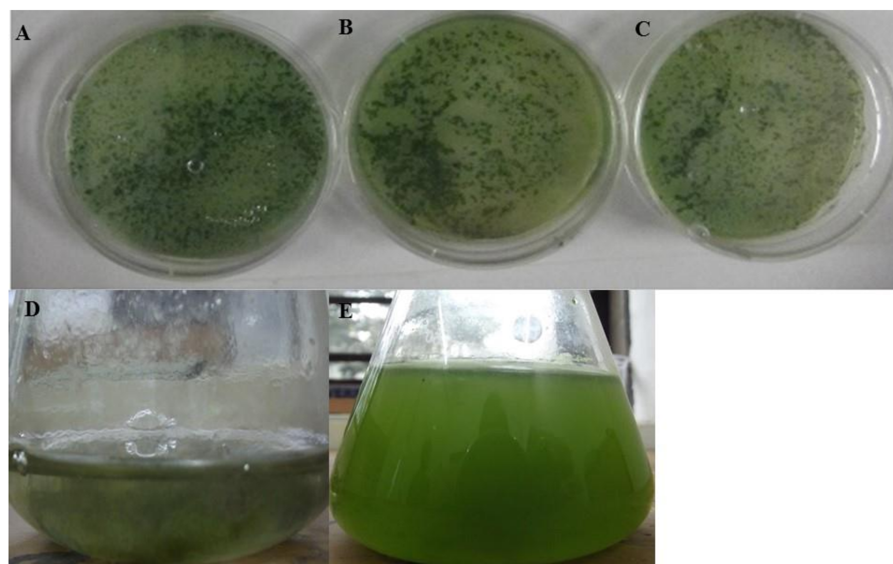

Figure 4. Comparison of algae growth in different culture media. A. BBM Agar B. Nutrient Agar C. Algae culture Agar D. Algae culture Broth E. BBM

Table 4. Nutrient ingredients of used culture media.

Ingredients	**Algae culture agar**	**Algae Culture Broth**	**BBM**	**Nutrient Agar**
	(Gms / Litre)	(Gms / Litre)	mg/L	Gms / Litre
Sodium nitrate	1.000	1.000	250	Absent
Dipotassium phosphate	0.250	0.250	75	Absent
Magnesium sulphate	0.513	0.513	75	Absent
Ammonium chloride	0.050	0.050	Absent	Absent
Calcium chloride	0.058	0.058	25	Absent
Ferric chloride	0.003	0.003	Absent	Absent
Agar	15.000	Absent	Absent	15.000
Monopotassium phosphate	Absent	Absent	175	Absent
Sodium chloride	Absent	Absent	250	5.000
Ethylenediaminetetraacetic Acid, Disodium Salt	Absent	Absent	50	Absent
Potassium hydroxide	Absent	Absent	31	Absent
Iron(II) Sulfate Heptahydrate	Absent	Absent	4.98	Absent
Sulfuric acid	Absent	Absent	1µl/L	Absent
Boric acid	Absent	Absent	11	Absent
Manganese(II) Chloride Tetrahydrate	Absent	Absent	1.81	Absent
Zinc Sulfate Heptahydrate	Absent	Absent	0.222	Absent
Sodium molybdate Pentahydrate	Absent	Absent	Trace amount	Absent
Copper(II) sulfate Pentahydrate	Absent	Absent	Trace amount	Absent
Cobalt(II) Nitrate Hexahydrate	Absent	Absent	Trace amount	Absent
Sodium hydroxide	Absent	Absent	Trace amount	Absent
Peptic digest of animal tissue	Absent	Absent	Absent	5.000
Beef extract	Absent	Absent	Absent	1.500
Yeast extract	Absent	Absent	Absent	1.500
Final pH (at 25°C)	7.0±0.2	7.0±0.2	6.8±0.2	7.4±0.2

3.3.4.1 Scale-up of algae culture

Algae were cultured first in 250 ml conical flask by maintaining mentioned parameters. Exponentially growing cultures were scaled up in 1-litre flask simultaneously in six to seven flasks. These cultures were finally shifted to bioreactor having the capacity of 30-40 litres (Figure 4). Cell cultures were monitored using spectrophotometer or hemocytometer on regular interval. The reactor was made up of thick transparent glass chamber covered from all sides. The compressed air was bubbled at the bottom of the reactors through a perforated and sterilized pumice stones, for mixing as well as gaseous exchange. The air flow rate was

controlled by the air compressor and was also filtered by filter attached to plastic tubes. Although, the reactors were kept inside the laboratory in window side facing the sun but the temperature, day to day-light duration, sunlight intensity and other physiological condition were not regulated.

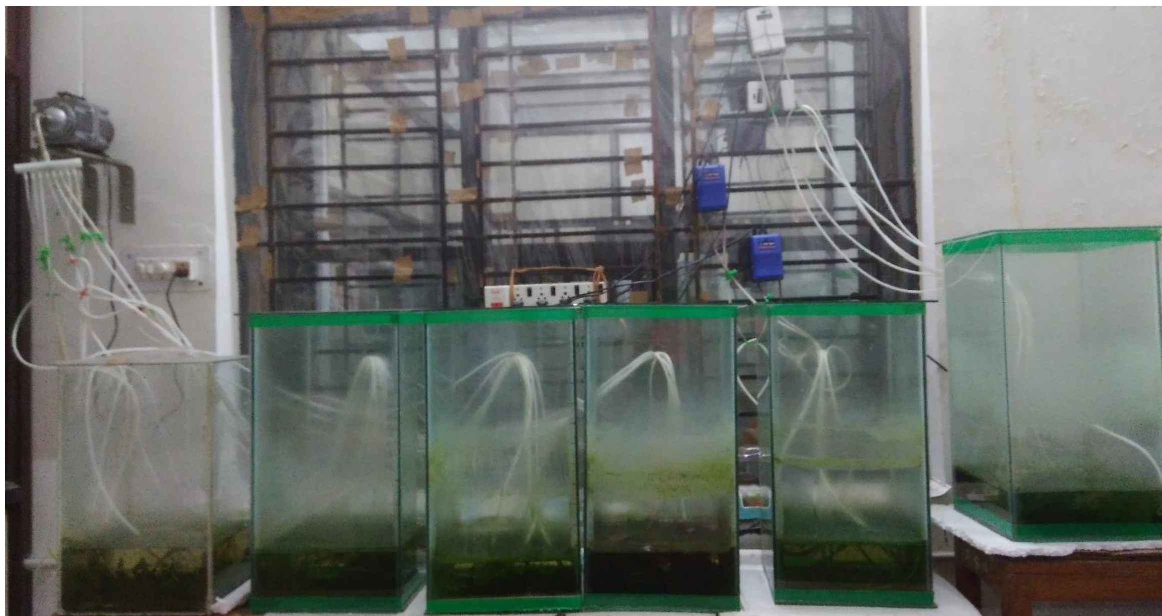

Figure 4. Experimental set-up for large scale algal growth.

3.3.4.2 Dewatering

For harvesting process, all the bioreactors were left unaerated to settle the algae. After few days supernatant water was removed. Further, cells were centrifuge at 4500 rpm for 10 min (Thermo Fischer, Germany). The supernatant could either be discarded or reused for further culture. The resulting algal paste was rinsed to remove residual salts. The microalgae paste provided all the biomass needed for the study. That could be either stored at 4 °C or dried.

3.3.4.3 Monitoring cell growth

Cell cultures were counted to know the growth phases of algae cells. These were monitored by cell count by the Haemacytometer or spectrophotometer by obtaining OD data of cell as well as chlorophyll. The Neubauer chamber is similar to glass slides having counting grids in the middle having noch to fill sample. The loading area having fixed size and loading samples should also be vortexed uniformly. As the cells were green therefore could be easily recognized under microscope otherwise lugol's reagent could be applied. The reagent not only strains the cells but also fix the motile cells. A linear dependence between absorbance and cell

counting is assumed while measuring absorbance at the wavelength of 680 nm in Perkin Elmer Lambda 25 (Santos-Ballardo et al. 2015).

Measurement of total chlorophyll content is also considered for growth and condition estimation of culture. The procedure includes, harvesting of biomass, washing with double distilled water and collection by centrifugation (~3000 ×g). After centrifugation the culture was mixed with 2 ml acetone. Glass beads were used to break the cell and were vortexed for 10 min. The extract was filtered through a membrane filter of 0.22 mm pore size (Merck Pvt Ltd., India). The absorbance at 650 and 665 nm (A650, A665) was measured using spectrophotometer (UV-3600 Plus, Shimadzu). The total chlorophyll content was estimated as dry mass percentage and concentration was calculated using following formula (Nakanishi and Deuchi 2014). The chlorophyll content should not be exposed to direct light.

$$\text{Total chlorophyll concentration in extract } (\mu g/ml) = 25.5 \times A_{650} + 4.0 \times A_{665}$$

Final growth evaluation was also determined by the dry cell weight of known volume of biomass. The cells were centrifuged, collected and washed with distilled water. The pre-weight filter paper was used to filter the biomass. After drying the biomass overnight at 60 °C it was weighed again.

3.3.5 Morphology study by microscopy

The cultures were identified based on the taxonomic keys mentioned in Anagnostidis and Komarek (1988), Desikachary (1959), Prescott (1962), Edward G. Bellinger (2010) and Whitford and Schumacher (1973) (Anagnostidis and Komárek 1985; Patidar et al. 2014; Prescott, n.d.; Bellinger, Edward G. 2015) . Cells were harvested by centrifugation (5000 × g for 5 min). The morphological study was performed under inverted microscope (Olympus Corporation Tokyo, Japan) and field-emission scanning electron microscope (FESEM). To estimate phyla of freshwater algae starch test with iodine indicator i.e. Lugol's reagent was also conducted. The reagent strains the cells depending upon species from yellow to dark brown and also applied as fixative. The morphology especially outer flagella could be more clearly visible. Lugol's iodine was prepared by dissolving 150 g potassium iodide with 50 g iodine in 980 ml distilled water, then the surplus addition of 20 ml glacial acetic acid. For the preparation of less quantity, the similar ratio should be used in appropriate quantity.

Sample preparation steps for FESEM includes algae samples vortexing and appropriate concentration dropping on glass slides. The specimen was fixed with glutaraldehyde (2-3%) followed by osmium tetroxide (1-2%). Dehydration steps included ethanol washing and overnight drying at room temperature (26-28 °C). Protocols for sample preparation were previously described by Dananjaya et al. (Dananjaya et al. 2016). The digital images of the gold-coated specimen were taken at 10,000-fold magnification by FESEM (Nova Nano SEM 450, USA).

3.3.6 Maintaining Microalgae Stocks

In this study algae stock cultures were stored either on the temporary basis by continuous subculture or by open encapsulation-vitrification methods. Strains were pinched from agar plates and streaked into agar slants or liquid broth. Sub-culturing rounds were done every month into fresh agar slants. However the broth media needs to be replaced every 15 days. They were all stored at 4 °C without agitation, 8:16 h light/dark photoperiod and 100 mol/sec/m^2 light intensity. The detailed protocol for open encapsulation-vitrification was described in chapter 4.

3.3.7 Lipid Estimation

3.3.7.1 Lipid staining

Nile red (9-(Diethylamino)-5H benzo [∞] phenoxa- zin- 5-one) is a red phenoxazone and lipid soluble dye which can be used to detect neutral lipids in vivo (Fig 5). Nile red is dissolved in Dimethyl sulfoxide (DMSO) so that it can pass through the thick and rigid cell walls in many green algae. Either dye was incubated with cells for inter-lipid accumulation visualisation or lipid was extracted and then was treated with dye for fluorescence microscopy/spectroscopy. 5 µL algal samples of known cell concentration were incubated with 3 µL of a 50 µgml^{-1} Nile red solution. Incubation was done at 30 °C for 10 min. Stained algal cells were observed under fluorescence microscopy (Huang, Chen, and Chen 2009).

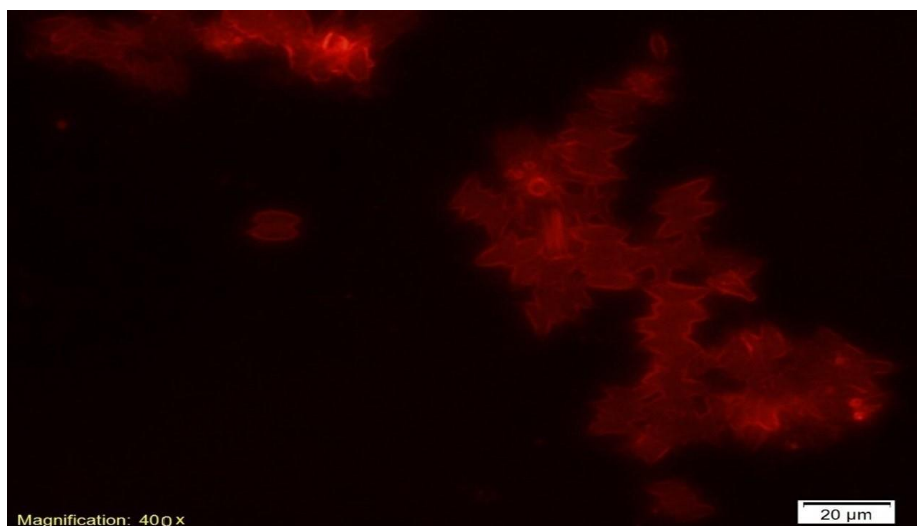

Figure 5. *Scenedesmus* cells stained by Nile red have characteristic red fluorescence.

3.3.7.2 Lipid Extraction

Total lipids were extracted from algal biomass by using a modified Folch method. The basic procedure was improved by replacing water by 1M NaCl to increase the yield of lipids [142]. The method includes the use of chloroform: MeOH in fixed 2:1 ratio for extraction of endogenous lipids from cells (Halim et al. 2011). The cells were homogenised for proper extraction before applying chloroform: MeOH binary mixture. The mixture was centrifuged and further addition of 1M NaCl and the binary mixture was repeated in the residue. Final residual biomass was collected, and evaporated in rotary evaporator. Extraction of biomass was repeated twice as described above. The organic extract was pooled into a weighed vial, dried and weighed to establish the total lipid.

3.3.7.3 Transesterification

Conversion of lipid into FAME was performed using a molar ratio of oil : MeOH: HCl :: 1 : 82 : 4 at reaction time of 6.4 h and the reaction temperature of 65 °C. The top organic phase, which contained FAMEs (Fatty acid methyl esters) was pipetted out for analysis using Gas Chromatography–Mass Spectroscopy (GC–MS) (Bagchi, Rao, and Mallick 2015).

3.3.7.4 Bio-diesel analysis

Fourier transforms infrared spectroscopic (FTIR) analysis of the algae and algal products were recorded on a Perkin-Elmer (USA/ RX-I) to determine its functional groups. It was used to obtain spectra from lipid, dried algae before and after lipid extraction in these experiments.

FTIR spectroscopy could identify the structure and chemical bonding of the functional groups. The physiological detection was accomplished by mercury cadmium telluride (MCT) detector attached to the instrument that sense and record the spectra frequency range from 400 to 4000 cm^{-1}. The dried analysis was done with potassium bromide (KBr) pellet along with dried sample by applying tonnes of pressure with the pelletizer. The liquid samples of algae oil and bio-diesel were also analyzed in a similar way by preparing the KBr pellets and then introduced into the spectrometer. Three absorption spectra were obtained for each sample, and the average spectra are reported.

The composition of algae bio-diesel (FAME) produced was determined by using GC-MS analysis on Agilent 7890B with DB-5 ms column (of diameter 0.250 µm and 30 m length) and flame ionization detector. A sample of 0.6 µl (0.5 mg of algae bio-diesel) was injected at 70 °C held for 3 min and ramped to 200 °C (at 10 °C/min) for the total run time of 32 min. GC separation was performed with the carrier gas (helium) at 1.5 mL/min flow rate. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV, having ion sources temperature at 200 °C with a scan range of m/z 40–700. FAMEs were identified based on their mass spectral characteristics and GC retention times and respective mass spectra were plotted and compared with the spectral data of the NIST library (Rout et al. 2015).

Experimental determination of fuel properties requires high quantity of oil which is lengthy and expensive. Therefore, bio-diesel properties were estimated by "Biodiesel Analyzer Ver. 1.1" (http://www.brteam.ir/biodieselanalyzer). This software can estimate 16 different fuel parameters based on the fatty acid methyl ester profile of the considered sample. The current version can easily run on windows platform and is freely available (Talebi, Tabatabaei, and Chisti 2014; Ramírez-Verduzco, Rodríguez-Rodríguez, and Jaramillo-Jacob 2012).

Chapter 4

Open encapsulation-vitrification for cryopreservation of algae

4.1 Introduction

Hence, for the first time, our study was designed to investigate the possible benefit of the open system of vitrification over the closed system of vitrification on the post-warming viability of algae. An encapsulation-vitrification procedure that was used previously for cryopreservation of algae was now evaluated in open and closed system for vitrification by using model organisms as green oleaginous microalgae *Oocystis sp*. and filamentous cyanobacteria *Anabaena sp*. Further, dose-dependent effects of two antioxidants viz. 2-mercaptoethanol (βME) and glutathione (GSH) on post-warming viability and re-growth of vitrified algae were also evaluated.

4.2 Materials and Methods

4.2.1 Isolation and culture of algae samples

Water samples were collected from local water deposits (22˚15'19.5"N 84˚54'14.5"E) and microalgae axenic culture was isolated by serial dilution and streak plating as described previously (Pasteur, n.d.). Isolated pure cultures were identified as per Prescott (Prescott, n.d.). Obtained strains (nitrkl/ch/5c and nitrkl/ch/4b) were maintained as per described parameters in chapter 3. Cells were harvested by centrifugation (5000 g for 5 min). The morphological study was performed under inverted microscope (Olympus Corporation Tokyo, Japan) and FESEM. Growth curve was based on cell count, repeated at least thrice and final average number was considered for final calculation.

4.2.2 Molecular Identification

Total genomic DNA was extracted from fresh samples as per described earlier by Sook-Young Park et al., 2014 (Park et al. 2014). The PCR reaction was carried out in a total volume of 10 µl containing 100 ng DNA, 5 pmol of forward and reverse primers, 1×PCR reaction buffer

containing Tris-HCl, $(NH_4)_2SO_4$, BSA; 2.5 mM $MgCl_2$ and 1 U Taq polymerase. PCR products were visualised on 2% agarose gels by ethidium bromide staining. The 16S rRNA gene for cyanobacteria and 18S rRNA gene for green algae were amplified using universal primers i.e. F: 5'-GAGTTTGATCCTGGCTCAG-3, R: 5'-AGAAAGGAGGTGATCCAGCC-3' and F: 5'- TGATCCTTCYGCAGGTTCAC -3' and R: 5'-ACCTGGTTGATCCTGCCAG-3' carried out with the following cycle: initial denaturation for 3 min at 94˚C, followed by 35 amplification cycles each consisting of 1.5 min denaturation at 94 °C, 1 min annealing at 59 °C, and a 2 min elongation at 72 °C, with a final 5 min elongation at 72 °C. Then the PCR products were sequenced by Eurofins Genomics India Pvt Ltd. Bangalore. The sequences were analysed using advanced BLAST search program on the NCBI Website. Phylogenetic analysis was also performed for further confirmation with the default setting of MEGA 4 using the neighbour-joining method.

4.2.3 Vitrification

Encapsulation-vitrification was executed as described previously with minor modifications (Zhang et al. 2009). Briefly, algae cell suspension (concentration: ~ 2×108 cells/ml) were mixed with 3% (w/v) sodium alginate (HiMedia Chemicals, Mumbai) solution. Eight to ten microliter of this solution was added drop-wise into 0.1 M calcium chloride (HiMedia Chemicals, Mumbai) solution with the help of a micropipette and maintained at room temperature for 30 min to obtain the microbeads. The algae-encapsulated microbeads were exposed to equilibration solution (15% glycerol + 7.5% ethylene glycol + 7.5% dimethylsulfoxide) for 30 min followed by vitrification solution (30% glycerol + 15% ethylene glycol + 15% dimethylsulfoxide) for 15 min at room temperature (~27 °C). In the case of open encapsulation-vitrification (OEV), the CPA-treated microbeads were transferred to test tubes and directly plunged into LN_2 to allow direct contact between the sample and LN_2. On the other hand, in the case of closed encapsulation-vitrification (CEV), the CPA-treated microbeads were transferred to cryovials, sealed and then plunged into LN_2 to avoid direct contact of the biological samples with the LN_2. The vitrified samples were stored in LN_2 for at least one week before warming and post-viability assessment. To assess the effects of antioxidants, βME (HiMedia Chemicals, Mumbai) or GSH (Sigma–Aldrich, Bangalore) were treated with the equilibration and vitrification solutions to a concentration of 10µM, 50µM and 100µM (Gupta, Uhm, and Lee 2010).

4.2.4 Warming and rehydration

After one week of storage in liquid nitrogen, the vitrified samples were transferred to the pre-heated water bath at 40 °C for 3 min. The microbeads were then collected and rehydrated through serial transfer to 2.0 M, 1.5 M and 0.5 M sucrose solutions for 15 min, 10 min and 5 min, respectively. Finally, the algae cells were released by maintaining the microbeads in 3M sodium citrate solution for 30 min under constant vortexing. Supernatant cells were separated carefully by centrifugation at 1000 g for 5 min (Gombotz and Wee 2012).

4.2.5 Assessment of viability

The viability of algae devitrified by warming was assessed by counting the green cells under an optical microscope (Olympus IX71, Japan). The living cells were counted after 0, 7 and 14 days of incubation period. The post-warming viability was further confirmed by 3'6'diacetyl fluorescein diacetate (FDA) assay. This evaluation was based on the esterase enzyme activity and plasma membrane integrity, described earlier (Z. C. Das et al. 2010). Briefly, cells were washed in Phosphate-buffered saline (PBS) for 1 min followed by incubation with 2.5 µg/ml FDA for 1 min. Stained cells were then washed three times with PBS to remove the traces of the dye and observed under ultraviolet illumination. Alive cells showed green fluorescence while dead ones were non-fluorescent. The viability and survival growth index were expressed with the comparison to unfrozen samples. The relative improvement in viability obtained in open system over the closed system of vitrification was estimated as,

$$\text{Relative Viability (\%)} = [(\text{Viability \% A} - \text{Viability \% B}) \div (\text{Viability \% A})] \times 100$$

(Where, A= open system, and B= close system, if open system> close system, else vice versa)

Total chlorophyll content was also measured before and after vitrification. Detailed protocol is described in chapter 3.

4.2.6 Statistical analyses

Each experiment was repeated at least three times with two replicates in each. The data were expressed as Mean ± SE. Statistical analyses were carried out by Chi-square test of ANOVA, as appropriate. The percentage values were subjected to Arc sine transformation before analyses. Differences at $p<0.05$ were considered significant.

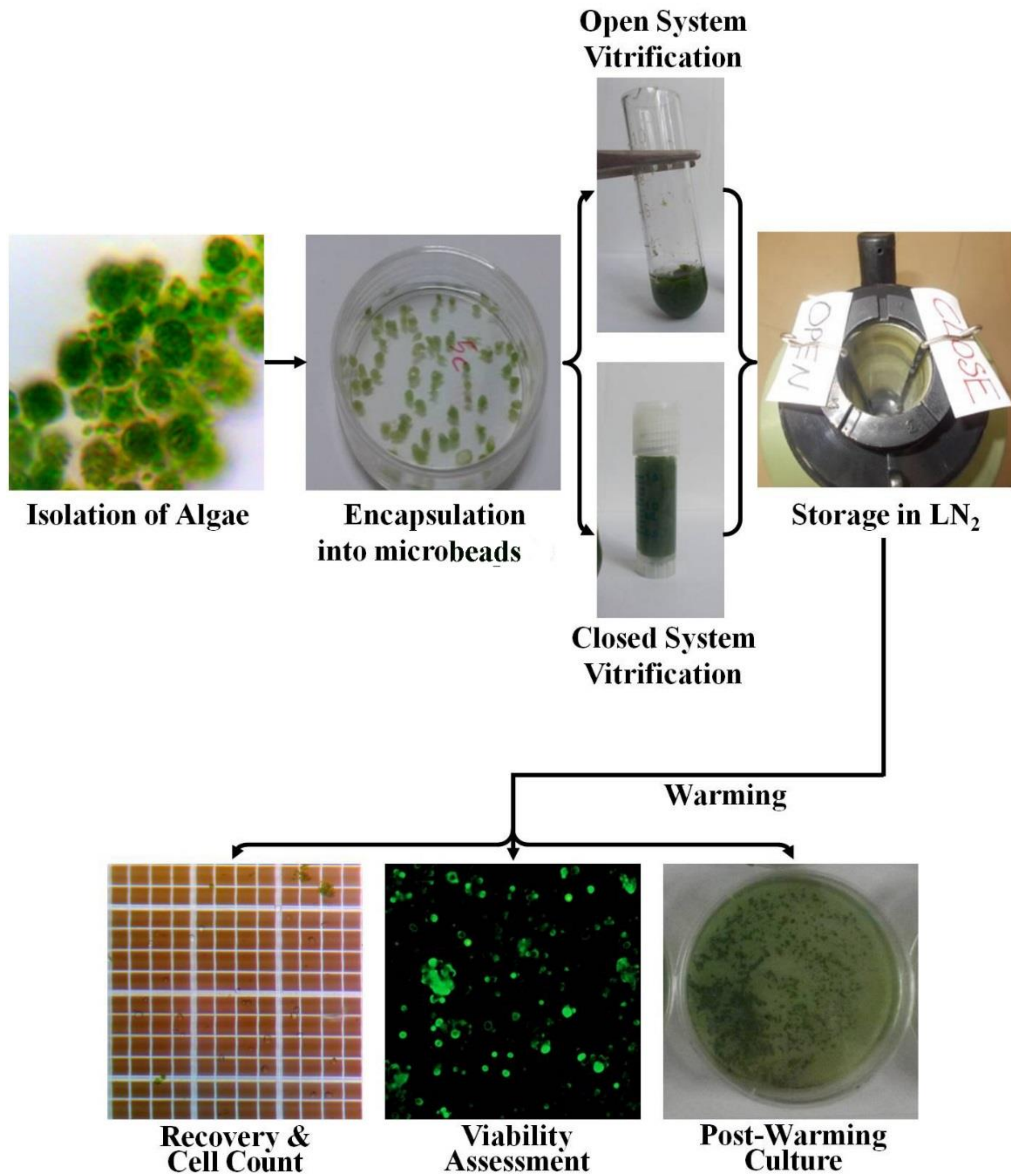

Figure 6. Brief outline of steps followed during experiment.

4.3 Result and Discussion

4.3.1 Isolation of algae

Algae have promising future, but in comparison to terrestrial culture, this is just the beginning. Thousands of new algae species have been isolated till date, and many more are yet to be annotated. Therefore, we explored this geographical locality (22˚15'19.5"N 84˚54'14.5"E). Moreover, isolated species have never been the subject of attention for improvement of cryopreservation protocols previously. Although, were the subject of attraction for novel research (Müller et al. 2007). Morphological features such as cell wall, chloroplasts and the cell dimensions were carefully observed. The results revealed that isolated axenic strains belong to the green algal lineage of genus *Oocystis* and *Cynobacterial* origin *Anabaena* (Figure 7). The *Oocystis sp*. appeared in different shades of clover green, surrounded by thick mucilage layer, oval or globular shape with autospore colonies present within a single mother cell, which released after rupturing of the cell wall (Figure 8A3). This feature is responsible for variation in cell size (7-50 µm long and 6-12 µm wide) during the different cell cycle. The cells showed the positive starch test, as estimated by Prescott standard method.

On the other hand, *Anabaena sp.* showed expected morphological features such as non-motile, filamentous, mucilage layer surrounding membrane, presence of hetrocysts, akinete, and showed negative starch test. FESEM analysis confirmed the morphometric data and estimated that mature *Oocystis sp.* and a single unit of *Anabaena* filament were >10µm in size (Figure 7). Molecular identification was also conducted by both 16S rRNA and 18S rRNA, as these are most commonly used genetic marker for the study of algal phylogeny and taxonomy. The concepts of 18S rRNA and green algal lineage have been revealed therefore the present study was designed for two different sets of conserved sequences (Krienitz et al. 2001). The 16S rRNA gene sequences of unknown *Anabaena sp.* were aligned with the sequences deposited in the GenBank database using a BLAST search. The results of the phylogenetic analysis have shown that strain belongs to the species *Anabaena variabilis* having the accession number AF247593.1 with 100 % query coverage. Similarly, other green algae isolates were identified as *Oocystis solitaria* having the accession number AF228686.1.

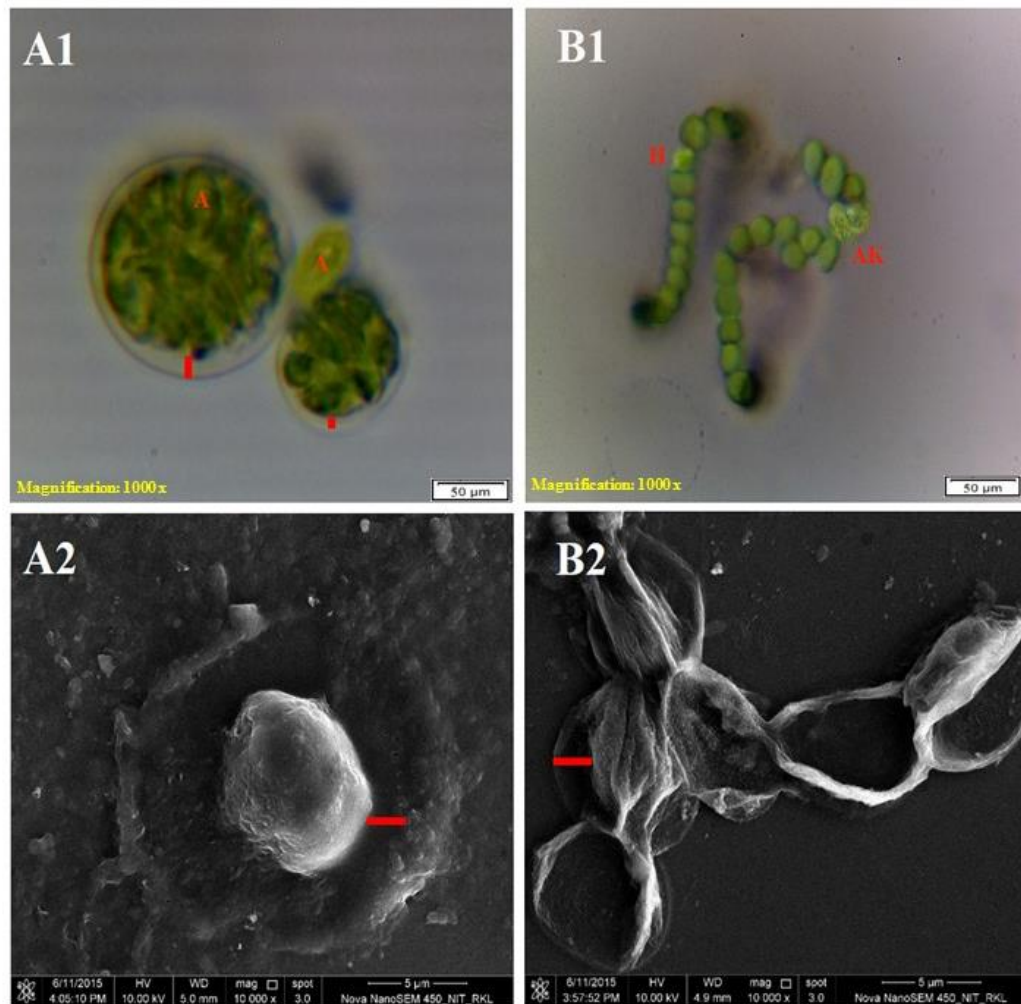

Figure 7. Morphological appearance of *Oocystis sp.* (A) and *Anabaena sp*. (B) isolated in the present study. Panel 1: Light Microscopy. Magnification: 1000x. Panel 2: FESEM. Magnification: 10,000x. A, Autospore. AK, Akinete. H, Heterocyte. Red line indicates the mucilage layer.

4.3.2 Comparison of open and closed systems of vitrification

In these set of experiments, we evaluated the possible advantage of OEV over the CEV systems. The *Oocystis sp.* was encapsulated in alginate microbeads, exposed to equilibration and vitrification solution and were subjected to either open or closed system of vitrification. Results revealed that, in comparison to controls, the percentage of living algae after warming was significantly higher in OEV than in CEV (58.20±1.21% vs. 26.66±1.61%; $p<0.05$). To further eliminate the possibility of confounding assessment due to encapsulation, a non-invasive and non-destructive FDA assay was employed. The FDA assay confirmed that OEV resulted in ~23% relative increment in post-warming viability over that of CEV (Table 5; Figure 8).

The outer mucilage membrane rupture which results in the increment of the high number of autospores and vitrified-warmed alga also suffered photosynthetic impairment, as indicated by the loss of chlorophyll, during early post-vitrification recovery (Figure 8). The chlorophyll concentration of control and OEV thawed cells were estimated as 2.13 and 1.81 % dry weight. However, loss of chlorophyll pigment and cell viability are interlinked parameters and increase in cell concentration were reported after weeks (Meador et al. 1998). However, several morphological changes including membrane damage during stress conditions were reported earlier (Krüger, Hölzel, and Luckas 2012). Further, regrowth was also determined after one and two weeks of culture duration and mentioned growth index were compared with cells preserved with antioxidant treatment (Fig 9, 10).

Summarizing, a log phase was observed after maintaining vitrified cells to one week. Probably, during this period, algae adopt itself to growth condition and recover from stress caused by vitrification treatments. Exponential phase was achieved in next week. The survived cells were nearly two-fold greater in OEV than CEV by comparing the growth curves of both the conditions (Fig 9, 10). The similar condition of *Oocystis pusilla* cells was reported which, were devoid of organismic characteristics on account of the unfavorable condition during several weeks' period. Thereafter, these cells have been increased to four-fold in next 14 days (Meador et al. 1998). Filamentous algae have been proven to be the most recalcitrant to cryopreservation and have shown far low post-freezing/post-warming viability (Day 2007). Thus, we evaluated if the OEV developed in this study can be used for filamentous algae, Anabaena sp. We observed that both systems of vitrification resulted in photosynthetic impairment, as indicated by the accumulation of phycobilin pigments (Harnischfeger and Codd 1977).

However, total chlorophyll concentration of control and OEV thawed cells were also calculated as 3.4% and 2.1 % dry weight respectively. Similar to *Oocystis sp*., cryo-injured filaments have also lost their chlorophyll pigments and appeared reddish brown (Figure 8B3). The vitrified-warmed cells also tend to form colony aggregates. Thus, uniform vortexing and counting of viable cells were difficult. As an approximation, 46% and 36% of viable cells were annotated in OEV than the CEV conditions respectively.

The cause for improved post-warming viability after the open system of vitrification instead of the closed system remains to be determined. Studies in the mammalian system have shown that direct contact between the biological samples and the LN_2 can significantly improve the post-warming viability due to the absence of insulating wall of the cryovial (Vajta, g10Rienzi, and Ubaldi 2015). Further, minimal volume of vitrification solution surrounding the microbeads may allow rapid cooling rate resulting in ultra-rapid vitrification and thereby, better success (Sakai, Engelmann, and International 2007).

An open system of vitrification may carry a risk of cross-contamination in LN_2 tanks as a number of viruses, bacteria and fungi are known to survive in LN_2 (Varma et al. 2015). Microbial contamination of embryos and semen during long-term banking in LN_2 was previously reported where 32 bacterial and one fungal species were identified from both randomly chosen commercial and research facility LN_2 tanks (Bielanski et al. 2003). This may be of particular concern in the case of inactive-stage storage, as these contaminating organisms may recover faster than the algae and affect their recovery and re-growth.

However, in other cases, contaminating partner organisms may act as symbionts in the actively growing cultures and therefore, may be an advantage (Amaral et al. 2013). Alternatively, filtered LN_2 and/or individual tanks may be used to prevent the spread of contaminating organisms which may require a spacious laboratory space.

Table 5. Post-warming viability (Mean±SE) of cryopreserved cells by open or closed system of vitrification.

Vitrification System	**Post-warming viability (%) of *Oocystis* sp. based on**		**Post-warming viability (%) of *Anabaena* sp. based on**	
	Morphology	**FDA**	**Morphology**	**FDA**
Open	58.20^{a}±1.21	56.74^{a}±1.45	46.60±2.43	46.89±6.7
Closed	26.66^{b}±1.61	26.69^{b}±1.03	36.6±9.76	32.36±10.5

Values with different superscript (a,b) within column differ significantly ($p<0.05$) Viability was calculated as percentage change in viable cells in comparison to those of controls.

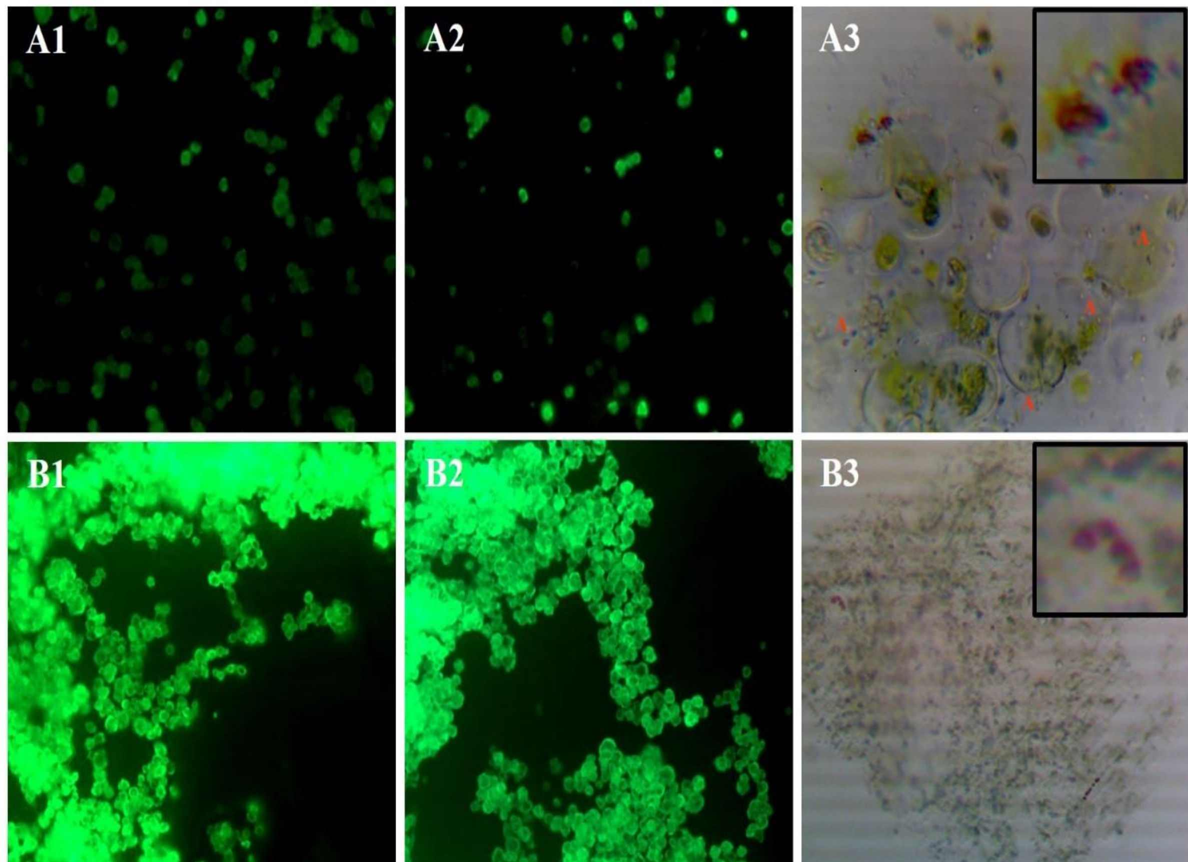

Figure 8. Post-warming viability of *Oocystis sp.* (Panel 1) and *Anabaena sp.* (Panel 2) vitrified by open (A1, B1) or closed (A2, B2) system of vitrification. Cells were stained with FDA after warming and were visualized under UV-illumination. A3, B3: Appearance of cells after 7 days of post-warming culture. Inset box shows a magnified dead cell. Magnification, 4000 x; A, Autospores.

4.3.3 Influence of bead size

Two different algae bead sizes were also prepared for viability determination, like other previous experiments. *Oocystis sp* was encapsulated in approximately, 1-1.5 mm diameter that gives the better result than relatively larger diameter beads i.e. 3-3.5 mm. The viability of smaller bead in OEV was ~58%, CEV was ~26% whereas larger bead in OEV was ~37% and CEV was ~20% (Table 6). However, the apparent reason behind this phenomena is yet to absolve but surface to volume ratio use to be higher in smaller geometric structure compare to larger identities. Therefore, more cells are likely to surround with LN_2 in smaller beads. As literature pertinent to this area is very rare but, future studies might enlighten further improvement in survival of living species during vitrification.

Table 6. Influence of bead size on Post-warming viability (Mean±SE) of cryopreserved cells.

Vitrification System	Bead size (3-3.5 mm)	Bead size (1-1.5 mm)
Open	37.5±2.10	58.20±1.21
Closed	20.15±8.12	26.23±1.61

4.3.4 Effect of antioxidants on post-warming viability

Free radicals are known to be generated during cryopreservation due to CPA and chilling injury. These free radicals can cause genetic alterations and reduce the post-warming viability of algal cells. Thus, in our next set of experiments, we evaluated the effect of antioxidants (βME and GSH) in improving the post-viability of alga cryopreservation in both the systems. The algae were encapsulated in alginate microbeads, exposed to equilibration and vitrification solution supplemented without (control) or with βME (10, 50, 100 µM) or GSH (10, 50, 100 µM). Results revealed that both the antioxidants had a positive response and were represented in growth index.

The best results for *Oocystis sp*. were obtained at the concentration of 50µM for βME (73.33±2.65%) and 100µM for GSH (78.67±1.56) in comparison to controls (58.2±1.21%) vitrified by the open system of vitrification ($p<0.05$). The positive effect of antioxidants on post-warming viability was also observed with the closed system of vitrification for both βME (46.66±2.66 vs. 26.66±1.61%; $p<0.05$) and GSH (49.33±1.0 vs. 26.66±1.61; $p<0.05$). Similarly, the viability of *Anabaena sp*. obtained in OEV with the treatment of βME (100 µM) and GSH (100 µM) was 63.33±3.66 and 60±5.11 respectively. CEV viability percentages were less in comparison to OEV i.e. 48.66±3.32, 50.66±3.23 for βME (100 µM) and GSH (100 µM) respectively (Table 7).

Further, growth index of these cells for two weeks was also observed. A short lag phase within seven days was noticed; subsequently, the remarkable exponential phase was achieved. As discussed previously, cells treated with GSH 100µM and βME 50µM having significantly higher survival and growth rate in *Oocystis sp*. than other conditions (Fig 9). Whereas, the

difference in optimum concentration of βME (100µM) and GSH (100µM) was observed in the case of *Anabaena* cells (Fig 10).

In both the algae species, it could be clearly observed that even after cryoinjury the recovery of cells was achieved after a log phase in the exponential rate. As per initial viability percentage obtained, the regrowth after two weeks duration varies according to initial treatment conditions. However, growth recovery after stress conditions were reported was argued as previous literature (Meador et al. 1998). Improved survival rate by GSH treatment in citrus shoot-tips was also reported (Z. C. Wang and Deng 2004).

Table 7. Influence of antioxidant treatment on post-warming viability (Mean±SE) of cryopreserved cells.

Antioxidant concentrations	***Oocystis sp.***		***Anabaena sp.***	
	OEV	**CEV**	**OEV**	**CEV**
Antioxidant (0 µM)	58.2±1.21	26.66±1.61	46.6±2.43	36.6±9.76
GSH (10 µM)	67.53±3.24	30.66±4.09	52±5.6	38.33±0.52
GSH (50 µM)	68.33±2.28	36.66±0.35	56±8.64	42.66±6.16
GSH (100 µM)	78.67±1.56	49.33±1.0	60±5.11	50.66±3.23
βME (10 µM)	66.66±4.55	30.66±2.09	52±4.99	39.66±2.14
βME (50 µM)	73.33±2.65	46.66±2.66	56.33±7.21	42.33±4.5
βME (100 µM)	68.01±3.98	40.66±1.02	63.33±3.66	48.66±3.32

However, little is known about the cell signaling pathway responses to oxidative stress at a subcellular level of plants as well as microbes (Zhang et al. 2009). Therefore, mechanism and behavior of proposed result are difficult to justify, but it could be postulated that addition of antioxidants to the vitrification solution may contribute to improved cryotolerance.

Figure 9. Growth index of vitrified-warmed *Oocystis sp*. in the presence of A: GSH (0, 10, 50, 100 µM) or B: βME (0, 10, 50, 100 µM). Panel 1: OEV; Panel 2: CEV.

Figure 10. Growth index of vitrified-warmed *Anabaena sp.* in the presence of A: GSH (0, 10, 50, 100 µM) or B: βME (0, 10, 50, 100 µM). Panel 1: OEV; Panel 2: CEV.

4.4 Conclusion

Taken together, the results of the present study reveal that open system of vitrification is superior to the closed system of vitrification for *Oocystis sp*. and *Anabaena sp*. Variation in bead size postulates the similar hypothesis that rapid cooling rate influences the cell viability. Further, the addition of βME (50 μM) or GSH (100 μM) significantly improved the post-warming viability of vitrified *Oocystis* algae. Whereas, the concentration of βME (100 μM) or GSH (100 μM) were observed as highest viability in *Anabaena sp*. Even after morphological stress, algal cells achieved exponential growth phase after a duration of lag phase. The developed vitrification protocol was successful protocol and better than existing vitrification for variable sized cells of *Oocystis sp*. as well as filamentous *Anabaena sp*. and hence, may be applied to a broad range of microalgae/alga.

Chapter 5

Pre-treatment and Bio-diesel production from fresh water algal culture

5.1 Introduction

The present era demands new technologies to overcome the situation of scarcity of fuel and biofuel is the excellent alternative. Although plenty of research confirmed that algal biomass is a promising source of biofuel, still large scale production is in infancy. Algae cultivation needs the selection of fast-growing, productive strains, optimized for the local climatic conditions for algal mass culture. Therefore, in the present study, we exploited this geographical environment for the selection of algal culture that could be successfully grown in the bioreactor. The precious cultures were preserved by developed OEV methodology for entire study. In this set of experiment, the water algae along with water depositories were purified and used for bio-diesel production. As the bio-diesel production proceeds through several steps therefore in the present work sample was pre-treated and amount of lipid extraction was compared. This energy conversion step also require innovations for economically feasible product development. The literature often omits the relevance of sample handling or pre-treatment before extraction in analytical processes. The best result among other compared treatment was further used in studies.

5.2 Materials and Methods

5.2.1 Algae culture

Water samples were collected from local water deposits (22°15'03.7"N 84°54'38.6" E), and microalgae culture was purified by serial dilution, streak plating, the addition of antibiotics and antifungal agents at least to remove bacterial and fungal contamination. The water samples from that site were also collected for analysis. Isolated mixed cultures were preliminarily identified as per Prescott. The morphological study was performed under inverted microscope (Olympus Corporation Tokyo, Japan). Obtained strains were maintained and cultured in large scale bioreactor under standard parameters mentioned earlier. Ten-milliliter samples were

collected daily from the cultures for monitoring the algae growth via chlorophyll OD spectroscopy. The detailed description of methodology was mentioned in chapter 3.

5.2.2 Water analysis

The water samples from area of algae collection were analyzed for various physico-chemical parameters like temperature, pH, turbidity, DO, TDS, TSS, BOD, COD, hardness, EC, total alkalinity, chloride as per described earlier in chapter 3.

5.2.3 Extraction pre-treatment

Algal cultures were left unaerated for few days for gravity settling. The concentrated cell cultures were dewatered further using a bench top centrifuge at 4500 g for 10 min. The resulting microalgae paste was rinsed with deionised water to remove residual salts. In experiments both the wet and dried microalgae was used. The microalgae paste was dried at 60 °C in an oven for overnight. A mortar and pestle were then used to grind the dried biomass into powder. The experiment using wet microalgae paste was obtained by centrifugation. Further cell disruption of wet paste was also done by putting the micro vial in liquid nitrogen and disrupting the with glass beads (Sigma-Aldrich, Bangalore, India) in a vortex mixer (Remi CM-101 Plus Cyclo Mixer) for 7 min. The procedure could be repeated until the sample forms a fine powder. Microalgae samples were stored at 5 °C for no longer than 1.5 months before they were used for lipid extraction.

5.2.4 Lipid Extraction

Total lipids were extracted from dry algal biomass by using a modified Folch's method. Petroleum ether extraction was also experimented using Soxhlet apparatus. Briefly, the microalgae powder was packed in a cellulose thimble inside the extraction chamber of the Soxhlet unit. Pure petroleum ether (300 mL) was used to extract the lipid for 7.5 h at the rate of 10 refluxes per hour. The experiment was repeated thrice. The extracted lipid was gravimetrically quantified and transferred into a sealed glass vial for storage in the dark at 5 °C for less than one month. Transesterification of lipids was performed and analyzed using GC–MS. Bio-diesel properties were also estimated by "Biodiesel Analyzer Ver. 1.1". (http://www.brteam.ir/biodieselanalyzer) [145,146]

5.3 Results and Discussion

5.3.1 Water analysis

Table 8 represents the Physico-chemical characteristics of water sample from algae collection area (22°15'03.7"N 84°54'38.6"E). As the sample of collection was during post monsoon period therefore the temperature of water sample was 26 °C i.e. neither cold nor hot. Similarly, pH concentration was found to be slight alkaline that might be favorable habitat for appearance of algae bloom. Turbidity was high to presence of dissolved microscopic organism and other suspended particle. TDS, TSS and hardness values depend on the presence of Calcium, Magnesium, Sodium, Potassium, Bicarbonate, Chloride and Sulphate ions in water. However, sample water is within the permissible range for drinking purpose might be due to absorbance of these particles by present algae or other organisms. The presence of ions also conducts electricity was analyzed in the present study. DO is amount of dissolved oxygen present in water so it is the direct idea of present aerobic biota. On the other hand amount of oxygen required by micro-organisms to degrade the organic matter is BOD whereas total oxygen required for oxidation of both the organic and in-organic substances by the reagents is COD. These all are interrelated terms and values in present study of DO, BOD, COD were found to be 8.4 mg/l, 3.8 mg/l and 15.6 mg/l respectively. As the location of collection was near swimming pool residing within campus hence this parameter was measured and found to be within permissible range.

Table 8. Physico-chemical characteristics of water sample from algae collection area

Parameters	**Location**	**Permissible limit for drinking water**
Temperature	26 °C	15 to 34 °C
pH	7.8	6.5 to 8.5
Turbidity	16.5 NTU	10 NTU
Total Dissolved Solids (TDS)	370 mg/l	500 mg/l
Total Dissolved Solids (TSS)	29 mg/l	-
Hardness	166 mg/l	300 mg/l
Electrical Conductivity (EC)	512 μmho/cm	300 μmho/cm
Dissolved Oxygen (DO)	8.4 mg/l	5.0 mg/l
Biochemical Oxygen Demand (BOD)	3.8 mg/l	5 mg/l
Chemical Oxygen Demand (COD)	15.6 mg/l	2.6 to 22.0 mg/l
Chloride	66 mg/l	250 mg/l

5.3.2 Growth and Identification of algae

The presence of species with different geographical, regional, ecological niche could be the probable region for distribution of the different biological organism. Therefore, in the present study, we collected samples from local water bodies within NIT Rourkela campus. Freshly collected samples were serially diluted and grown in BBM along with antibiotic treatment. Antibiotic and antifungal treatment to isolate axenic algal culture has been employed successfully over the past years.

Most algae tolerate higher concentrations of antibiotics than bacteria but as in environment wide range of bacteria exists; therefore, it is necessary to use the antibiotics mixture. Similarly, algae also differ in their sensitivity, but closely related species sometimes have quite related tolerances (A. K. Jones, Rhodes, and Evans 1973). In present work, gentamicin, vancomycin, penicillin and nystatin were used in combination as per prescribed dose in earlier literature.

Growth index of mixed algae culture in thirty litres bioreactor was measured in order to monitor the cell condition on the routine basis. The culture was maintained in BBM and was routinely provided as per requirement of cell condition. The exponentially growing culture in the incubator was transferred in bioreactor but still it follows the general trend of lag phase followed by log phase (Fig. 11).

Figure 11. Growth curve of algae culture used in experiment.

Morphological features of algae culture such as cell wall, chloroplasts, and the cell dimensions were carefully observed. Few predominant species in the mixed algae culture were found to be *Chlorella, Anabaena, Euglena, Oocystis and Sphaerocystis*. The microscopic image with and without lugol's reagent was shown in Fig 12. The morphological feature of *Chlorella* includes single-cell green algae, spherical shaped, about 2-10 μm in diameter, without flagella, positive starch test (Bellinger, Edward G. 2015). *Anabaena* was identified as filamentous structure, presence of hetrocysts, surrounded by mucilage layer, non-motile and negative starch test with lugol's reagent.

Optical microscopic observation of samples showed solitary, motile, green eukaryotic cell surrounded by a thin outer wall, centrally located nucleus, containing several green and red bodies like chloroplasts, negative starch test, typically about 30-60 µm long and 6-10 µm wide. These characteristics are very similar to those described for the *Euglenoid* sp. (Sittenfeld et al. 2002). The observation reveals that the few species belongs to *Oocystis* genus as it is green, colonies within a thin mucilaginous envelope, oval or globular shape, autospores colonies released by rupture of the mother cell wall, positive starch test, 7-50 μM diameter during the different phase of cell cycle (Bellinger, Edward G. 2015).

The study examined *Sphaerocystis* sp. as immotile, green algae, spherical colony, globose cells embedded in outer mucilage cover, sometimes colonies are even more than 1 mm diameter, chloroplasts are cup-shaped, mostly found in freshwater and positive starch test (Prescott, n.d.). These species had a high growth rate, survived well in uncontrolled conditions and displayed wide temperature tolerances over the period of the study. Isolation, purification, and maintenance of monoculture is hectic and wastage of infrastructure and economy. Cell culture banks especially invest money and manpower to maintain those cultures, but still few organisms are difficult to preserve alone. Therefore, they are stored with symbionts (Amaral et al. 2013). That problem could be automatically reduced if we directly utilize the mixed algal culture.

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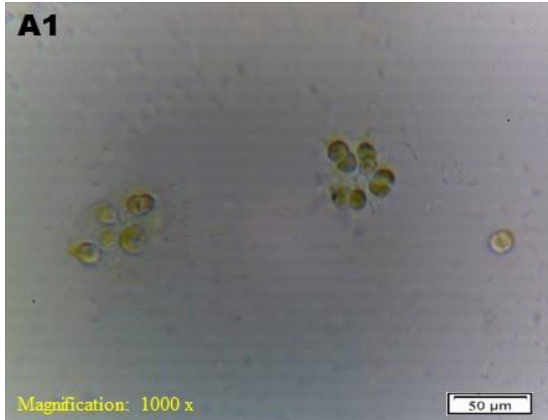
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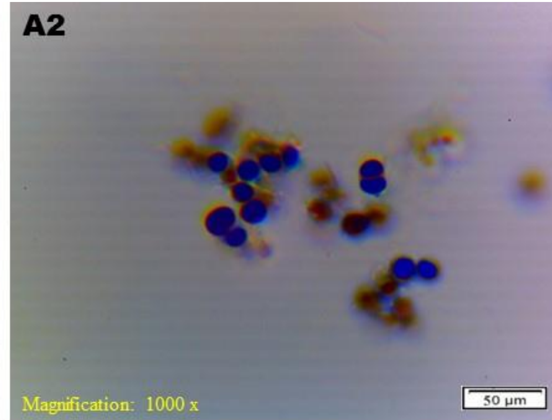
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B1
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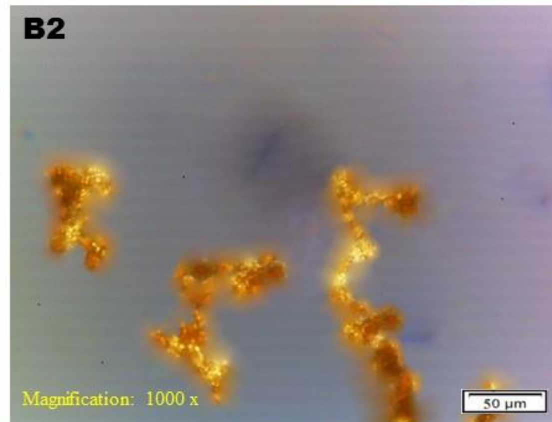
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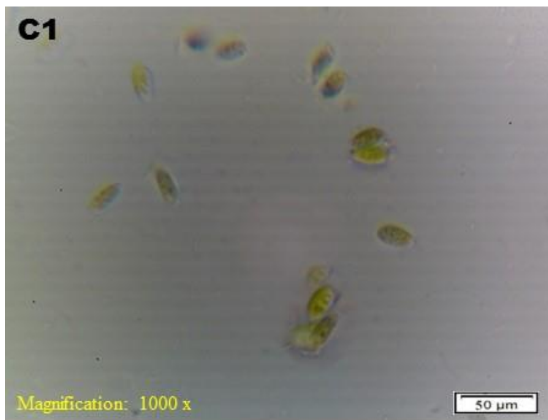
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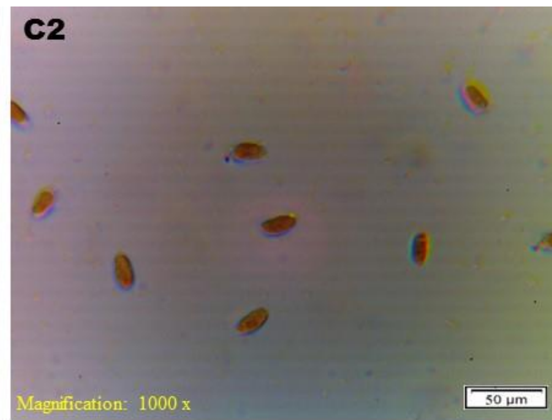
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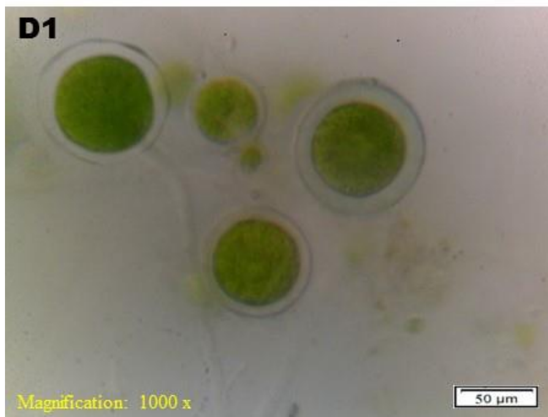
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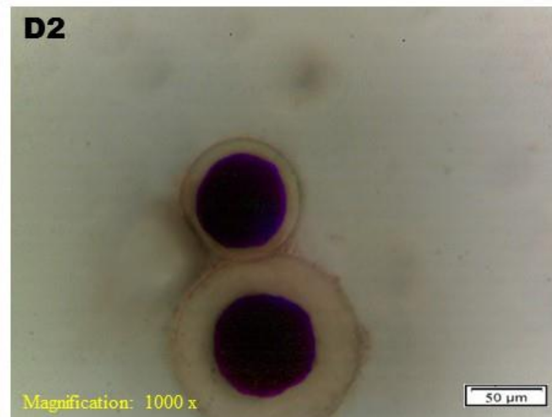

D2
Magnification: 1000 x
50 µm

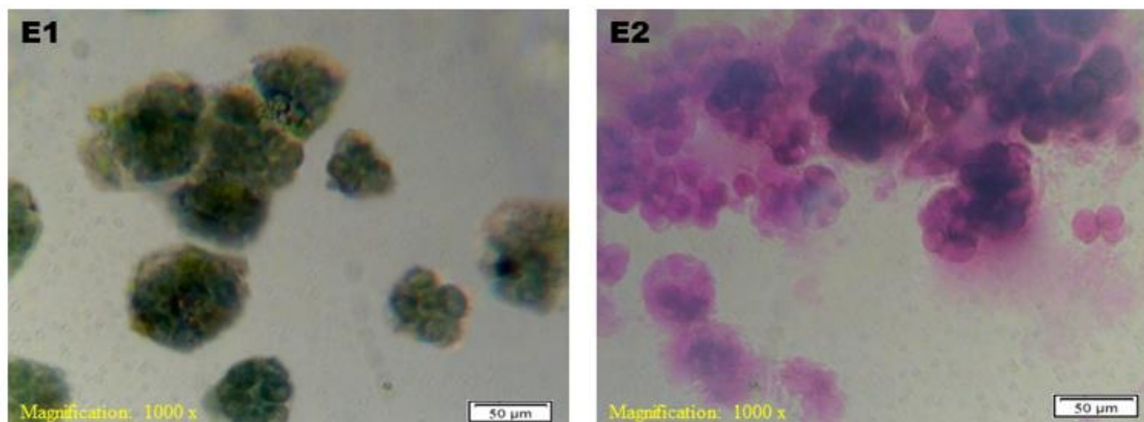


Figure 12. Microscopic view of predominant species present in the study. Panel 1 and Panel 2 represents the image of before and after addition of lugol's reagent. (A1, A2) *Chlorella sp.* (B1, B2) *Anabaena sp.* (C1, C2) *Euglena sp.* (D1, D2) *Oocystis sp.* (E1, E2) *Sphaerocystis sp.*

5.3.3 Selection of lipid extraction methodology

In this part of the experiment, the algal cells were subjected to lipid extraction from different viable techniques. Different methods of extraction were compared on the basis of extracted lipid percentage that could be useful to increase the reliability of results on lipid content. The combination of dried algal biomass with the Folch method was the most effective extraction method among those taken into consideration which led to a yield >27% (Fig. 13). While the traditional Soxhlet extraction achieved just 15%.

Moreover, solvent polarity matters a lot while extraction of lipid from the cell. As the algae contain various polar and non-polar lipid within cell that could be better extracted by combination of solvent mixtures. Chloroform and MeOH binary mixtures were reported to extract higher oily fraction from different cell and tissues previously (Folch, Lees, and Stanley 1957). Pre-treatment using dried and wet biomass did not show significant differences i.e. ~27 and ~25%, respectively. Lipids were also extracted from wet biomass by cell disruption. Lipid yield was higher in comparison to uncrushed algal biomass. It was clear from the result that, breaking down algal cell walls is essential to enhance extraction of intracellular compounds. Based on these results, it was decided to extract the lipid fraction using Folch method from dry biomass as this protocol could be applied to extract the highest oily fraction. The previous literature also states the similar finding (Ramluckan, Moodley, and Bux 2014).

Figure 13. Lipid yield obtained by different techniques of extraction. Grey bar indicates wet biomass and black bar indicates dried biomass. F (Folch's method of lipid extraction), CD (Cell disruption), Pet Eth (lipid extraction using Petroleum ether as solvent)

However, derivation of pure lipid from the mentioned protocol could not be claimed. As the intracellular pigments are also actively soluble with applied solvent therefore co-extracted into crude oil. However, presence of chlorophyll and other pigments are known to decrease the transesterification conversion and combustion efficiency of bio-diesel. However, little literature available regarding the purification of these pigments and even by using existing protocol is yet not satisfactorily accomplished. However according to literatures pigments are highly unstable in the presence of high light irradiance, acids, bases, and oxygen (Li et al. 2016). Therefore during further transesterification reaction the pigment degradation may occur.

5.3.4 FAME profile analysis

Further, transesterification reaction yielded very high values of myristic acid (14:0), which constituted about 8% (mol/mol) of the overall lipid fraction (Table 9). A total of ~10 different fatty acid substrate from C12-C24 were identified by mass spectrometry (Fig 14). Out of these fatty acids, the majority are low carbon chain compounds which are very suitable for bio-diesel production. According to retention time, Linolenic acid (C18:3), Linoleic acid (C18:2) and Oleic acid (C18:1) consists of more than 50% of total FAs. Moreover, the variety of fatty acids were present in total FAME fractions. These varieties could be due to the presence of the

different variety of algae community (Lang et al. 2011). Basic properties and application of these compound have also been described in Table 9 (Ramluckan, Moodley, and Bux 2014).

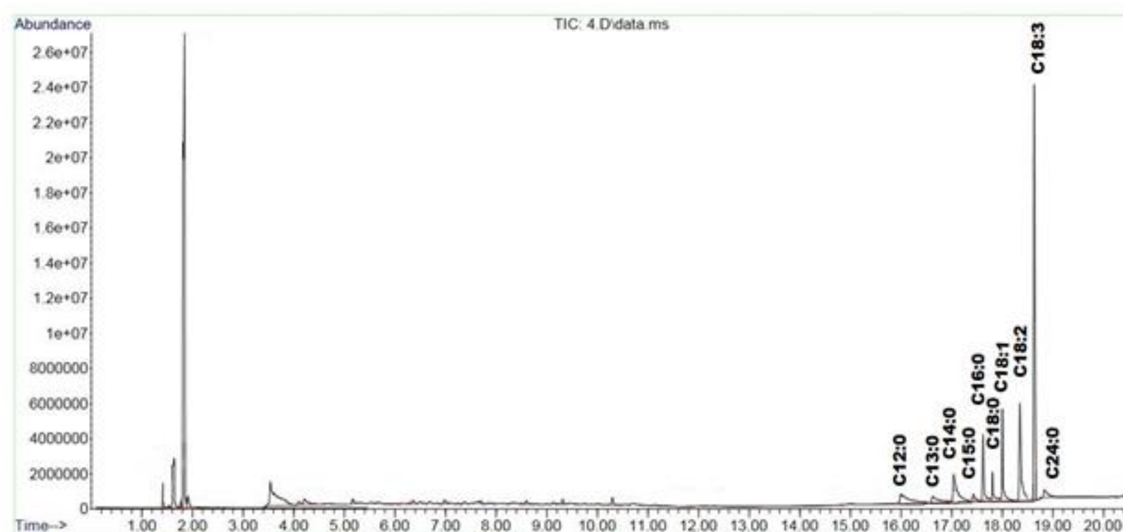


Figure 14. Chromatograms of FAMEs obtained during experiment.

Table 9. Fatty acid profile detected at various nutrient conditions.

Fatty acid	**Lipid number**	**Relative content (%)**	**Application**
Lauric acid	C12:0	4.56%	Antibacterial, antioxidant and antiviral inhibitor
Tridecyclic acid	C13:0	3.58%	Ingredient in methyl ester formation
Myristic acid	C14:0	8.46%	Manufacturing of Biofuels, cosmetics and tropical medicines
Pentadecanoic acid	C15:0	1.66%	Combustion, marker in butter fat composition
Palmitic acid	C16:0	6.32%	Biofuels and cosmetics preparation
Stearic acid	C18:0	3.44%	Biofuels and dietary supplements preparation
Lignoceric acid	C24:0	2.85%	Widely used in research.
Oleic acid	C18:1	9.12%	Used in food industry
Linoleic acid	C18:2	11.01%	Making of soaps, emulsifiers, beauty products, anti-inflammatory agent
Linolenic acid	C18:3	31.94%	Main component in drying of oils

Determination of bio-diesel properties is useful parameter to know the characteristics of the fuel (Table 10). The Biodiesel Analyzer Version 1.1 requires only FAME data input that reduces the task of obtaining the heavy amount of oil for these important analyses (Prathima Devi and Venkata Mohan 2012). Variation in fatty acids profile effects the overall bio-diesel quality.

Table 10. Comparative analysis of Bio-diesel properties. (Atabani et al, 2012; Sivaramakrishnan et al, 2012; Jain et al, 2010; Knothe, 2002; Islam et al, 2013)

Bio-diesel Properties	**Present study**	***Amphidinium sp.***	***Bidulphia sp.***	***Phaeodactylum tricornutum***	***Picochlorum sp.***	***Nannochlopsis oculata.***	***Extubocellulus sp.***	***Scenedesmus dimorphos***	***Franceia sp.***	***Mesotaenium sp.***	**Jatropha**	**Rapeseed**	**Soybean**
SFA	30.87	-	-	-	-	-	-	-	-	-	-	-	-
MUFA	9.12	-	-	-	-	-	-	-	-	-	-	-	-
PUFA	42.95	-	-		-	-		-		-	-	-	-
DU	95.02	-	-	-	-	-	-	-	-	-	-	-	-
LCSF	8.05	11.3	2.7	2.8	5.5	3.7	3	3.8	3.1	1.6	-	-	-
SV	175.89	188.2	210	204	195	203	209	195.7	197.5	200	198.55	168–187	189–195
IV	115.56	159	87.9	114	135	80.6	65.1	183.7	205.5	202	104	94–120	128–143
CN	51.33	42.9	54.6	50.3	49.0	57.9	60.9	37.1	33.3	33.4	51.6	54.4	37.9
CFPP (°C)	8.81	19.1	-7.9	-7.8	0.7	-4.8	-7	-4.6	-6.7	-11	0	-13	-4
CP (°C)	-1.67	-	-	-	-	-	-	-	-	-	2.7	-3.3	0.9
OS (hours)	5.34	-	-	-	-	-	-	-	-	-	2.3 (at 110 °C)	7.6 (at 110 °C)	2.1 (at 110 °C)
HHV (MJ/Kg)	32.41	40.3	40.0	39.8	39.9	39.8	40.1	40.2	40.4	40.2	38.2	39.7	39.6
Kinematic Viscocity (μ) (mm^2/s)	0.96	4.1	3.7	3.7	4.0	4.2	3.9	3.6	3.5	3.4	52.76	37.00	32.60
Density (ρ) (kg/m^3)	730	900	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	879.5 (at 15 °C)	882 (at 15 °C)	913.8 (at 15 °C)

Normally, PUFAs are present in more quantity in algae that decreases the stability of fuel, but on the other hand it has low melting point and better cold weather properties in comparison to other bio-oil (Talebi, Tabatabaei, and Chisti 2014). In the present study, the values of estimated SFA, MUFA, PUFA, the DU and LCSF are 30.87, 9.12, 42.95, 95.02 and 8.05 respectively. The estimated SV of bio-diesel from algae is 175.89 which is very near to Jatropha i.e. 198.85. Similarly, IV of our sample is nearly equivalent to soybean i.e. 115.56 and 120.52 respectively (Gopinath, Puhan, and Nagarajan 2009). According to the European specification, the minimum CN of 51 is required to meet the specification, but like other bio-diesel, algae bio-diesel is also just in boundary line. CFPP and CP values are very similar to palm.

Oxidative stability not only depends on FAME content but also varies from the age of bio-diesel, storage condition and so on. Therefore, the present result could be difficult to justify. But generally, oxidative stability of algal bio-diesel differs from vegetable oil profiles which are slightly astonishing. Higher Heating Value (HHV) is lower in biomass due to high oxygen content. Our result follows the same trend and has even less HHV than coconut bio-diesel. The kinematic viscosity by ASTM D6751 and EN 14214 standard is within the range of 1.9–6.0 mm^2/s and obtained value is 0.96. Whereas 860–900 kg/m3 is for density but bio-diesel from algae do not satisfy the range hence extra effort is required in these particular case (Hoekman et al. 2012).

5.4 Conclusion

Concluding, the study was about to culture local freshwater algal species so that it can thrive in uncontrolled conditions easily. The observation revealed that *Chlorella sp., Anabaena sp., Euglena sp., Oocystis sp.* and *Sphaerocystis sp*. were dominant species to adapt the environment throughout the large scale culture experiments. The best feasible lipid extraction methodology was developed, and Folch's method from dried algal sample was finally selected by repeating the experiment thrice. FAME conversion states that the final product was the mixture of lower carbon chain compounds which are very suitable for biofuel production. Bio-diesel properties were very similar to other plant derived oils. However, algae biomass pre-treatment, crude oil refining, modification in lipid extraction or direct transesterification technologies could improve the quality of bio-diesel and will be economically more feasible.

Chapter 6

Bio-diesel production from airborne algae

6.1 Introduction

The atmosphere is considered to harbour a wide mixture of microorganisms in all possible locality, such as air, water and soil. Airborne alga are those considered species which spread in environment by various air localization movement. Few studies also suggests that, availability of species depends mostly upon the nearly found local soil or water microbial diversity. Therefore, screening air samples could directly provides the idea of indigenous species availability in this geographical locality.

Mainly, available literature describes the assurance of different algal species and its consequences related to health. The work described here is based on the positive utilization of these algae as the source of bio-diesel. This is the first report which describes the use of airborne algae as raw material for bio-diesel production. The report will be beneficial for open large culture system as most often the problem of contamination during culture has been noticed. The source of most of these contaminations is air. Therefore, this work is the step towards limiting the problem from other algae. Further, these strains could survive and adapt better in the environment during the climate change. The study includes the collection of airborne algae species throughout the year. Its preliminary identification up to genus level, scale up, lipid extraction, transesterification and characterization of liquid was conducted.

6.2 Materials and methods

6.2.1 Sample collection

The algae samples were collected from different locations within the NIT premises such that it may cover the entire campus. Major renovation work was carried out throughout the year from August 2014 to November 2015 for observation of variation in algal species with the difference in climatic variation every month. The data was separated on monthly basis and samples were collected on every possible weekend at morning time. Detailed collection

procedure, parameter for culture maintenance, large-scale culture condition, aseptic techniques were used for day-to-day experiments for purification of algae colonies, and morphological identification procedures was mentioned in chapter 3.

6.2.2 Lipid extraction

For determining initial lipid content of algae cells concentration was adjusted by haemocytometer using optical microscope such that suspension consists of not more than 8×10^6 cells per millilitre. Further, Five millilitres of diluted algal suspension was incubated with at least 2 μg ml^{-1} concentration of Nile red solution in DMSO and agitated on a vortex vigorously. The suspensions were examined on a spectrofluorometer (Horiba Jobin Yvon, USA/Fluoromax 4P) with a 480 nm narrow band excitation filter and a 570 ~ 590 nm emission filter and the following conditions were applied: excitation slit: 10 nm, emission slit: 10 nm, photomultiplier: 400 V. Relative fluorescence intensity was determined after subtraction of autofluorescence of alga and Nile red at similar wavelength. Lipid Extraction by modified Folch's method, temporary storage condition and FAME conversion were mentioned in chapter 3.

6.2.3 Analytical methods

The elemental analysis of carbon, hydrogen, nitrogen and sulphur of algae bio-diesel was carried out using CHNS analyser (Elementar Vario El Cube, Germany) and the oxygen content was calculated by difference. Dried and powdered algal samples were acid digested in a microwave digester. For acid digestion, 0.5g of the sample were digested in 6ml of HNO_3 (65%) and 2ml of H_2O_2 (30%). The mixture was digested for 15 minutes at a temperature of 200 °C (Fisher Scientific, USA). The digested product was then cooled and filtered. Standard solutions and samples were analyzed in an AAS using Intensitron (Hollow Cathode) lamp.

The absorbance of AAS was detected in flame mode. The concentration of elements were detected using standards (Kumar, Dasgupta, and Das 2014). The remaining elements (which) were detected by flame atomic absorption spectrometer. Functional groups analysis obtained using FTIR spectrometer, composition of algae bio-diesel (FAME) was determined by using GC-MS analysis, and bio-diesel properties were also estimated by "Biodiesel Analyzer Ver. 1.1" (Details were described in Chapter 3.) The sample was detected by HPLC (Shimadzu LC

solution, DGU-20A) to detect sugars, acids and sugar alcohols present in MeOH layer in Agilent Hi-Plex column having size 6.5 × 300 mm length and 8 µm (p/n PL1F70-6850) thickness. 10 µL of sample was injected with flow rate 0.4 mL/min having the mobile phase of 100% DI water with temperature 85 °C. Peaks were detected by RI detector.

6.3 Results and Discussion

6.3.1 Effect of climate

Rourkela is situated in north-western Odisha at 84.54 E longitudes and 22.12 N latitude and about 219 m above sea level in Sundargarh district. The area is rich in mineral ores and having many industries. The weather condition uses to be tropical monsoon climate where the minimum and maximum temperature ranges from about 8-45 °C. All summer, rainy, winter seasons could be experienced from March to June, June to September and November to February respectively. Along with that, the city has been exposed to air pollutants from a long time coming majorly from steel plant, thermal power plant, refractories, sponge iron industries, automobile exhaust and small scale industries. The air contains the significant percentage of SO_2, CO, NO_2 and heavy metals (S. Das and Prasad 2010).

Morning light intensity, air temperature, relative humidity and rainfall were estimated and summarized in table 11. As usual light intensity on earth surface was high during summer, followed by winter and low at rainy season due to the cloud, fog, etc. The temperature trend is different from light intensity. The temperature was recorded highest during summer, followed by rainy season. Relative humidity was highest during rainy season, followed by winter and least during summer. Due to high temperature and sun rays the water vapors in atmosphere was evaporated. Rainfall use to highest at the end of June till mid of September due to arrival of monsoon at this time.

The climatic variation is relative to the abundance of microbial biota. The air samples collected in Petri dishes were incubated for 2-3 weeks and resultant microbes and algae are shown in Fig 15. From the graph it is clearly visible that maximum no. of samples were found during the month of October and November (Fig 16). The reason behind is favorable climate. During the rainy season, the number was nil due to environmental, natural cleaning phenomena i.e.

rain. This natural activity eliminates contaminated particles from the air. These air suspensions and consequently the algae are present in the air due to surface localization via air flow or atmospheric turbulence from distant surfaces. Places from low and high latitudes from countries Germany, Holland, USA (inc. Hawaii), India, Taiwan, Mexico and Sweden were previously reported (Schlichting 1969; Rosas, Roy-ocotla, and Mosifio 1989; Marshall and Chalmers 1997). Similar fluctuating results were found by Van Overeen while samplings were taken from an aeroplane. In months of April, May and June the algae samples were found in negligible number and therefore was eliminated from study.

Table 11. Physiological condition during sample collection.

Parameter	**Month**											
	Jan	**Feb**	**March**	**April**	**May**	**Jun**	**July**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
Light intensity ($kWh/m^2/day$)	6-6.5	6-6.5	6-6.5	6.5-7	6.5-7	4-4.5	2.5-3	2-2.5	3-3.5	5-5.5	5.5-6	5.5-6
Temperature (˚C)	8-15	15-20	20-25	30-35	37-45	35-40	32-37	30-35	25-30	20-25	18-23	10-15
Average rainfall (mm)	10.8-15.1	3.3-21	10.4-27	37.5-67.5	32.6-70.2	214.1-237.8	294.8-337	264-362.1	236-237	24.7-111.6	6.2-27.7	1.1-4.8
Relative humidity (%)	70-75	60-70	60-40	40-30	35-25	50-70	80-90	80-85	75-80	80-70	80-70	65-75

Figure 15. Airborne algae after incubation of 7-14 days.

Figure 16. Occurrence of airborne algae and *Cyanobacteria* on year 2014-2015.

6.3.2 Identified Algae

In total, 17 taxa of algae and cyanobacteria were recorded in this study including two unidentified algae (Figure 17). The highest number of taxa were *Chlorophyta* (5 taxa) followed by *Cyanobacteria* (4 taxa), *Bacillariophyta* (3 taxa) species and from *Euglenozoa* (2 taxa) were also recognized. Green algae observed includes *Scenedesmus dimorphus, Scenedesmus ovalternus, Scenedesmus bijugatus, Chlorella sp., Pteromonas sp., Oocystis sp., Spherocystis sp*. and *Oedogonium sp*. Filamentous cyanobacteria enlisted in this study are *Anabaena, Pseudanabaena sp*., whereas unicellular includes *Gloeocapsa sp*. and *Microcystis sp*. Bacillariophyceae includes diatoms and *Striatella sp*. Whereas *Euglena sp*. and *Phacotus sp*. from class Euglenozoa were also noticed. Two unidentified species are also detected. One of them are unicellular but having similarity with individual filaments of *Zygnema* algae. The dominant species were shown in Fig 17. Among them *Chlorella, Oocystis* and *Spherocystis* were found to be available throughout the year.

Three of different *Scenedesmus* species were found. *Scenedesmus dimorphus* are species found in 4 and 8 celled colonies having sharp ends. 4-celled colonies occur in linear form while 8-celled colonies arranged in sub alternating series. Average individual cell size measured to be 6.6 μm long, 1.65 μm wide whereas average 8 celled colony size ranges from 23.1 μm long to

6.6 µm wide. *Scenedesmus ovalternus* described as colonies of 2 or 4 or 8 or16 cells attached side by side, arranged linearly or zigzag. The shape of the cell varies from elliptical or spindle or as the crescent. Few species may consist of terminal spiny projections having the smooth cell wall, but some species may even content granulated or dented or rigid cell wall. Whereas *Scenedesmus bijugatus* colonies use to be arranged in a single linear series having 2 or 4 or 8 cells. The morphology of individual cells were flat, curved and ovoid or broad rounded ends. Colonies of 4 Cells were measured to be 13.2 µm -23.1 µm long, 13.2 µm. wide on the other hand individual cell was 9.9 µm -13.2 µm long and 3.3 µm -5 µm wide (Bellinger, Edward G. 2015).

Chlorella sp. is mainly found in freshwater habitat. Under a microscope, the individual cells shaped spherical with size 3-8 microns in diameter, may or may not found in colonies. The cells seem to have cleavage furrow in middle of the cell. These species were known to accumulate high lipid and used as many other purposeful resources. *Peteromonas* also belongs to Chlorophyta group. The microscopic lateral view looks ellipsoidal whereas from inside view the walls looks more or less parallel or slightly intended with angular corners, in polar view, it looks rhomboidal, hexagonal or angular and also having truncated anterior end. Size varies from 9-20 µm wide and 12-18 µm long and thickness about 7µm. Interior of cells consists of pyrenoid or eyespot. It is rapidly floating organism. *Sphaerocystis* alga found to be solitary, spherical algal cells. Individual cells are embedded in thick, transparent coverings of jelly-like mucilaginous sheaths.

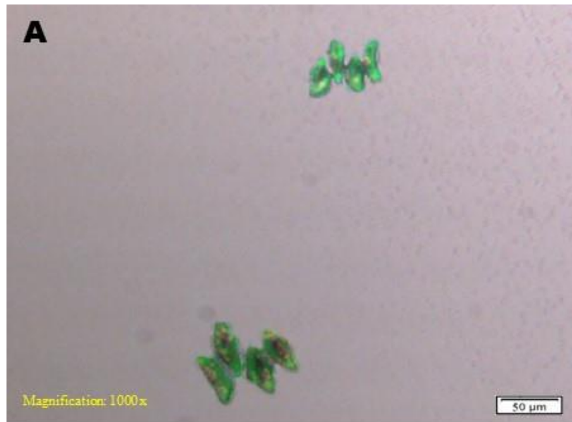
Oocystis sp. were colonial freshwater green algae with ovoid or lemon-shaped cells, usually in groups of 2, 4 or 8, and enclosed in a large, inflated, remnant mother cell wall. Having green, parietal, variable in shape chloroplasts whereas cell is covered with thick mucilage layer. After breakage of mother cell many autosphore cell releases. *Oedogonium sp*. is the only freshwater filamentous green algae found in this study. These cells could be branched or unbranched, and cell division occurs through formation of cell cap within the filaments. The cells were elongated and cylindrical. The cell wall was thick, rough and rigid.

Anabaena is the group of *Cynobacterial* origin having filamentous structure, presence of heterocysts and akinetes. It is well known for nitrogen fixation, rich sources of

pharmacological and accumulation of secondary metabolites. As per name suggested the species *Pseudanabaena* is having morphological similarity with *Anabaena*. The features include very fine filaments either solitary or agglomerated, very thin mucilaginous covering, without any branching, 0.8-3 µm wide, cylindrical filaments, cell end cylindrical or rounded or blunt or sharp at the end. *Gloeocapsa sp*. is another colonial cyanobacteria found mainly in freshwater. The mother cells use to be cover around thick mucilage sheath. After appropriate maturation this wall ruptures and autospores releases similar to *Oocystis*. These two species have close similarities the only visible difference noticed could be the thickness of mucilage layer and number of autospore released. *Microcystis sp*. are cyanobacteria most frequently responsible for harmful algal blooms. Characteristics include very tiny cells, mostly transparent, spherical shaped, organized into colonies that become perforated or irregularly shaped over time, rapid cell division and entire colonies bound in mucilage sheath.

Diatoms occur in innumerable species, and characteristics include solitary, colonial, filamentous; siliceous wall, grooves or rows of dots which form certain patterns, solitary cells often showing a gliding or jerky movement. *Navicula sp*. were found to be solitary, 'boat-shaped', actively motile, single rod-shaped pyrenoid along with vertical length. The genus *Mastogloia* is characterised by a drum-shaped or cigar-shaped, yellow-brown coloration non-flagellate individual cells, and developed depression at both the end.

Striatella sp. are rarely found species having very less number of species in this genus. It is having whitish or transparent background with triangular or irregular shape structure inside of the base. Inside transverse, disc varies from male to female from brown to dark yellow. The found species was motile. Here we find *Euglena* and *Phacus sp*. that belongs to Euglenozoa phylum. *Euglena* is single-celled organisms, live mostly in fresh water, oval or irregular shaped, consists of red eyespots and the presence of flagella for movement. *Phacus* species are unicellular, very light green in colour, almost spherical in shape, twisted, flagella, eyespot, chloroplasts usually small, discoid, and numerous pyrenoids inside the cell.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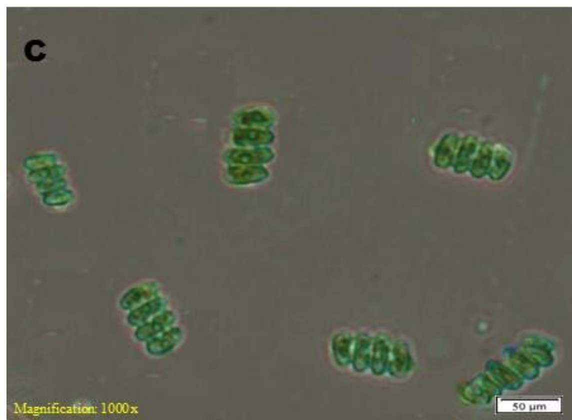
50 µm

B
Magnification: 1000x
50 µm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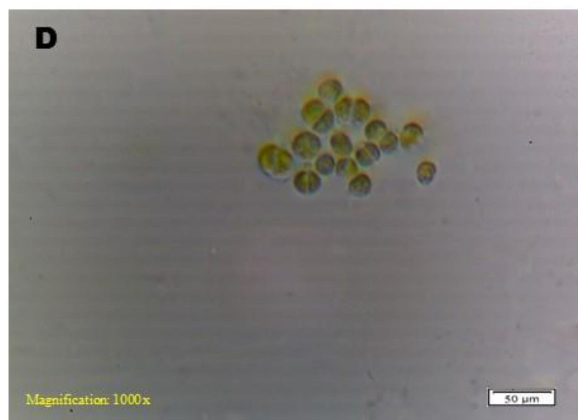
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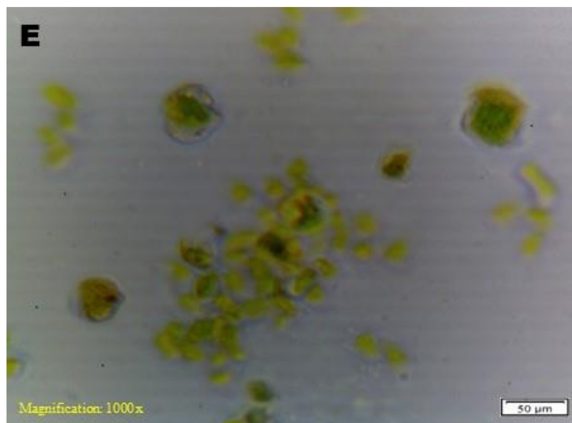
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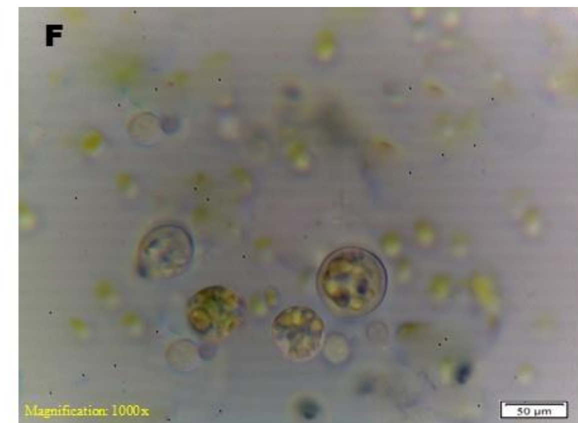
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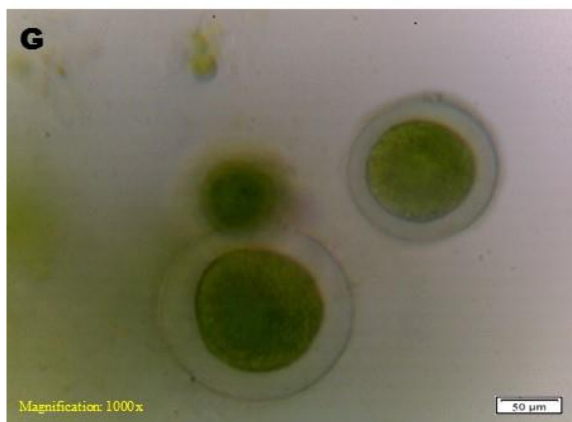
50 µm


G
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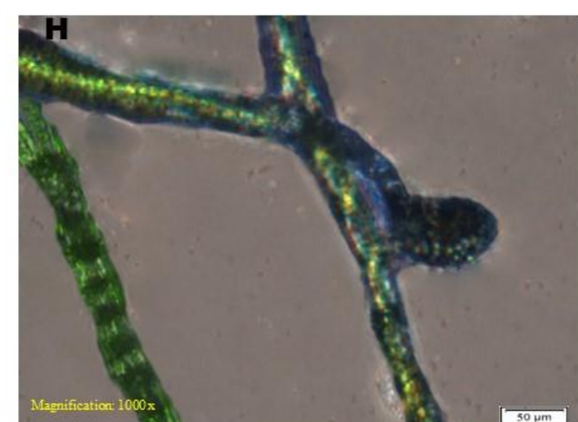
50 µm


H
Magnification: 1000x
50 µm

I
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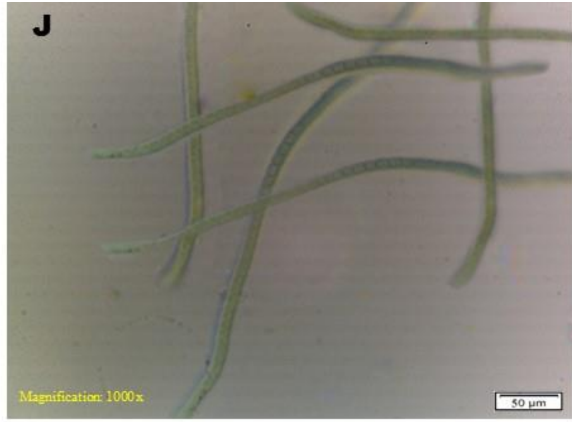
J
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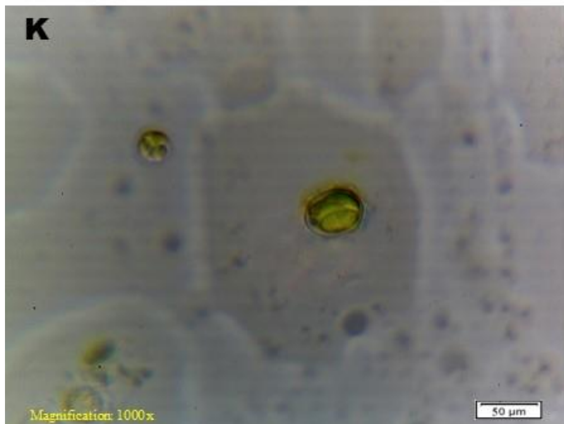
K
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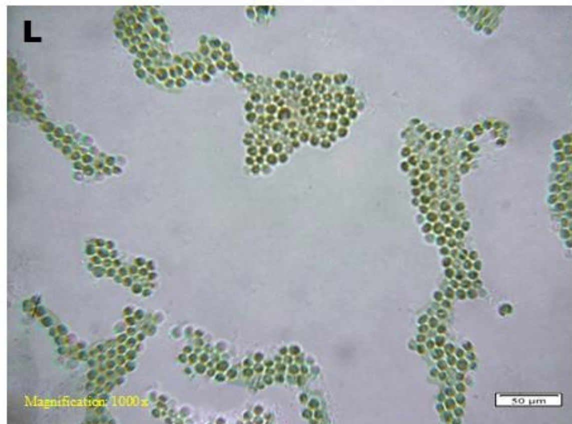
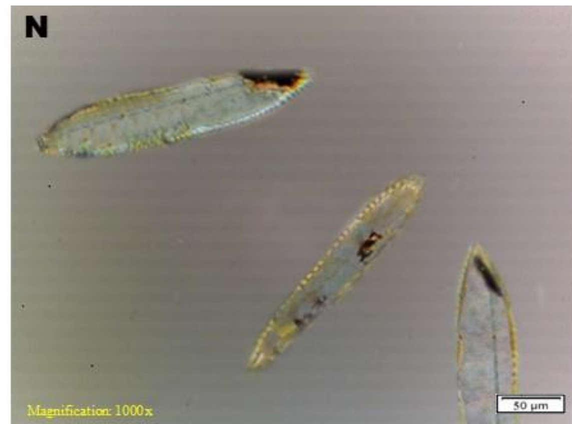
50 µm


L
Magnification: 1000x
50 µm

M
Magnification: 1000x
50 µm


N
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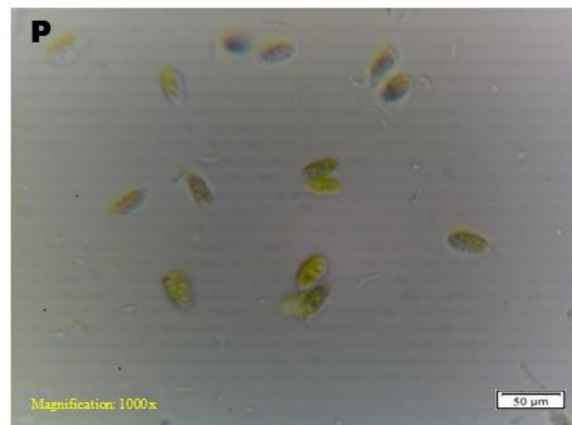
50 µm

O
Magnification: 1000x
50 µm


P
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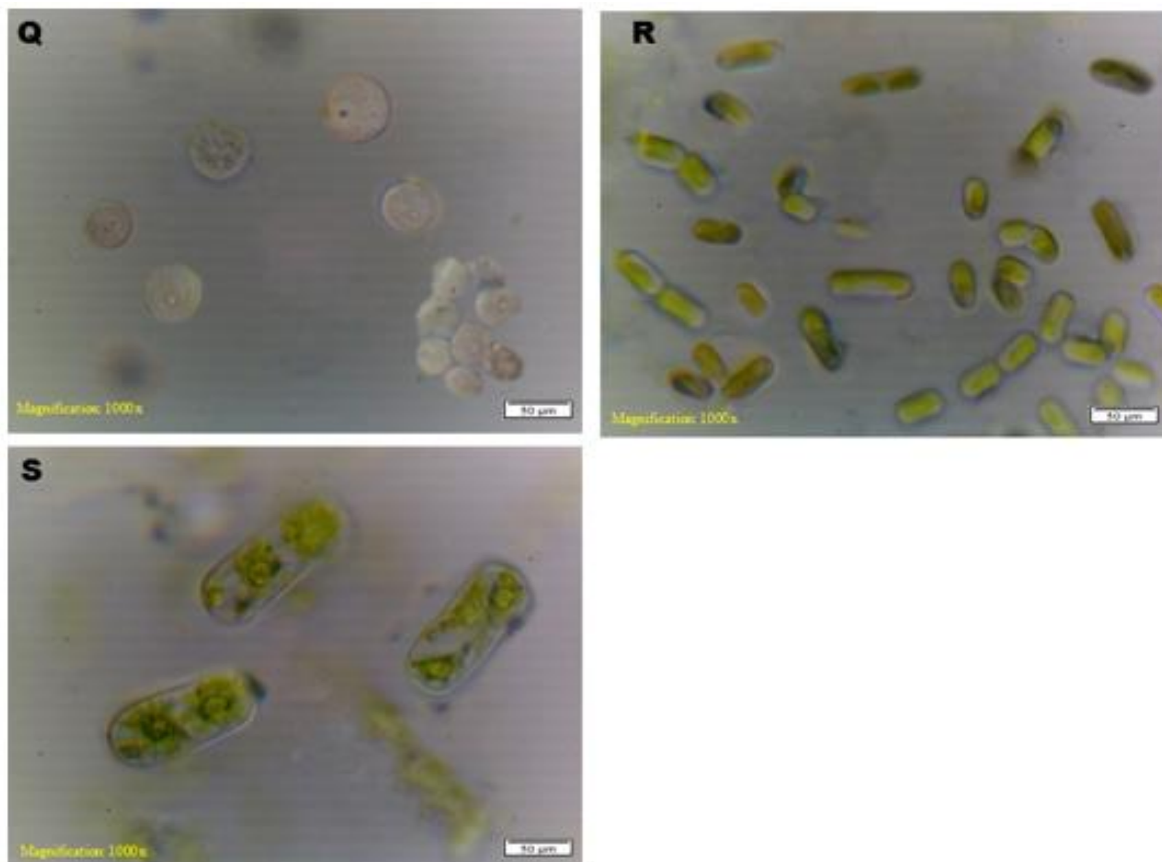


Figure 15. Micrograph of sample under 100x resolution. *Chlorophyta*: *Scenedesmus dimorphus* (A), *Scenedesmus ovalternus* (B), *Scenedesmus bijugatus* (C), *Chlorella sp*. (D), *Pteromonas* (E), *Sphaerocystis sp*. (F), *Oocystis sp*. (G), *Oedogonium sp*. (H), *Cyanobacteria*: *Anabaena sp*. (I), *Pseudanabaena sp*. (J), *Gloeocapsa sp*. (K), *Microcystis sp*.(L), *Bacillariophyceae: Naviculoid diatoms* (M), *Mastogloia elliptica* (N), *Striatella sp*. (O), *Euglenozoa*: *Euglena sp*. (P), *Phacus sp*. (Q), Unidentified species (R,S)

6.3.3 Lipid estimation

Fluorescence emission of Nile strain algae strains was estimated at 580 nm which is characteristics of the strained cell and was different than other unstrained algae cell or only Nile strain. The techniques are used to get the preliminary idea of lipid content within the cell. This is non-destructive technique and according to study is directly correlated with lipid content obtained after extraction. So, the samples which do not or have limited fluorescence exhibition could be eliminated from the experiment. This practice not only saves the experimental time and labor but also gives preliminary support for further calculative step or modification in next successful planning. As shown in Table 12 both the relative fluorescence intensity at 572~582 nm of the stained cells and extracted lipid content determined by gravimetry. Among the strains tested, the cells collected in the month of October has the

highest lipid content of 30.59 % and also has the highest fluorescence intensity (55.43). Whereas samples from the month of March was not found suitable to carry further experiments.

Table 12. Comparison of lipid content by gravimetric analysis and relative fluorescence intensities.

Month	**Lipid content determined by gravimetry (%)**	**Relative fluorescence intensity**
Feb	15.76	6.13
Jan	17.22	10.89
Dec	18.65	14.14
Aug	20.83	20.5
Sept	25.91	31.61
Nov	28.34	40.2
Oct	30.59	55.43

Notes: Stained algae cells were excited at 480 nm and their relative fluorescence intensity were detected at the 570~580 nm emission wave band.

6.3.4 Analysis of FAME

6.3.4.1 CHNS analysis

The CHNS, AAS and Flame photometry analysis show that the elemental composition of airborne algae bio-diesel of various month (Table 13, 14). Comparatively, algae bio-diesel having the higher carbon content that could be probable estimation for HHV. Lower nitrogen and sulphur content could decipher to lower the nitrogen and sulphur percentages in the bio-diesel, and the lesser will be the exhaust emissions (NO_x and SO_2) while using it in a diesel engine. Oxygen and carbon content varies month wise. As the culture constitutes mixed culture, therefore, it is difficult to estimate correct reason of this deviation. However, elemental composition varies from species to species and also upon the further downstream processing to convert into bio-diesel. As few samples also content filamentous organism so Folch's method of lipid extraction may not be suitable for every sample. In general, the metal content in algae reflects the metal percentage of surrounding area; therefore, the metal analysis was done (Conti and Cecchetti 2003). Results were presented in table 13 which shows the comparatively higher iron and magnesium content which normally use to be higher in plant

material.

Table 13. CHNS analysis values of algal samples (wt %).

Month	C	H	N	S	O (by difference)
Feb	64.75	7.86	0.45	0.14	26.8
Jan	62.58	9.39	0.36	0.11	27.56
Dec	71.62	10.56	0.12	0.09	17.61
Aug	68.89	11.03	0.22	0.08	19.78
Sept	70.13	14.99	0.47	0.12	14.29
Nov	77.23	15.23	0.18	0.12	7.24
Oct	79.23	12.40	0.07	0.08	8.22

Table 11. Common metal contents present in algae of different months.

Elements	Feb	Jan	Dec	Aug	Sept	Nov	Oct
AAS							
Iron	65	88	79	65	74	114	87
Zinc	38	54	46	48	39	58	43
Magnesium	63	78	80	76	97	84	134
copper	5	17	14	9	11	6	9
Flame photometry							
Sodium	39	51	72	49	79	62	67
calcium	15	23	12	30	28	17	11
potassium	29	39	47	38	41	34	28

6.3.4.2 FTIR

In FTIR spectroscopy, the functional group of FAME was determined by passing infrared radiation which was absorbed by the entire molecule and the spectrum emitted was computationally recorded. The FTIR spectrum of the liquid product obtained was presented in Fig. 18 and Table 15. It is clearly visible that biomass from different month has different FTIR spectra. Few common bands are present in every sample whereas few bands are either spliced or merged from source absorption peak. From the analysis, it could be concluded that most

frequent groups were Ester, Alcohol, Alkane, Alkene and carboxylates. As the lipid was transesterified therefore presence of ester is quite obvious. Other functional groups may substitute the side chain belongs to ester. Samples from the month of Oct and Nov were having more functional verities whereas the sample from the month of Feb even doesn't consist Ester. Therefore on this basis that sample was also eliminated from further analysis. The broad, strong band of H–bonded O–H stretch stretching vibration between 3200 and 3600 cm^{-1} of the liquid product indicates the presence of phenol and alcohols. Whereas C─H stretching vibration on 2960 cm^{-1} form methyl bond. Similarly, the 2850 cm^{-1} and 1640 cm^{-1} indicates the presence of methylene and Alkene. The bands at 1580 cm^{-1} with C═C or C═O stretching indicates the presence of Aromatic or carboxylates group. On the other hand, 1380 cm^{-1} and 1350 cm^{-1} with N─O stretching show the presence of Aliphatic or Aromatic nitro compounds in the product. Most of the ester compound observed on 1110 cm^{-1}and 1016 cm^{-1}. The results were further consistent when compared with the results of GC–MS.

Table 12. Month wise comparison of functional groups present in transesterified product.

Absorption peak (cm^{-1})	Type of vibration	Functional group	Months					
			Jan	Aug	Sept	Oct	Nov	Dec
1016	C─O	Ester	√	√	√	√	√	
1060	C–O stretch	Alcohol		√				
1110	C─O	Ester			√	√	√	
1350	N─O	aromatic nitro compounds	√		√	√	√	√
1380	N─O	Aliphatic nitro compounds		√	√	√	√	√
1450	C═C	aromatic				√		
1580	C═C, C═O	Aromatic, carboxylates	√	√	√	√	√	√
1640	C=C stretch (Medium)	Alkene	√	√	√	√	√	√
2850	C─H	methylene			√	√	√	√
2960	C─H	Methyl			√	√		
3365	H–bonded O–H stretch (Broad strong band)	Alcohol	√	√	√	√	√	√

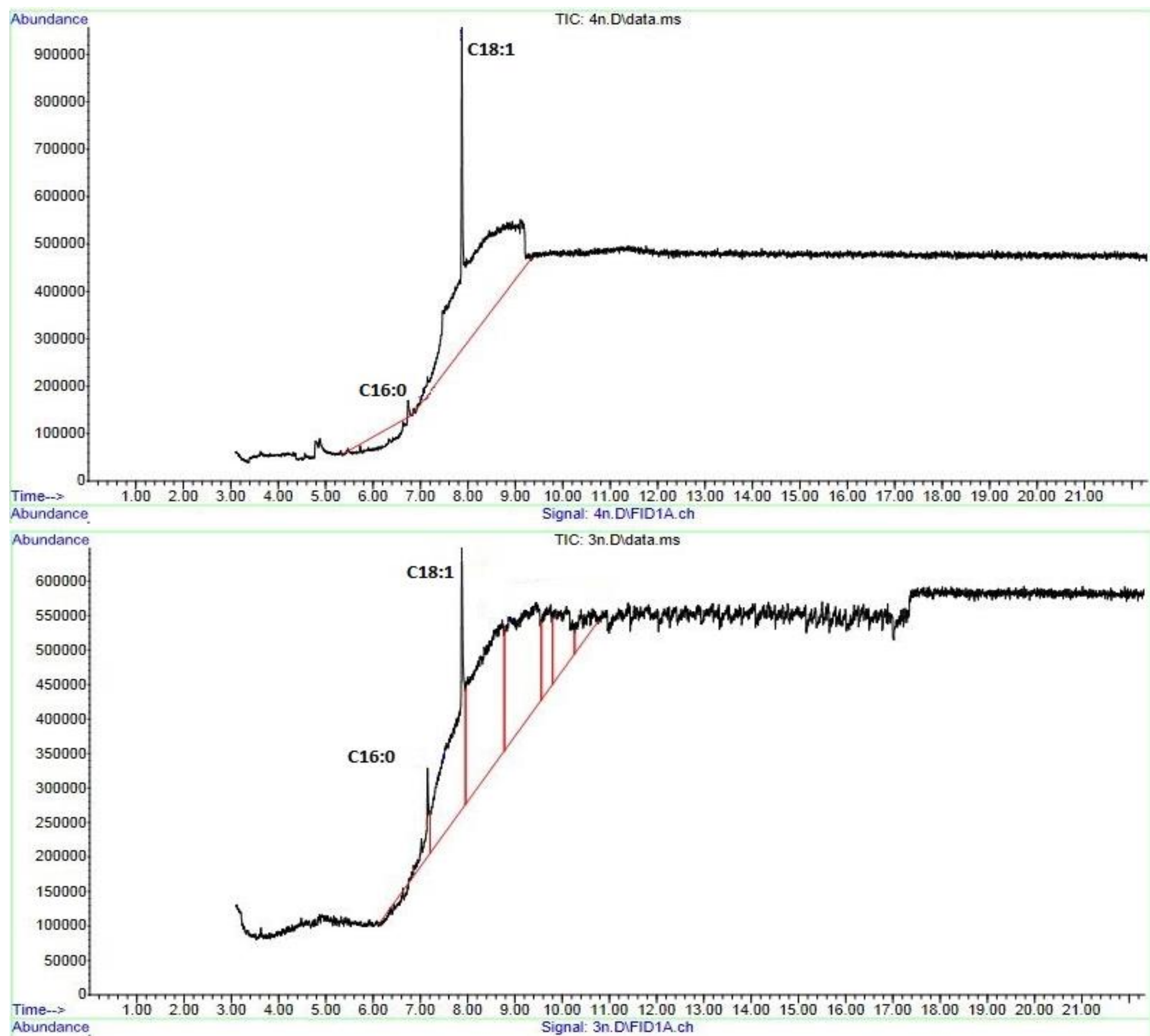
Figure 16. Month wise comparison of FTIR spectra of transesterified product.

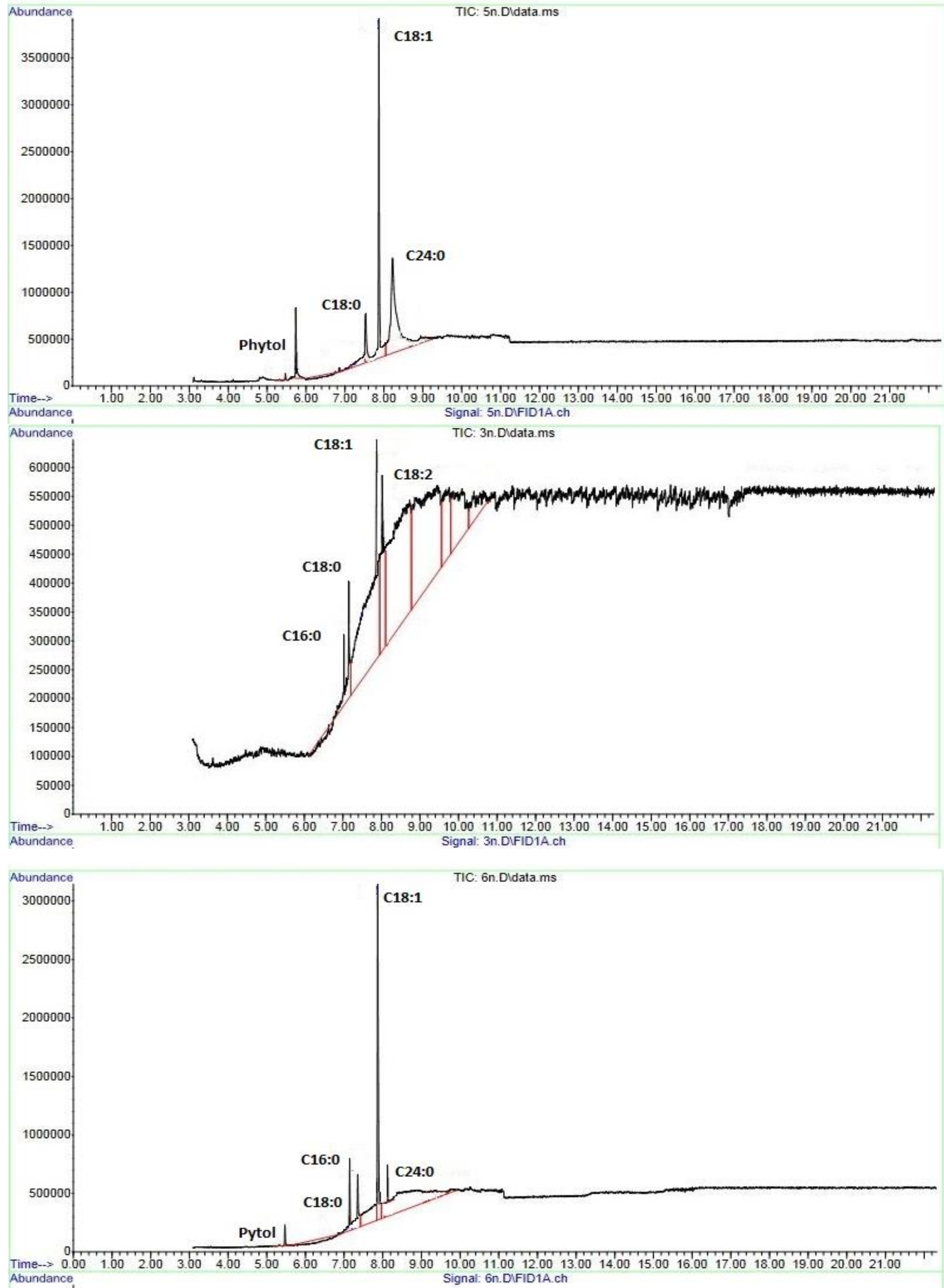
6.3.4.3 GC-MS

According to finding and further analysis entirely six different samples from month January, August, September, October, November and December were analyzed. During transesterification reaction, the converted FAME was found to be very high values of Oleic acid (18:1), which constituted about 30 to 50 % of the overall lipid fraction (Fig 19). A total of ~6 different fatty acid substrate from C16-C24 were identified by mass spectrometry. Out of these fatty acids, the majority are low carbon chain compounds which are very suitable for bio-diesel production. According to retention time, Oleic acid (C18:1) and Palmitic acid (C16:0), consists of more than 40% of total FAs.

Overall total lipid, as well as FAME quantity in the month of October, is more in comparison to others. Further followed by samples found in the month of November, September, August, December and January. As per environmental data described earlier the moderate season between rainy and the start of winter is the best-suited climate for the growth of such microbes. As per FAME profile, different esters are also more at that climate because of the presence of more variety of algae in air. As the variation was more, therefore, FAME content and variation is different (Lang et al. 2011). On the other hand month of January and December, only a few verities of microalgae were found. However, microalgae are considered more efficient for the production of bio-diesel so those months could also be considered suitable for sample collection. However, oleic acid was the common fraction found in all samples in relatively higher amount. Phytol fraction in good quantity was also detected in few samples. Normally phytol is formed after chlorophyll degradation and commonly found in the lipid fraction of fresh chlorophyll content biomass samples (J. Jones et al. 2012). Chromatogram of GC-MS obtained of different months has been presented in Fig 20.

Figure 19. Month wise comparison of FAME content





Abundance
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C18:1
C24:0
C18:0
Phytol
Time-->
Signal: 5n.D\FID1A.ch
TIC: 3n.D\data.ms
C18:1
C18:2
C18:0
C16:0
Signal: 3n.D\FID1A.ch
TIC: 6n.D\data.ms
C18:1
C16:0
C24:0
C18:0
Pytol
Signal: 6n.D\FID1A.ch

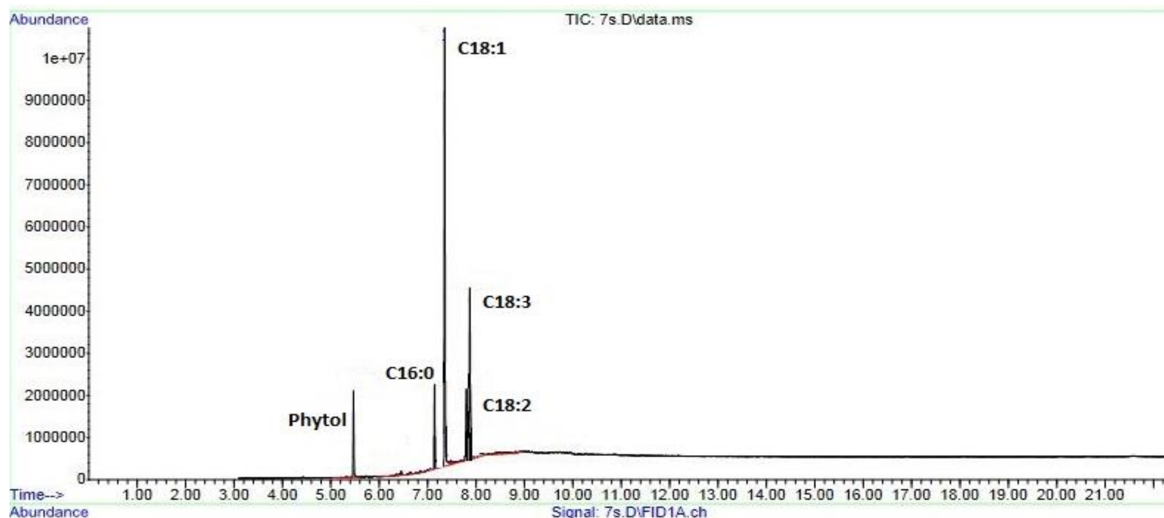


Figure 17. GC–MS chromatogram (A) January (B) December (C) August (D) September (E) November and (F) October

6.3.4.4 Fuel Properties

Determination of bio-diesel properties is useful parameter to know that the obtained product could be further suitable to be used as fuel in future (Table 16). The Biodiesel Analyzer Version 1.1 requires only FAME data input that reduces the task of obtaining the heavy amount of oil for these necessary analyses (Talebi, Tabatabaei, and Chisti 2014). Variation in fatty acids profile effects the overall bio-diesel quality. Normally PUFAs are present in more quantity in algae that decreases the stability of fuel, but on the other hand, it has low melting point and better cold weather properties in comparison to other bio-oil (Prathima Devi, Venkata Subhash, and Venkata Mohan 2012). In the present study, the values of estimated SFA for the month of Jan, Dec, Aug, Sept, Nov, Oct were 10.37, 17.26, 31.21, 35, 44.52 and 17.31. Whereas MUFAs were maximum in January i.e. 49.9 due to the presence of high amount of oleic acid. PUFAs were absent in most of the samples except for the month of September and October due to the presence of Linoleic acid. The DU and LCSF for the month of Jan, Dec, Aug, Sept, Nov, Oct were 49.9, 43.2, 49.36, 87, 40.6 and 106.17 respectively.

The estimated SV of bio-diesel from algae for the month of Jan, Dec, Aug, Sept, Nov, Oct were 121.578, 123.342, 150.054, 191.252, 170.345 and 182.288 which is very near to Jatropha i.e. 198.85, Mahua i.e. 187, Safflower seed i.e. 190.23, etc. Similarly, IV of our sample for the months of Jan, Dec, Aug, Sept, Nov, Oct were 44.872, 38.847, 44.387, 78.582, 36.509 and 115.618 which are nearly equivalent to Palm i.e. 60.54, Mahua i.e. 80.21 respectively

(Gopinath, Puhan, and Nagarajan 2009). According to the European specification, minimum CN of 51 is required to meet the specification and in all these samples meet this criterion well. CP is very similar to other Palm, Jatropha, Pongamia bio-diesels (Sarin et al. 2009). HHV is lower in biomass due to high oxygen content. Our result follows the same trend and has even less HHV than coconut bio-diesel. The kinematic viscosity by ASTM D6751 and EN 14214 standard is within the range of 1.9–6.0 mm2/s and obtained values of all samples reside within this range. Whereas 860–900 kg/m3 is for density but bio-diesel from algae do not satisfy the range hence extra effort is required in these particular case (Hoekman et al. 2012).

Table 13. Properties of Bio-diesel

Bio-diesel Properties	**Jan**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
Saturated Fatty Acids % (SFA)	10.37	31.21	35	17.31	44.52	17.26
Monounsaturated Fatty Acids % (MUFA)	49.9	49.36	33	38.79	40.6	43.2
Polyunsaturated Fatty Acids % (PUFA)	0	0	27	33.69	0	0
Degree of Unsaturation (DU)	49.9	49.36	87	106.17	40.6	43.2
Long-Chain Saturated Factor (LCSF)	1.037	46.46	11.9	1.731	22.467	1.726
Saponification Value (SV) mg/g	121.578	150.054	191.252	182.288	170.345	123.342
Iodine Value (IV)	44.872	44.387	78.582	115.618	36.509	38.847
Cetane Number (CN)	81.097	72.687	57.157	50.228	70.126	81.81
Cloud Point (CP) (˚C)	0.463	-4.992	2.372	4.113	7.537	4.087
Higher Heating Value (HHV) (MJ/Kg)	23.839	32.305	37.571	35.367	33.775	23.884
Kinematic Viscocity (μ) (mm^2/s)	2.317	3.767	3.722	3.136	3.538	2.313
Density (ρ) (kg/m^3)	527	701	831	791	740	528

6.3.4.5 HPLC

According to literature, sugars might be present in MeOH layer after Folch's lipid extraction method (Folch, Lees, and Stanley 1957). Therefore, HPLC analysis was performed to know the possible monosaccharides and acid. Standards were matched, the concentration was also calculated from the obtained pick and summarised in Table 17. Mostly maltotriose, arabinose, cellobiose, glucose, xylose, inulin, stachyose along with acids was detected in the significant

amount. Maltotriose in the month of October, Melizitose in November, arabinose, glucose, cellobiose in September, August and January were found in comparatively high quantity. Whereas in December none of these saccharides were found in significant quantity. Mentioned saccharides were commonly found sugars in algae.

Melizitose consists of three glucose molecules and were in practice to be extracted from algae. Arabinose is an aldopentose having five carbon atoms and were successfully extracted along with glucose, xylose from Anabaena cylindrical (From and Alga 1954). Cellobiose is a disaccharide and was detected in cell wall of the marine green alga Ulva lactuca (L.) Thuret. Inulins are a group of naturally occurring polysaccharides produced by many types of plants and algae (Shono 1984). Stachyose is a tetrasaccharide and was also detected in blue-green algae (Liliana, Cardemil and Wolk, Peter 1978). Similar to fatty acid profiles, quantity and quality of saccharides were not consistent due to the presence of different algae species. The types of saccharides obtained were also reported.

Table 14. HPLC data for probable sugar, acids and other water soluble compound analysis.

Retention time (min)	**Possible structure**	**Concentration (%)**					
		Jan	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
5.54±0.06	xylose	-	-	-	0.1	-	-
6.94±0.05	Inulin	-	-	-	0.65	-	0.47
7.26±0.15	Stachyose	2.9	0.03	4.76	-	0.02	1.87
7.65±0.05	Maltotriose	-	-	-	2.87	-	-
7.95±0.04	Melezitose	-	-	-	-	5.1	-
7.94±0.11	Cellobiose	6.68	6.15	4.3	0.32	-	-
8.98±0.19	Citric acid	-	0.21	-	-	0.15	0.11
9.06±0.12	Glucose	5.7	2.82	6.2	2.88	-	-
10.04±0.12	mannose	-	-	4.34	-	-	0.2
10.7±0.13	fructose	-	-	-	-	0.12	-
11.57±0.34	arabinose	-	4.69	5.47	1.3	1.4	0.47
13.44±0.36	glycerol	0.02	0.01	0.06	0.36	0.07	0.02
15.69±0.41	acetic acid	0.09	0.01	3.1	-	1.1	0.03
17.35±0.43	Propionic acid	-	0.0005	-	0.02	0.002	-
22.38±0.49	ethanol	-	-	0.32	-	-	0.1
24.77±0.51	DMSO	-	-	0.04	-	-	-
Total Saccharides		15.28	13.68	25.07	8.13	6.63	3.1
Total acids		0.09	0.22	3.1	0.02	1.26	0.14
Others		0.02	0.01	0.42	0.36	0.07	0.12

6.4 Conclusion

This study was about to collect, identify and culture airborne microalgae throughout the year in the locality of Rourkela. The dominant species to adapt the environment throughout the large scale culture experiment were *Scenedesmus dimorphus, Scenedesmus ovalternus, Scenedesmus bijugatus, Chlorella sp., Pteromonas, Sphaerocystis sp., Oocystis sp., Oedogonium sp.* among Chlorophyta phyla. Whereas among cyanobacterial origin *Anabaena, Pseudanabaena sp., Gloeocapsa sp.*, *Microcystis sp.* were found. *Naviculoid Diatoms, Mastogloia elliptica, Striatella sp.*, belongs from Bacillariophyceae phylum. Species from Euglenozoa identified were *Euglena sp.* and *Phacus sp*. Other than this two unidentified species were also observed.

Few species were previously reported to be found in the air but others are reported first time in this study may be due to the difference in geographical environment. Climatic condition observation reveals that in rainy season presence of microbes and algae in the air were negligible as rainwater cleanse the air naturally and eliminates the present contamination. On the other hand abundance of algae could be found during post monsoon season. Lipid estimation was conducted by Spectrofluorometry by Nile red fluorescence dye. Lipid fraction obtained from modified Folch's method, and further transesterification was conducted. Characterization of FAME by CHNS, FTIR, GC-MS and biofuel basic properties were performed to know the suitability of extracted oil as bio-diesel. Further, probable sugars, acids and alcohols were also estimated from MeOH layer after lipid extraction. The overall analysis obtained provides that airborne algae oil fraction is suitable to use as bio-diesel. As the airborne algae are also oleaginous in nature, hence these could not be considered as contamination or reason for low lipid extraction during large scale open culture system. The study also favours the large open scale culture system over photobioreactor because if the algae could survive and thrive in air therefore without any special physical parameter control the large scale culture would be economically more feasible.

Chapter 7

Bio-diesel and co-product production from soil algal biomass

7.1 Introduction

The major TAG pool from cells was extracted in chloroform layer. As MeOH is also used as the solvent, therefore, many polar cellular components also get dissolved in that layer. However, description of those dissolved components is not much studied. Algae is known to consist many useful varieties of the compound. Therefore, leftover residues should also be utilised. Lipid-extracted biomass residues for maltodextrin production, animal feed and other feed as nutritional co-products were proposed earlier (Bryant et al. 2012; Lam, Tan, and Lee 2014). Co-product production is economically feasible for industrialisation and therefore are major focused on research. As data shows that carbohydrates or polysaccharides are also the major part of algae component hence the present study focuses on the conversion of bio-diesel from algae lipid, identification of common water soluble components (polysaccharides), and characterization of cell debris as bio-char. This set of work involved the sudden appeared soil algae biomass. The data also revealed that most of the species were also present frequently in air samples.

7.2 Materials and Method

7.2.1 Algae Strain

Algae soil samples were collected from NIT, Rourkela premises (22˚15'19.5"N 84˚54'14.5" E). The soil samples from that site were also collected for analysis of pH, moisture content, organic matter content and percentage of calcium carbonate. Isolated mixed cultures were morphologically identified under inverted microscope (Olympus Corporation Tokyo, Japan) (Prescott. 1954). Obtained strains were maintained, monitored and cultured in large scale bioreactor under standard parameters mentioned earlier. The detailed description of methodology was mentioned in chapter 3.

Protein estimation was done by the standard AOAC method, i.e., Kjeldahl method using Kelplus Nitrogen Estimation System, Model- Classic-DX VATS E. Dried and finely ground algae extract was digested in boiling concentrated sulphuric acid and caustic soda. Final titration value was done to estimate the protein percentage using copper sulphate and potassium permanganate as the catalyst. Lipid content was determined by the standard AOAC procedure, i.e, Soxhlet method using Socsplus Extraction System, Socsplus series SCS-06 AS DLS TS using petroleum ether as the reagent. Similarly, fibre content was estimated by the standard AOAC procedure using Fibre Extraction System, Fibraplus Series FES-06 AS DLS TS.

7.2.2 Lipid and other Co- Product Extractions

Lipid was determined by modified Folch's method and transesterified into FAME. The composition of algae bio-diesel and Phytol produced was determined by using GC-MS analysis. Phytol is produced from the hydrolysis of chlorophyll (Biller, Riley, and Ross 2011). Bio-diesel properties were also estimated by "Biodiesel Analyzer Ver. 1.1" (Talebi et. al. 2013, Verduzco et. al. 2012). MeOH layer was analysed in HPLC (MODEL no.) to detect sugars, acids and sugar alcohols in Agilent Hi-Plex column having size 6.5 × 300 mm length and 8 μm (p/n PL1F70-6850) thickness. 10 μL of sample was injected with flow rate 0.4 mL/min having the mobile phase of 100% DI water with temperature 85 °C. Peaks were detected by RI detector. Further, the presence of chlorophyll pigment was also analysed spectrophotometrically. The MeOH extracts were filtered through a membrane filter of 0.22 mm pore size (Merck Pvt Ltd., India). The absorbance at 650 and 665 nm (A650, A665) was measured using UV-Vis spectrophotometer (UV-3600 Plus, Shimadzu). The total chlorophyll content estimation was described in chapter 3.

7.2.3 Bio-char Preparation and characterization

The cell debris after solvent extraction was used further as feedstock for biochar preparation. As the algal biomass was treated with various chemicals previously hence was washed repeatedly followed by drying for moisture removal. For physical activation, powdered samples were heated in the muffle furnace at 450 °C at the rate of 20 °C/min for the constant hold of 2 h.

After cooling of the furnace, the sample was transferred directly to a desiccator and weighed finally. The obtained product was used for various analysis and stored in the airtight bottles for further use in the batch sorption experiments (Nautiyal, Subramanian, and Dastidar 2016). To perform the proximate analysis, ASTM D-271-48 standard was used to determine its ash content, volatile matter, moisture content and fixed carbon. The elemental analysis was conducted in a CHNS analyser (Vario EL, Germany). The TGA analyser (NETZSCH, STA409C, Germany) was performed in the nitrogen atmosphere under the flow rate of 30 ml/min. The temperature was increased from room temperature till 600 °C at the constant heating rate of 20 °C/min. The identification of functional group in both biomass and biochar was performed with the help of an FTIR instrument (NICOLET, Impact 410) with a resolution of 4 cm^{-1}. The X-ray diffractometer (XRD) analysis was conducted on an analytical XRD diffractometer (RIGAKU Mini flex, Japan). The XRD was functioned at a voltage of 30 kV with a current density of 15 mA. The scanning range was from $2\theta = 10°$ to 70° with the scan speed of 0.05°/s. EDX attached with SEM was also analysed to know surface morphology and metal composition at the surface.

Bio-char study alone was also conducted to know pH and pore size. A digital pH meter (SYSTRONICS, 802) was used to measure the pH value of the biochar. The biochar was mixed with deionized water in the ratio of 1:20 (w/v) to form a homogeneous suspension, and the pH was determined after 1.5 h. The BET surface area of residual algae biochar was measured using BET surface area analyser (Quantachrome/AUTOSORB-1). Liquid phase adsorption study using Methylene blue was also conducted. 100 ppm of Methylene blue stock solution was prepared, and 30ml of the stock solution was taken in the separate flask along with 0.2 gram of biochar. The solution was under constant shaking at 100 r.p.m. for 20 min. The filtered solution was subjected to UV-Visible spectroscopy. The final amount of dye adsorbed Qe (mg/gm) and percentage of adsorption were calculated.

7.3 Results and discussion

7.3.1 Soil Analysis

In this study soil samples along with algae were collected on the campus of National Institute of Technology, Rourkela (22°15'22.4"N 84°54'18.5" E). The study includes physicochemical

properties of soil like pH, moisture content, organic matter content and percentage of calcium carbonate. Nutrient enriched the soil, and other related parameters were the cause behind the appearance of algae in that area. Therefore, physical and chemical analysis of the soil was accomplished to know the parameter of algae growth (Table 18).

Mainly, soil contains carbon, hydrogen, oxygen, nitrogen and trace amount of sulphur and other elements. These organic content are even less than 10% of soil by weight but influence greatly on fertility of land and have relative terms with other physiological parameters. Like, availability of organic matter increases for flora and fauna with favourable temperature, oxygen, and moisture conditions. However, the only stable organic matter could be detected during soil analyses.

Total carbonates present in 100g of dry soil is determined to know the percentage of calcium carbonate. The available water or moisture for algae growth known as the moisture content of soil. This moisture uses to be entrapped within the soil pores. These water act as the solvent to dissolve the nutrients which further absorbed during growth. Depending upon planted crop the requirement of soil pH also varies, therefore, alkaline and acidity of soil both are useful. However, a certain pH buffer range maintenance is always important as all other biochemical cycle depends on pH of the surrounding water. In our study organic matter content, the percentage of calcium carbonate, moisture content, pH of collected soil was 1.3622, 16.64, 9.89 and 6.336 respectively. The organic matter content reflects that the soil was moderately nutrient rich and not exposed from organic waste from longer period (Anikwe and Nwobodo 2002). As the texture of soil was sandy clay, so other moisture percentages, calcium carbonate and pH properties were similar accordingly.

Table 15. Physicochemical properties of collected soil.

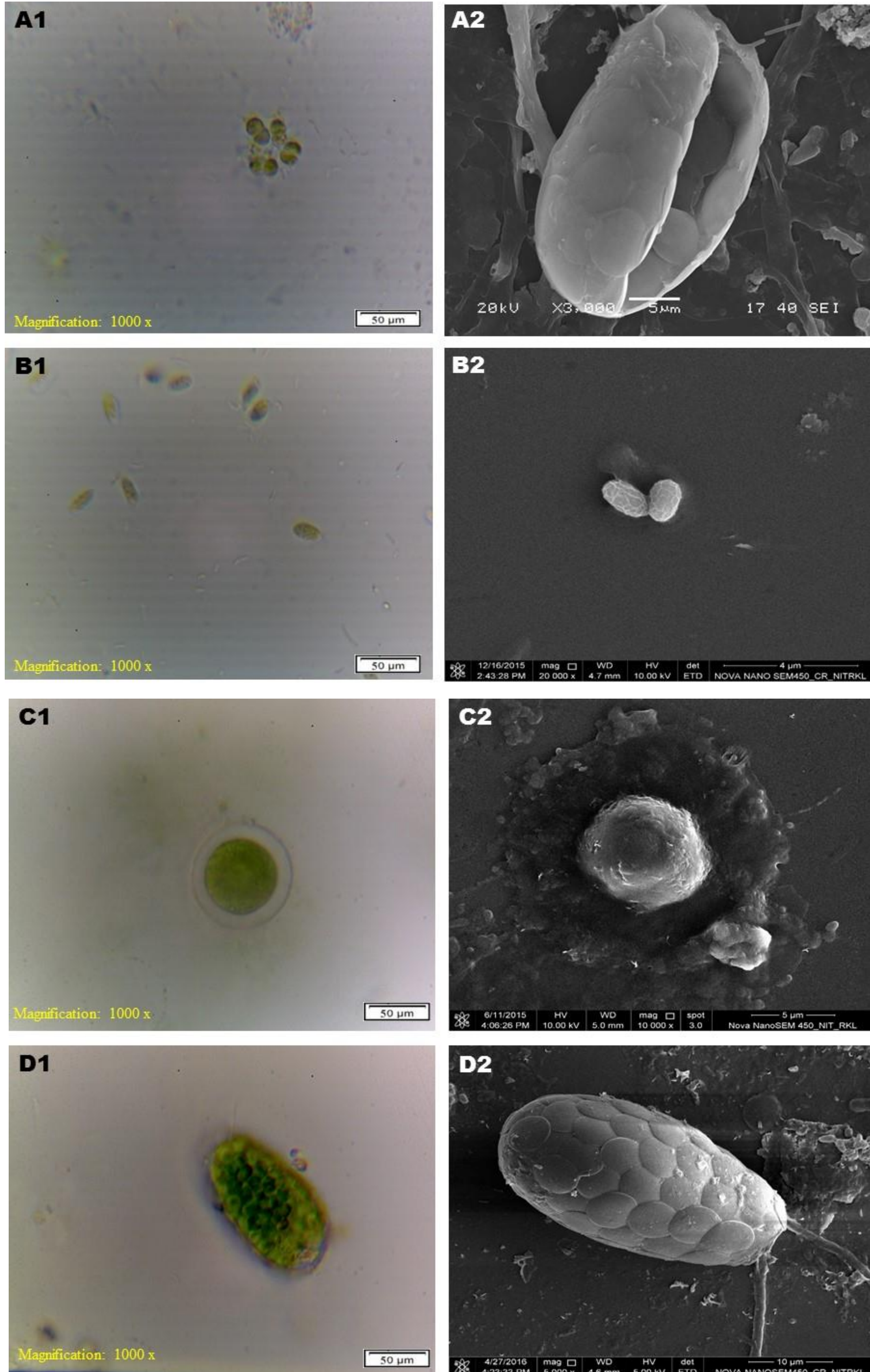
Parameter	Value
Organic matter content (%)	3.36
percentage of calcium carbonate (mg/gm)	16.64
Moisture content (%)	9.89
pH	6.34

7.3.2 Algae Identification

In total, six taxa of algae were recorded including one unidentified alga (Fig. 21). Among them Chlorophyta phylum includes *Chlorella sp., Oocystis sp*., filamentous cyanobacteria enlisted were *Anabaena, Pseudoanabaena sp*., whereas *Euglena sp*. from class Euglenozoa were also noticed. One unidentified species was also detected. *Chlorella sp*. are mainly found in freshwater habitat. Under a microscope, the individual cells shaped spherical with size 3-8 microns in diameter, may or may not found in colonies. The cells seem to have cleavage furrow in middle of the cell.

These species were known to accumulate high lipid and used as many other purposeful resources. *Oocystis sp*. were colonial freshwater green algae with ovoid or lemon-shaped cells, usually in groups of 2, 4 or 8, and enclosed in a large, inflated, remnant mother cell wall. Having green, parietal, variable in shape chloroplasts whereas cell is covered with thick mucilage layer. After breakage of mother cell many autosphore cell releases. *Anabaena* is the group of *Cynobacterial* origin having filamentous structure, presence of heterocysts and akinetes.

It is well known for nitrogen fixation, rich sources of pharmacological and accumulation of secondary metabolites. As per name suggested the species *Pseudanabaena* is having morphological similarity with *Anabaena*. The features include very fine filaments either solitary or agglomerated, very thin mucilaginous covering, without any branching, 0.8-3 µm wide, cylindrical filaments, cell end cylindrical or rounded or blunt or sharp at the end. *Euglena* is single-celled organisms, live mostly in fresh water, oval or irregular shaped, consists of red eyespots and the presence of flagella for movement.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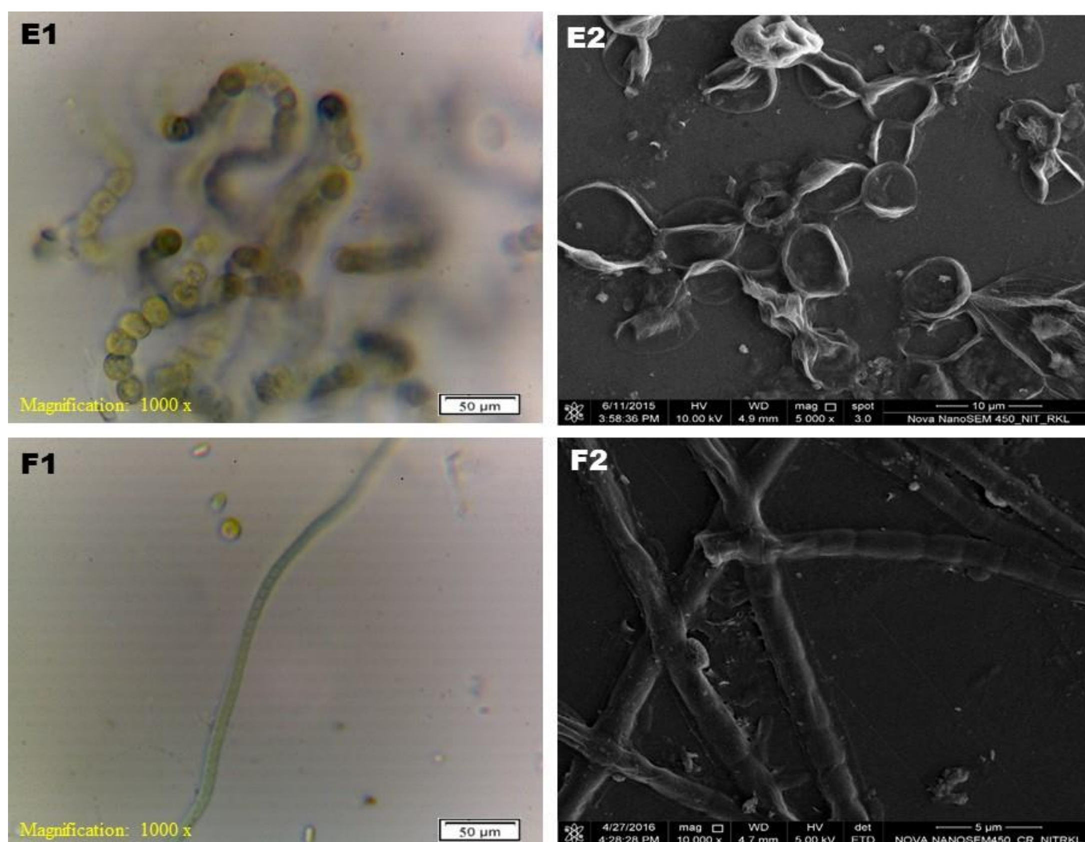

Figure 18. Algae identification by optical microscopy (100x) magnification (left) and SEM analysis (> 3000x) magnification (right). (A) *Chlorella*, (B) *Euglena*, (C) *Oocystis,* (D) Unknown, (E) *Anabaena*, (F) *Pseudoanabaena* species.

7.3.3 Nutrient profile of raw algae

Nutrient content of raw algae biomass was also determined (Table 19). Nutrition analysis showed that the mixed culture algae have higher content of protein (i.e. 38.2 wt %), followed by carbohydrate (i.e. 25.6 wt%) and lipid (i.e. 21.4 wt%). As petroleum ether was used in the instrument as solvent therefore in further lipid extraction by modified Folch's method the result was comparatively higher (i.e. 24.3 wt%).

The study was designed to search the probability for utilisation of all the three layers obtained after lipid extraction (fig 22). The bottom chloroform layer was further transesterified into FAME and was detected through GC-MS analysis. The cell debris was physically activated for the preparation of biochar, and supernatant MeOH layer was analysed by HPLC for detection of saccharides, alcohol, acids, chlorophyll and other components.

Table 16. Nutritional profile (wt%) of raw algae biomass

Nutritional profile (wt%)	
Protein	38.2
Lipid	21.4
Carbohydrate	25.6
Others	14.8

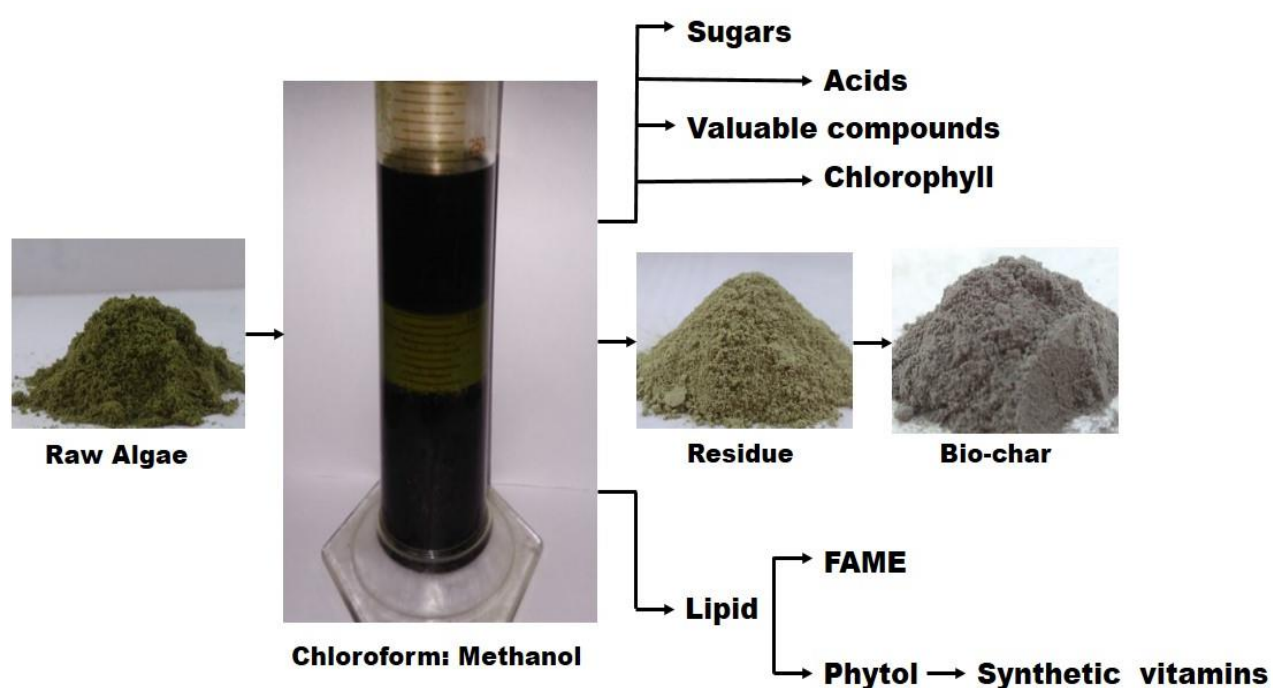

Figure 19. Schematic diagram of expected products by utilizing all the layers during lipid extraction.

7.4 FAME analysis

7.4.1 GC-MS

Conversion of FAME by methoxide for various fractions were detected by GC-MS (Fig 23). The analysis shows the very high yield of Linolenic acid (C18:3), followed by Palmitic acid (C16:0) and Oleic acid (C18:1). The presence of short chain fatty acids is known to be suitable as bio-diesel. The fatty acid profiles also reveal the presence of high microalgae content in the mixture.

Mixed algae culture exists naturally as they survive as symbiotic species relationship. Hence in the present work mixed algae culture were used by the only removal of other bacterial and fungal contaminants. As growth rate and lipid content of microalgae is higher in comparison to macroalgae, therefore, detection of FAME profile is similar to microalgae in the analysis. This type of culturing process also reduces the workload and time duration of axenic culture isolation, preservation, contamination risk and overall economic burden of research and industry.

The presence of phytol was also identified in comparatively high quantity. Phytol is an acyclic diterpene alcohol and could be used for the manufacture of synthetic vitamins such as vitamin E and vitamin K1. It is accumulated in cells as secondary metabolites due to hydrolysis of cellular chlorophyll pigments. Vitamin E is an important nutritional supplement for humans and is obtained commercially by isolation from natural sources. Synthetic vitamin E is typically obtained by reacting trimethylhydroquinone with phytol in the presence of acid catalyst, which is an important source than compared to the natural sources (Huo and Negishi 2001). Research is in progress to isolate phytol, a precursor of vitamin E from various sources for considering it as a dietary source as well as a source of natural products - to be drug candidates.

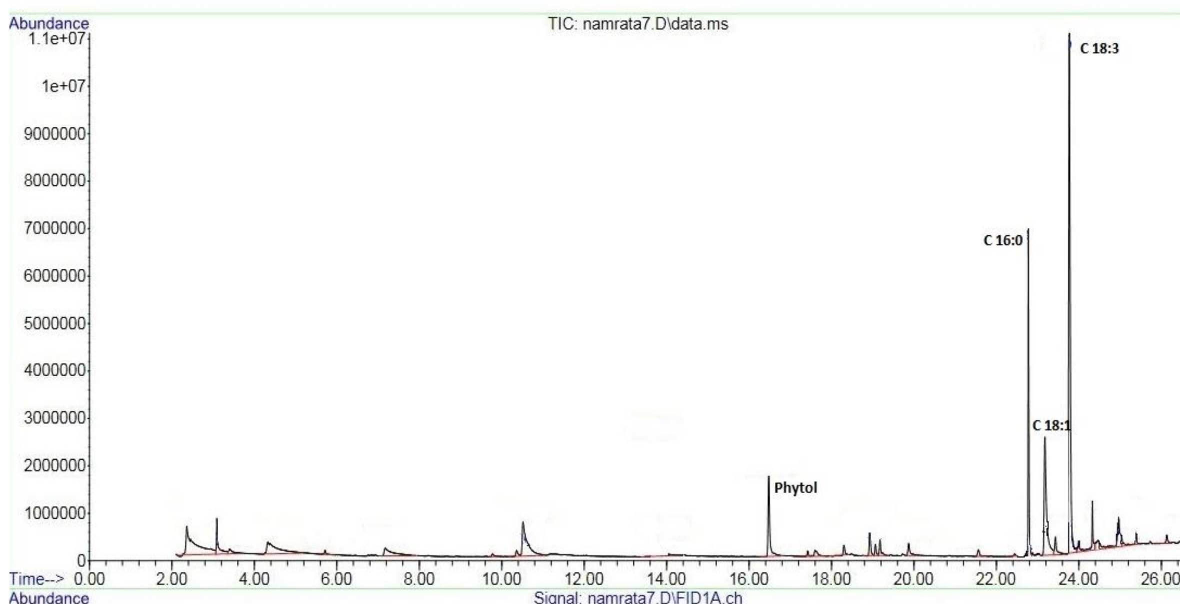

Figure 20. GC–MS chromatogram of transesterified product

7.4.2 FTIR

In FTIR spectroscopy, the functional group of FAME was determined by passing infrared radiation which was absorbed by the entire molecule and the spectrum emitted was computationally recorded. The FTIR spectrum of the liquid product obtained was presented in Fig. 24 and Table 20. The broad, strong band of H–bonded O–H stretch stretching vibration between 3200 and 3600 cm^{-1} of the liquid product indicates the presence of phenol and alcohols.

Figure 21. FTIR analysis of transesterified product

Table 17. FTIR analysis of transesterified product

Absorption peak (cm^{-1})	Type of vibration	Functional group
3365	H–bonded O–H stretch (Broad strong band)	Alcohol
1640	C=C stretch (Medium)	Alkene
1580	C═C, C═O	Aromatic, carboxylates
1380	N─O	Aliphatic nitro compounds
1350	N─O	aromatic nitro compounds
1016	C─O	Ester

Similarly, the 1640 cm^{-1} indicates the presence of Alkene. The bands at 1580 cm^{-1} with C═C or C═O stretching indicates the presence of Aromatic or carboxylates group. On the other hand, 1380 cm^{-1} and 1350 cm^{-1} with N─O stretching show the presence of Aliphatic or Aromatic nitro compounds in the product. Most of the ester compound observed on 1016 cm^{-1}. The results were further consistent when compared with the results of GC–MS.

7.4.3 Detection from MeOH layer

7.4.3.1 Chlorophyll

Chlorophyll pigment is also a worthful bioactive compound produced from plant materials. Chlorophyll compounds could be used as food colouring natural agent and are full of antioxidant and antimutagenic proportion. Within intracellular layers, it is associated with phospholipids, polypeptides and tocopherols embedded with hydrophobic membrane. Due to its medicinal properties, it is widely used in pharmaceutical items. It is known to accelerate wound healing by stimulating tissue growth and inhibiting bacterial contamination in applied area (Oswald and Golueke 1960). Ointments containing chlorophyll relief from pain and also improves clearing affected area after applying (Cady and Morgan 1948). Therefore, it is recommended for the treatment of diseases like ulcers, oral sepsis, proctology, post-operative wounds from rectal surgery, etc. (Horwitz 1951). It has also been reported that it also act as cancer prevention agent by trapping of mutagens in the gastrointestinal tract (Ferruzzi and Blakeslee 2007). The chlorophyll concentration in MeOH layer of the present study was estimated as 2.86 % dry weights respectively.

7.4.3.2 FTIR

Functional groups present in MeOH layer was identified by FTIR analysis. Mainly, four sharp peaks were involved that represents the group from Alcohol, carboxylic acids, nitro compounds and fluoroalkanes. It was also identified from 3373.48 cm^{-1} absorption peak that estimated alcoholic group would be in high concentration. The peak at 1015.86 cm^{-1} indicates the functional group of fluoroalkanes. According to literature, algae are natural sources of organofluoride. These compounds have application in pharmaceuticals but may act as toxins depending upon species and amount of bioaccumulation (Gribble 2002).

Table 18. FTIR analysis for compounds present in MeOH layer

Absorption peak (cm^{-1})	**Type of vibration**	**Functional group**
3373.48	O─H (broad band)	Alcohol
1583.44	C═O	carboxylic acids/derivates
1350.01	N─O	nitro compounds
1015.86	C─X	fluoroalkanes

Figure 22. FTIR analysis of compounds soluble in MeOH layer

7.4.3.3 HPLC

According to literature, sugars might be present in MeOH layer after Folch's lipid extraction method. Therefore, HPLC analysis was performed to know the possible monosaccharides and acid. Standards were matched, the concentration was also calculated from the obtained pick and summarised in Table 22. Mostly saccharides like Stachyose, Maltotriose, Glucose, Fructose, acids like Acetic acid, Butyric acid, and other components like DMSO and Glycerol peaks were detected in MeOH cellular extract. Mentioned saccharides were commonly found sugars in algae. Stachyose is a tetrasaccharide and was also detected in blue-green algae (Liliana, Cardemil and Wolk, Peter 1978).

Melizitose consists of three glucose molecules and were in practice to be extracted from algae. Mild acids are also naturally occurring substances. The unusual DMSO and glycerol peaks were also previously reported in algae. It has also been described that although the reason behind DMSO occurrence is not clear yet often these substances are frequently reported (P. a. Lee, de Mora, and Mora 1999).. Glycerol is the commercial product and currently been extracted from algae (Muscatine 1967). Similar to fatty acid profiles, quantity and quality of saccharides were not consistent due to the presence of different algae species.

Table 19. HPLC analysis of MeOH layer for detection of sugar, acids and other water soluble products

Peak no.	Ret. Time	chemical	Concentration available (%)
1	7.21	Stachyose	0.57

2	7.365	Stachyose	0.28
3	7.83	Maltotriose	1.4
4	9.31	Glucose	1.24
5	10.42	Fructose	0.93
6	12.59	N.A.	N.A.
7	13.64	Glycerol	0.04
8	14.03	Glycerol	0.12
9	15.77	Acetic acid	0.7
10	16.62	N.A.	N.A.
11	19.895	N.A.	N.A.
12	23.115	Butyric acid	0.61
13	24.19	N.A.	N.A.
14	25.11	DMSO	0.0002
15	25.62	DMSO	0.0002

7.4.4 Detection from Solid Fraction

The study includes the comparative analysis of raw algae, residual algae after lipid extraction and algae biochar by including results of proximate and elemental analyses, thermogravimetric analysis, FTIR, X-ray diffraction, SEM and EDX. However, Fig 29 shows the clear visible changes in physical status among these. Table 23 lists the results of proximate and elemental analyses for the raw algae, residual algae after lipid extraction and algae biochar used in the study. The percentage of volatile matter, fixed carbon and ash in raw algae and residual algae do not differ much except for the decrease in moisture content in residual algae. After thermal treatment, it has been reported for the increment of fixed carbon on the contrary to volatile matter which decreases in comparison to feedstock. Due to the escape of volatile matter, the crystalline surface converts to porous morphology. Table 23 of elemental analysis confirms the reduction in hydrogen, nitrogen, sulphur and oxygen content than raw and residual algae biomass. In the absence of oxygen several reactions of dehydration, decomposition and elimination release the volatile matter (Adinata, Wan Daud, and Aroua 2007).

Table 20. Proximate and Elemental analysis of raw, residual and bio-char of algae biomass

Proximate Analysis (wt%)			
Parameters	**Raw Algae biomass**	**Algae residual biomass**	**Algae Bio-char**
Moisture	4.68	3.88	1.65
Volatile matter	78.1	72.32	21.46
Fixed carbon	9.33	9.92	59.88
Ash	12.66	11.32	11.01
Elemental analysis (wt%)			
Carbon	48.10	48.33	63.36
Hydrogen	6.97	6.12	3.56
Nitrogen	10.14	9.12	7.11
Sulphur	0.66	0.61	0.37
Oxygen	34.13	35.82	25.60

7.4.4.1 TGA

The TGA profiles of raw algae, residual algae and algae biochar are shown in Fig. 26. The TGA profile clearly shows the difference in volatile matter, moisture content, fixed carbon and overall weight loss difference among this three status of algae. The first moisture loss in raw algal biomass was maximum up to 100 °C due to the release of physisorbed water. However, this weight loss decreased in residual algae but mostly negligible in biochar. From the TG curve, it has been noticed that the main weight loss of the raw algae biomass starts at around 200 °C. This can be attributed to the decomposition of undegraded cellulose content in the raw algae (Hariz et al. 2015). The highest peak in the TGA curve can be found at about 242 °C, which is due to the decomposition of cellulose. The comparatively less decomposition rate with respect to other agricultural biomass is due to the negligible amount of lignin in algae.

Figure 23. Comparative TGA analysis of raw, residual and bio-char of algae biomass

Lignin content normally does not contribute more to high oil content as the structure of lignin is complex and are not easily degradable. However, in present graphs, it is clearly observed that moisture and volatile content were decreased in both residual and bio-char of algae. Therefore, these volatile materials in relatively higher content were successfully converted into oil fraction. As fixed carbon content was also higher in biochar so utilising biochar as fertiliser would be much clearer decision than raw algae or residual algae. However, after

filtration and proper treatment it could be treated as ruminant food.

7.4.4.2 FTIR

The cellular component of algae involves carbohydrates, proteins, lipids, etc. constituting many functional groups such as hydroxyl, phenolic, aldehydic, ketonic, carboxylic, etc. FTIR spectra obtained will help in investigating the present functional groups and comparative changes on the surface of raw algae, algae residue and algae bio-char. Fig. 27 and Table 24 show the FTIR spectra of raw algae, residual algae and algae bio-char respectively.

On comparison with the spectrum, the disappearance of some bands due to the breaking of the chemical bonds and the change in the chemical nature during the activation process prior and after biochar formation. The major peaks obtained in the spectrum of raw algae were a broad singlet peak at 3374.23 cm^{-1} due to O-H bond stretching vibrations. Other peaks observed in the spectra were 1582.24 cm^{-1} , 1380 cm^{-1}, 1349.82 cm^{-1}, 1260.52 cm^{-1}, 1016.04 cm^{-1}, 794.96 cm^{-1} and several peaks between 633.61-537.72 cm^{-1} . C═O stretching vibration was due to 1582.24 cm^{-1} which represent carboxylic acids. Absorption peaks at 1380 cm^{-1}, 1349.82 cm^{-1}, 1260.52 cm^{-1} belong to alkyl, nitro compounds and methyl functional groups respectively. Aliphatic amines, aromatic functional groups were also observed at 1016.04 cm^{-1} and 794.96 cm^{-1}. Several repetitive peaks observed between 633.61-537.72 cm^{-1} which represent various C─X vibration or chloroalkanes functional group. Accordingly, after lipid extraction, several fractions were removed from the algae, and few peaks were omitted and shifted from the spectrum. The absorption peak at 1640 cm^{-1}, 1040 cm^{-1}, 795.02 cm^{-1} were probably shifted from other groups and belonged to dienes, alcohols and aromatics respectively. But the C─X vibration belongs to chloroalkanes functional group exists in several peaks between 607.29 to 544.58 cm^{-1}. The FTIR analysis of biochar shows various peaks of chloroalkanes between 614.41-537.83 cm^{-1}.

Table 21. Comparative FTIR analysis of raw, residual and bio-char of algae biomass

Absorption peak (cm^{-1})	**Type of vibration**	**Functional group**

Raw algae biomass		
3374.23	alcohols	O—H
1582.24	carboxylic acids	C═O
1380	alkyl	C—H
1349.82	nitro compounds	N—O
1260.52	Alkyl (methyl)	C—H
1016.04	aliphatic amines	C—N
794.96	aromatic	C—H
633.61-537.72	chloroalkanes	C—X
Residual algae biomass		
1640	dienes	C═C
1040	alcohols	C—O
795.02	aromatic	C—H
607.29-544.58	chloroalkanes	C—X
Algae Bio-char		
614.41-537.83	chloroalkanes	C—X

Figure 24. FTIR analysis of A) raw, B) residual and C) bio-char of algae biomass

7.4.4.3 XRD

The X-ray diffraction patterns of raw algae, residual algae and algae biochar are given in Fig. 28. It was observed that, the patterns of raw algae, residual algae and algae bio-char exhibit well-defined peaks (2ϴ) at 28.46, 40.62, 50.26, 58.76, 66.48 and 73.78. Peaks are sharp for all the three cases but based on the intensity it could be concluded that algae residue is having most crystalline structure, followed by algae biochar and raw algae. Further SEM analysis consists the similar results. After lipid extraction, the powdered biomass converted to the tough crystalline structure. Physical activation of that residue created pores in the surface of those crystals hence are have less intense peaks. Whereas raw algae powder is having a bit amorphous structure. Therefore, derived biochar is not much suitable as the adsorbent

Figure 25. XRD pattern of raw, residual and bio-char of algae biomass

7.4.4.4 SEM-EDX

The Fig. 29 clearly provides the idea of the surface changes after lipid extraction and physical activation of algae powder. All the micrographs were taken under 2500 X resolution SEM. The image is consistent with results of XRD. Raw algae biomass was little amorphous in nature but after lipid extraction the elimination of lipid and other components the surface morphology changes to the crystalline phase.

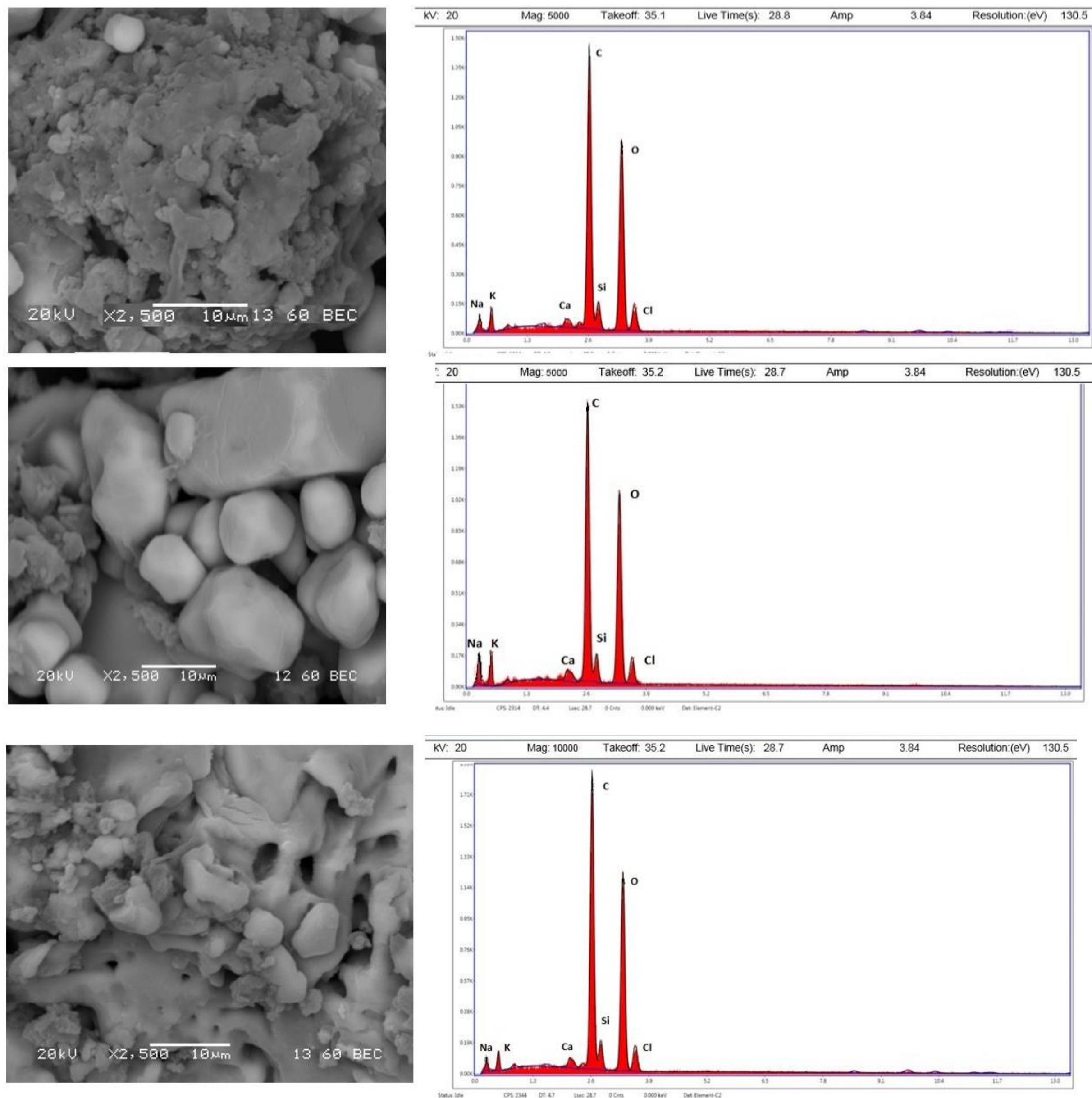

Figure 26. SEM-EDX analysis of raw, residual and bio-char of algae biomass.

Further, volatile matters escape during physical activation of biomass, left pores behind the surface of the biochar (Nsamba et al. 2015). These findings were consistent with BET analysis for surface area measurement where the average surface area was found to be 47.6 m^2/g. Heterogeneous distribution of macropores and a rough texture obtained could be useful in many liquid–solid adsorption processes, provides more adsorption sites for ions, nutrients and water retention. However, algae biochar could also be utilised as fertiliser in farm to improve nutrient supplement in soil. Enclosed EDX analysis reveals the presence of high percentage of chlorine in all three statuses. Other than this silicon, potassium, calcium, sodium were also basic metals present as the non-organic feature of algae. The pH analysis reveals that the char was highly alkaline i.e. 9.4 and therefore could be used to reduce the acidity of the soil. The identified metals could enrich soil if utilised as fertilisers. Algae residue after lipid extraction still contents high amount of nutrients, therefore, are planned by industries to use as animal feed, poultry feed or in aquaculture.

7.4.5 Effect of time as adsorbent

Liquid phase adsorption study using Methylene blue with respect to time was calculated. More than 90% of the dye was absorbed after the interval of one hour. During first ten mins the absorption capacity of char was rapid and has absorbed more than 70% of dye. After that pores of the absorbent may be bounded by the dye, so further absorption was slow.

Figure 27. Effect of contact time on the amount of methylene blue adsorption.

7.5 Conclusion

The following conclusions drawn from the present study report the prospects of using the algae as the source of not only lipid but also various other co-products. The current method of solvent extraction do not utilise the other high-value nutrient products and are wastage of resources. Therefore, the present study focuses on all the possible characterization and utilisation of three layers obtained during Folch's method of lipid extraction. The locally available algae biomass having *Chlorella, Euglena, Oocystis, Anabaena, Pseudomonas* and one unreported species and were also reported in previous chapter 4, 5 and 6. Samples were subjected to grow and extracted with chloroform and MeOH. Bottom chloroform layer consists of lipid was further transesterified for FAME production.

GC-MS results revealed the presence of oleic acid, palmitic acid, linoleic acid and phytol. Phytol is hydrolysis components of the chlorophyll molecule, and precursor of vitamin E and K. Research is in progress to extract phytol from algae for nutraceutical purposes. The MeOH layer was analysed with the help of Hi-Plex Columns in HPLC. Various carbohydrates, Acids and other commercially valuable components were traced. Mainly saccharides like Stachyose, Maltotriose, Glucose, Fructose, acids like Acetic acid, Butyric acid, and other components like DMSO and Glycerol were identified in the significant amount. Therefore, MeOH layer could be further purified and utilise as the source of carbohydrates and other valuable chemicals. The cell debris was further physically activated to utilise it as bio-char.

Comparative characterization of raw algae, residual algae and algae biochar by proximate, elemental, TGA, FTIR, XRD, SEM-EDX were done. The results show that volatile matter depleted after lipid extraction but fixed carbon increases. As cellulosic materials were less in algae in comparison to other agricultural product therefore after lipid extraction the utilisation of residual algae for human consumption will not be a suitable idea. Peaks of FTIR study identified many chloroalkanes repeat in all the three status which was consistent with EDX analysis. EDX shows the presence of high amount of carbon, oxygen along with few inorganic substitutes like chlorine, calcium, etc. SEM and XRD pattern reveal the surface morphology of raw, residue and bio-char of algae. The residual algae are much crystalline in comparison

to other two may be due to the extraction of intracellular components and hence could not be utilised directly as the adsorbent. The further physical treatment creates the pores in crystalline surface hence could be used as the adsorbent. Organic and inorganic enriched biomass could also be utilised for the purpose of fertiliser for agricultural purpose. Further, methylene blue dye adsorption study was also conducted to know the time and capacity of biochar as the adsorbent. Summarising the facts that algae is very valuable biomass and wisely utilisation could provide various value added products for human benefit.

Chapter 8

Conclusion and Recommendation for Future

8.1 Summary and Conclusions

The renewable energy resources are the ultimate future resources to drive the current demand of the population. Although new repositories are still being discovered fossil fuel underneath the earth crust have limited stock. Several alternatives are in progress to satisfy the need, but every research solution has its pros and cons. Solar energy, wind energy, hydro energy are also renewable energy sources but are confined to a limited area according to geographical distribution. On the other hand, biofuel could be derived from the tremendous source and even from waste which will even be added to our environmental polluting reagent. It is liquid fuel and could be easily stored and utilised like Petro fuel. New products are yet added today as biofuel resource, but they are mainly agricultural based.

The main limitation of these resources is competition with food, other human consumables, utilisation of farmland or other infrastructure, seasonally based availability, etc. Therefore, algae were considered as the third generation biofuel product. It is having all advantage of agricultural biofuel resources along with the extra advantage of all the limitation caused by other agricultural resources. Briefly, it can be cultural in any land or even in waste water of industries or other minerals enriched effluent which ultimately pollutes usable water depositories.

It is unlike seasonally based crop and will not be competitive with food or other consumables. As it is organic so after extraction, the residue could be utilised as the fertilizer, animal feed, gasification for surplus energy, etc. Algal species are the most diverse organism and occupy the position in three kingdoms among five kingdom classification system. Therefore, properties are also species specific. But unlike other microbes very rare diseases have been reported from algae. The conversation of this biotics into fuel is still infancy.

Although the idea is novel but still needs research supports and efforts to convert this imagination into real usable items. Each step from species selection, cultivation in large scale, harvesting, extraction procedures, residual benefits needs optimization. Some United States based companies like Saffire, Alginol, etc are claiming for successful and practice bio-diesel production, but the cost is too high in the market at present time. Overall, it is known that the algae as fuel resources would be practically possible, eco-friendly, renewable, but still, some hurdles needs to be sort out.

In the present work, we also tried to derive opinion about the possibilities of algae as bio-diesel resources. We emphasize our work on locally available algal diversities to add the extra potential client in repositories. Day to day new species has been added to our knowledge which could be utilise in future for human benefits. Moreover, the local varieties could survive and thrive well in its environment so the large scale culture could be easy. We also find the similar results as expected. The species were grown successfully throughout the study period without maintenance of temperature and light to the dark ratio in 30-40 litre fabricated reactors. The study hints that the culture cost of maintenance in photobioreactors could be reduced from the budget by usage of appropriate species. However, it has been mentioned elsewhere that Asian climate is best suited for such type of large open culture practice. The samples were collected from air, water and soil from the same locality to compare the availability of entire geographical niche.

Further, a cryopreservation methodology was developed for preservation of these species with higher viability. Few pre-treatment and lipid extraction methodologies were compared to obtained higher lipid fraction from samples. The dried biomass with binary mixture during solvent extraction was the best among other considered treatments hence was followed in next experiments. The further working sets involved air and soil algal biomass. The screening of air samples throughout the year presented the idea of indigenous algal varieties of this locality, appearance or abundance period, environment correlation etc. The results also revealed that algae samples obtained from water or soil samples were also dominated algal species in air. Finally, soil samples were subjected for lipid along with other co-product extraction.

Following are the brief summary of the conclusions made in the respective chapters.

- The introduction chapter 1 includes the basic idea regarding the problem of conventional fuel, present solutions, biofuel aspects, benefits of algae as biofuel resources.

- Literature chapter 2 includes the current market condition, strategies, of algae biofuel in Indian and world's scenario. It also includes the brief review on the previous study of local algal variety utilised as biofuel resources, airborne algae species reported, utilisation of algae for bio-diesel and other co-product production and preservation efforts of these valuable algal resources till date.

- The experimental chapter 3 includes the enlisted material utilised during the study, main procedure details of sample area study, sample collection methodology, isolation, identification, microscopy techniques, culture conditions, lipid extraction, FAME conversion and analytical study details.

- Chapter 4 was based on preservation technique to store these precious species with high viability percentage. The chapter was basically on the development of new method i.e. open encapsulation-vitrification which was compared with conventional closed encapsulation-vitrification technique. The rapid rate of cooling was the advantage of the presented technique. Cell count via haemocytometer, fluorescence dye (FDA) and chlorophyll estimation were conducted using *Oocystis sp* and *Anabaena sp* as model organism. Collectively, the results of the present study reveal that an open system of vitrification is superior to a closed system of vitrification for *Oocystis sp*. and *Anabaena sp*. Variation in bead size postulates the hypothesis that rapid cooling rate influences cell viability. Furthermore, addition of βME (50 mM) or GSH (100 mM) significantly improved the post-warming viability of vitrified *Oocystis* algae. Whereas concentrations of βME (100 mM) or GSH (100 mM) provided the highest viability for *Anabaena* sp. Even after morphological stress, algal cells achieved an exponential growth phase after a lag period. The developed vitrification protocol was successful and superior to existing vitrification methods for variable sized cells of

Oocystis sp. as well as filamentous *Anabaena* sp. and hence, may be applied to a broad range of microalgae/algae.

- Chapter 5 includes the study about to culture local freshwater algal species so that it can thrive in outdoor conditions easily. The observation revealed that *Chlorella sp., Anabaena sp., Euglena sp., Oocystis sp*. and *Sphaerocystis sp*. were dominant species to adapt the environment throughout the large scale culture experiment. Physico-chemical properties of collected water such as temperature, pH, turbidity, TDS, TSS, hardness, EC, DO, BOD,COD, chloride content were also analyzed and comparied with permissible drinking water range. Further, the best feasible lipid extraction methodology was developed by comparison of dry vs. wet algae biomass, cell disruption vs. without disruption and Folch's method vs. conventional soxhlet apparatus has also experimented in this study. Folch's method from dried algal sample was finally selected by repeating the experiment thrice. FAME conversion states that the final product was the mixture of lower carbon chain compounds which are very suitable for biofuel production. Bio-diesel properties were very similar to other plant derived oils. However, algae biomass pre-treatment, crude oil refining, modification in lipid extraction or direct transesterification technologies could improve the quality of bio-diesel and will be economically more feasible.

- Chapter 6 also includes the study about to collect, culture and utilize airborne microalgae throughout the year in the locality of Rourkela. The study is unique in itself as till date only presence of algae in the air was reported but no literature describes its utilization as bio-diesel. The study includes the comparison of month wise data regarding overall weather condition, and its effect on the availability of airborne algae. The climatic condition observation reveals that in rainy season presence of algae in the air were negligible as rainwater cleanse the air naturally and eliminates the present contamination. On the other hand abundance of algae could be found during post monsoon season. The microscopic and elemental examination describes the species level identification and metal content within the cell. The dominant species to adapt the environment throughout the large scale culture experiment were *Scenedesmus sp., Chlorella sp., Pteromonas, Sphaerocystis sp., Oocystis sp., Oedogonium sp*. among

Chlorophyta phyla. Whereas among *Cyanobacterial* origin *Anabaena, Pseudanabaena sp., Gloeocapsa sp., Microcystis sp*. were found. *Naviculoid diatoms*, *Mastogloia elliptica, Striatella sp*., belongs from *Bacillariophyceae* phylum. Species from *Euglenozoa* identified were *Euglena sp*. and *Phacus sp*. Other than this two unidentified species were also observed. Few species were previously reported to be found in the air but others are reported first time in this study may be due to the difference in geographical environment. The CHNS, AAS and Flame photometry analysis were not drastically variable month wise. However, higher carbon percentage among organic matters, higher Mg, K content among macro elements and higher iron content among micro elements were noticed.

In next set of experiments, these cultures were grown and lipid was extracted using modified Folch's protocol. The presence of lipid was examined before extraction by fluoro spectroscopy. The remaining methanol layer were also analyzed, and the presence of saccharides, acids and few other chemicals were also reported. Strains from month of October was found to content highest lipid content. HPLC data revels the presence of maltotriose, arabinose, cellobiose, glucose, xylose, inulin, stachyose along with acids in the significant amount.

Finally, lipid extracted from chloroform was followed by FAME conversion. Characterization of FAME by FTIR, GC-MS and biofuel basic properties were performed to know the suitability of extracted oil as bio-diesel. Common functional group detected were Ester, Alcohol, Alkane, Alkene and carboxylates. Oleic acid (C18:1) and Palmitic acid (C16:0) were constituent overall higher fatty acid fraction within algal samples. Observed saponification value, cloud point were close to Jatropa oil whereas lower HHV and kinematic viscosity in comparison. The report will be beneficial for open large culture system as most often the problem of contamination during culture has been noticed. As the considered airborne algae were also oleaginous in nature hence it favors the large scale open culture system.

- Chapter 7 includes the study of the prospects of using the algae as source of not only lipid but also various other co-products. Present method of solvent extraction do not utilize the other high value nutrient products and are wastage of resources. Therefore, present study focuses on all the possible characterization and utilization of three layer obtained during Folch's method of lipid extraction. The locally available algae biomass obtained by suddenly appeared soil algae which includes *Chlorella, Euglena, Oocystis, Anabaena, Pseudomonas* and one unreported species.

  Further were grown in appropriate conditions and lipid extracted with chloroform and methanol. Bottom chloroform layer consists of lipid was further transesterified for FAME production. GC-MS results revealed the presence of oleic acid, palmitic acid, linoleic acid and phytol. Phytol are hydrolysis components of chlorophyll molecule and precursor of vitamin E and K. Research reports the progress to extract phytol from algae for nutraceutical purposes. The methanol layer was analysed with help of Hi-Plex Columns in HPLC. Various carbohydrates, acids and other commercially valuable components were traced. Mainly saccarides like Stachyose, Maltotriose, Glucose, Fructose, acids like Acetic acid, Butyric acid, and other components like DMSO and Glycerol were identified in significant amount.

  Therefore, methanol layer could be further purified and utilize as source of carbohydrates and other valuable chemicals. The cell debris were further physically activated to utilize it as bio-char. Comparative characterization of raw algae, residual algae and algae bio-char by proximate, elemental, TGA, FTIR, XRD, SEM-EDX were done. The results shows that volatile matter depleted after lipid extraction but fixed carbon increases. Peaks of FTIR study identified many chloroalkanes repeat in all the three status which was consistent with EDX analysis. EDX shows the presence of high amount of carbon, oxygen along with few inorganic substitutes like chlorine, calcium etc. SEM and XRD pattern revels the surface morphology of raw, residue and bio-char of algae.

  The residual algae is much crystalline in comparison to other two may be due to extraction of intracellular components and hence could not be utilize directly as

adsorbent. Further physical treatment creates the pores in crystalline surface hence could be used as adsorbent. As algae biomass is enriched source of organic and inorganic compound hence could also be utilized as purpose of fertilizer in agricultural purpose. Further, methylene blue dye adsorption study was also conducted to know the time and capacity of bio-char as adsorbent. Summarizing the facts that algae is very valuable biomass and wisely utilization could provide various value added products for human benefit.

- Chapter 8 includes the major summary, conclusion, discussion of the presented study and also future recommendations.

8.2 Recommendations for future

- Viabilities of cyanobacteria, filamentous or macroalgae are not much higher with current cryopreservative techniques so much focus would improve the algae depositories.
- Variation in bead size also reflects the results and much work has not been reported in literature so further work may improve the cryopreservation techniques.
- Residual biochar obtained in the study was not much porous hence should also be chemically treated before production.
- Simple genetic manipulation technique like random mutagenesis or targeted gene knock out in algal culture can further improve the strain.
- Co-product such as chlorophyll, pytol, saccharides extracted in this study can be further isolated and purified.

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Dissemination

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- **Namrata Kumari**, Raghubansh Kumar Singh. Bio-diesel production from airborne algae. Communicated in Algal Research, Elsevier Publication.
- **Namrata Kumari**, Raghubansh Kumar Singh. Bio-diesel and co-product production from soil algal biomass. Communicated in Energy Conversion and Management, Elsevier Publication

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- Tanmya Rout, Debalaxmi Pradhan, R.K. Singh, **Namrata Kumari**. Exhaustive study of products obtained from coconut shell pyrolysis. Journal of Environmental Chemical Engineering (2016) 4:3696–3705
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Conferences and Workshop

- **Namrata Kumari**, R.K. Singh, Contribution of Genetics to decipher Algae biomass in to Energy. "International conference on Frontiers in Chemical Engineering (ICFCE-2013)" 9-11 Dec 2013, NIT, Rourkela
- **Namrata Kumari**, Rajesh Kumar, Raghubansh Kumar Singh, Copper removal from mixed algae culture. "Sustainable technology for cleaner environment (STCE-2016)"13 -14 Feb 2016. The Institution of Engineers (India) Rourkela Local Centre, NIT Campus, Rourkela.
- Participated in five days short term courses on "Distributed Multimode Renewable Energy Systems (DMRES-2016)" 11th – 15th April, 2016. Department of Mechanical Engineering, NIT, Rourkela

Biography

Namrata Kumari is PhD research scholar in Department of Chemical Engineering, National Institute of Technology (NIT), Rourkela from October 2012. During doctoral degree she published research paper in the peer-reviewed international scientific journals, attended national and international conferences, workshop and served as teaching assistantship. Her area of interest was utilization of valuable algae resources for fuel production. She is also member of Society for Cryobiology. She obtained her M. Tech. degree in Biotechnology from Uttarakhand Technical University with 69% marks. During her master's programme she got opportunity to work in Centre for Cellular and Molecular Biology (CCMB), Hyderabad where she also worked hard along with team for peer reviewed international journal. Before that she also obtained B.Tech. Degree in Biotechnology from IASE University with 80% marks. She was born and brought up at Bokaro (Jharkhand) and completed her schooling from Jawahar Navodaya Vidyalaya, Bokaro.