# Screening of microalgae for lipids and their extraction and characterization

A

DISSERTATION REPORT

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

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# CERTIFICATE

This is to certify that the thesis entitled "**Screening Of microalgae for Lipids and their Extraction and Characterisation**" submitted by Himangi Agarwal in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her under his supervisor. The report has not been submitted for the award of any other degree or certificate in this or any other University or institute.

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# CANDIDATE'S DECLARATION

I hereby declare that the work presented in the dissertation entitled **"Screening of microalgae for lipids and their extraction and characterization"** in partial fulfillment of the requirement for the award of the degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab, is an authentic record of my own work during the period of six months from Jan 2012 to June 2012, under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology and Environmental Science, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other University.

Date: 16.07.2012

Place: Patiala

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Place: Patiala Himangi Agarwal

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# LIST OF ABBREVIATIONS

BGA - Blue green algae
DW - Distilled water
gm - gram
kg - kilogram
$K_2HPO_4$ - Dipotassium hydrogen phosphate
L / Ltr - Litre
mg - milligram
$MgSO_4$ - Magnesium Sulphate
ml - millilitre
µg - microgram
(–N) - without nitrogen
(+N) - with nitrogen
NaCl - Sodium chloride
w/v - weight by volume
HCl - Hydrochloric acid

# ABSTRACT

Nine microalgae (*Nostoc muscorum*, *Spirulina.sp, Oscillatoria* R2, *Chlorella* Cp4, *Chlorella* R5, *Nannochloropsis oculata*, *Botrycoccus braunii*, *Scendesmus armatus*, *Chlamydomonas moewsii*) were screened for containing lipid content. Overall it was observed showed that all the algal cultures showed increase in biomass and lipid content with increase in time interval of 7 days respectively. *Nannochloropsis oculata* showed highest lipid content from 0.32 mg/ml on 7th day to 0.42 mg/ml on 28th day with increase in wet biomass from 0.26 mg/ml on 7th day to 0.46 mg/ml on 28th day and increase of dry biomass from 0.04 mg/ml on 7th day to 0.13 mg/ml on 28th day in lab conditions respectively. Variation was found when *Nannochloropsis oculata* was grown in open condition, where it showed increase in lipid content from 0.32 mg/ml on 7th day to 0.42 mg/ml on 28th day with the increase in wet biomass from 0.26 mg/ml on 7th day to 0.46 mg/ml on 28th day and dry biomass from 0.04 mg/ml on 7th day to 0.08 mg/ml on 28thday respectively. Chlorophyll content was found to be highest in *Spirulina.sp* (2.6 µg/ml). Increase in lipid content by *Spirulina.sp* and *Nostoc muscorum* in open tubs as well as lab conditions was slow. Microalgal were disrupted by homogenization and ultrasonication to release lipids . Maximum lipids were released when cells were homogenized and further ultrasonicated.

Four microalgae algae (*Nannochloropsis oculata, Nostoc muscorum, Spirulina.sp, Oscilltoria* R2) that were found to contain high lipid content and were selected for lipid extraction and characterization by HPLC. HPLC characterization indicated that in lipid of all the four microalgae contained fatty acid fraction similar to that are present in olive oil. *In situ* tranesterification was used for making biodiesel. *Nannochloropsis oculata* appeared to be potential source for biodiesel production followed by *Oscillatoria R2* as both contains high lipid content and grows rapidly in lab as well as open conditions.

# Chapter 1

# INTRODUCTION

Microalgae are sunlight driven factories that converts carbon dioxide to potential biofuels, foods and high value bioactives. Increase in the escalating price of petroleum and concern about global warming associated with burning of fossil fuels has necessitated to look for biofuels as an emerging source of energy (Gavrilescu and Chisti, 2005). Microalgae are considered to be a good source of biofuel (Sawayama *et al*., 1995). It has been reported that microalgae double their biomass within twenty four hours and oil content in microalgae significantly exceed 80% of dry biomass (Metting, 1996; Spolaore *et al*., 2006). Technical challenges have been reported for the cultivation of high oil content algae. Large scale production of algae can be done either in open ponds or closed photobioreactors. The most economical culturing set-up would be an open system, however these systems are not feasibile for culturing high oil content algae due to invasive species and temperature fluctuations. The closed system is although more costly but controlled conditions can optimize oil yields. The microlagal biomass generated in closed bioreactors can be gasified or pyrolysed to produce a range of biofuels and agri-char as a part of carbon dioxide sequesteration strategy. Algae stores energy in the form of lipid in controlled enironment such as nutrient starvation and carbon uptake. Overall optimization of oil yield is a limiting factor (Chisti *et al*., 2007).

The 'Economics of Climate Change' and the Intergovernmental Panel on the Climate Change 'AR4 Synthesis Report' have reported that development of carbon dioxide fuel is one of the most urgent challenges facing our society (Stern report, 2006; IPCC, 2007). The major problem with current first generation biofuel systems is as production capacities increases, their competition with agriculture for arable land used for food production also increases. A range of second generation biofuel-production systems are more water efficient and require much less arable land. Lignocellulosic technologies and microalgae are of particular interest (Chisti, 2008; Hankamer *et al*., 2007; Schwab *et al*., 2007). Microlalgae provides several different types of renewable biofuel which include methane produced by anaerobic digestion of the algal biomass (Spolaore *et al*., 2006), biodiesel from microalgal oil (Roessler *et al*., 1994; Banerjee *et al*., 2002; Gaverilescu and Chisti, 2005) and photobiologically produced biohydrogen (Ghiradi *et al*., 2000; Kapdan and Kargi, 2006). Microalgae are known to have lower impact on the environment and the world's food supply, the conventional biofuel producing crops. Biodiesel, an alternative diesel fuel, is made from renewable biological sources such as vegetable oils and animal fats. It is biodegradable and nontoxic, has low emission profiles and so is environmentally beneficial (Krawczyk, 1996). Biodiesel has many benefits over petroleum diesel. Not only is it renewable, but it is a domestic resource, and therefore lends itself towards American economic security. If biodiesel is produced from photosynthetic sources, the effect of carbon dioxide produced from biodiesel combustion is minimal on the greenhouse gas (GHG) effect (Agarwal and Das 2001).It is a biodegradable and nontoxic substance (Zhang, Dube et al. 2003) Continued and increasing use of petroleum will intensify local air pollution and magnify the global warming problems caused by carbon dioxide (Shay, 1993), in such a case biodiesel fuel has the potential to reduce the level of pollutants and the level of potential carcinogens (Krawczyk, 1996). Oil from algae, bacteria and fungi also have been investigated. (Shay, 1993). Microalgae have been examined as a source of methyl ester diesel fuel (Nagel and Lemke, 1990). Biodiesel is produced currently from plant and animal oils, but not from microalgae. This is likely to change as several companies are attempting to commercialize microalgal biodiesel (Knothe et al., 1997; Fukuda et al., 2001; Barnwal and Sharma, 2005; Demirbas, 2005; Van Gerpen, 2005; Felizardo et al., 2006; Kulkarni and Dalai, 2006; Meher et al., 2006).

Technology involved in biodiesel production from microalgae can be made economically viable if harvesting and drying the microalgal biomass can be made cost effective. Another challange in biodiesel production is lipid extraction from algal biomass on large scale as present solvent based extraction does not support large scale biodiesel production due to high cost. Engineering aspects at biochemical and genetic level can be considered as a potent strategy for biodiesel production (Courchesne *et al*., 2009). Genetic and metabolic engineering in microalgae for biodiesel production is a blooming strategy where introduction and expression of genes encoding rate limiting enzyme of lipid biosynthetic pathway are concerned (Armbrust *et al*., 2004; Derelle *et al*., 2006; Bowler *et al*., 2008; Beer *et al*., 2009).

Global atmospheric carbon dioxide increase and depletion of mineral oil reserves require the rapid development of carbon neutral renewable alternatives. Biggest challenge over the next few years in the biodiesel production from microalgal oil will be to isolate and screen highest lipid containing microalgae and their large scale mass multiplication. Therefore the present investigation was aimed at screening of microalgae with higher lipid content so as to get some channels for the replacement of petroleum oil with the biodiesel.

The main objectives of the present are :

1. Screening of different microalgae for lipid content
2. Mass cultivation of microalgae with highest lipid content for extraction of lipids
3. Transesterification of algal oil using alkali

# Chapter 2

# REVIEW OF LITERATURE

## 2.1 Microalgae as a second generation feedstock

Microalgae can provide several types of renewable biofuels that are composed mainly of carbohydrates, proteins and lipids. Algae having the ability to synthesize triacylglycerides are considered as a second generation feedstock for biofuels production (Khan *et al*., 2009).

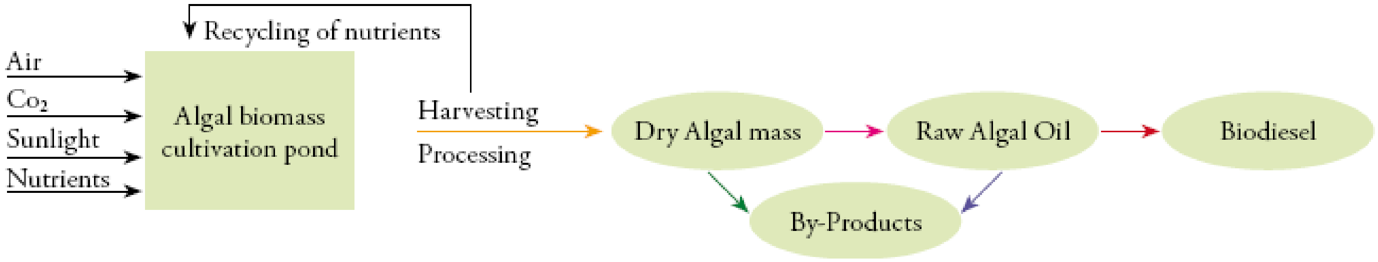
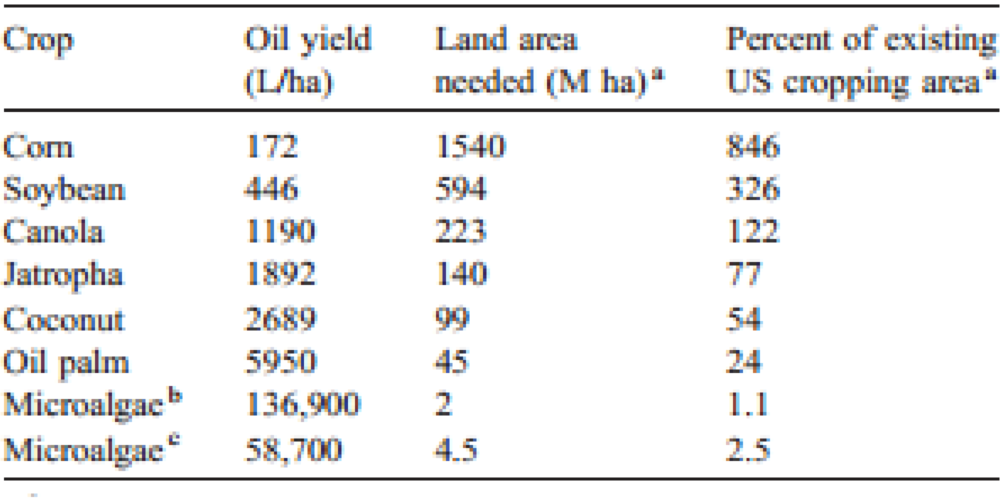


**Figure 1: Different stages and process in algal biomass production for biodiesel**

The lipid content of algal oil can be processed into biodiesel, carbohydrates into ethanol and proteins into animal feed or human nutritional supplements. Also, by anaerobic digestion of the algal biomass they can provide biogas and fertilizers (Rosenberg *et al*., 2008). An important algal characteristic for biodiesel production is the suitability of lipids in terms of type, chain length, degree of saturation and proportion of total lipid made up by triglycerides. The most extensive research into the development of biofuels from algae was performed by the National Renewable Energy Laboratory (NREL) from 1978 to 1996. Shay *et al*.,1993 reported more than 3000 species of algae under the program known as the Aquatic Species Program (ASP) whi9ch focused on the production of biodiesel from high lipid content algae grown in ponds and utilizing waste $CO_2$ from coal fired power plants. Studies have revealed that microalgae sequester $CO_2$ from flue gasses emitted from fossil fuel power plants and other sources, reducing emissions of a major greenhouse gas, around 1 kg of algal biomass requiring about 1.8 kg of $CO_2$ (Khan *et al*., 2009). Algae are reported as the only viable source to have a significant impact on diverting demand away from petroleum and soil crops demand inputs for cropping area include irrigation, planting, fertilization, and harvesting which can be greatly minimized with an aquatic crop, algae, with a well-designed system (Vasudevan and Briggs, 2008).

## 2.2 Potential of microalgae as source of biodiesel

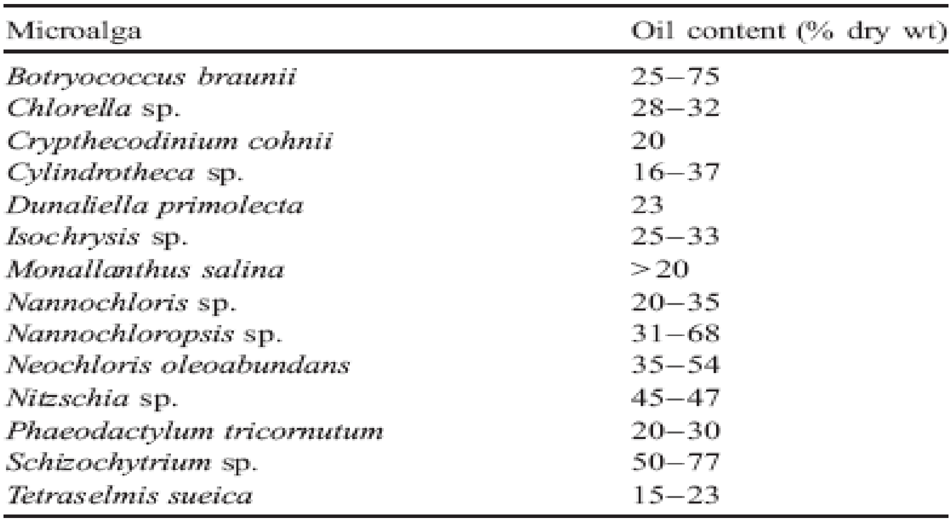
Reports have shown that biodiesel is produced currently from plant and animal oils, but not from microalgae but biodiesel production from microalgae is a proven fuel and technology related to it is known for years (Knothe *et al*., 1997; Fukuda *et al*., 2001; Barnwal and Sharma, 2005; Demirbas, 2005; Van Gerpen, 2005; Felizardo *et al*., 2006; Kulkarni and Dalai, 2006; Meher *et al*., 2006). Surveys have told that replacing all the transport fuel consumed with biodiesel will require 0.53 billion $m^3$ of biodiesel annually at the current rate of consumption which can be possible only if microalgae are used as source for biodiesel production (Chisti, 2007).



| Crop | Oil yield (L/ha) | Land area needed (M ha)[a] | Percent of existing US cropping area[a] |
|---|---|---|---|
| Corn | 172 | 1540 | 846 |
| Soybean | 446 | 594 | 326 |
| Canola | 1190 | 223 | 122 |
| Jatropha | 1892 | 140 | 77 |
| Coconut | 2689 | 99 | 54 |
| Oil palm | 5950 | 45 | 24 |
| Microalgae[b] | 136,900 | 2 | 1.1 |
| Microalgae[c] | 58,700 | 4.5 | 2.5 |

**Table 1: Comparison of some sources of biodiesel (Chisti, 2007)**

Microalgae are reported to be the only source of biodiesel that has the potential to completely replace fossil diesel as it has been shown that microalgae grow extremely rapidly and many of them are exceedingly rich in oil. Metting (1996) and Spolaore *et al.,* (2006) have proven that microalgae commonly double their biomass within 24 hr whereas oil content in microalgae with oil levels 20-50% can exceed 80% by weight of dry biomass.



| Microalga | Oil content (% dry wt) |
|---|---|
| *Botryococcus braunii* | 25–75 |
| *Chlorella* sp. | 28–32 |
| *Crypthecodinium cohnii* | 20 |
| *Cylindrotheca* sp. | 16–37 |
| *Dunaliella primolecta* | 23 |
| *Isochrysis* sp. | 25–33 |
| *Monallanthus salina* | >20 |
| *Nannochloris* sp. | 20–35 |
| *Nannochloropsis* sp. | 31–68 |
| *Neochloris oleoabundans* | 35–54 |
| *Nitzschia* sp. | 45–47 |
| *Phaeodactylum tricornutum* | 20–30 |
| *Schizochytrium* sp. | 50–77 |
| *Tetraselmis sueica* | 15–23 |

**Table 2: Oil content of some algae (Chisti 2007; Gouveia and Oliveira 2009)**

Ratledge (1993) and Ratledge &Wynn (2002 ) have stated that method required for production of oil rich algal biomass are inexpensive. It has been studied that genetic and metabolic engineering are likely to have the greatest impact on improving the economics of production of microalgal diesel (Roessler *et al.*, 1994; Dunahay *et al.*, 1996). According to Leon- Banares *et al.*, 2004 commercial application of genetically manipulated algae has been reported but limitation in this field is mainly due to the inability to silence foreign genes causing instability in the gene expression.

## 3.3 Large scale production of algae

Mass production of microalgae is much more costly than oil crops. Essential elements that should be added in the growth medium of microalgae include nitrogen (N), phosphorus (P), iron and in some cases silicon (Chisti Y and Moo-Young, 2009). Various formula for minimal nutritional requirement for growing algae are given by Grobbelaar, 2004. Molina *et al.*, 1999 have used water supplemented with nitrogen and phosphate fertilizers for growing sea water algae. Reports have told that microalgal biomass contains approximately 50% carbon by dry weight, this carbon is derived mainly from carbon dioxide (Sanchez *et a*l., 2003) from which some of the carbon dioxide can be potentially be used for the production of the biofuels (Sawayama *et a*l., 1995; Yun *et al.*, 1997). The temperature should be between 15 and 30°C (~60-80 °F) for optimal growth (Hu *et al.*, 2007). According to Molina *et al.*, 1999 use of continuous culture during daylight is generally used in large scale production of algae, in which fresh culture is fed constantly and microalgal culture is withdrawn at same rate. For large-scale production of microalgae, algal cells are continuously mixed to prevent the algal biomass from settling (Molina, Fernandez *et al.*, 2000). Two common practical method for large scale production of microalgal biomass are raceaway ponds (Terry and Raymond, 1985; Molina , 1999) and the closed photobioreactors (Molina *et al.*, 2003; Tredici, 1999; Sanchez *et al.*, 1999).

## 3.3.1 Raceway ponds

The largest raceway-based biomass production facility as reported occupies an area of 440,000 m$^2$ (Spolaore *et al.*, 2006). Raceways are perceived to be less expensive than photobioreactors, because they cost less to build and operate but the disadvantage of raceway ponds is that the biomass concentration remains low due to contamination with unwanted algae and loss of carbon dioxide to the atmosphere they are even poorly mixed and aerated (Terry and Raymond, 1985).

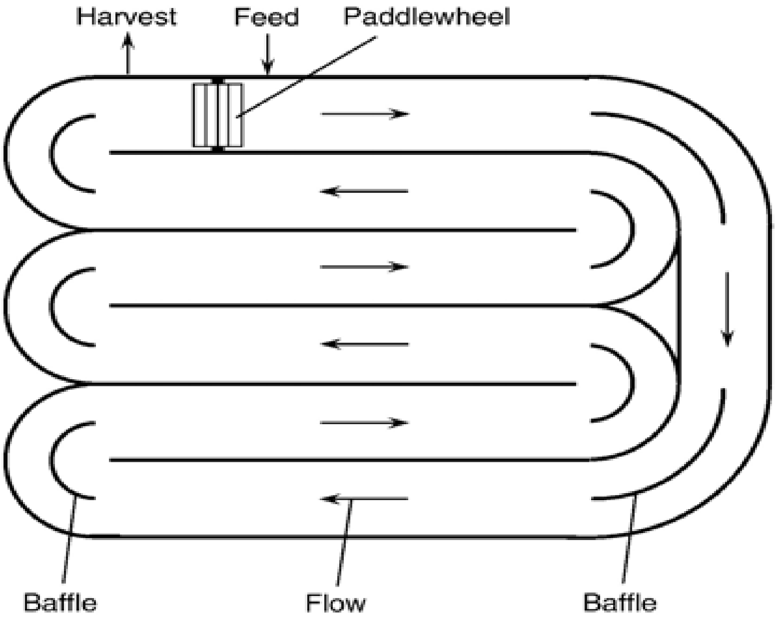


**Figure 2: Arial view of raceway ponds (Chisti Y, 2007)**

## 3.3.2 Photobioreactors

Successful use of photobioreactors have been reported for mass production of microalgae (Molina *et al.*, 1999; Tredici, 1999; Pulz, 2001; Carvalho *et al.,* 2006). In the photobioreactors solar collectors are organized in a manner to maximize the sunlight capture (Molina *et al.*, 2001; Sanchez *et al.*, 1999). Many variants of photobioreactors are there but are not commonly used due to high cost (Molina *et al.*, 1999;

Tredici, 1999; Pulz, 2001; Carvalho *et al.*, 2006). Additional carbon dioxide injection points may be necessary at intervals along the tubes, to prevent carbon limitation and an excessive rise in pH (Molina *et al.*, 1999).

## 3.4 Biomass estimation of microalgae

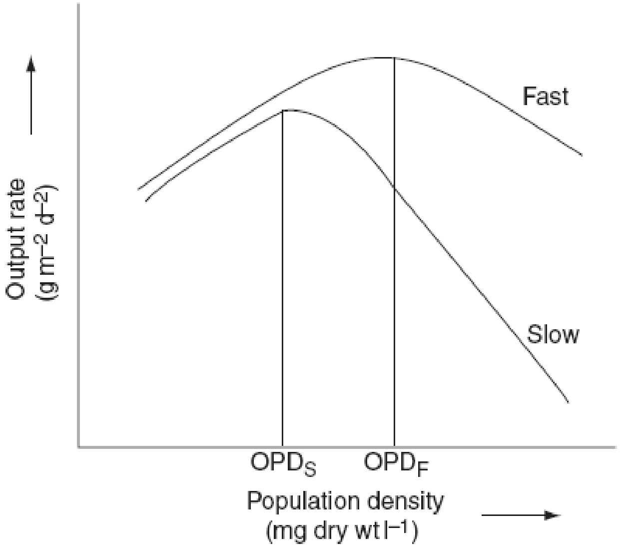


**Figure 3: The effect of stirring rate on culture productivity in relation to the population density (Richmond and Grobbelaar, 1986)**

Richmond & Grobbelaar, 1986 estimated the biomass of *Spirulina* in open conditions and showed that a relatively slow stirring rate in an open raceway, affecting a flow rate considerably less than 30 cm sec$^{-1}$, plays havoc on the output rate of cell mass in Spirulina cultures as the population density increases far above optimal, a situation which may readily occur on large-scale. Hu *et al.*, 1996 reported the basic principle of the graph that higher the intensity of the light source, the higher (potentially) becomes the optimal population density and the more significant the degree to which the extent of mixing may exert on the output rate of cell mass of spirulina culture.

## 3.5 Lipid extraction from microalgae

Various method have been developed for the lipid extraction from the microalgae. Use of diethyl ether as an extraction solvent with ultrasoniction, which prevents oxidative modification of lipids has been reported by Mecozzi *et al.*, 2002. Pernet and trembley have suggested grinding followed by ultrasonication for complete extraction of lipid from microalgae. Application of ultrasonication and microwaves during lipid extraction enhances the yield of lipid (Cravatto *et al.*, 2008; Virot *et al.*, 2008). Various methods such as autoclaving, bead beating, sonication and 10% NaCl solution extractant for lipid extraction have been compared by Lee *et al*., 2010. Lipids are basically located in the cytoplasm of algal cells in the form of droplets, therefore two mechanism for lipid extraction could be diffusion through cell wall or disruption of the cell wall (Silverberg *et al*., 1976). The commonly applied method suggested by Bligh and Dyer, 1959 involves dissolving microbial cell suspension in water with a mixture of chloroform and methanol which leads to extraction as well as partitioning of lipid. Lipid extraction from microalgae using n-hexane as suitable solvent has been reported by Soxhlet *et al*., 1879.

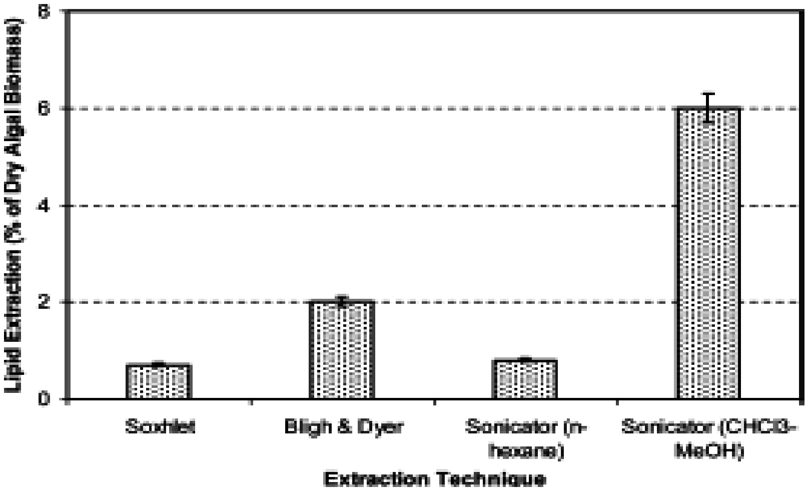


**Figure 4: Extent of lipid extraction with different techniques (Ranjan *et al*., 2010)**

Lipid extraction by ultrasonication of microbial cells followed by Bligh and Dyer method has been reported by Ranjan *et al*, 2010. Vimalarasan *et al*, 2010, reported efficient oil extraction from *O. annae* by ultrasonication in combination with organic solvents (50.9%) than homogenization (40.4%). Ranjan *et al*., 2010 performed lipid characterization by high performance liquid chromatography (HPLC) using olive oil as standard.

## 3.6 Transesterification

Transesterification requires 3mol of alcohol for each mole of triglyceride to produce 1 mol of glycerol and 3mol of ethyl esters. The reaction is equilibrium. Industrial processes use 6mol of methanol for each mole of triglyceride (Fukuda *et al*., 2001). Transesterification is catalyzed by acids, alkalis (Fukuda *et al*., 2001; Meher *et al*., 2006) and lipase enzymes (Sharma *et al*., 2001). Use of lipases offers important advantages, but is not currently feasible because of the relatively high cost of the catalyst (Fukuda *et al*., 2001).

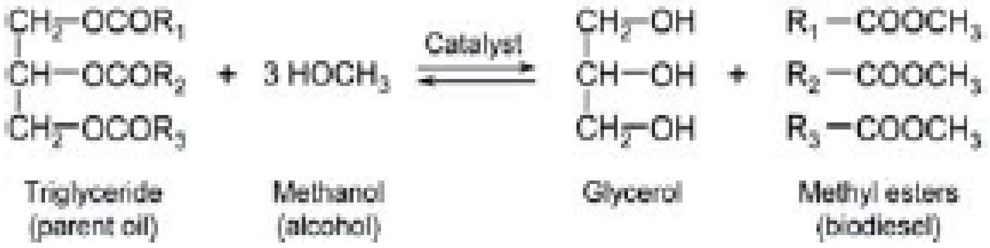


**Figure 5: Transesterification of oil to biodiesel** (Chisti Y, 2007)

Ruoyu Xu and Yongli Mi, 2010 studied *in situ* transesterification in *Spirulina* which involved combining oil extraction and transesterification in one step. Haas *et al*., 1998 studied sodium hydroxide catalyzed insitu tranesterfication. Boocock *et al.*, 1998 demonstrated use of tetrahydrofuran or methyl tertiary butyl ester in speeding up single step reaction. Ehimen *et al.*, 2009 studied variables that affected sulphuric acid catalyzed *in situ* transesterification of lipids of *Chlorella*.

## 3.7 Challenges and prospects in biodiesel production

The major challenge in biodiesel production from microalgae is its mass production which is very costly (Chisti Y, 2007). A major decision to be made is whether to use closed photobioreactors or open ponds (Borowitzka , 1999; Lehr and Posten , 2009). The second challenge is in lipid extraction which is due to difficulty in releasing of lipids from algal intracellular location in most energy-efficient and economical way possible and avoiding the use of large amounts of solvent such as hexane, and utilizing as much of the carbon in the biomass as liquid biofuel, potentially with the recovery of minor high-value products (Stuart A *et al*., 2010). Economics of producing microalgal biodiesel need to improve substantially to make it competitive with petrodiesel, but the level of necessary improvement appears to be attainable (Chisti Y, 2007).

# Chapter 3

# Material and Methods

## 3.1 Culture methods

Different microalgal cultures *Spirulina.sp*, *Nostoc muscorum* (ARM442) were procured from National Centre for Conservation and Utilisation of Blue Green Algae, 22, Division of Microbiology, IARI, New Delhi. The cyanobacterial cultures were maintained and grown routinely in batch culture in BG-11 media (Stanier *et al.*, 1971). *Spirulina.sp* was grown in CFTRI media. Chlorella cultures (*Nannochloropsis oculata*, *Botryococcus Braunii*, *Scendesmus armatus*, *Chlamydomonas moewsii*) were grown in Fogg's media (Fogg, 1949). Waste water isolates *Oscillatoria* R2, *Chlorella* R5, *Chlorella* Cp4 were grown in BG-11+N media. For maximal growth of microalgae controlled environmental conditions are necessary. The cultures were maintained at light intensity (approx. 3200 lux), temperature 27±1ºC. Light intensity, temperature and ph were selected as parameters to be manipulated to yield a combination that promoted the maximum growth of microalgae. All the growth media were prepared in double distilled water and were sterilized in autoclave at 121ºC at 15psi (1.06 $kg/m^2$ pressure) for 20 minutes. The glasswares were sterilized in hot air oven at 180ºC for 1-2 hours.

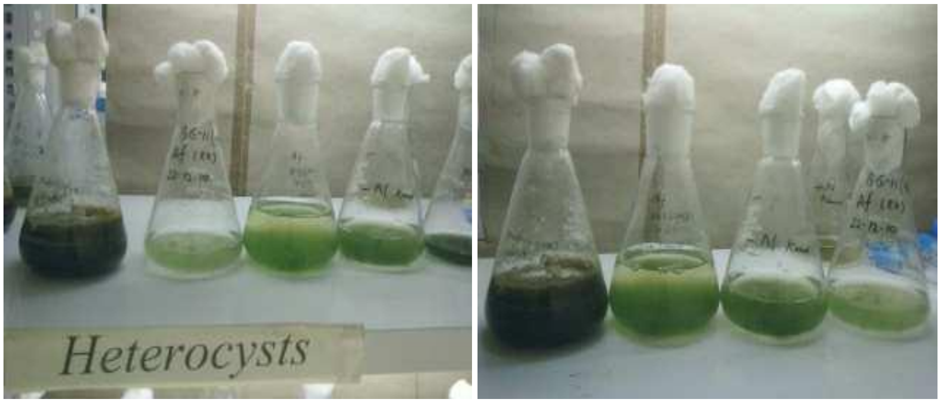


**Figure 6 : Algal cultures in racks**

**Composition of CFTRI media**

| CONSISTUENTS | gram/L |
|---|---|
| 1. Sodium bicarbonate | 4.5 |
| 2. Di-Potassium hydrogen phosphate | 0.5 |
| 3. Sodium nitrate | 1.5 |
| 4. Potassium sulphate | 1.0 |
| 5. Sodium chloride | 1.0 |
| 6. Magnesium sulphate | 1.2 |
| 7. Calcium chloride | 0.4 |
| 8. Ferrous sulphate | 0.01 |
| 9. A5 Micronutrient solution | 1.0ml |

**A5 Micronutrient solution composition**

| CONSISTUENTS | gram/L |
|---|---|
| 1. Boric acid | 2.86 |
| 2. Manganese chloride | 1.81 |
| 3. Zinc sulphate | 0.222 |
| 4. Sodium molybdate | 0.017 |
| 5. Copper sulphate | 0.079 |

**Composition of BG11+N**

| CONSISTUENTS | gram/L |
|---|---|
| 1. Sodium Nitrate | 1.5 |
| 2. Di sodium hydrogen phosphate | 0.04 |
| 3. Calcium chloride | 0.036 |
| 4. Citric acid | 0.006 |
| 5. Ferric ammonium citrate | 0.006 |
| 6. EDTA (di sodium magnesium salt) | 0.001 |
| 7. Sodium carbonate | 0.02 |
| 8. Trace metal mix | 1.0 ml |

## Trace metal mix composition

The trace metal mix contain the following constituents in gram/L

| CONSISTUENTS | gram/L |
|---|---|
| 1. Boric acid | 2.86 |
| 2. Manganese chloride | 1.81 |
| 3. Zinc sulphate | 0.222 |
| 4. Sodium molybdate | 0.039 |
| 5. Copper sulphate | 0.079 |
| 6. Cobalt nitrate | 0.0492 |

BG11+N and BG11-N media was used for growing non-heterocystous and heterocystous algal strains respectively. The growth temperature was maintained from 28ºC-37ºC and pH was maintained at 7-7.27. Composition of BG11+N media. In case of heterocystous algae, BG11-N medium was prepared which did not contain sodium nitrate salt.

## Composition of Fogg’s media

| **CONSISTUENTS** | **gram/L** |
|---|---|
| **1**. Pottasium dihydrogen phosphate | 0.2 |
| **2.** Magnesium sulphate heptahydrate | 0.2 |
| **3**. Calcium chloride dehydrate | 0.1 |
| **4.** A5 micronutrient solution | 1ml |
| **5.** Fe-EDTA | 1ml |

Fogg’s media was used for growing chlorella cultures. The temperature was maintained at 28ºC -37ºC and the pH was maintained in the range of 7-7.27. Composition of Fogg’s media is:

## Fe-EDTA solution composition

| **CONSISTUENTS** | gram/L |
|---|---|
| **1.** EDTA | 26.1 |
| **2**. 1N Pottasium hydroxide | 268ml |
| **3.** Ferrous sulphate heptahydrate | 24.9 |

The Fe-EDTA solution was made by dissolving all the components by stirring continuously and the solution was aerated overnight (around 16-18hrs).

## 3.2 Growth studies

### 3.2.1 Dry biomass estimation of microalgae (Richmond and Gobbelaar, 1986)

**1.** The whatmann filter was soaked in water to saturate and dried overnight.
**2.** The weight of the dried filter paper was noted as the initial reading.
**3.** The cultures were homogenized by vigorous shaking and 10ml of aliquot of culture was taken and filtered through previously dried paper.

**4.** This is again left for drying and transferred to hot air oven maintained at about 60ºC, till constant weight was recorded at room.

**5.** The difference between initial and final reading of the weight gave the dry biomass in mg/10ml.

### 3.2.2 Chlorophyll estimation (Mckiney, 1941)

**1.** 10ml of algal suspension was filtered through whatmann filter paper no. 42 and washed with double distilled water.

**2.** Algal biomass along with filter paper is transferred to Oakridge centrifuge tubes and the level of methanol was marked on the Oakridge centrifuge tubes.

**3.** The Oakridge centrifuge tubes were tightly capped, vigorously shaken and kept in water bath at 60ºC for 30min, which led to the extraction of chlorophyll into the solution.

**4.** Samples were removed from the water bath and allowed to cool at room temperature, made the volume again to 10ml by adding 96% methanol

**5.** Samples were then centrifuged at 8000rpm for 10 minutes.

**6.** Pigment of solution was analyzed using spectrophotometer (in terms of absorbance) by comparing a sample of unknown transmission against a blank (96% methanol alone) of 100% transmission at 650nm and 665nm.

**Calculations:**

Total chlorophyll = $2.55 \times 10^{-2}.E_{650} + 0.4 \times 10^{-2}.E_{665}$ mg/ml

Where, $E_{650}$ = absorbance at 650nm wavelength

$E_{665}$ = absorbance at 665nm wavelength

## 3.3 Harvesting of algal biomass

Microalgae were harvested after 23 – 25 days of inoculations when thick sheath of algae was observed. Thick sheath of algal biomass was collected through sieving procedure and collected in the glass petridish. The algal biomass was dried and crushed to powder using mortar and pestle. Dried Algal powder was collected for lipid estimation and extraction.

### 3.3.1Cell disruption with tissue homogeniser:

Disruption of algal cells was done in a small tissue homogeniser in which 10ml of algal cultures was taken and manually operated to homogenise the algal cultures.

**Figure 7: Tissue homogeniser**

### 3.3.2 Cell disruption with ultrasonicator

Disruption of algal cell was also done by using ultrasonicator to disrupt the cell wall and to simplify the lipid extraction. The probe of ultrasonicator was properly wiped with ethanol.

**1.** 25ml of algal culture was taken in small 50ml glass beaker along with three four glass beads.

**2.** All the parameters were set before the operation of the instrument i.e energy (90,000kJ), Amplitude (65Hz) and Pulse on time (20 sec) & pulse off time (10sec) and operation time was set for 30 minutes respectively

**3.** The sonicated algal cultures were collected and observed under the microscope to identify the disrupted cells and finally these sonicated algal cultures were used for lipid estimation.

## 3.4 Mass cultivation of algal cultures

The mass cultivation of algal cultures was carried out in an open condition in plastic tubs and tanks in polyhouse. Tubs and tanks were filled with normal tap water and the level was maintained till 3-4 inches. Water level and pH was checked regularly during the mass multiplication of algal cultures. For non heterocystous algal cultures pinch of sodium nitrate was added whereas in other algal cultures pinch of $MgSO_4.10\ H_2O$ and $K_2HPO_4$ was added. Starter cultures of *Nostoc muscorum*, *Spirulina.sp*, *Oscillatoria*

*R2, Nannochloropsis oculata* were inoculated in each tub followed by addition of few drops of malathion to prevent insect breeding in the tubs.

## 3.5 Lipid estimation (Folch et al., 1957)

**REAGENTS:**

1. Palmitic Acid-Dissolve Palmitic Acid in chloroform to a final concentration of 1mg/ml
2. Dichromate solution- Dissolve 2.5grams of $K_2Cr_2O_7$ in 1 liter of conc.$H_2SO_4$.
3. Perchloric Acid ($HClO_4$), 0.2 N
4. Chloroform: Methanol (2:1)

**Procedure:**

**1.** Collected enough cells from centrifugation, discard the supernatant. Place the tube containing cell pellets on ice and add 10 ml of ice cold 0.2 N $HClO_4$.Thoroughly resuspend the cell pellets by vortexing or inversion.

**2.** After 15 minutes at 4ºC, Centrifuge the sample in a refrigerated centrifuge and carefully remove the supernatant. Procedure can be repeated with a further 10 ml of 0.2 N $HClO_4$.

**3.** Added 10 ml of chloroform-methanol solution to the pellet from $HClO_4$ extraction. Resuspended the pellet from $HClO_4$ extraction. Resuspended the pellet and allow the suspension to stand 5 min at room temperature.

**4.** Centrifuged the sample at room temperature. Centrifuge the sample and remove and retain the supernatant. Added 0.2 volumes of water to the combined chloroform-methanol extracts of the cells.

**5.** Shaked the solutions for 5 minutes to mix well and centrifuge to separate the phases. Collected the organic (lower) phase leaving behind the precipitate that forms at the interphase. Discarded the aqueous phase.

**6.** Evaporated the chloroform –methanol solution under a stream of nitrogen to a final volume of 2ml. Transferred 0.05, 0.1, 0.15, 0.2, 0.3 ml of lipid standard solutions to the marked screw capped tubes.

**7.** Transferred 0.1, 0.2, 0.5 ml of unknown lipid sample to the marked tubes. Evaporate all tubes to dryness under vacuum or stream of nitrogen.

**8.** Added 2 ml of dichromate solution to all tubes and cap with Teflon lined caps. Placed all tubes in a boiling water bath for 45 minutes.

**9.** Shaked the tubes 2 or 3 times during the heating. Cooled the tubes, Removed 1 ml from each, dilute to 100 ml with water.

**10.** Read the absorbance of each tube at 350 nm against water as blank. Plot a standard curve with the unknown graphically.

## 3.6 Lipid extraction from microalgae

### 3.6.1 Lipid extraction for HPLC

1. 0.5g dry algal biomass was placed in a 50ml conical polypropylene tube.
2. A mixture of 4ml water, 10ml methanol and 5ml chloroform was introduced to the tube, and stirred for 2min to obtain a monophasic system on a vortex mixer.
3. Again 5ml volume of chloroform was added and stirred for 30s on the vortex mixer. Finally 5ml water was added and stirred for 30s on the vortex mixer.
4. The homogenate obtained after mixing on the vortex mixer was centrifuged at 2000rpm in a table top centrifuge for 10min at room temperature to form a two phase system (methanol and water on top, chloroform at bottom).
5. The chloroform layer was recovered and placed into another 50ml conical polypropylene tube with a pipette. The recovered volume of the chloroform layer in the tube was measured.
6. The microalgal oil was collected after drying under a nitrogen stream.
7. The lipid extracted was characterized by High Performance Liquid Chromatography (HPLC).

### 3.6.2 HPLC

Lipids extracted from dry algal biomass were characterized with the help of HPLC analysis. The sample were centrifuged at 8000rpm for 5min and then filtered through 0.22µm filter. HPLC () was carried out with purified sample. Samples (20µl) were injected and separated on C8 column () in methanol: water (50:50) solvent system at a flow rate of 1ml/min. The eluates were monitored at 280nm with online diode array detector. Each sample of *Nostoc muscorum, Spirulina.sp, Oscillatoria R2, Nanochloropsis oculata* was compared against olive oil (0.1M) used as a standard in HPLC analysis.

### 3.7 *In Situ* Transeterifiction

**1.** 0.5g quantity of dry algal biomass was placed in a 50ml conical polypropylene tube.

**2.** 15ml ml of chloroform:methanol(1:1) and 1ml of methanol with 0.0485g of pottasium hydroxide was added to the conical polypropylene tube.

**3.** Continous stirring was done for 1minute too homogenise the ystem.

**4.** The tube was kept at room temperature (24ºC) without stirring. Two phases are formed in the tube.

**5.** After an hour 0.0878g hydrochloric acid was added to stop the reaction.

## Chapter 4

# Results

### 4.1 Standard curve of palmitic acid

Standard curve of palmitic acid was prepared by Folch method (Folch *et al*, 1957). Concentration of palmitic acid: 1mg palmitic acid in 1ml chloroform

| Volume (ml) | Absorbance(350nm) |
|---|---|
| 0.05 | 0.003 |
| 0.1 | 0.009 |
| 0.15 | 0.019 |
| 0.20 | 0.025 |
| 0.30 | 0.039 |
| 0.40 | 0.050 |
| 0.50 | 0.065 |

**Table 3: Standard curve of palmitic acid**

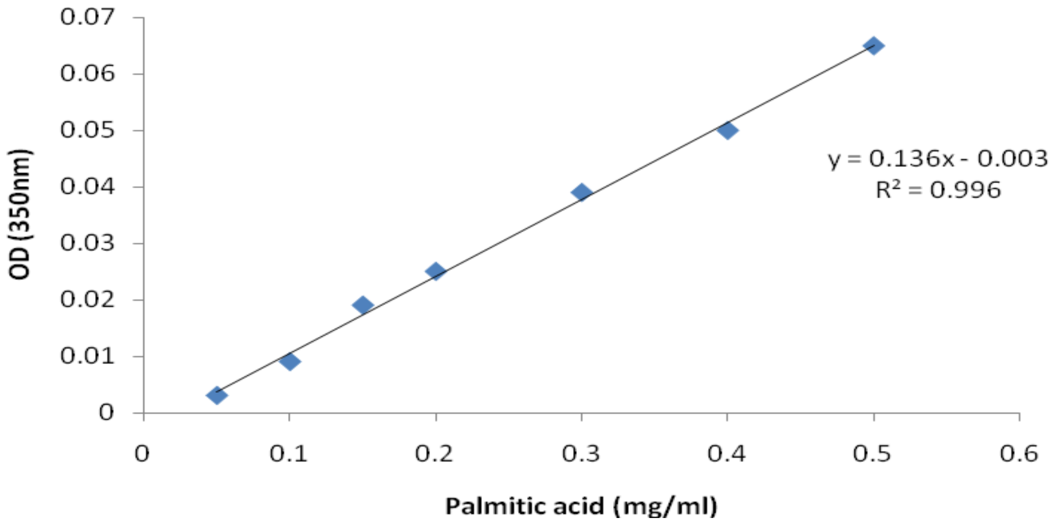


**Figure 8: Standard curve of palmitc acid**

## 4.2Lipid estimation of different microalgal cultures

10ml of microalgal cultures were taken in 15ml centrifuge tube and lipid content was estimated. Table 4 shows the lipid estimation in nine algal species. *Nannochloropsis oculata* contains the highest lipid content that is 0.42 mg/ml followed by *Botryococcus braunii*, which contains 0.41 mg/ml of lipid. *Chlorella* R5 has the least amount of lipid i.e. 0.15 mg/ml. Similarly *Nostoc muscorum, Spirulina.sp, Oscillatoria* R2, *Chlorella* Cp4, *Scenedesmus armatus, Chlamydomonas moewsii* contains 0.22, 0.27, 0.38, 0.21, 0.40, 0.34 mg/ml respectively.

| ALGAL CULTURES | VOLUME | | |
|---|---|---|---|
| | 0.1ml | 0.2ml | 0.5ml |
| *Nostoc muscorum* (ARM442) | 0.12±0.003 | 0.17±0.005 | 0.22±0.003 |
| *Spirulina.sp* | 0.12±0.008 | 0.15±0.01 | 0.27±0.005 |
| *Oscillatoria* R2 | 0.19±0.003 | 0.23±0.01 | 0.38±0.01 |
| *Chlorella* Cp4 | 0.14±0.003 | 0.19±0.01 | 0.21±0.003 |
| *Chlorella* R5 | 0.09±0.008 | 0.13±0.005 | 0.15±0.003 |
| *Nannochloropsis oculata* | 0.28±0.005 | 0.35±0.02 | 0.42±0.02 |
| *Bortyococcus braunii* | 0.25±0.01 | 0.33±0.008 | 0.41±0.01 |
| *Scenedesmus armatus* | 0.20±0.02 | 0.34±0.01 | 0.40±0.01 |
| *Chlamydomonas moewsii* | 0.27±0.008 | 0.29±0.003 | 0.34±0.008 |

**Table 4: Lipid estimation in algal cultures**

### 4.2 Lipid estimation after disruption of algal cell

The algal cells were disrupted using two techniques: tissue homogenizer and ultrasonicator. The combination of two was also used to check the extent of cell disruption.

### Lipid estimation in algal cells disrupted by tissue homogenizer

Table 5 shows lipid content in microalgae after disruption with tissue homogenizer. A small tissue homogenizer was used for disruption of algal cells. Maximum lipid content was observed in *Nannochloropsis oculata* 0.46 mg/ml and *Nostoc muscorum* contained the least lipid content (0.28 mg/ml). The average lipid content is 0.35 mg/ml. The increase in average lipid content shows the disruption of algal cells and release of lipid droplets.

| Algal cultures | Volume | | |
|---|---|---|---|
| | 0.1ml | 0.2ml | 0.5ml |
| *Nostoc muscurum*(ARM442) | 0.14±0.01 | 0.21±0.01 | 0.28±0.01 |
| *Spirulina.sp* | 0.11±0.01 | 0.14±0.003 | 0.24±0.01 |
| *Oscillatoria R2* | 0.14±0.02 | 0.27±0.01 | 0.40±0.01 |
| *Nannochloropsis oculata* | 0.31±0.02 | 0.37±0.004 | 0.46±0.02 |

**Table 5: Lipid estimation in algae after disrupting with tissue homogenizer**

### 4.3.2 Lipid estimation of algal cells disrupted by using ultrasonicator

Table 6 shows the lipid content in microalgae that were treated with ultrasonicating waves. The lipid content in *Nannochloropsis oculata, Oscillatoria R2, Nostoc muscorum, Spirulina.sp* was 0.48 mg/ml, 0.44 mg/ml, 0.34 mg/ml, 0.30 mg/ml respectively. Greater release of lipids was observed in all the four microalgal cultures.

| Algal cultures | Volume | | |
|---|---|---|---|
| | 0.1ml | 0.2ml | 0.5ml |
| *Nostoc muscurum*(ARM442) | 0.15±0.01 | 0.19±0.01 | 0.34±0.01 |
| *Spirulina.sp* | 0.14±0.01 | 0.16±0.01 | 0.30±0.02 |
| *Oscillatoria* sp.*R2* | 0.18±0.01 | 0.25±0.01 | 0.44±0.01 |
| *Nannochloropsis oculata* | 0.25±0.01 | 0.34±0.01 | 0.48±0.0 |

**Table 6: Lipid estimation after treatment with ultrasonicating waves**

### 4.3.3 Lipid estimation of algal cells disrupted both with tissue homogenizer and ultrasonicator

Table 7 shows the lipid content in microalgae after they were treated with both tissue homogenizer as well as ultrasonicating waves. *Nannochloropsis oculata* contains maximum lipid content (0.50mg/ml ) followed by *Oscillatoria* sp.R2 (0.47mg/ml). The increase in lipid content shows more disruption of cell takes place when both disruption techniques are used in combination

| Algal cultures | Volume | | |
|---|---|---|---|
| | 0.1ml | 0.2ml | 0.5ml |
| *Nostoc muscurum* (ARM442) | 0.14±0.006 | 0.23±0.009 | 0.37±0.01 |
| *Spirulina.sp* | 0.14±0.009 | 0.20±0.006 | 0.34±0.003 |
| *Oscillatoria* sp.*R2* | 0.21±0 | 0.27±0.02 | 0.47±0.01 |
| *Nannochloropsis oculata* | 0.23±0.01 | 0.35±0.009 | 0.50±0.02 |

**Table 7: Lipid estimation after treatment with tissue homogeniser and ultrasonicating waves**.

## 4.4 Chlorophyll estimation in microalgae

Chlorophyll was measured in µg/ml as shown in Table13, Figure 9. Chlorophyll was measured after 28 days of inoculation. Maximum chlorophyll was observed in *Spirulina.sp* i.e 2.6 µg/ml and minimum was observed in *Oscillatoria* sp.R2 i.e. 2 mg/ml.

| Algal cultures | R1(µg/ml) | R2(µg/ml) | R3(µg/ml) | Mean±SE |
|---|---|---|---|---|
| *Nostoc muscurum* (ARM442) | 2.4 | 2.4 | 2.3 | 2.4±0.1 |
| *Spirulina.sp* | 2.6 | 2.7 | 2.7 | 2.6±0.03 |
| *Oscillatoria R2* | 2.0 | 2.0 | 2.1 | 2.0±0.1 |
| *Nannochloropsis oculata* | 2.1 | 2.1 | 2.0 | 2.1±0.03 |

**Table 8: Chlorophyll estimation in algal cultures**

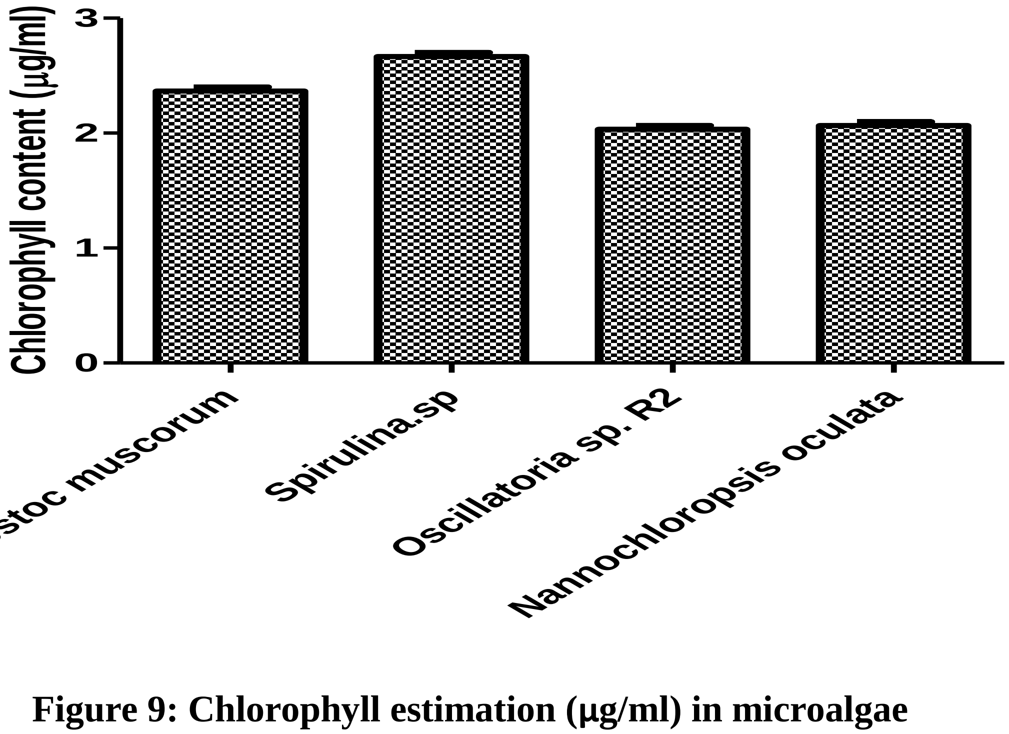


**Figure 9: Chlorophyll estimation (µg/ml) in microalgae**

## 4.4 Mass cultivation of microalgae

### 4.4.1 Small scale cultivation of microalgae (Lab scale )

Bg11+N, CFTRI and Fogg's media (100ml) were inoculated with different microalgae and incubated at 26±2°C under discontinuous illumination at 16:8 h light/dark cycles at 2500-3000 lux light intensity. Algal cultures were grown aseptically in 1litre, 2litre and 5litre flasks and after the microalgae has attained adequate growth the flasks were transferred to polyhouse.

Figure 10: Algal cultures

### 4.4.2 Large scale cultivation of microalgae in tubs and tanks

Large scale production of algae carried out by transferring cultures from 5litre flasks to plastic tubs and iron tanks in a polyhouse. A pinch of $MgSO_4$ and $K_2HPO_4$ was added before the inoculation of algae. Few drops of malathion was added to prevent insect breeding. Inoculation was done in plastic tubs and iron tanksformassproduction.

Figure 11: Algal cultures grown in tubs and iron tanks

## 4.5 Growth study of microalgae in lab conditions

### 4.5.1 Biomass estimation

**Wet biomass estimation (Lab scale)**

Growth of *Nannochloropsis oculata, Oscillatoria R2, Spirulina.sp, Nostoc muscorum* was measured at lab scale in trems of wet biomass in mg/ml. The peak growth upto 28days was observed at weekly intervals for each culture. Maximum growth was observed in *Nannochloropsis oculata* that is 0.46 mg/ml followed by *Oscillatoria R2* i.e. 0.40 mg/ml. (Table 9; Figure 12)

| Wet biomass in mg/ml | | | | |
|---|---|---|---|---|
| Algal cultures | 7days | 14 days | 21 days | 28days |
| *Nostoc muscurum* (ARM442) | 0.21±0.2 | 0.24±0.02 | 0.30±0.02 | 0.35±0.03 |
| *Spirulina.sp* | 0.18±0.01 | 0.21±0.01 | 0.31±0.02 | 0.39±0.02 |
| *Oscillatoria R2* | 0.20±0.01 | 0.27±0.03 | 0.33±0.03 | 0.40±0.03 |
| *Nannochloropsis oculata* | 0.26±0.2 | 0.31±0.03 | 0.34±0.03 | 0.46±0.01 |

**Table 9: Wet biomass estimation at lab scale of different microalgae**

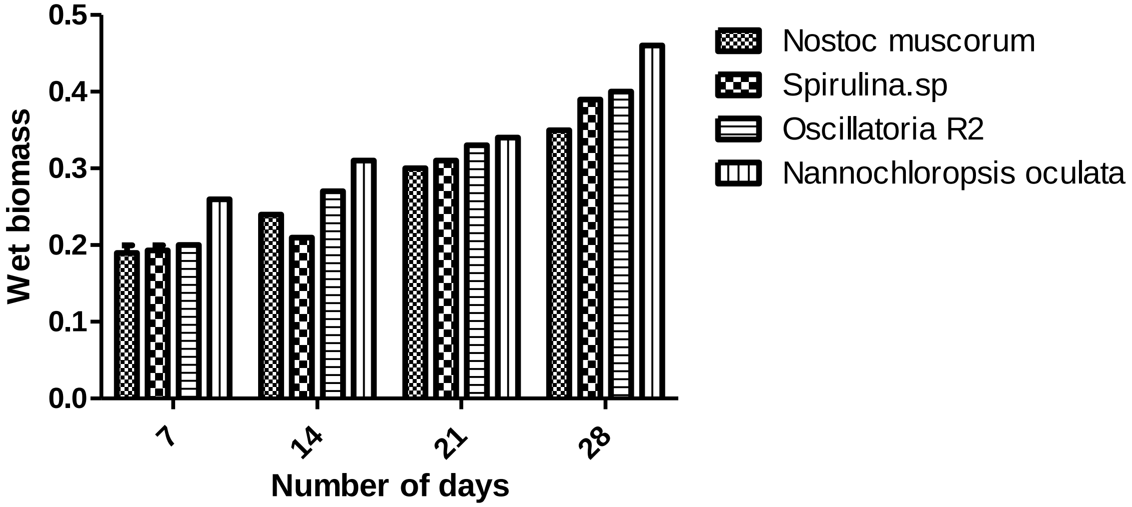


**Figure 12: Wet biomass of microalgal cultures at regular intervals in lab conditions**

### Dry biomass estimation (lab scale)

Dry biomass for each microalgal culture was measured at lab scale in mg/ml. With increase in time, the biomass of algal inoculants increased from the date of inoculation. Maximum growth was observed in *Nannochloropsis oculata* i.e. 0.13 mg/ml whereas minimum growth was observed in *Nostoc muscorum* i.e. 0.08 mg/ml. (Table 10; figure 13).

| Dry biomass in mg/ml | | | | |
|---|---|---|---|---|
| Algal cultures | 7days | 14 days | 21 days | 28days |
| *Nostoc muscurum* (ARM442) | 0.03±0.02 | 0.04±0.04 | 0.05±0.02 | 0.08±0.05 |
| *Spirulina.sp* | 0.01±0.03 | 0.03±0.03 | 0.06±0.04 | 0.09±0.06 |
| *Oscillatoria R2* | 0.03±0.01 | 0.03±0.06 | 0.07±0.03 | 0.11±0.04 |
| *Nannochloropsis oculata* | 0.04±0.2 | 0.07±0.07 | 0.10±0.01 | 0.13±0.01 |

**Table 10: Dry biomass of different microalgae at lab scale**

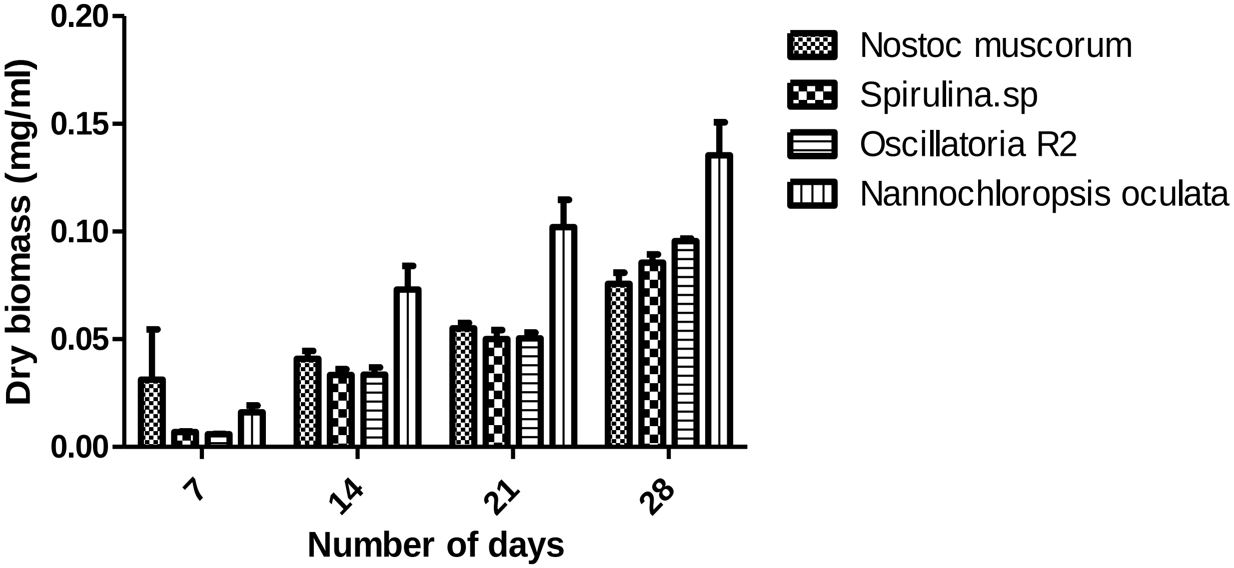


**Figure 13: Dry biomass of algal cultures at regular intervals in lab conditions**

**Table 11: Lipid estimation at regular interval of time (lab conditions)**

| Volume of organic layer 0.1 ml | | | | |
|---|---|---|---|---|
| Algal culture | Incubation time in days | | | |
| | 7 days | 14 days | 21 days | 28 days |
| Nostoc muscurum (ARM442) | 0.09±0.02 | 0.12±0.01 | 0.14±0.01 | 0.20±0.01 |
| Spirulina sp. | 0.06±0.03 | 0.09±0.01 | 0.13±0.01 | 0.18±0.01 |
| *Oscillatoria R2* | 0.12±0.06 | 0.15±0.02 | 0.17±0.01 | 0.22±0.01 |
| *Nannochloropsis oculata* | 0.10±0.02 | 0.16±0.01 | 0.21±0.02 | 0.27±0.03 |
| Volume of organic layer 0.2 ml | | | | |
| Nostoc muscurum (ARM442) | 0.16±0.01 | 0.18±0.01 | 0.21±0.01 | 0.22±0.02 |
| Spirulina sp. | 0.14±0.01 | 0.16±0.01 | 0.23±0.01 | 0.23±0.02 |
| *Oscillatoria R2* | 0.16±0.01 | 0.20±0.01 | 0.29±0.02 | 0.31±0.03 |
| *Nannochloropsis oculata* | 0.20±0.01 | 0.22±0.03 | 0.25±0.01 | 0.31±0.03 |
| Volume of organic layer 0.5 ml | | | | |
| Nostoc muscurum (ARM442) | 0.25±0.01 | 0.27±0.01 | 0.31±0.01 | 0.32±0.01 |
| Spirulina sp. | 0.22±0.01 | 0.25±0.01 | 0.29±0.01 | 0.32±0.01 |
| *Oscillatoria R2* | 0.28±0.01 | 0.30±0.01 | 0.35±0.01 | 0.42±0.01 |
| *Nannochloropsis oculata* | 0.32±0.01 | 0.34±0.02 | 0.39±0.01 | 0.42±0.02 |

At different intervals lipid content of microalgae was measured. With time the lipid content of microalgae increased as the biomass of microalgae was increased. Maximum lipid content in 0.1 ml layer of lipid was found in *Nannochloropsis Oculata* 0.24 mg/ml. lipid content in *Nostoc muscorum, Spirulins.sp, Oscillatoria* R2 was 0.18 mg/ml, 0.19 mg/ml, 0.24 mg/ml respectively. Similar results were observed when 0.2ml of organic layer of different algal cultures were compared. Maximum lipid was observed in Nannochloropsis oculata i.e. 0.31 mg/ml. the average lipid content in algal cultures was 0.27 mg/ml. With increase in the volume of organic layer the amount of lipid increased in all the four algal cultures. The average layer increased to 0.42 mg/ml. Maximum lipid was present in Nannochloropsis oculata (0.42 mg/ml) and Oscillatoria R2 (0.42 mg/ml) (Table 11; Figure 14,15,16)

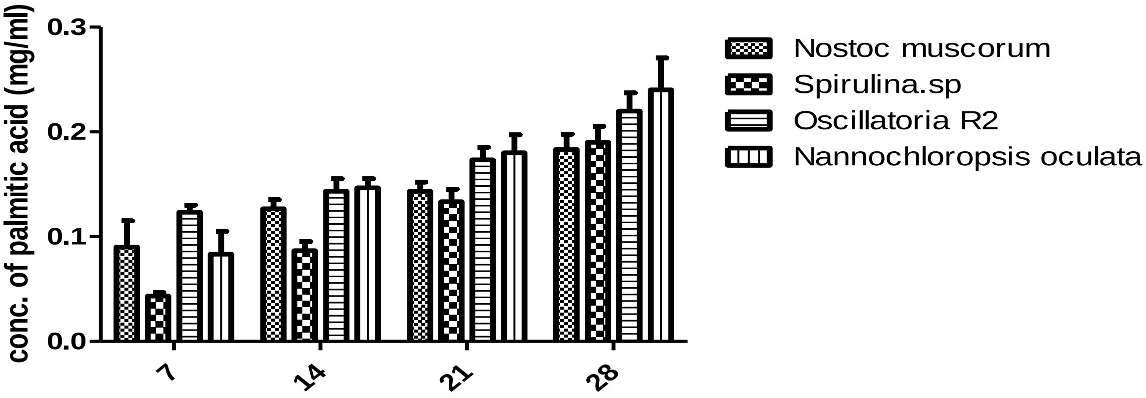


**Figure 14: Estimation of lipid in 0.1ml of organic layer in microalgae at regular intervals**

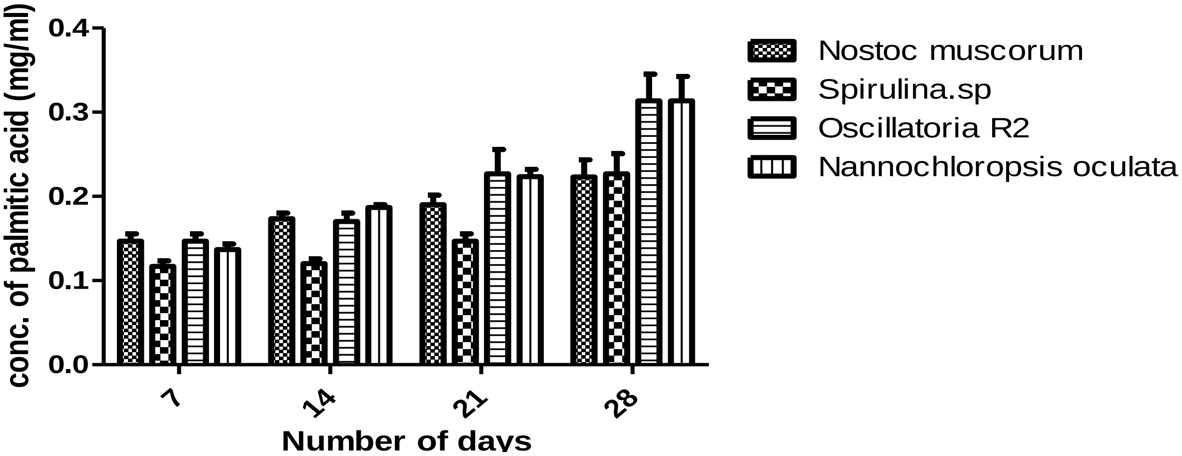


**Figure 15: Estimation of lipid in 0.2ml of organic layer in microalgae at regular intervals**

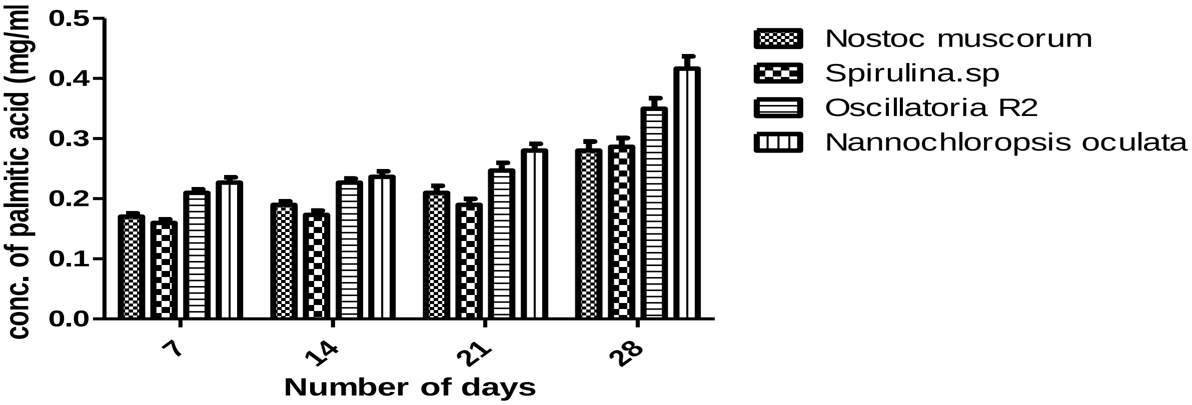


**Figure 16: Estimation of lipid in 0.5ml of organic layer in microalgae at regular intervals**

## 4.6 Growth studies of microalgae in open conditions

It was noted that in tubs inoculated with *Nostoc muscorum, Oscillatoria* R2, *Nannochloropsis oculata* the Ph ranged from 7-7.5, whereas in *Spirulina.sp* it was 8.9-9.3. The water temperature in each tub was in the range 23°C-32°C and water level was between 2-3 inches.

| Variation of pH in tubs | | | | |
|---|---|---|---|---|
| Algal culture | Incubation time in days | | | |
| | 7 days | 14 days | 21 days | 28 days |
| Nostoc muscurum (ARM442) | 7.04 | 7.3 | 7.4 | 7.6 |
| Spirulina sp. | 8.9 | 9.0 | 9.1 | 9.3 |
| *Oscillatoria R2* | 7.1 | 7.2 | 7.3 | 7.4 |
| *Nannochloropsis oculata* | 7.2 | 7.3 | 7.4 | 7.5 |
| Variation of temperature (°C) in tubs | | | | |
| *Nostoc muscurum* (ARM442) | 24 | 26 | 29 | 32 |
| *Spirulina sp.* | 25 | 27 | 29 | 29 |
| *Oscillatoria R2* | 23 | 26 | 27 | 31 |
| *Nannochloropsis oculata* | 26 | 27 | 30 | 31 |
| Variation of water levels (in inches) in tubs | | | | |
| Nostoc muscurum (ARM442) | 3 | 2.9 | 2.7 | 2.6 |
| Spirulina sp. | 3 | 2.8 | 2.7 | 2.5 |
| *Oscillatoria R2* | 3 | 2.7 | 2.7 | 2.5 |
| *Nannochloropsis oculata* | 3 | 2.9 | 2.6 | 2.6 |

**Table 12: Observation of pH, temperature, water level at different interval of time in tubs**

### 4.6.1 Biomass estimation

**Wet biomass estimation (open condition)**

With the increase in the time interval of 7 days, wet biomass was observed in *Nannochloropsis oculata* from 0.21 mg/ml on day 7 to 0.57mg/ml on day 28. Similarly *Nostoc muscorum*, *Spirulina.sp*, *Oscillatoria* R2 showed 0.20 mg/ml, 0.22mg/ml, 0.15 mg/ml, 0.21 mg/ml on day 7 to 0.46 mg/ml, 0.49 mg/ml, 0.53 mg/ml on day 28 respectively (Table 13; Figure 17)

| Algal cultures | Time interval in days | | | |
|---|---|---|---|---|
| | 7 | 14 | 21 | 28 |
| *Nostoc muscurum* (ARM442) | 0.20±0.01 | 0.33±0.01 | 0.42±0.01 | 0.46±0.02 |
| *Spirulina.sp* | 0.22±0.01 | 0.27±0.03 | 0.36±0.02 | 0.49±0.02 |
| *Oscillatoria R2* | 0.15±0.01 | 0.30±0.01 | 0.39±0.02 | 0.53±0.03 |
| *Nannochloropsis oculata* | 0.21±0.02 | 0.29±0.03 | 0.41±0.02 | 0.57±0.01 |

**Table 13: wet biomass estimation in open condition**

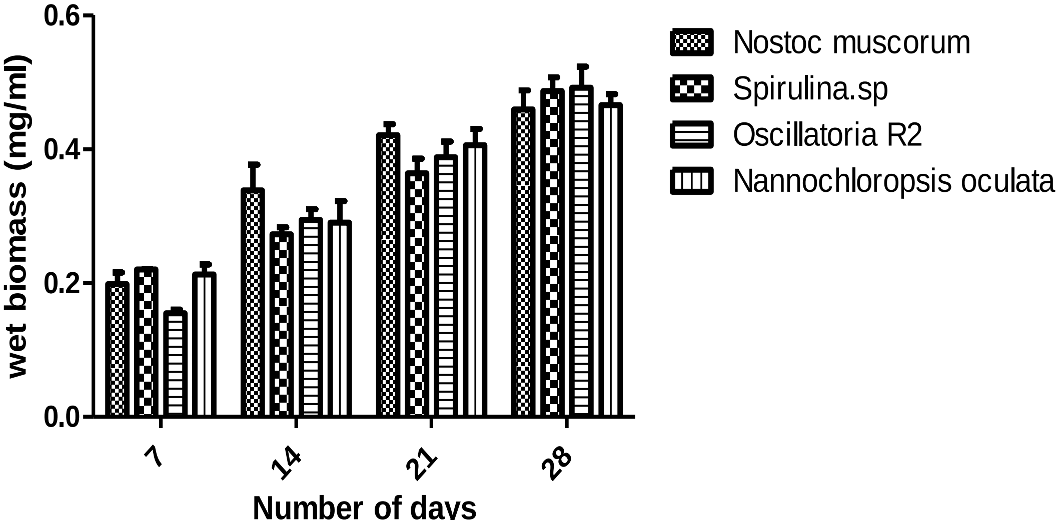


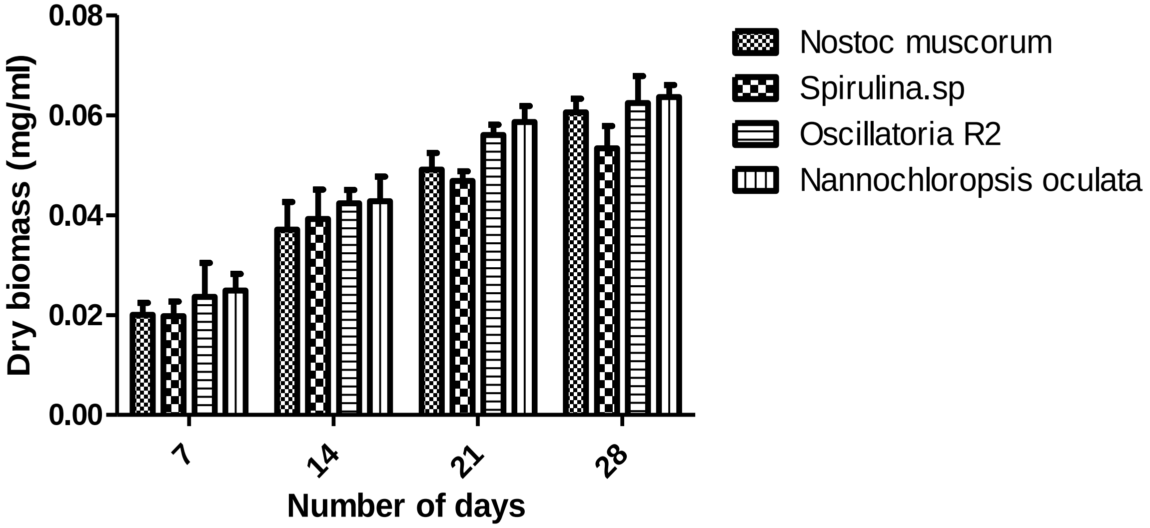
**Figure 17: wet biomass estimation at regular interval in open condition**

### Dry biomass estimation (open condition)

With the increase in the time interval of 7 days, wet biomass was observed in *Nannochloropsis oculata* from 0.04 mg/ml on day 7 to 0.08 mg/ml on day 28. Mean biomass production was highest in tub containing *Nannochloropsis oculata* i.e. 0.08 mg/ml harvested after 28 days of innoulation.the mean biomass in all ponds after 7, 14,21.28 days was 0.03 mg/ml, 0.05 mg/ml, 0.06 mg/ml, 0.07 mg/ml respectively (Table 14;Figure 18)

| Algal cultures | Time interval in days | | | |
|---|---|---|---|---|
| | 7 | 14 | 21 | 28 |
| *Nostoc muscurum* (ARM442) | 0.02±0.02 | 0.04±0.01 | 0.05±0.03 | 0.06±0.02 |
| *Spirulina.sp* | 0.01±0.03 | 0.03±0.01 | 0.04±0.02 | 0.05±0.04 |
| *Oscillatoria R2* | 0.03±0.06 | 0.05±0.02 | 0.06±0.01 | 0.07±0.05 |
| *Nannochloropsis oculata* | 0.04±0.01 | 0.06±0.04 | 0.07±0.03 | 0.08±0.02 |

**Table 14: Dry biomass stimation in open condition**



.

**Figure18: Dry biomass of algal cultures at regular intervals in open conditions**

### 4.6.2 Lipid estimation

| Volume of organic layer 0.1 ml | | | | |
|---|---|---|---|---|
| Algal culture | Incubation time in days | | | |
| | 7 days | 14 days | 21 days | 28 days |
| Nostoc muscurum (ARM442) | 0.04±0.01 | 0.06±0.02 | 0.15±0.01 | 0.19±0.02 |
| Spirulina sp. | 0.02±0.01 | 0.04±0.02 | 0.13±0.01 | 0.17±0.01 |
| *Oscillatoria R2* | 0.09±0.01 | 0.11±0.01 | 0.20±0.01 | 0.22±0.01 |
| *Nannochloropsis oculata* | 0.12±0.02 | 0.14±0.03 | 0.22±0.01 | 0.24±0.01 |
| Volume of organic layer 0.2 ml | | | | |
| Nostoc muscurum (ARM442) | 0.12±0.01 | 0.15±0.01 | 0.20±0.01 | 0.24±0.01 |
| Spirulina sp. | 0.09±0.02 | 0.12±0.01 | 0.18±0.01 | 0.22±0.02 |
| *Oscillatoria R2* | 0.17±0.01 | 0.20±0.01 | 0.24±0.01 | 0.28±0.02 |
| *Nannochloropsis oculata* | 0.15±0.01 | 0.22±0.01 | 0.28±0.02 | 0.32±0.01 |
| Volume of organic layer 0.5 ml | | | | |
| Nostoc muscurum (ARM442) | 0.16±0.01 | 0.20±0.01 | 0.24±0.01 | 0.28±0.01 |
| Spirulina sp. | 0.17±0.03 | 0.18±0.01 | 0.23±0.01 | 0.29±0.01 |
| *Oscillatoria R2* | 0.19±0.01 | 0.21±0.01 | 0.31±0.01 | 0.33±0.01 |
| *Nannochloropsis oculata* | 0.18±0.01 | 0.21±0.01 | 0.33±0.01 | 0.39±0.02 |

**Table 15: Lipid estimation at regular intervals (open condition)**

Microalgal cultures (*Nannochloropsis oculata, Nostoc muscorum, Spirulina.sp, Oscillatoria* R2) were grown in open conditions and lipid content was checked at regular intervals till 28 days of inoculation. The lipid content of algal biomass increased with different interval of time. In 0.1 ml organic layer highest lipid content was found in *Nannochloropsis oculata* i.e. 0.12 mg/ml, 0.14 mg/ml, 0.22 mg/ml, 0.24 mg/ml on 7 , 14, 21 and 28 days respectively. Similarly lipid content was measured in 0.2ml organic layer with different time interval of 7,14.21.28 days. The lipid content increased with the volume of organic layer. The maximum lipid content was found in *Nannochloropsis oculata* of 0.15mg/ml, 0.22 mg/ml, 0.28mg/ml 0.32 mg/ml whereas lowest lipid content was found in *Spirulina.sp* of 0.09 mg/ml, 0.12mg/ml, 0.18mg/ml, 0.22mg/ml on 7, 14, 21 ,28 days respectively. The lipid content was measured in 0.5ml organic layer. The lipid content in all algal cultures were observed till 28 days of inoculation and compared with biomass. Lipid content in the different algal samples were observed at regular intervals of 7days for one month and was compared against biomass obtained at same rate interval of gowth. (Table 15; Figure 19, 20, 21)

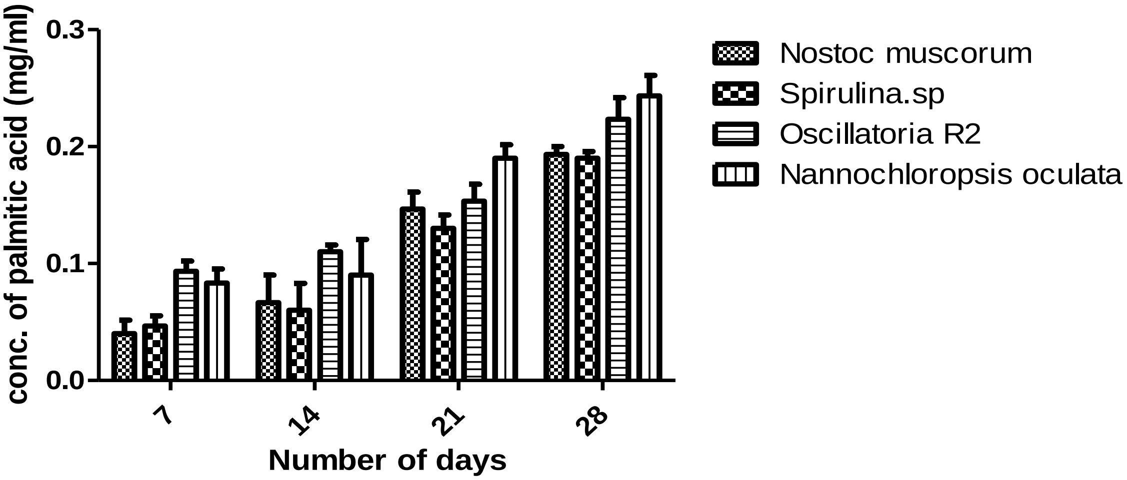


**Figure 19: Estimation of lipid in 0.1 ml of organic layer in microalgae at regular intervals**

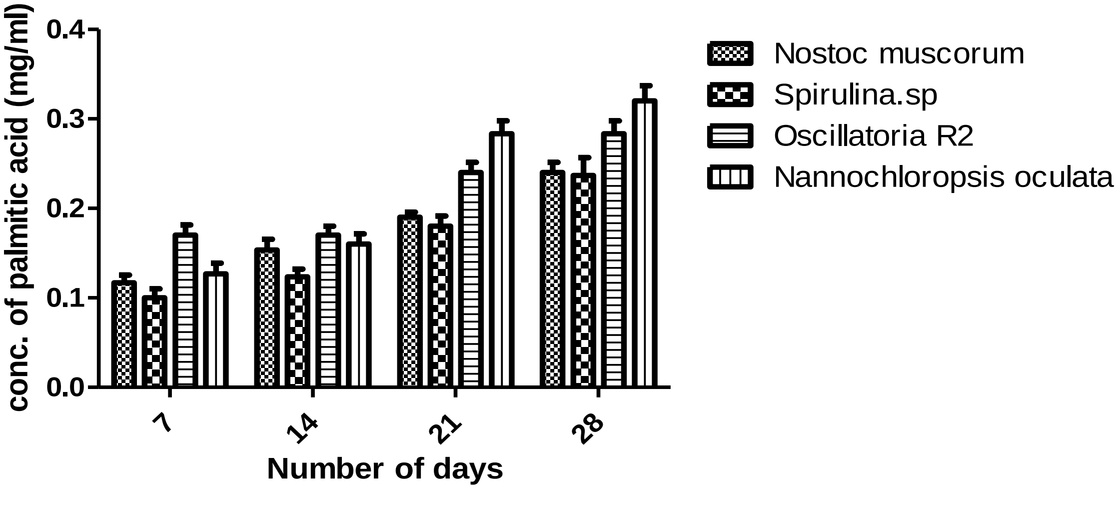


**Figure 20** : **Estimation of lipid in 0.2 ml of organic layer in microalgae at regular intervals**

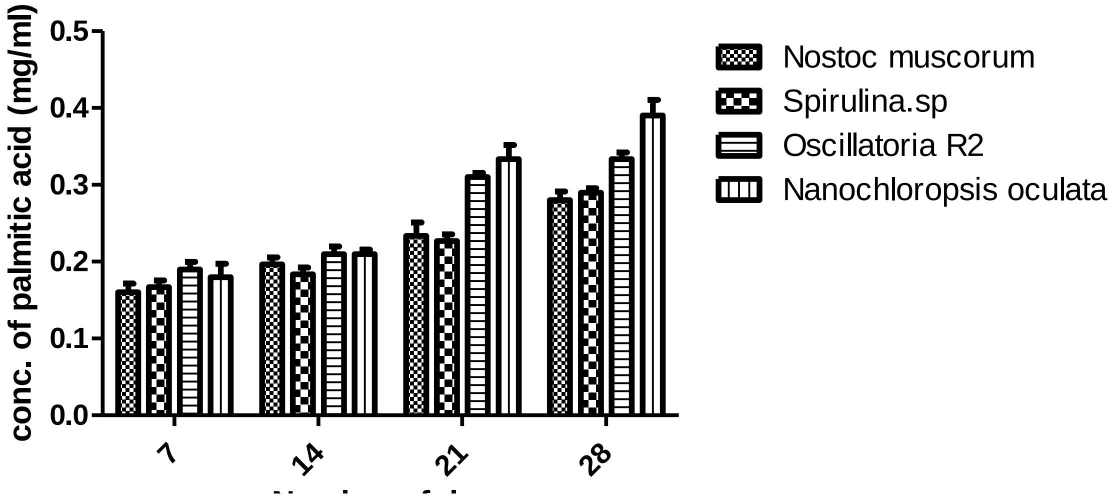


Figure 21: Estimation of lipid in 0.5 ml of organic layer in microalgae at regular intervals

### 4.7 Lipid characterisation by HPLC

Olive oil (0.1M) was run as refrence standard and different peaks at retention time 1.39, 2.72, 2.86, 3.44, 3.97 were observed and was compared with chromatograms of different algal samples.

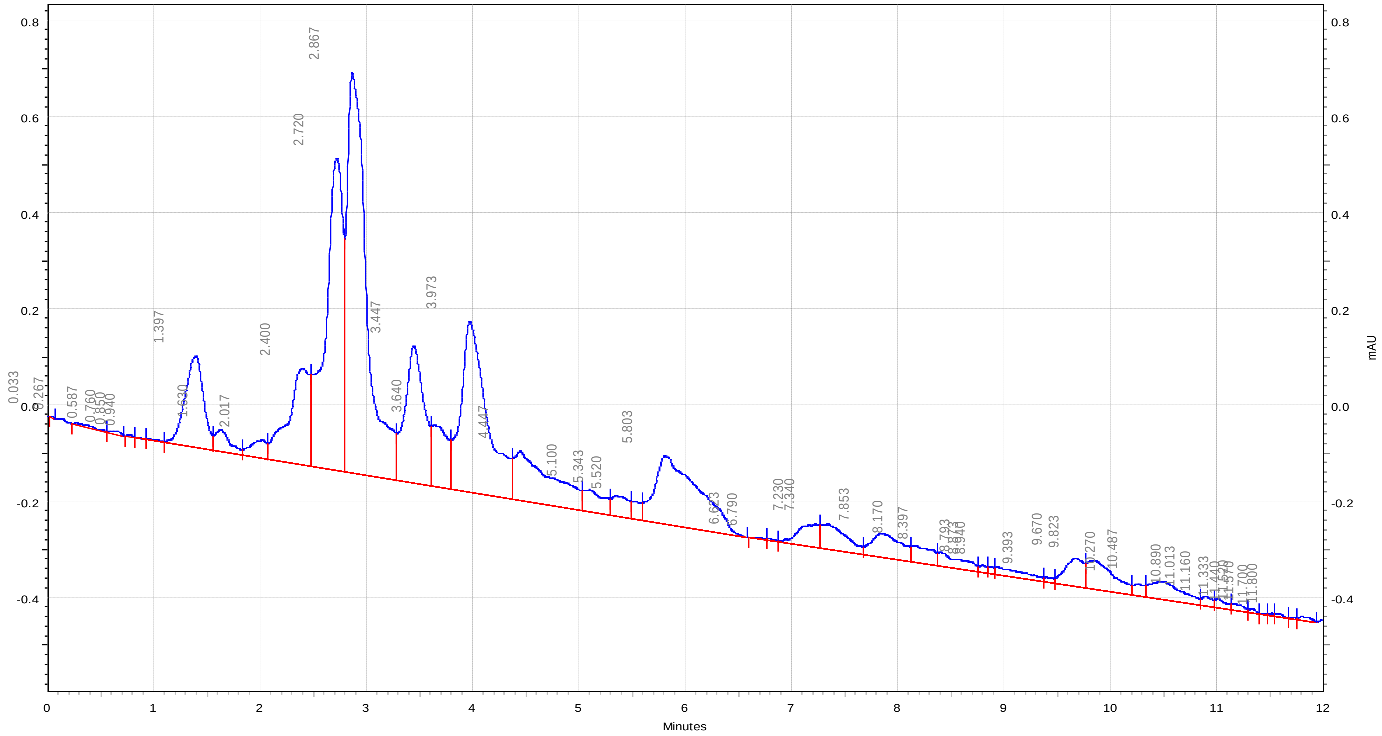


Figure 22: Chromatogram of olive oil

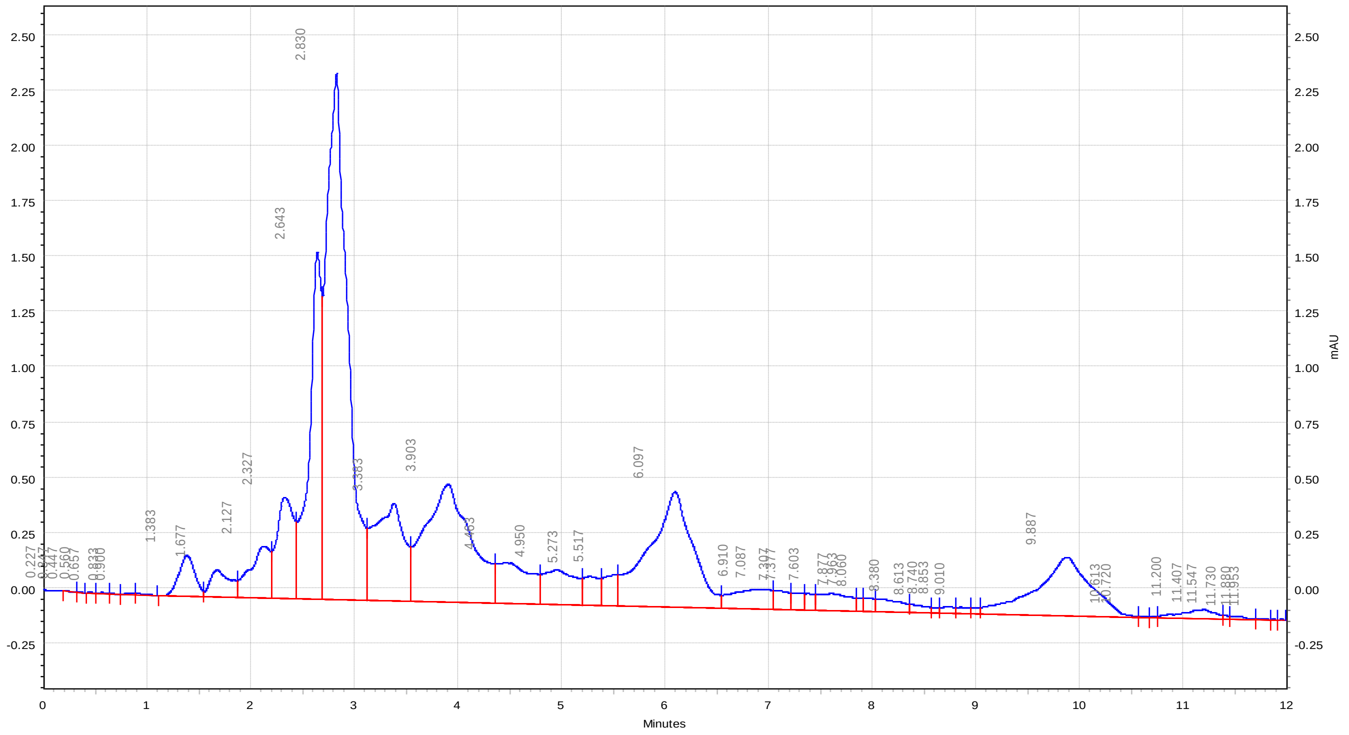


**Figure 23: Chromatogram of *Spirulina.sp***

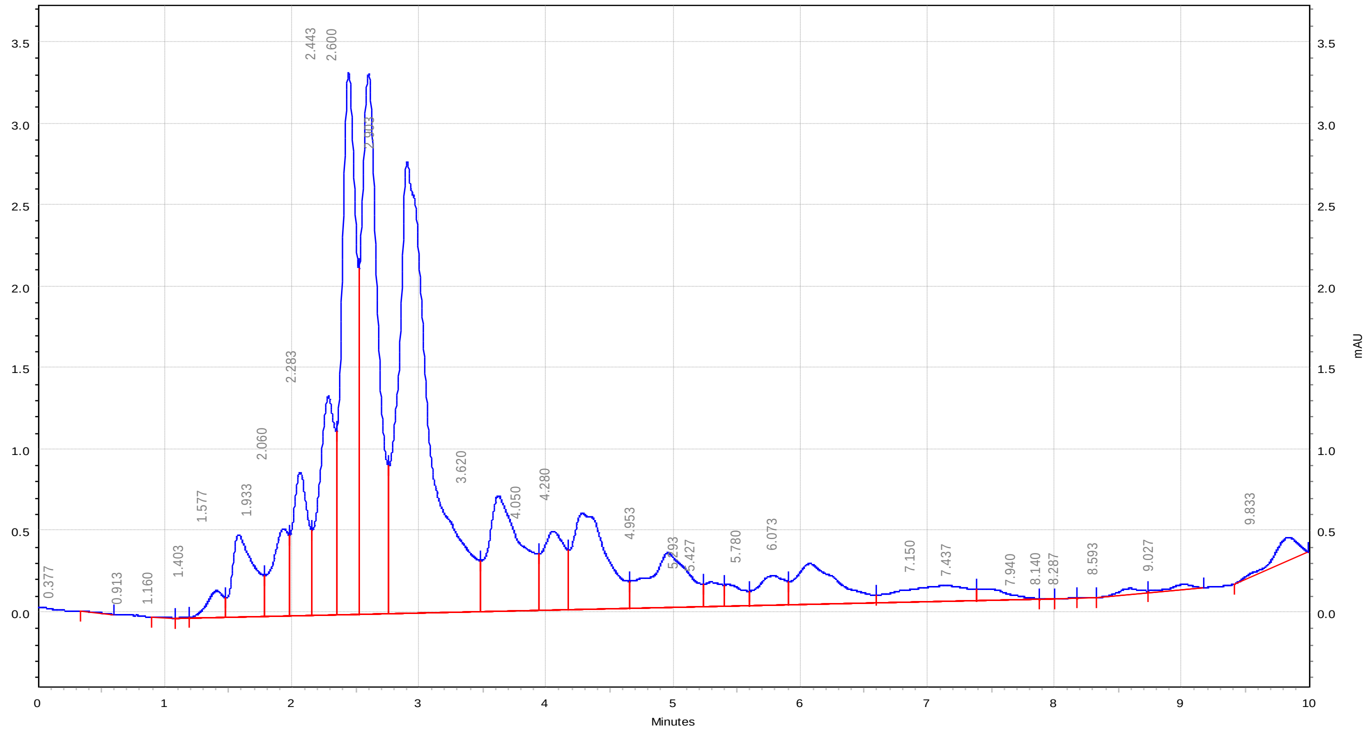


**Figure 24: Chromatogram of *Oscillatoria R2***

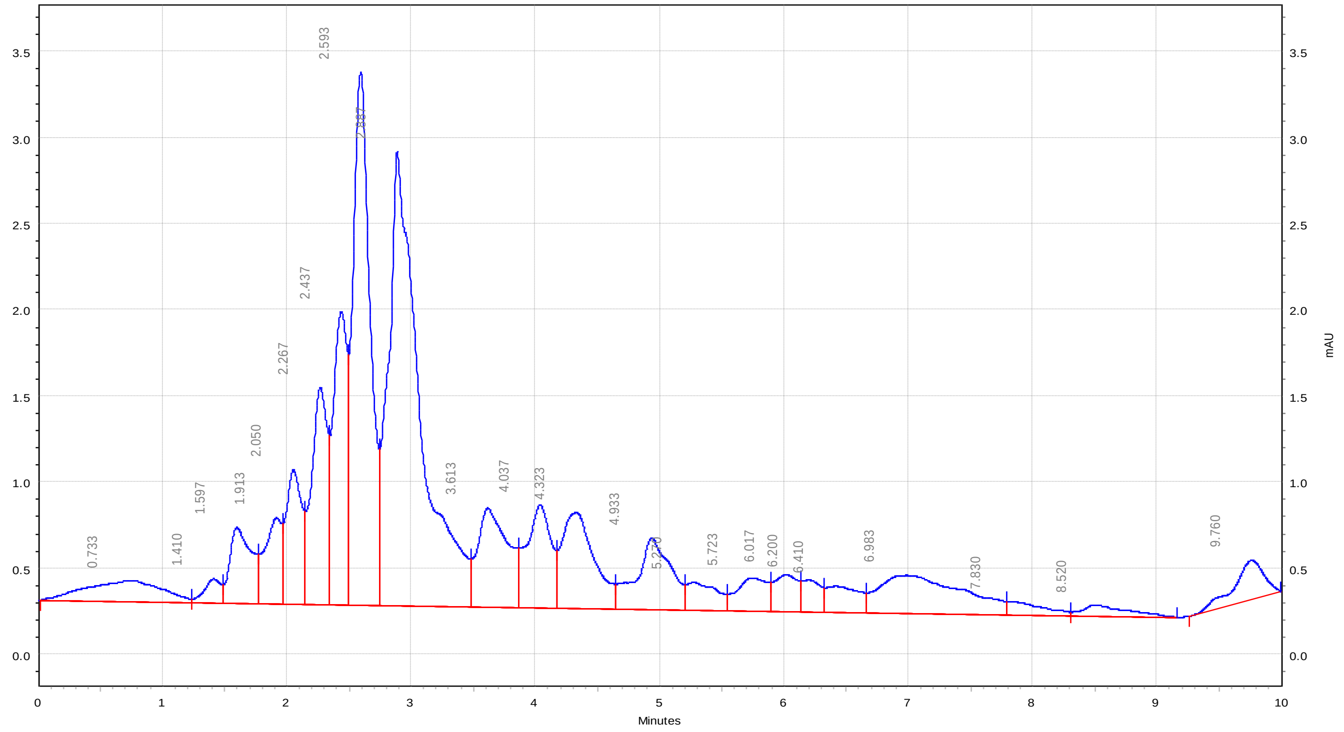


Figure 25: Chromatogram of *Nanochloropsis oculata*

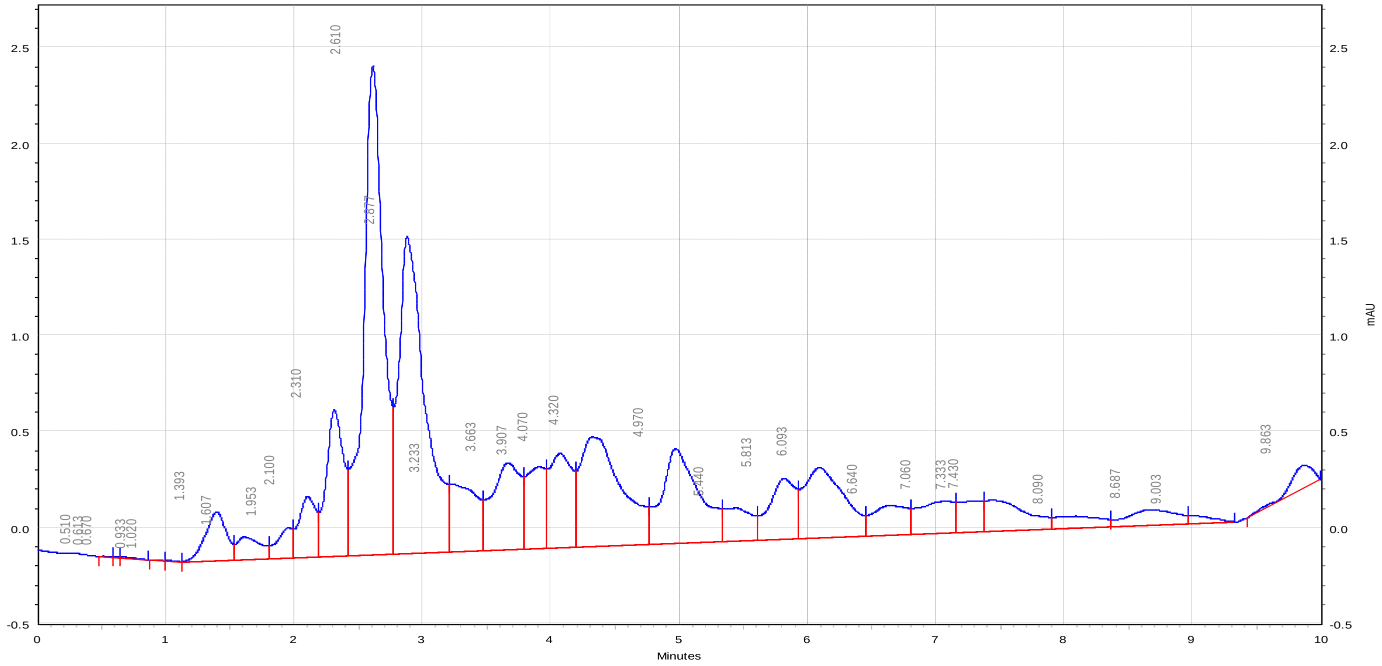


Figure 26: Chromatogram of *Nostoc muscorum*

HPLC chromatograms clearly indicated that the lipid extracted from different *Spirulina.sp* (Figure 23 ), *Oscillatoria* R2 (figure), *Nannochloropsis oculata* (Figure 24), *Nostoc muscorum* (Figure 25 ), have similar peaks that appeared in chromatogram of olive oil (Figure 26). Therefore the lipid extracted from different microalgae contained identical fatty acid fractions that were present in olive oil. (Table 16; Figure 22-26)

| **Retention times (min)** | | | | | |
|---|---|---|---|---|---|
| **Lipid Samples** | Olive oil | *Nostoc muscorum* | *Spirulina.sp* | *Oscillatoria* R2 | *Nannochloropsis oculata* |
| | | | | | |
| Peak 1 | 1.39 | 1.39 | 1.37 | 1.57 | 1.59 |
| Peak 2 | 2.72 | 2.60 | 2.64 | 2.60 | 2.43 |
| Peak 3 | 2.86 | 2.87 | 2.82 | 2.90 | 2.59 |
| Peak 4 | 3.44 | 3.66 | 3.38 | 3.61 | 2.88 |
| Peak 5 | 3.97 | 4.07 | 3.89 | 4.28 | 4.3 |

**Table 16: Comparison of retention times of microlagal oil with olive oil**

# Chapter 5

# Discussion

## 5.1 Screening of microalgae for lipid content

Total lipid content was determined observed in different microalgae such as *Nostoc muscorum*, *Spirulina.sp*, *Oscillatoria* R2, *Chlorella* Cp4, *Chlorella* R5, *Nannochloropsis oculata*, *Botrycoccus braunii*, *Scendesmus armatus*, *Chlamydomonas moewsii*.. *Nannochloropsis oculata* showed maximum lipid content (0.42 mg/ml) and the minimum lipid content was shown by *Chlorella* R5 (0.15mg/ml). Average lipid content in microalgal cultures was found 0.33 mg/ml which is in confirmation with earlier studies where it had been reported 0.018 mg/ml, 0.041 mg/ml lipid in *Chlorella* species, *Nannochloropsis* respectively (Mata *et al.*, 2010). Three different methods were applied for disrupting the cell wall, tissue homogenization and utrasonication and the combination of the two method. After treatment with tissue homogenizer increase in lipid content was observed whereas maximum lipid was in *Nannochloropsis oculata* (0.46mg/ml) and lowest was observed in *Spirulina.sp* (0.24 mg/ml). The average lipid content in microalgae was 0.35mg/ml. The mechanism of cell disruption by ultrasound is probably associated with the cavitation phenomena associated with ultrasonication.which results in release of lipid droplets (Yusuf Chisti and Moorey Moo-yong, 1986). In present investigation ultrasonication was carried out to disrupt the algal cells and the maximum lipid content was obtained in *Nannochloropsis oculata* (0.48 mg/ml) and minimum in *Spirulina.sp* (0.30 mg/ml). The average lipid content in homogenized cells of Nannochloropsis oculata was 0.46 mg/ml and after cell disruption with ultrasonication it increased to 0.48 mg/ml whereas when cells were homogenized olowed by ultrasonication the lipid increased to 0.50 mg/ml. This means that when cells are homogenised and further disrupted with ultrasonictation there is maximum release of lipids.

## 5.2 Growth studies of microalgae

Microalgal cells doubles every few hours during their exponential growth period (Metting, 1986) which was also found during the present studies. Microalgae grow very quickly compared to terrestrial crops (Chisti, 2007). The present experiment carried out on four high lipid containing microalgae in terms of growth studies which involve biomass estimation and chlorophyll estimation showed that the biomass estimation of microalgal strains estimated at different interval of time that is 7, 14, 21, 28 days of inoculation, increased with the time interval of 7 days. *Nannochloropsis oculata* showed maximum growth in terms of wet weight (0.04 mg/ml)as well as dry weight (0.13 mg/ml) followed by *Oscillatoria R2* with wet weight of (0.40 mg/ml) and dry weight of (0.11 mg/ml) respectively.The chlorophyll content as a parameter for growth was also estimated in µg/ml. Maximum chlorophyll content was observed in *Spirulina.sp* (2.6 µg/ml) followed by *Nostoc muscorum* (2.4 µg/ml) respectively. Overall with the increase in the time interval the biomass and chlorophyll increased in all tha microalgae but maximum biomass increase was shown by *Nannochloropsis oculata* and maximum chlorophyll content in *Spirulina.sp*.

### 5.3 Mass cultivation of microalgae

Biomass estimation was done in all tubs at different interval of time on wet weight basis was done at regular intervals of 7, 14, 21, 28 days and average wet weight was found to be 0.19mg/ml, 0.29mg/ml, 0.39mg/ml, 0.51mg/ml respectively. Similarly average dry weight was estimated at interval of 7, 14, 21, 28 days and was found to be 0.03 mg/ml, 0.05 mg/ml, 0.06 mg/ml and 0.07 mg/ml respectively. Lipid estimation was also carried out at regular intervals and it was observed that as the biomass of algal inoculants increased the lipid content also increased. The maximum biomass and lipid content was attaind after 28 days of inoculation in all the four microalgal cultures (*Nostoc muscorum, Spirulina.sp, Oscillatoria* R2, *Nannochlorpsis oculata*). Overall in the all the microalgae the lipid content increased with biomass at interval of 7 days.

### 5.4 Lipid extraction from microalgae

Extraction of lipid from microalgal mass forms an rate determing step in overall process. Several methods for lipid extraction from  microalgae have been reported such as soxhlet method, bligh and dyer method, and supercritical fluid extraction (Amrita R *et al*., 2010). In the present experiment the microalgal oil extraction was conducted in accordance with little variation in Bligh and Dyer method. The extracted microalgal oil was characteized using High Performace Liquid Chromatography (HPLC) using olive oil as refrence standard. Olive oil has various retention times (1.39, 2.72, 2.86, 3.44, and 3.97). Similarly extracted lipid from Nostoc muscorum showed similar peaks at (1.39, 2.60, 2.80 and 4.3), *Spirulina.sp* at (1.37, 2.64, 2.82, 3.38 and 3.89 ), *Oscillatoria R2* at ( 1.57, 2.60, 2.90, 3.61 and 4.28) and *Nannochloropsis* *oculata* at (1.59, 2.43, 2.59, 2.88 and 4.31) respectively, that indicates the presence of identical fatty acid fractions in standard and the cyanobacterial cultures.

### 5.5 *In situ* transesterification

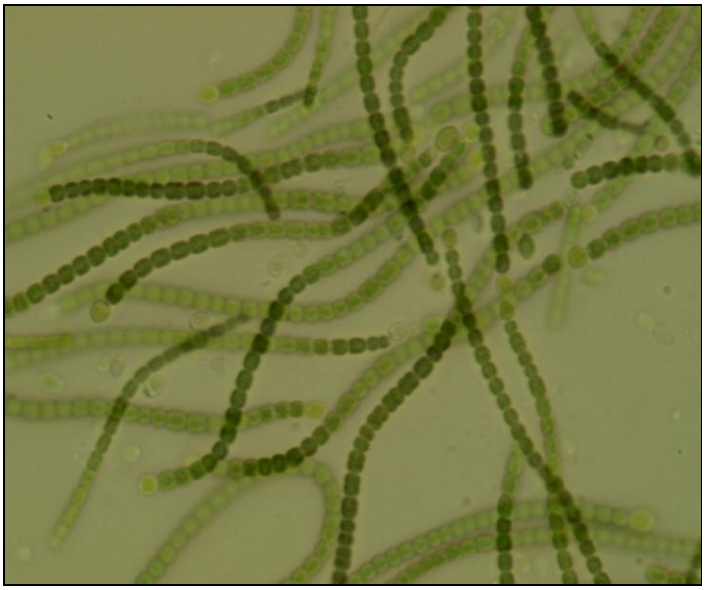
Tranesterification was done directly using algal biomass rather than microalgal oil with least energetic demand without stirring and heating as stated by Ruoyu Xu, 2010. The in situ tranesterification was performed in accordance with protocol given by ruoyu xu, 2010. The layer of biodiesel was observed with pH 7 further characterization is required for confirmation.

# CHAPTER 6

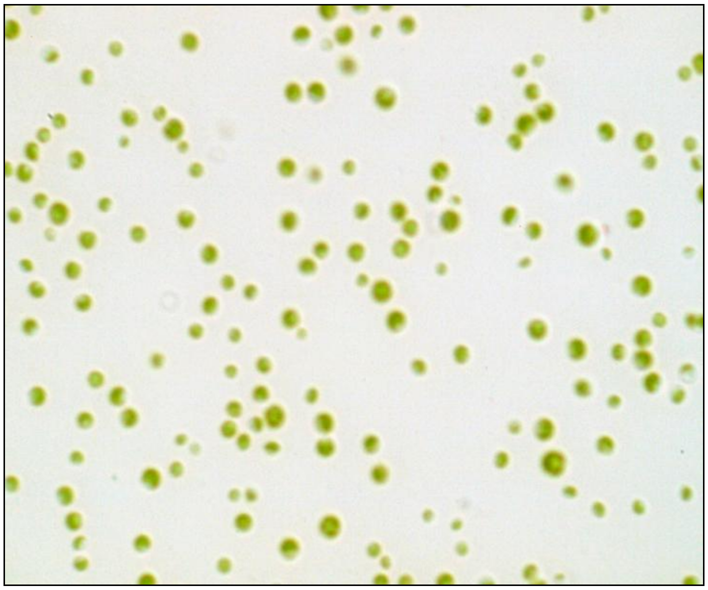
# CONCLUSION

1. Microalgae are considered as second generation biofuel for which microalgae biodiesel must have higher lipid content. *Nannocloropsis oculata* showed rapid growth and was found to contain maximum lipid. *Oscillatoria* R2 also contained comparable lipid out of the nine microalgae tested.
2. The maximum growth in terms of dry biomass as well as wet biomass was observed in *Nannochloropsis oculata* and *Oscillato*ria R2 in lab conditions as well as in open conditions.
3. During mass cultivation of four microalgae maximum increase in lipid content with biomass with regular interval of time was observed in *Nannochloropsis oculata* and minimum was observed in *Oscillatoria.sp* (R2)
4. From the present study it was concluded that the microalgal (*Nannochloropsis oculata, Oscillatoria R2, Nostoc muscorum, Spirulina.sp*) oil contains the fractions similar to that are present in the olive oil. The confirmation of the fact was done by performing high performance liquid chromatography (HPLC).
5. *Insitu* transesterification was also done using algal dry biomass directly. Layer of biodiesel appeared in all the species.

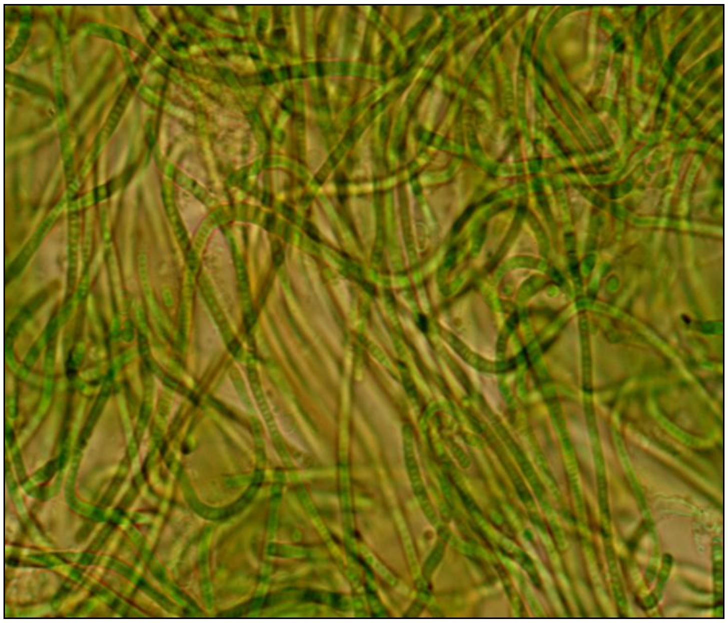
# Photomicrographs



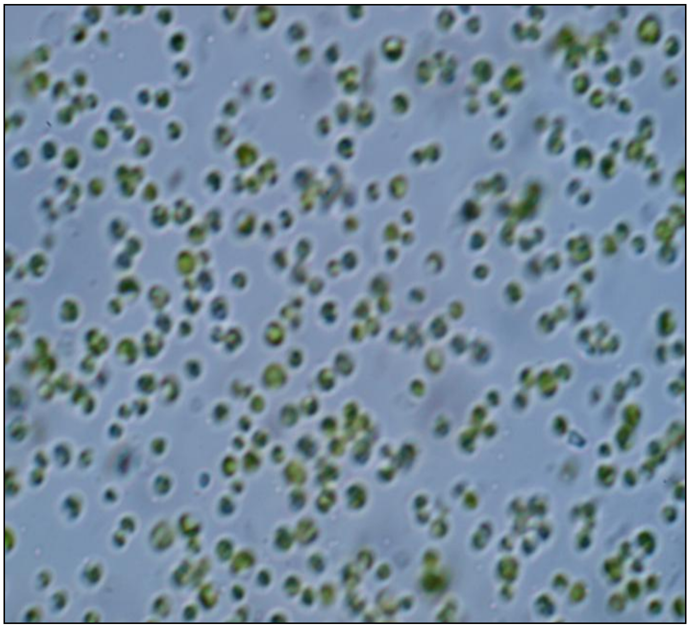
***Nostoc muscorum***



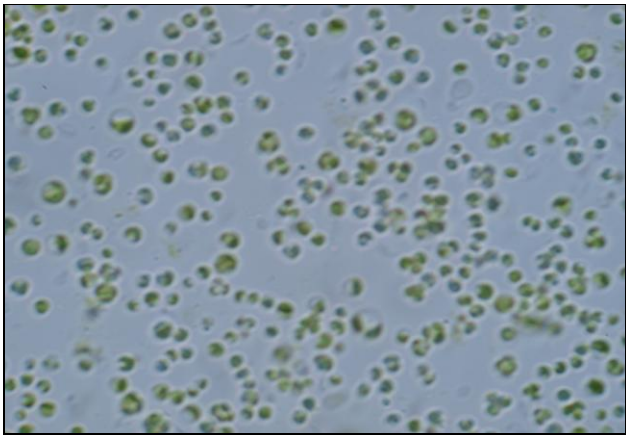
***Chlorella* Cp4**



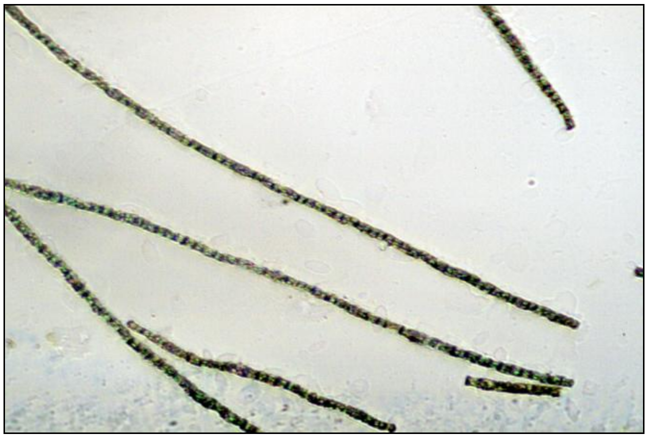
***Oscillatoria* R2**



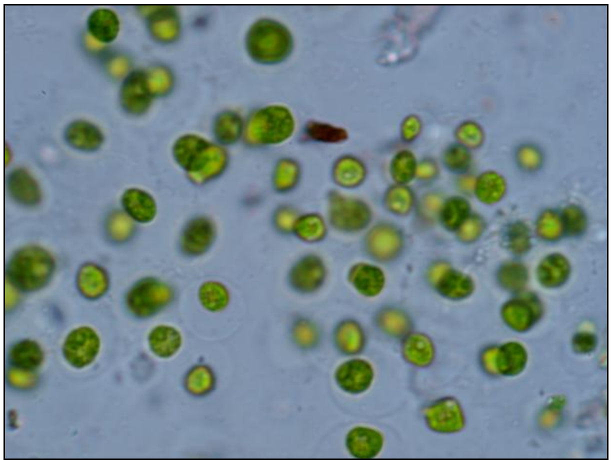
***Botryococcus braunii***



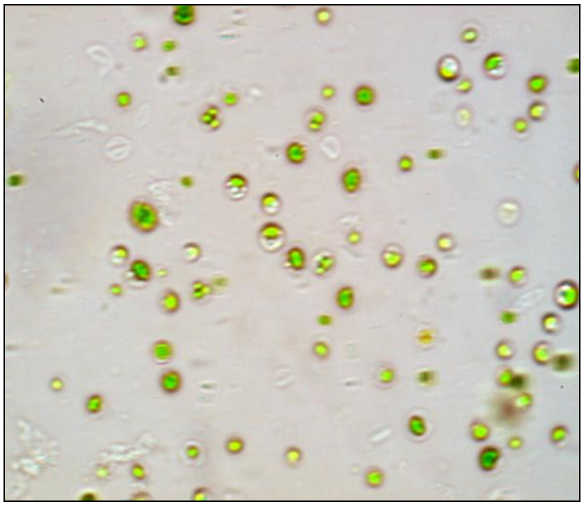
***Scendesmus armatus***



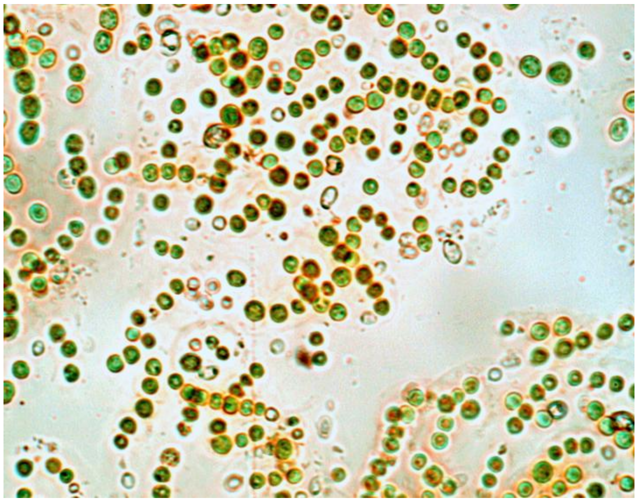
***Spirulina.sp***



***Chlamydomonas moewsii***

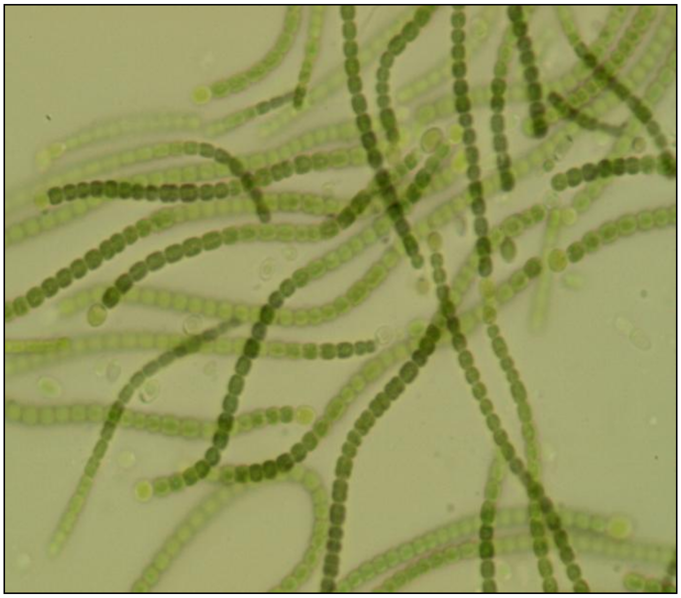


**Nannochloropsis oculata**



**E:** ***Chlorella*** **R5**

## *Nostoc muscorum*



**Before cell disruption**

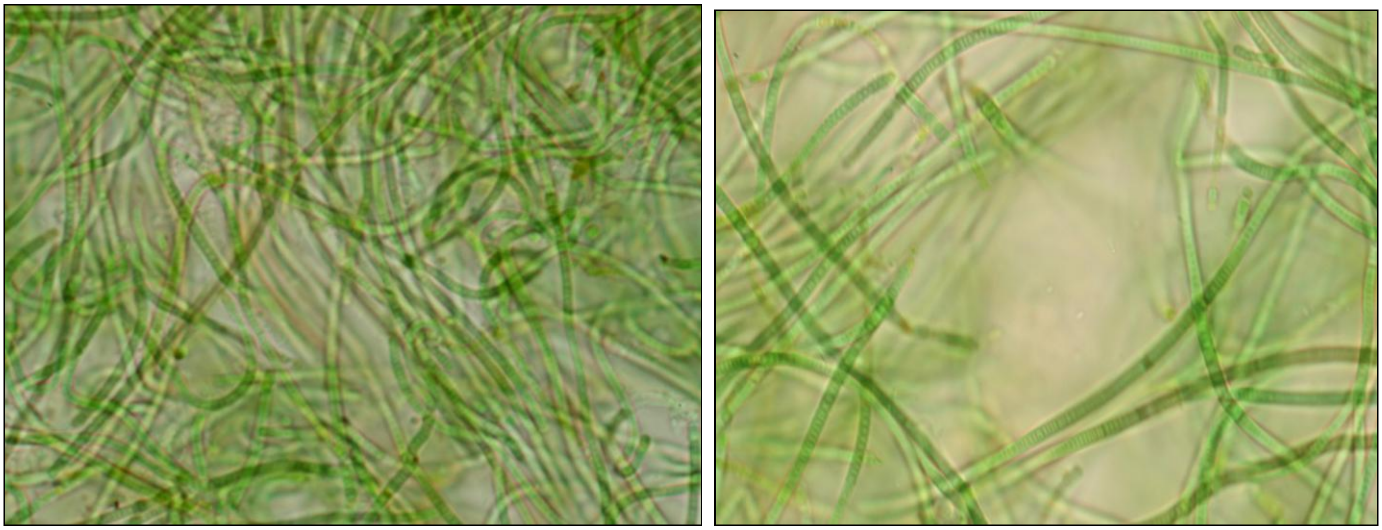
**After cell disruption**

## *Spirulina.sp*

**Before cell disruption**

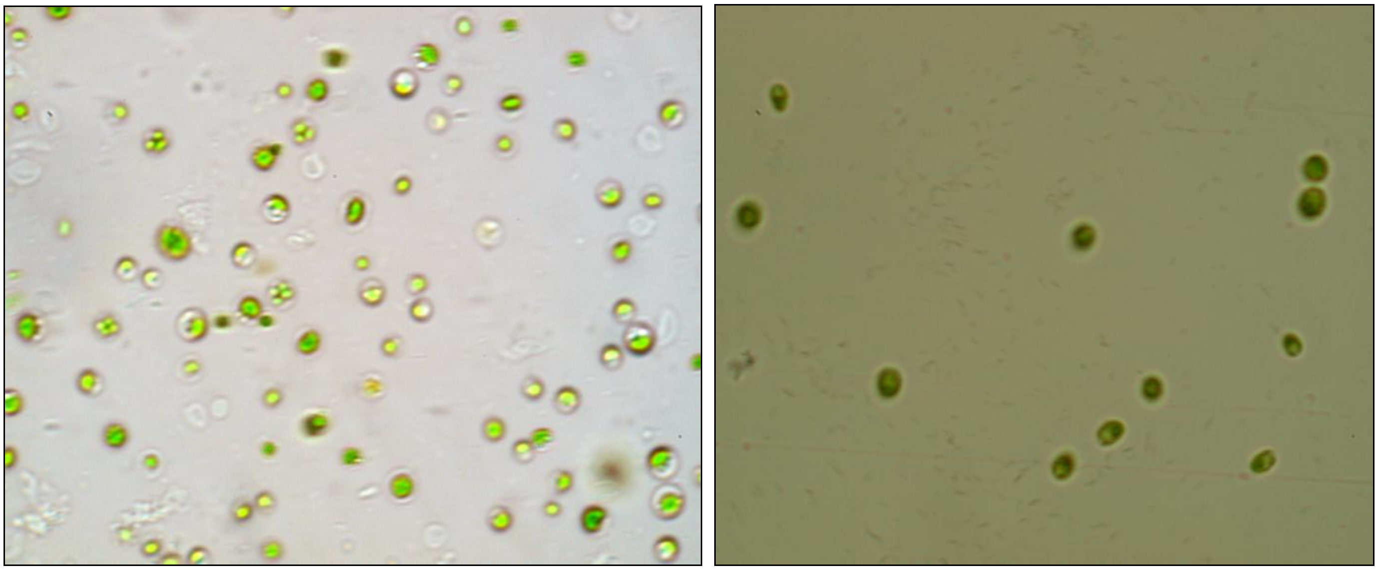
**After cell disruption**

### *Oscillatoria* R2



**Before cell disruption** **After cell disruption**

### *Nannochloropsis oculata*



**Before cell disruption** **After cell disruption**

# CHAPTER 7

## REFRENCES

1. Agarwal A.K. and Das L. M. 2001. "Biodiesel development and characterization for use as a fuel in compression ignition engines." *Journal of Engineering for Gas Turbines and Power-Transactions.* Vol.123 (2): 440-447.
2. Armbrust E.V., Berges J.A., Bowler C., Green B.R., Martinez D., Putnam N.H., Zhou S., Allen A.E., Apt K.E., Bechner M., Brzezinski M.A., Chaal B.K., Chiovitti A., Davis A.K., Demarest M.S., Detter J.C., Glavina T., Goodstein D. and Hadi B. 2007. "Photosynthetic biomass and $H_2$ production by green algae: from bioengineering to bioreactor scale-up." *Physiolgia Plantarum.* Vol.131:10–21.
3. Banerjee, R., Chowdhury A.R., Misra, G., Sudarsanam, G., Verma, S.C., Srivastava, G.S., 2002 "Jatropha seed oils for energy." *Biomass.* Vol.8:277–282.
4. Barnwal B.K. and Sharma M.P. 2005. "Prospects of biodiesel production from vegetables oils in India." *Renewable and Sustainable Energy Review.* Vol.9:363–78.
5. Beer L.L., Boyd E.S., Peters J.W. and Posewitz M.C. 2009. "Engineering algae for biohydrogen and biofuel production." *Current Opinion in Biotechnology.* Vol.20(3): 264-271.
6. Bligh E. G. and Dyer W. M. 1959. "A Rapid Method of Lipid Extraction and Purification." *Candian Journal of Biochemistry and physiology.* Vol.37: 91-111.
7. Boocock D., Konar S., Mao V., Lee C. and Buligan S. 1998. "Fast formation of high-purity methyl esters from vegetable oils." *Journal of the American Oil Chemist's Society.* Vol.75:1167–1172.
8. Borowitzka M.A. 1999. "Pharmaceuticals and agrochemicals from microalgae." *Europeon Journal of Phycology.* Vol.35(3):313–52.
9. Bowler C., Allen A.E. and Badger J.H. 2008. "The Phaeodactylum genome reveals the evolutionary history of diatom genomes." *Nature.* Vol.456(7219): 239-244.
10. Carvalho A.P., Meireles L.A. and Malcata F.X. 2006. "Microalgal reactors: a review of enclosed system designs and performances." *Biotechnology Progres.s* Vol.22:490–506.

**11.** Chisti Y. 2007 “Biodiesel from microalgae.” *Biotechnological Advances.* Vol.25: 294-306.

**12.** Chisti Y. and Moo-Young M. 1989. “On the calculation of shear rate and apparent viscosity in airlift and bubble column bioreactors.” *Biotechnology and Bioengineering.* Vol.34(2):13.

**13.** Chisti, Y. (2008). "Biodiesel from microalgae beats bioethanol." *Trends in Biotechnology*. Vol.26 (3):126-131.

**14.** Courchesne N.M.D., Parisien A., Wang B. and Lan C.Q. 2009. “Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches.” *Journal of Biotechnology*. Vol.141 (1, 2): 31-41.

**15.** Cravatto G., Boffa L., Mantegna S., Perego P., Avogadro M. and Cintas P. 2008. “Improved Extraction of Vegetable Oils under High Intensity Ultrasound and/or Microwaves.Ultrasonication.” *Sonochemistry*. Vol.15:898.

**16.** Demirbas A. 2005. “Biodiesel production from vegetable oils via catalytic and non-catalytic supercritical methanol transesterification methods.” *Progress in Energy and Combustion Science*. Vol.31(5–6):466–87.

**17.** Derelle E., Ferraz C., Rombauts S., Rouze P. and Worden A.Z. 2006. “Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features.” *Proceeding of the National Academy of Science.* Vol.103(31): 11647-11652

**18.** Dunahay T.G., Jarvis E.E., Dais S.S. and Roessler P.G. 1996. “Manipulation of microalgal lipid production using genetic engineering.” *Applied Biochemistry and Biotechnology*. Vol.57–58:223–31.

**19.** Ehimen E., Sun Z. and Carrington C. 2009. “Variables affecting the in situ transesterification of microalgae lipids.” *Fuel.* Vol.89:677–684.

**20.** Felizardo P., Correia M.J.N., Raposo I., Mendes J.F., Berkemeier R. and Bordado J.M. 2006. “Production of biodiesel from waste frying oil.” *Waste Management.* Vol.26(5):487–94.

**21.** Fogg, G. E. 1949. “Growth and heterocyst production in *Lemm. II* in relation to carbon and nitrogen metabolism” *Annales Botanici.* Vol.13: 241- 249.

**22.** Folch J., Lees M. and Stanley G.H.S., 1957. “Lipid extraction from microalgae.” *Journal of Biological Chemistry*. Vol.226: 497-509.

**23.** Fukuda H., Kondo A. and Noda H. 2001. “Biodiesel fuel production by transesterification of oils.” *Journal of Bioscience and Bioengineering.* Vol.92:405–16.

**24.** Gavrilescu M. and Chisti Y., 2005. “Biotechnology- a sustainable alternative for chemical industry” *Biotechnology Advance.s* Vol.23:471-499.

**25.** Ghirardi M.L., Zhang J.P., Lee J.W., Flynn T., Seibert M. and Greenbaum E., 2000. “Microalgae: a green source of renewable $H_2$.” *Trends in Biotechnology*. Vol.18:506–11.

**26.** Gouveia, L. and Oliveira A. C. 2009. "Microalgae as a raw material for biofuels production." *Journal of Industrial Microbiology & Biotechnology*. Vol.36 (2): 269-274.

**27.** Grobbelaar, J. U. 2004 "Algal biotechnology: real opportunities for Africa." *South African Journal of Botany* Vol.70 (1):140-144.

**28.** Haas M., Scott K., Marmer W. and Foglia T. 2004. “In situ alkaline transesterification: an effective method for the production of fatty acid esters from vegetable oils.” *Journal of American Oil Chemist’s Society*. Vol.81:83–89.

**29.** Hankamer B**.**, Lehr F., Rupprecht J., Mussgnug J. H., Posten C**.** and Kruse O.**,** “Photosynthetic biomass and H2 production by green algae: From bioengineering to bioreactor scale-up.” *Physiogial Plantarum.* Vol. 131:10-21.

**30.** Hu Q., Guterman H. and Richmond A. 1996. “A flat inclined modular photobioreactor (FIMP) for outdoor mass cultivation of photoautotrophs.” *Biotechnology and Bioengineering* Vol.51(1): 51–60.

**31.** Kapdan I.K. and Kargi F. 2006. “Biohydrogen production from waste materials.” *Enzyme Microbial Tehnology.* Vol. 38:569-82.

**32.** Khan S.A., Hussain Z., Prasad S. and Banerjee U.C. 2009. “Prospects of biodiesel production from microalgae in India.” *Renewable Sustainable Energy Review*. Vol.13:2361–72.

**33.** Knothe G., Dunn R.O. and Bagby M.O. 1997. “Biodiesel: the use of vegetable oils and their derivatives as alternative diesel fuels.” *ACS Symposium Series.* Vol. 666:172–208.

**34.** Krawczyk T., 1996. “Biodiesel : Alternative fuel makes inroads but hurdles remain.” I*nform.* Vol.7:801-829.

**35.** Kulkarni M.G. and Dalai A.K. 2006. “Waste cooking oil — an economical source for biodiesel: A review.” *Industrial and Engineering Chemistry Research*. Vol. 45:2901–13.

**36.** Lee J., Yoo C., Jun S.Y., Ahn C.Y. and Oh H.M. 2010. “Comparison of several methods for effective lipid extraction from microalgae.” *Bioresource Technology*. Vol.101:S75–S77.

**37.** Lehr F. and Posten C. 2009. “Closed Photo-Bioreactors as Tools for Biofuel Production.” *Current Opinion in Biotechnology*. Vol. 20 (3):280-285.

**38.** León-Bañares R., González-Ballester D., Galváan A. and Fernández E. 2004. “Transgenic microalgae as green cell-factories.” *Trends in Biotechnology.* Vol. 22:45–52

**39.** Mata, T. M., Martins A. A., and Caetano N. S. 2010. “Microalgae for biodiesel production and other applications: A review.” *Renewable & Sustainable Energy Reviews.* Vol. 14(1): 217-232.

**40.** McKinney G. 1941. “Absorption of light by chlorophyll solutions.” *Journal of Biological Chemistry.* Vol. 140: 315-322.

**41.** Mecozzi M., Amici M., Romanelli G., Pietrantonio, E. and Deluca A. 2002. “Ultrasound Extraction and Thin Layer Chromatography Flame Ionization Detection Analysis of Lipid Fraction in Marine Mucilage Sample.” *Journal of Chromatography A.* Vol.963:363.

**42.** Meher L.C., Vidya Sagar D. and Naik S.N. 2006. “Technical aspects of biodiesel production by transesterification — a review.” *Renewable and Sustainable Energy Reviews.* Vol.10:248–68.

**43.** Metting B. and Pyne J.W. 1986. “Biologically-active compounds from microalgae.” *Enzyme Microbial Technology.* Vol. 8:386–94.

**44.** Metting F.B., 1996. “Biodiversity and application of microalgae.” *Journal of Industrial Microbiology.* Vol.17:477–89.

**45.** Molina E. and Fernandez J. 2000. “Tubular photobioreactor design for algal culture.” *Science.* Vol. 306(Issue 5693): 79-86.

**46.** Molina Grima E., 1999. “Microalgae, mass culture methods.” *Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation.* Vol. 3:1753–69.

**47.** Molina Grima E., Acién Fernández F.G., García Camacho F. and Chisti Y. 1999. “Photobioreactors: light regime, mass transfer, and scaleup.” *Journal of Biotechnology.* Vol.70:231–47.

**48.** Molina Grima E., Belarbi E.H., Acién Fernández F.G., Robles Medina A. and Chisti Y. 2003. “Recovery of microalgal biomass and metabolites: process options and economic.” *Biotechnology Advances.* Vol. 20:491–515.

**49.** Molina Grima E., Fernández J., Acién Fernández F.G. and Chisti Y. 2001. “Tubular photobioreactor design for algal cultures.” *Journal of Biotechnology.* Vol. 92: 113–31.

**50.** Nagle N. and Lemke P. 1990. “Production of methyl-ester fuel from microalgae.” *Applied Biochemistry and Biotechnology.* Vol.24 (24-25):355-361.

***51.*** Pernet F. and Tremblay R. 2003. “Effect of ultrasonication and grinding on the determination of lipid class content of microalgae harvested on filters.” *Lipids.* Vol.38:1191–5.

**52.** Ranjan a., Patil C. and Moholkar V.S. 2010. "Mechanistic assessment of microalgal lipid extraction." *Industrial and Engineering Chemistry Research.* Vol.49:2979-2981.

**53.** Ratledge C., 1993. "Single cell oils — have they a biotechnological future?" *Trends in Biotechnology.* Vol.11:278–84.

**54.** Ratledge C. and Wynn J.P. 2002. "The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms." *Advances in Applied Microbiology.* Vol. 51:1–51.

**55.** Richmond A., 2004. "Biological principles of mass cultivation- Handbook of microalgal culture: biotechnology and applied phycology." *Blackwell* 125–77.

**56.** Roessler P.G., Brown L.M., Dunahay T.G., Heacox D.A., Jarvis E.E. and Schneider J.C. 1994. "Genetic-engineering approaches for enhanced production of biodiesel fuel from microalgae." *ACS Symposium Series.*Vol.566:255–70.

**57.** Rosenberg J.N., 2009. "A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution." *Current Opinion in Biotechnology.* Vol. 19:430-436.

**58.** Ruoyu X. and Yongli M., 2010. "Simplifying the process of microalgal biodiesel production through *in situ* transesterification technology." *Journal of American Oil Chemist's Society.* Vol. 88:91–99.

**59.** Sánchez M.A., Contreras G.A., García C.F., Molina G.E. and Chisti Y. 1999. "Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae." *Journal of Biotechnology.* Vol. 70:249–70.

**60.** Sawayama S., Inoue S., Dote Y. and Yokoyama S.Y. 1995. "$CO_2$ fixation and oil production through microalga." *Energy Conversion and Management.* Vol. 36: 729–31.

**61.** Schwab A. W., Bagby M. O. and Freedman B. 1987. "Preparation and properties of diesel fuels from vegetable oils." *Fuel.* Vol. 66:1372–1378.

**62.** Sharma R., Chisti Y. and Banerjee U.C., 2001. "Production, purification, characterization, and applications of lipases." *Biotechnology Advances.* Vol.19: 627–62..

**63.** Shay E.G., 1993. "Diesel fuel from vegetable oils: status and opportunities." *Biomass bioenergy.* Vol. 4: 227-242.

**64.** Silverberg B. A., Stokes P. M. and Ferstenberg L. B., 1976. "Intranuclear Complexes in a Cooper-Tolerant Green Alga." *Journal of Cell Biology.* Vol. 69:210.

**65.** Soxhlet F. 1879. "The Weight Analytic Determination of Milk Fat (in Germany)." *Polytechnisches Journa.* Vol. 232: 461.

**66.** Spolaore P., Joannis-Cassan C., Duran E. and Isambert A. 2006. “Commercial applications of microalgae.” *Journal of Bioscience and Bioengineering.* Vol.101:87–96.

**67.** Stanier R.Y., Kunisawa R., Mandal M. and Cohen B. G., 197.1 “Purification and properties of unicellular BGA (order *croococcales*) bacteroid.” B*iological Reviews.* Vol. 35 : 171-205.

**68.** Stuart A.S., Davey M.P., Dennis J.S., Horst I., Howe C.J.,Smith D.J.L. and Smith A.G. 2010. “Biodiesel from microalgae: challenges and propects.” *Current Opinion in Biotechnology*. Vol.21:277-286.

**69.** Terry K.L. and Raymond L.P. 1985. “System design for the autotrophic production of microalgae” *Enzyme Microbial Technology*. Vol. 7:474–87.

**70.** Tredici M.R. 1999. “Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation.” Vol.1:395–419.

**71.** Van Gerpen J., 2005. “ Biodiesel processing and production.” *Fuel Process Technology*. Vol.86:1097–107.

**72.** Vasudevan P. T. and M. Briggs. 2008. “Biodiesel production current state of the art and challenges.” *Journal of Industrial Microbiology and Biotechnology*. Vol.35:421-430.

**73.** Vimalarasan A., Pratheeba N., Ashok K.B., Sivakumar N. and Varalakshmi P. 2011. “Production of biodiesel from *cyanobacteria* by alkali and enzyme mediated transesterification” J*ournal of Industrial and Scientific Research.* Vol.70:959-962.

**74.** Virot M., Tomao V., Ginies C., Visinoni F. and Chemat F. 2008. “Microwave Integrated Extraction of Total Fats and Oils.” *Journal of Chromatography A.* Vol. 57:1196-1197.

**75.** Yun Y.S., Lee S.B., Park J.M., Lee C.I. and Yang J.W. 1997. “Carbon dioxide fixation by algal cultivation using wastewater nutrients.” *Journal of Chemical Technology and Biotechnology*. Vol. 69:451–5.

**76.** Zhang Y., Dube M.A., Mclean D.D. and Kates M. 2003. “Biodiesel production from waste cooking oil- Process design and technological assessment.” *Bioresource Technology.* Vol.89(1):1–16.