Lipid estimation of different algal species

A

DISSERTATION REPORT

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

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CERTIFICATE

This is to certify that the thesis entitled "**Lipid estimation of different algal species**" submitted by Radhika Tanwar in partial fulfilment of the requirements for the award of degree of Master of Science in Microbiology to Thapar University, Patiala is a record of the students own work carried out by her under her 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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I hereby declare that the work presented in the dissertation entitled **"Lipid estimation of different algal species"** in partial fulfilment of the requirement for the award of the degree of Masters in Microbiology, 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 January 2013 to June 2013, 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.

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LIST OF ABBREVIATIONS

BGA	Blue green algae
DW	Distilled water
g	Grams
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
$HClO_4$	Perchloric acid
Kg	Kilogram
K_2HPO_4	Dipotassium hydrogen phosphate
$K_2Cr_2O_7$	Potassium dichromate
L	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

ABSTRACT

In the present investigation eight different microalgae were screened for growth and lipid content grown under both indoor and outdoor conditions. Three heterocystous, filamentous cyanobacteria *Nostoc muscorum* (ARM 442), *Anabaena variabilis* (ARM 441), *Tolypothrix tenuis* (ARM 443) were selected along with *Spirulina sp.* and other microalgae such as *Nannochloropsis oculata, Botryococcus braunii, Scenedesmus armatus* and Isolate no. 5 *(PS 1).* To maximise lipid extraction, the algal cells were homogenised and ultrasonicated. Overall it was observed that all the algal cultures showed increase in biomass and lipid content with increase in time interval of 7 days respectively. *Nostoc muscorum* (ARM 442) showed highest lipid content 0.47 mg/ml with increase in wet biomass from 0.16 mg/ml as observed on 0 day to 0.43 mg/ml on 28th day and increase of dry biomass from 0.03 mg/ml on 0 day to 0.15 mg/ml on 28th day under lab conditions respectively, whereas lipid content in *Spirulina sp.* when cultivated under open conditions, showed increase in lipid content from 0.02 mg/ml on 0 day to 0.17 mg/ml on 28th day in 0.1 ml organic layer, 0.05 mg/ml on 0 day to 0.35 mg/ml on 28th day , 0.12 mg/ml on 0 day to 0.57 on 28th day, with the increase in wet biomass from 0.23mg/ml on 0 day to 0.55 mg/ml on 28th day and dry biomass from 0.05 mg/ml on 0 day to 0.19 mg/ml on 28th day respectively. Overall chlorophyll content in both open and closed conditions was observed to be maximum in *Spirulina sp.* by (0.5µg/ml) in open and (1.47 µg/ml) in closed conditions. Microalgae were disrupted by homogenization and ultrasonication to release lipids. Maximum lipids were released when cells were homogenized and further ultrasonicated. *In situ* tranesterification was done for making biodiesel. *Spirulina sp.* (0.4 mg/ml) appeared to be potential source for biodiesel production followed by *Nannochloropsis oculata* (0.4 mg/ml) and *Botryococcus braunii* (0.24 mg/ml) as they contain high lipid content and grow rapidly in indoor and outdoor conditions.

1. INTRODUCTION

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions. The algae used for biodiesel production are usually unicellular and double its biomass in less than 24 hours under good conditions and green algae consist of around 50% lipid content (Christi, 2007; Schneider, 2006). This high yield, high density biomass is ideal for intensive agriculture and may be an excellent source for biodiesel production. Microalgal biomass contains approximately 50% carbon by dry weight (Mirón *et al*., 2003). All of this carbon is typically derived from carbon dioxide. Producing 100 t of algal biomass fixes roughly 183 t of carbon dioxide. Biodiesel production can potentially use some of the carbon dioxide that is released in power plants by burning fossil fuels (Sawayama *et al*., 1995; Yun *et al*., 1997). Ideally, microalgal biodiesel would be carbon neutral, as all the power needed for producing and processing the algae would come from biodiesel itself and from methane produced by anaerobic digestion of biomass residue left behind after the oils have been extracted. Large-scale production of microalgal biomass generally uses continuous culture during daylight. In this method of operation, fresh culture medium is fed at a constant rate and the same quantity of microalgal broth is withdrawn continuously (Grima *et al*., 1999). As much as 25% of the biomass produced during daylight, may be lost during the night because of respiration. The extent of this loss depends on the light level under which the biomass was grown, the growth temperature, and the temperature at night.

All of the current annual production of vegetable oils and waste fats, if dedicated to fuel production, would come close to meeting these targets but that demand creates competition between food and fuel. If algal feedstock can be developed that are cost effective with high oil content, they can play a significant role in meeting these alternative fuel objectives (Krawczyk, 1996). 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). 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 has been investigated (Shay, 1993). 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). Producing microalgal biomass is generally more expensive than growing crops.

Currently, algal biofuel production has not been commercialized due to high production cost. This is mainly due to the production methods used to grow and harvest the algae, rather than the reaction methods of creating the biofuel, which are the same as when using traditional plant oils (Eriksen, 2008). Indeed, microalgal oil costs considerably more than soybean oil currently, mainly due to the existing infrastructure for terrestrial crops and the lack of investment in large scale culture of aquatic microalgae (Eriksen, 2008). In the present investigation eight different microalgae were screened for lipid content and its extraction.

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2. LITERATURE REVIEW

2.1 Microalgae as a source of biodiesel

The idea of using microalgae as a source of fuel is not new (Chisti, 1980–81; Nagle and Lemke,1990;Sawayama *et al*., 1995), but it is now being taken seriously because of the escalating price of petroleum and more significantly, the emerging concern about global warming that is associated with burning fossil fuels (Gavrilescu and Chisti, 2005). Biodiesel is produced currently from plant and animal oils, but not from microalgae. But several companies are attempting to commercialize the production of biodiesel from microalgae. Various sources for commercial biodiesel production are soya beans, canola oil, animal fat, palm oil, corn oil, waste cooking oil (Felizardo *et al*., 2006; Kulkarni and Dalai, 2006) and jatropha oil (Barnwal and Sharma, 2005). Unlike other oil crops, microalgae grow extremely rapidly and many are exceedingly rich in oil. Microalgae commonly double their biomass within 24 hrs. It has been estimated that 0.53 billion m^3 of biodiesel would be needed to replace current US transportation consumption of all petroleum fuels (Christi, 2007). Neither waste oil nor seed oil can come close to meeting the requirement for that much fuel; therefore, if biodiesel is to become a true replacement for petroleum, a more productive source of oil is needed (Scott and Bryner, 2006; Christi, 2007). Biodiesel belongs to the second generation biofuels. The advent of second generation biofuels is intended to produce fuels from lignocellulosic biomass, the woody part of plants that do not compete with food production. Sources include agricultural residues, forest harvesting residues or wood processing waste such as leaves, straw or wood chips as well as the non-edible components of corn or sugarcane (Brennan L., *et al*, 2010).

2.2 Second generation biofuel systems

Algal biomass contains three main components: carbohydrates, protein and lipids/natural oil. Because the bulk of the natural oil made by microalgae is in the form of triacylglycerides which is the right kind of oil for producing biodiesel, microalgae is the exclusive focus in the algae-to-biofuel arena (Danielo, 2005).

Microalgae	**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 1: Oil content of some microalgae (Chisti, 2007)

It has been reported that microalgae grow quickly as compared to terrestrial crops. They commonly double every 24 h. During the peak growth phase, some microalgae can double every 3.5 hours (Chisti 2007). Oil contents of microalgae are usually between 20-50% (dry weight) as shown in Table 1, while some strains can reach as high as 80% (Metting 1996; Spolaore *et al*. 2006).

A range of second generation biofuel-production systems are more water-efficient and require much less arable land. Of particular interest are lignocellulosic technologies and microalgae (Chisti, 2007; Hankamer, *et al.*, 2007; Schaub, *et al.*, 2007). Microalgae are already reported to produce 15-300 times more oil for biodiesel production than traditional crops on an area basis. Furthermore, compared with conventional crop plants which are usually harvested once or twice a year, microalgae have very short harvesting cycle(1-10 days depending on the process), allowing multiple or continuous harvests with significantly increased yields. Microalgae can be cultivated as an integrated concept with wastewater treatment to optimize the energetic and financial input for the production process. Greater light capture and conversion efficiencies ultimately lead to reduced fertilizer and nutrient inputs.

2.3 Potential of microalgal biodiesel

Unlike other oils crops, microalgae grow extremely rapidly and many are exceedingly rich in oil. These are sunlight-driven cell factories that convert CO_2 to potential biofuels, foods, feeds and high-value bioactives (Spolarore *et al.,* 2006). Microalgae can provide several different types of renewable biofuels. These include methane produced by anaerobic digestion of algal biomass, biodiesel derived from microalgal oil and photosythetically produced biohydogen. The idea of using microalgae as a source of fuel is not new but it is now being taken seriously because of the escaping price of petroleum and more significantly the emerging concern about global warming that is associated with burning fossil fuels (Belarbi *et al.,* 2000). Microalgae commonly double their biomass within 24 h and biomass doubling time during exponential growth may be as short as 3.5 h. Oil content in microalgae can exceed 80%by weight of dry biomass. Oil levels of 20-50% are quite common and production of methyl ester or biodiesel from microalgal oil (Belarbi *et al.,* 2000).

2.4 Microalgae having high lipid content

High lipid productivity of dominant, fast-growing algae is a major prerequisite for commercial production of microalgal oil-derived biodiesel. However, under optimal growth conditions, large amounts of algal biomass are produced, but with relatively low lipid contents, while species with high lipid contents are typically slow growing (Sharma. *et al*, 2012**).** Lipids produced by microalgae generally include neutral lipids, polar lipids, wax esters, sterols and hydrocarbons, as well as prenyl derivatives such as tocopherols, carotenoids, terpenes, quinines and pyrrole derivatives such as the chlorophylls. Lipids produced by microalgae can be grouped into two categories, storage lipids (non-polar lipids) and structural lipids (polar lipids). Storage lipids are mainly in the form of TAG made of predominately saturated FAs and some unsaturated FAs which can be transesterified to produce biodiesel. Structural lipids typically have a high content of polyunsaturated fatty acids (PUFAs), which are also essential nutrients for aquatic animals and humans (Sharma. *et al*, 2012). According to the Solar Energy Research Institute (SERI) rapport, the most promising species for fuel production are *Botryococcus braunii* due to its important quantities of hydrocarbons, *Nannochloropsis salina* with its high quantities of ester fuel production and

Dunaliella salina due to its high quantities of fatty acids (Feinberg, 1984). The National Renewable Energy Laboratory (NREL) in United States affirms that *Spirulina*, *Dunaliella*, *Scenedesmus*, and *Chlorella* are the most popular strains that have been produced at commercial or large scale (>0.1 ha).

One who has grown microalgae under laboratory or outdoor condition is well aware of the fact that to obtain high lipid content, external stress or lipid induction techniques need to be applied. Many microalgae produce saturated and unsaturated fatty acids naturally under ideal growth conditions, which have high nutritional value, but are less ideal for biofuels (Miao, X., *et al, 2006;* Hu, Q., *et al*, 2008).

Crop	**Oil yield (L/ha)**	**Land area needed (M ha)[a]**	**Per cent 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 2: Comparison of some sources of biodiesel (Chisti, 2007)

2.5 Microalgae for biodiesel production

Several study suggested that microalgae have long been a topic of interest for liquid fuel production. This is due to many factors, including their high lipid content ability to grow on non arable land and/or salt water, their fast rate of growth, and the fact that they do not compete with food crops (Baum, 1994).

2.5.1 Biofuels

Algae can be used to generate energy in several ways. One of the most efficient ways is through the utilization of the algal oils to produce biodiesel. Some algae can produce hydrogen gas under specialized growth conditions. The biomass from alga can also be burned, similar to wood, to generate heat and electricity. Compared with terrestrial crops, which take an entire season to grow and contain a maximum of about 5% dry weight of oil, microalgae grow quickly and contain high oil content (Chisti, 2007). This is where microalgae come into play in producing biodiesel. Some species of microalgae produce long-chain fatty acids that are useful as jet fuel, known as Jet A or

Jet A-1 fuels. Indeed, a Virginia Atlantic Boeing 747-400 made a test flight in February 2008 using a blend of 20% biofuel. The U.S. Department of Defence (DOD) is sponsoring research in the creation of JP-8 jet fuel from algae. Several other studies say that 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 (Das *et al.,* 2001, Zhang *et al,* 2003; Dube *et al.*2003). It is a biodegradable and nontoxic substance, and so spills are not nearly the concern as when dealing with petroleum fuels. If biodiesel is produced from photosynthetic sources, the effect of carbon dioxide produced from biodiesel combustion is minimal on the greenhouse gas (GHG) effect. Its higher flash point over petroleum diesel makes it safer to transport, and its extensive lubrication properties can extend engine life when used in conventional diesel engines (Das *et al.,* 2001, Zhang *et al,* 2003; Dube *et al.* 2003).

2.5.2 Potential of using microalgae as biodiesel feedstock

Microalgae have the capability to be an alternative to petroleum. The National Renewable Energy Laboratory (NREL) has categorized approximately 300 species of microalgae that could be potential fuel sources (Dedyukhina *et al,* 2006). Microscopic algae are the most efficient photosynthetic organisms on the planet; their fast growth rate makes them an ideal feedstock. Microalgae biomass productivity has been estimated to be 50 times that of switch grass, the world's fastest growing terrestrial plant (Demirbas, 2009). According to the NREL's Aquatic Species Program (ASP) Close-Out Report, microalgae have yields vastly exceeding terrestrial plants because they are unicellular, and their cultivation in a liquid environment allows them better access to resources (Patil *et al,* 2008). The conclusion of the close-out report, although recommending other steps to reduce costs associated with producing algae, stated that microalgae are capable of "synthesizing 30 times more oil per hectare than the terrestrial plants used for the fabrication of biofuels" (Sheehan *et al,* 1998). It is important to note that this estimation was with the use of ponds to cultivate the algae, which require much more surface area for equal biomass production than any of the other growth methods discussed previously. A newer report states that microalgal farming can potentially be more cost effective than conventional farming (Li *et al,* 2008). However, this recent study also pointed out the high harvest and drying costs due to the low cell concentrations, but claims that these will be overcome via technology development.

Under heterotrophic growth and genetic modification, microalgae appeared to produce even higher percentages of lipid and at a growth rate making it viable for biodiesel production (Xu, Miao et al, 2006). In addition, manipulations of medium composition such as using N- deficient medium can also increase lipid production (Gouveia et al, 009).

2.5.3 Biodiesel production from microalgal biomass

Once the oils have been extracted, then they are reacted with methanol and a catalyst, and the process for the creation of biodiesel is the same as when using vegetable oil. If direct methanolysis (transesterification using methanol prior to extraction) is performed, then filtering and washing steps are required to remove the destroyed algal tissue from the biodiesel (Chisti, 2007). Oil is extracted from dry algal biomass through a number of ways. The least expensive extraction is simply through cold pressing. Up to 70% of the oil contained within the algae can be extracted this way (Danielo, 2005). Use of organic solvents increases the extraction level to 99%, but there is an increase in the cost of processing. Using direct transesterification allows for a single step process that extracts algal oil and reacts with methanol to make biodiesel.

2.6 Large scale production of microalgae

Mass production of microalgae is much more costly than oil crops. Because they are photosynthetic, they need a light source, carbon dioxide, water, and inorganic salts. The water temperature should be between 15°C and 30°C (approximately 60°F to 80°F) for optimal growth. The growth medium must contribute the inorganic elements that help make up the algal cell, such as nitrogen, phosphorus, iron, and sometimes silicon (Grobbelaar, 2004). For large-scale production of microalgae, algal cells are continuously mixed to prevent the algal biomass from settling (Grima *et al*, 1999) and nutrients are provided during daylight hours when the algae are reproducing. However, up to one-quarter of algal biomass produced during the day can be lost through respiration during the night (Chisti, 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. Two common methods for large scale production of microalgal biomass are raceway ponds (Terry and Raymond, 1985; Molina, 1999) and the closed photobioreactors (Molina *et al*., 2003; Tredici, 1999; Sanchez *et al*, 1999).

2.6.1 Raceway ponds

Open ponds cost less to build and operate than enclosed photobioreactors; this culture system has its intrinsic disadvantages. Because they are open-air systems, they often experience a lot of water loss due to evaporation. Thus, open ponds do not allow microalgae to use carbon dioxide as efficiently, and biomass production is limited (Chisti, 2007). Biomass productivity is also limited by contamination with unwanted algal species as well as organisms that feed on algae. In addition, optimal culture conditions are difficult to maintain in open ponds, and recovering the biomass from such a dilute culture is expensive (Grima *et al*, 1999).

2.6.2 Photobioreactors

Photobioreactors have been successfully used for producing large quantities of microalgal biomass (Grima *et al*., 1999; Tredici, 1999; Pulz, 2001; Carvalho *et al*., 2006). Enclosed photobioreactors have been employed to overcome the contamination and evaporation problems encountered in open ponds (Grima *et al*, 1999).

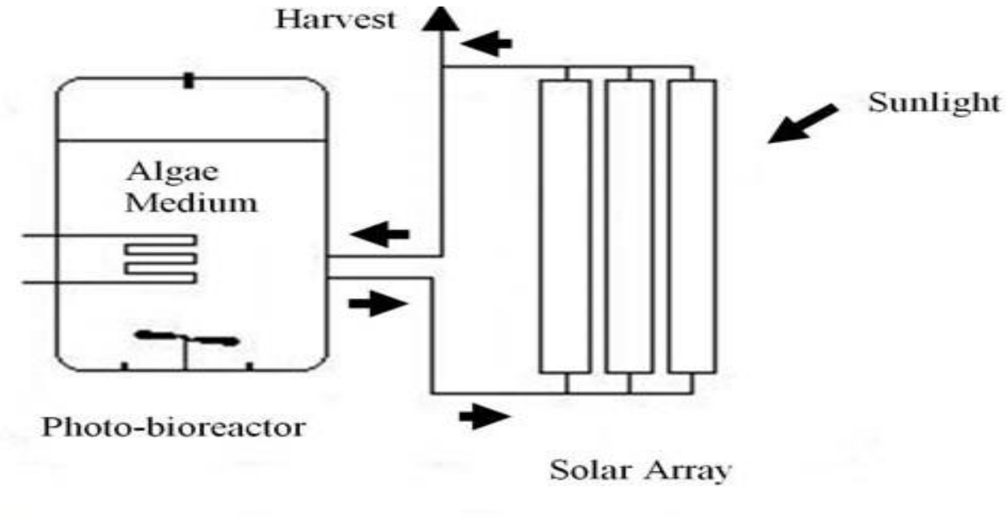


Figure 1: A simplified photo-bioreactor design (Modified from Christi, 2007).

2.7 Transesterification

Transesterification is an equilibrium reaction that requires 3 moles of alcohol for each mole of triglyceride to produce 1 mole of glycerol and 3 moles of methyl esters. Industrial processes use 6 moles of methanol for each mole of triglyceride (Fukuda *et al*, 2001). This large excess of methanol ensures that the reaction is driven in the direction of methyl esters, i.e. towards biodiesel. Yield of methyl esters exceeds 98% on a weight basis (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).

2.8 Current limitations for algal biodiesel production

The major limitation for algal biodiesel is the production cost and energy input required while producing and harvesting the microalgal biomass. Other studies reported due to high moisture content of microalgae drying before using it for energy production involves great energy usage (Sawayama *et al*, 2005).While the academic world is still debating the cost factor, some companies such as Aurora Biofuels ®, ValcentInc®, and Solazyme® are turning algae into biofuel; at a profit. However, both of these companies are doing it on a small scale; it remains to be seen how influential algae will be in the future of transportation fuels, and bio-energy overall (Danielo, 2005). Another limitation in the use of algal biomass is the extraction of lipids from the cells before it can undergo transesterification into biodiesel. As mentioned before, algal tissue is much different from animal tissue, for which the Bligh and Dyer (1959) method was developed. Research has reported that the lipid in algae is more difficult to extract with these methods. While direct transesterification methods have existed for years, there is little research into extraction or direct transesterification of algal biomass. Direct transesterification using *Botryococcus braunii* yielded highest lipid content when extracted with bead beater and chloroform (Yoon *et al.,* 1998).

Some biofuel companies

- ADM olmuhle Hamburg, part of Archer Daniels Midland
- Germany Dieseter industry – part of Bunge limited, France
- Blue marbel energy, Seattle Washington, USA
- Chremec Stockholm, Sweden
- Dupont Danisco, USA
- Solazyme, South San Francisco California, USA

3. MATERIALS AND METHOD

Different algal cultures *Spirulina sp., Nostoc muscorum* (ARM 442), *Anabaena variabilis* (ARM 441), *Tolypothrix tenuis* (ARM 443) were procured from National Centre for Conservation and Utilisation of Blue Green Algae, Division of Microbiology, IARI, New Delhi. The heterocystous algae like *Scenedesmus armatus, Botryococcus braunii* were maintained and grown routinely in batch culture in BG-11 media (Stanier *et al.*, 1971). *Spirulina sp.* was grown in CFTRI media. *Nannochloropsis oculata* was grown in Fogg's media (Fogg, 1949). Compositions of different media used for growth of algae are given below:

Composition of CFTRI media

CONSTITUENTS	g/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	1.0 ml

Micronutrient solution

The composition of A5 micronutrient solution is as follows:

Composition of micronutrient solution

CONSTITUENTS	g/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

The medium was prepared using double distilled water and the pH was maintained in the range of 9.0. All the growth media, solutions were sterilized by autoclaving at 121˚C temperature at 15 psi for 15 minutes.

Culture methods

The heterocystous and non – heterocystous cyanobacterial cultures were maintained and grown in BG – 11 medium (-N) and BG – 11 (+N) respectively.

BG – 11 medium (+N)

Nutrient media BG – 11 (+N)

CONSTITUENTS	**g/L**
1. Sodium nitrate	1.5
2. Di– Potassium 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

The trace metal mix contains the following constituents in g/L.

Composition of trace metal mixture

CONSTITUENTS	**g/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

The medium was prepared in the double distilled water and the pH was kept in range of 7.1 – 7.3 for the optimal growth of the culture. The growth media was sterilized in the autoclave at 121˚C temperature at 15 psi for 15 minutes. For BG -11 (-N) Sodium nitrate is not added.

Composition of Fogg's media

CONSTITUENTS	**g/L**
1. Potassium 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.

Fe-EDTA solution composition

CONSTITUENTS	**g/L**
1. EDTA	26.1
2. 1N Potassium 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).

3.1 Growth Studies

3.1.1 Dry biomass estimation of microalgae (Richmond and Grobbelaar, 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.

3.1.2 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 30 minutes, 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 8000 rpm 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 = $2.55 \times 10^{-2}.E_{650} + 0.4 \text{ x} 10^{-2}.E_{665}$mg/ml

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

E_{665} = absorbance at 665nm wavelength

3.2 Harvesting of algal biomass

Algal biomass was harvested after 23 – 25 days of incubation when thick sheath of algae was observed. Biomass obtained were dried and crushed to powder using mortar and pestle. Further dried algal powder was used for lipid analysis and extraction.

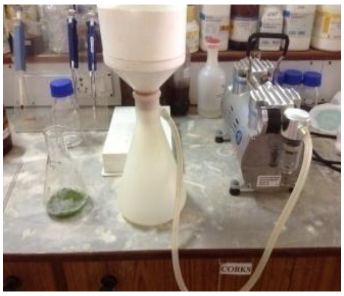


Fig 2: Filtration to obtain algal biomass

Fig 3: Algal biomass collection

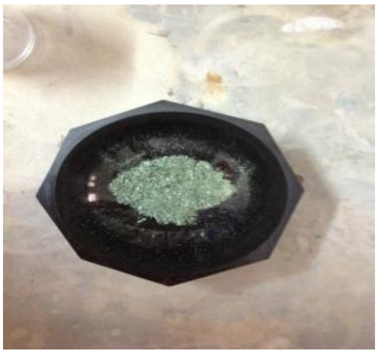


Fig 4: Air drying

Fig 5: Powdered form obtained after crushing

3.2.1 Cell disruption with tissue homogeniser: Tissue homogeniser (50ml) was used for random disruption of algal cells in which 10ml of algal culture was taken and homogenised manually for 15 to 20 minutes.

3.2.2 Cell disruption with ultrasonicator

Ultrasonicator was used for disruption of algal cells by breaking 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.

Fig 6: Ultrasonicator

3.3 Mass cultivation of algal cultures

The mass cultivation of algal cultures was carried out in plastic tubs in open conditions and in large carboys (10L) in closed conditions. The tubs 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 *Botrycoccus braunii, Spirulina sp., Nannochloropsis oculata* were inoculated in each tub followed by addition of few drops of Malathion to prevent insect breeding in the tubs. 8 – 10 L of BG 11 media was prepared in large carboys, inoculated and kept in growth room at temperature ±28˚C and lux intensity of 3000. Regular shaking and observation was done for 0, 7, 14, 21 and 28 days to observe the growth parameters of microalgae like pH, temperature, chlorophyll content, lipid content and biomass.

Figure 7: Algal cultures grown in tubs

3.3.1 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 1 L, 2 L and 5 L flasks and after the microalgae have attained adequate growth the flasks were transferred to polyhouse.

Fig 8: Algal cultures

3.3.2 Large scale cultivation of microalgae in tubs and large carboys

Large scale production of algae carried out by transferring cultures from 5L flasks to plastic tubs and large carboys. A pinch of $MgSO_4$ and K_2HPO_4 was added before the inoculation of algae. Few drops of Malathion were added to prevent insect breeding. Inoculation was done in plastic tubs in open conditions and large carboys in closed conditions for mass production.

Fig 9: Algal cultures grown in tubs and large carboys

3.4 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 2.5g of $K_2Cr_2O_7$ in 1L 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, discarded the supernatant. Placed the tube containing cell pellets on ice and added 10 ml of ice cold 0.2 N $HClO_4$. Thoroughly resuspended 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. Resuspend the pellet from $HClO_4$ extraction. Resuspend the pellet and allow the suspension to stand for 5 minutes 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. Shake the solutions for 5 minutes to mix well and centrifuged 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 capped with Teflon lined caps. Placed all tubes in a boiling water bath for 45 minutes.

9. Shake the tubes 2 or 3 times during the heating. Cooled the tubes, removed 1ml from each, diluted 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.

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	0.021

0.1	0.044
0.2	0.102
0.3	0.141

Table 3: Standard curve of palmitic acid

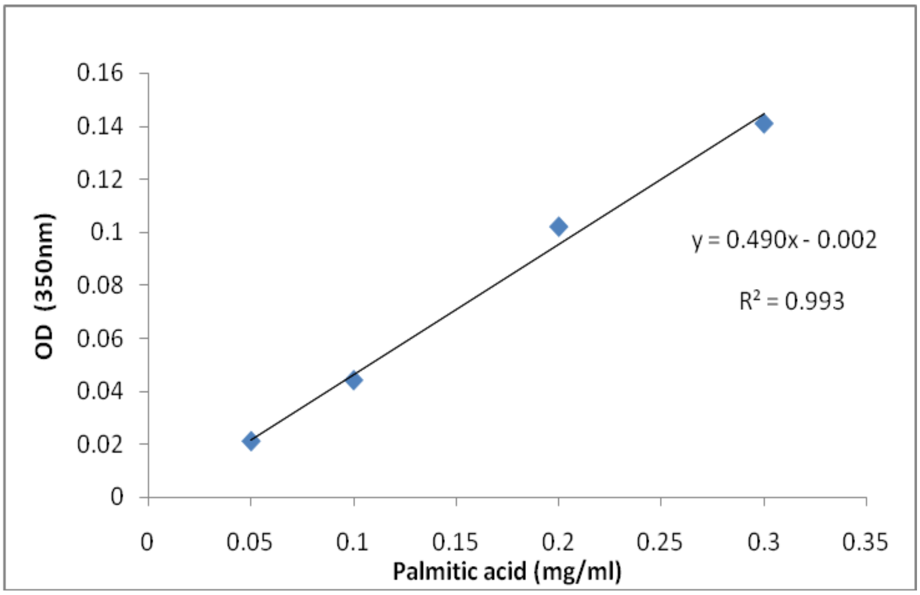


Figure 10: Standard curve of palmitic acid

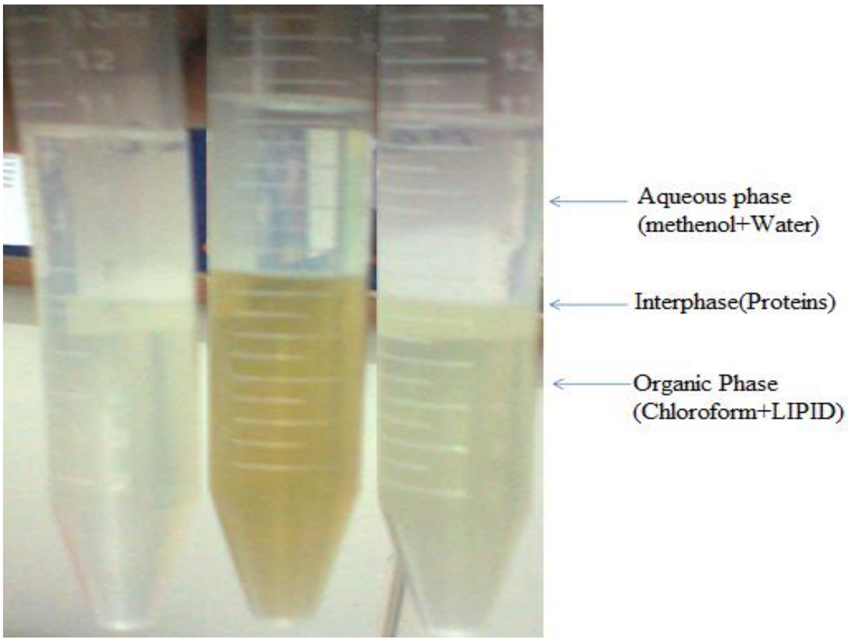


Fig 11: Different layers formed during lipid estimation of algal cultures

3.5 *In Situ* Transesterification

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

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

3. Continuous stirring was done for 1 minute to homogenise the system.

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

5. After an hour, 0.0878g hydrochloric acid was added to stop the reaction equal to approximately 200µl of concentrated HCl having specific gravity of 1.18.

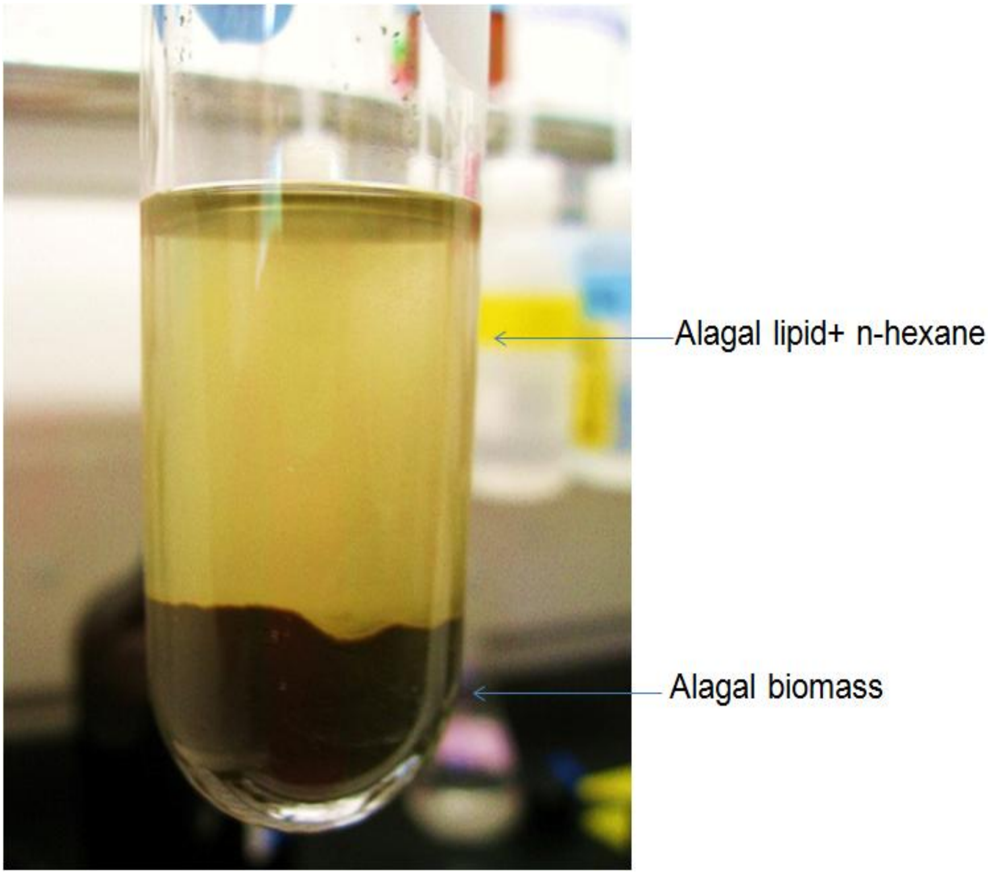


Fig 12: Lipid and solvent layer formed during transesterification of *Spirulina sp*.

4. RESULTS

In the present investigation eight different microalgae were screened for growth and lipid content grown under both indoor and outdoor conditions. Three heterocystous, filamentous cyanobacteria *Nostoc muscorum* (ARM 442), *Anabaena variabilis* (ARM 441), *Tolypothrix tenuis* (ARM 443) were selected along with *Spirulina sp.* and other microalgae such as *Nannochloropsis oculata, Botryococcus braunii, Scenedesmus armatus* and Isolate no. 5 *(PS 1).* Growing algal cultures were checked for pH, temperature and water level. To maximise lipid extraction, the algal cells were homogenised and ultrasonicated**.**

4.2 Lipid estimation of different microalgal cultures

Lipid estimation done in eight algal species showed that *Nostoc muscorum* (ARM 442) had highest lipid content of 0.47 mg/ml followed by *Scenedesmus armatus* (0.3 mg/ml), whereas, the least amount of lipid was observed in *Anabaena variabilis* (ARM 441) (0.06 mg/ml). The lipid content in *Tolypothrix tenuis* (ARM 443)*, Spirulina sp., Nannochloropsis oculata, Botryococcus braunii,* Isolate no. 5 *(PS1),* was observed to be 0.07, 0.09, 0.13, 0.15, 0.25 mg/ml respectively. Among the heterocystous algal cultures the highest lipid content was observed in *Nostoc muscorum* (ARM 442) i.e. 0.47 mg/ml and the least lipid content was observed in *Anabaena variabilis* (ARM 441) (0.06 mg/ml) **(Table 4; Fig 13).**

Algal cultures	**Lipid (mg/ml)**		
	0.1ml	**0.2 ml**	**0.5ml**
Anabaena variabilis (ARM 441)	0.02±0.01	0.03±0.02	0.06±0.03
Tolypothrix tenuis (ARM 443)	0.03±0.01	0.05±0.01	0.07±0.03
Isolate no. 5 *(PS 1)*	0.09±0.03	0.19±0.02	0.25±0.02
Nostoc muscorum (ARM 442)	0.3±0.02	0.45±0.02	0.47±0.03

Table 4: Lipid estimation in heterocystous algal cultures

Among the non – heterocystous algal cultures the highest lipid content was observed in *Scenedesmus armatus* (0.3 mg/ml) followed by *Botryococcus braunii* (0.15 mg/ml) and the least lipid content was observed in *Spirulina sp.,* i.e. 0.09 mg/ml. *Scenedesmus* sp. subjected to nitrogen or phosphorus limitation showed an increase in lipids as high as 30% and 53%, respectively(Xin,*et al*.,2010).*Nannochloropsis oculata* subjected to nitrogen limitation showed an increase in lipid content as high as 15.31% (Converti *et al,* 2009).In *Botryococcus braunii,* increased temperature resulted in a decrease of the relative content of unsaturated intracellular fatty acids (Sushchik *et al*, 2003) **(Table 5; Fig 14).**

Algal cultures	**Lipid (mg/ml)**		
	0.1ml	**0.2ml**	**0.5ml**
Nannochloropsis oculata	0.06±0.01	0.10±0.01	0.13±0.03
Botryococcus braunii	0.08±0.01	0.1±0.03	0.15±0.03
Spirulina sp.	0.05±0.01	0.07±0.01	0.09±0.02
Scenedesmus armatus	0.1±0.02	0.2±0.03	0.3±0.02

Table 5: Lipid estimation in Non – heterocystous algal culture

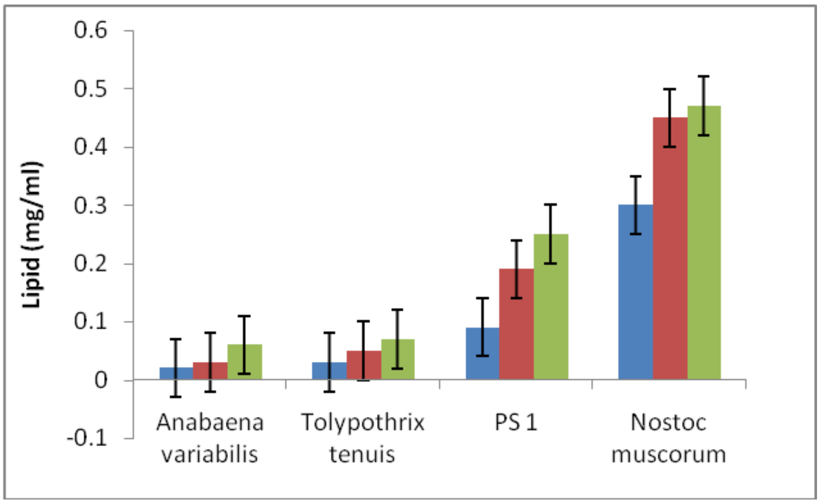


Fig 13: Lipid estimation in heterocystous algal cultures

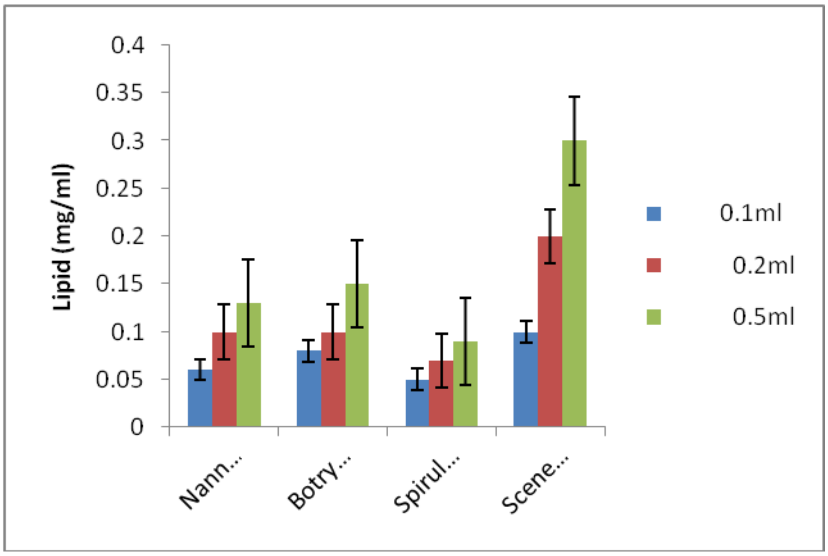


Fig 14: Lipid estimation in Non –heterocystous algal cultures

4.3 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.

4.3.1 Lipid estimation in algal cells disrupted by tissue homogenizer

Table 6 shows lipid content in microalgae obtained after disruption with tissue homogenizer. Maximum lipid content was observed in *Nannochloropsis oculata* 0.15 mg/ml and *Botryococcus braunii* contained the least lipid content (0.05 mg/ml). The average lipid content is 0.11 mg/ml. The lipid content in *Spirulina sp.* was observed to be 0.14 mg /ml **(Fig 15)**. The increase in average lipid content shows the disruption of algal cells and release of lipid droplets.

Algal cultures	Lipid (mg/ml)		
	0.1 ml	**0.2 ml**	**0.5 ml**
Spirulina sp.	0.03±0.02	0.04±0.02	0.14±0.03
Nannochloropsis oculata	0.05±0.03	0.11±0.02	0.15±0.02
Botryococcus braunii	0.006±0.002	0.02±0.01	0.05±0.02

Table 6: Lipid estimation in algae after disrupting with tissue homogenizer

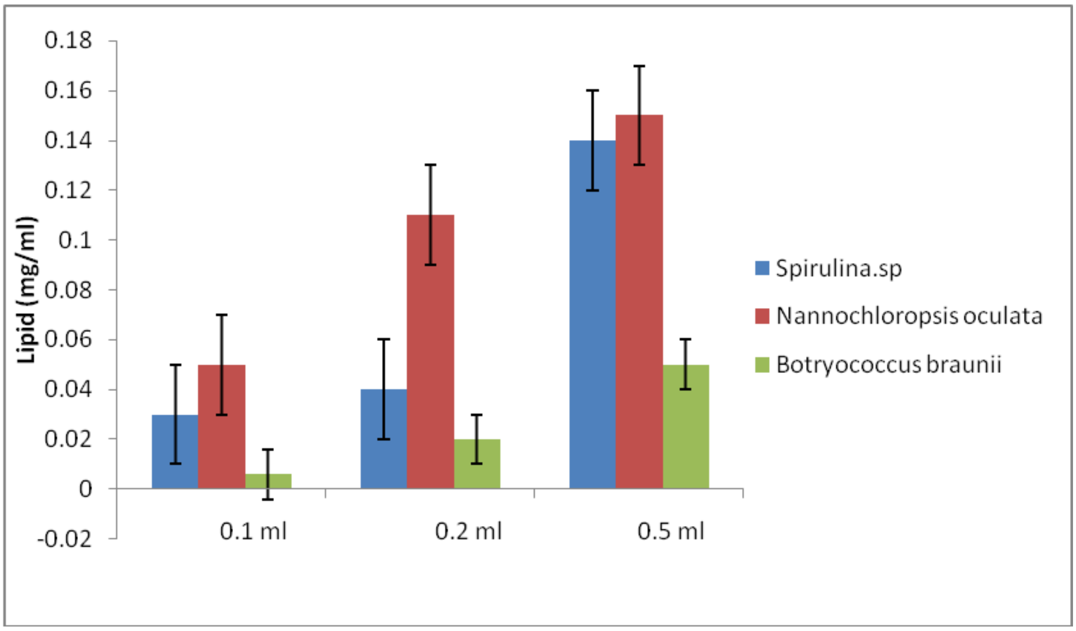


Fig 15: Lipid estimation in algae after disrupting with tissue homogenizer

4.3.2 Lipid estimation of algal cells disrupted by using ultrasonicator

Table 7 shows the lipid content in microalgae after ultrasonication. The lipid content in *Nannochloropsis oculata, Spirulina sp., Botryococcus braunii* was 0.19 mg/ml, 0.15mg/ml, 0.07 mg/ml respectively. Hence ultrasonication method of homogenisation is reported for greater release of lipids as observed in all the three microalgal cultures. The highest lipid content after ultrasonication was observed in *Nannochloropsis oculata* (0.19 mg/ml) and the least lipid content was observed in *Botryococcus braunii* (0.07 mg/ml) **(Fig 16)**. The lipid estimation of algal cells disrupted by ultrasonicator shows the following trend:

Botryococcus braunii <Spirulina sp. <Nannochloropsis oculata

Algal cultures	**Lipid (mg/ml)**		
	0.1 ml	**0.2 ml**	**0.5 ml**
Spirulina sp.	0.02±0.01	0.07±0.02	0.15±0.02
Nannochloropsis oculata	0.04±0.02	0.06±0.03	0.19±0.03
Botryococcus braunii	0.03±0.02	0.06±0.02	0.07±0.06

Table 7: Lipid estimation after ultrasonication

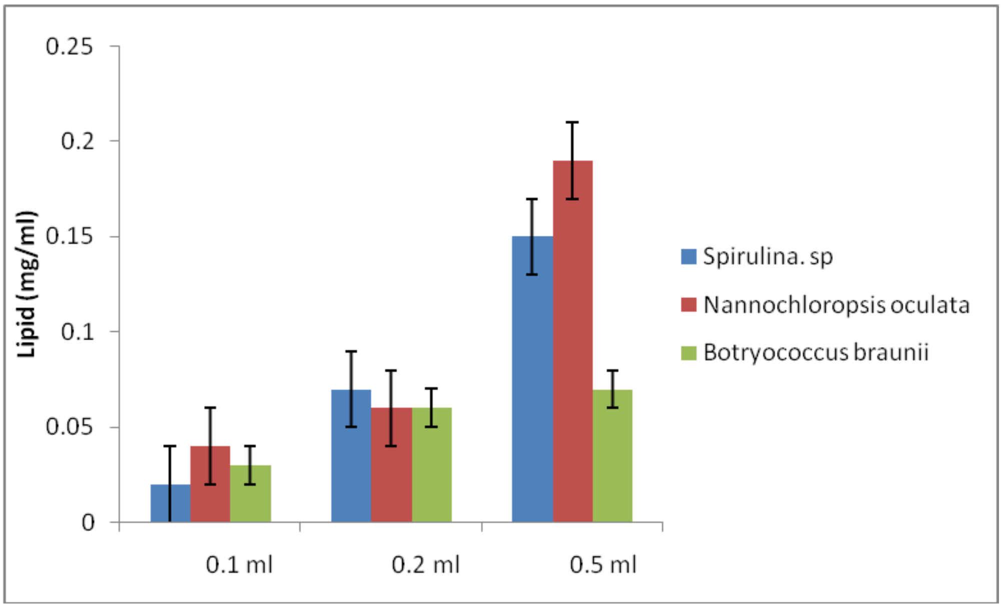


Fig 16: Lipid estimation after ultrasonication

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

Table 8 shows the lipid content in microalgae after tissue homogenization as well as ultrasonication. *Nannochloropsis oculata* contains maximum lipid content (0.21mg/ml) followed by *Spirulina sp.* (0.2 mg/ml). The minimum lipid content was observed in *Botryococcus braunii* (0.16 mg/ml) **(Fig 17)**. The increase in lipid content shows more disruption of cell takes place when both disruption techniques are used in combination.

Algal cultures	**Lipid (mg/ml)**		
	0.1 ml	**0.2 ml**	**0.5 ml**
Spirulina sp.	0.09±0.02	0.14±0.02	0.2±0.02
Nannochloropsis oculata	0.02±0.01	0.09±0.02	0.21±0.02
Botryococcus braunii	0.006±0.002	0.05±0.03	0.16±0.03

Table8: Lipid estimation after treatment with tissue homogeniser and ultrasonicator

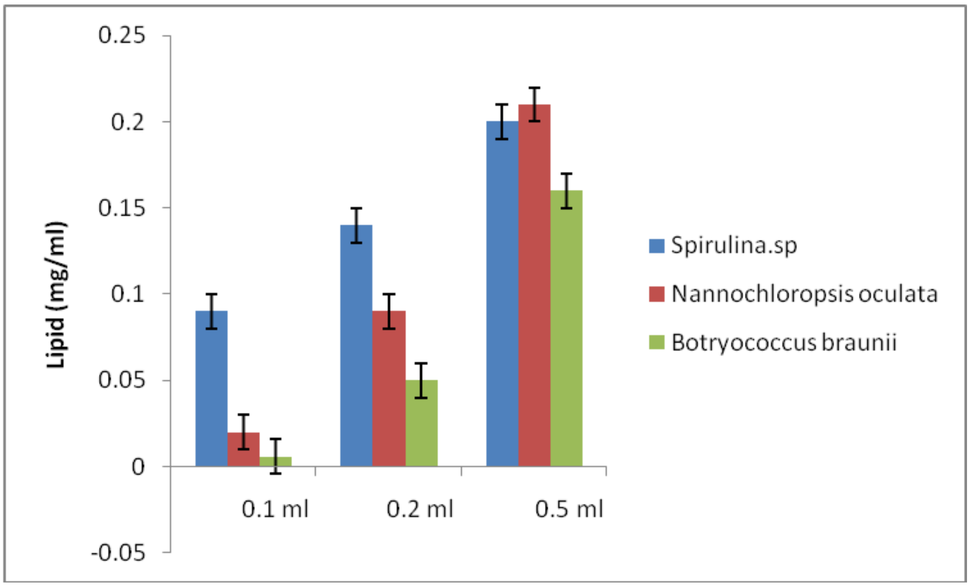


Fig 17: Lipid estimation after treatment with tissue homogeniser and ultrasonicator

4.4 Chlorophyll estimation in microalgae

Chlorophyll (µg/ml) was measured after 28 days of incubation. Maximum chlorophyll was observed in *Nannochloropsis oculata* i.e. 1.41 µg/ml and minimum was observed in Isolate no.5 *(PS 1)* i.e. 0.21µg/ml **(Table 9; Fig 18)**. The order was:

Scenedesmus armatus **<** **Isolate no.5** **<** ***Spirulina sp.*** **<** ***Tolypothrix tenuis*** **(ARM 443) <** ***Botryococcus braunii*** **<** ***Nostoc muscorum*** **(ARM 442) <** ***Anabaena variabilis*** **(ARM 441) <** ***Nannochloropsis oculata.***

Algal cultures	R1(µg/ml)	R2(µg/ml)	R3(µg/ml)	Mean±SD
Anabaena variabilis (ARM 441)	1.01	0.51	0.58	0.7±0.27
Tolypothrix tenuis (ARM 443)	0.4	0.21	0.43	0.34±0.12
Nannochloropsis oculata	1.44	1.38	1.42	1.41±0.03
Botryococcus braunii	0.44	0.46	0.67	0.52±0.13
Isolate no.5 *(PS 1)*	0.15	0.18	0.30	0.21±0.08
Spirulina sp.	0.24	0.26	0.27	0.25±0.01
Nostoc muscorum (ARM 442)	0.57	0.66	0.66	0.63±0.05
Scenedesmus armatus	0.02	0.03	0.05	0.03±0.01

Table 9: Chlorophyll content of different algae after 28 days of incubation

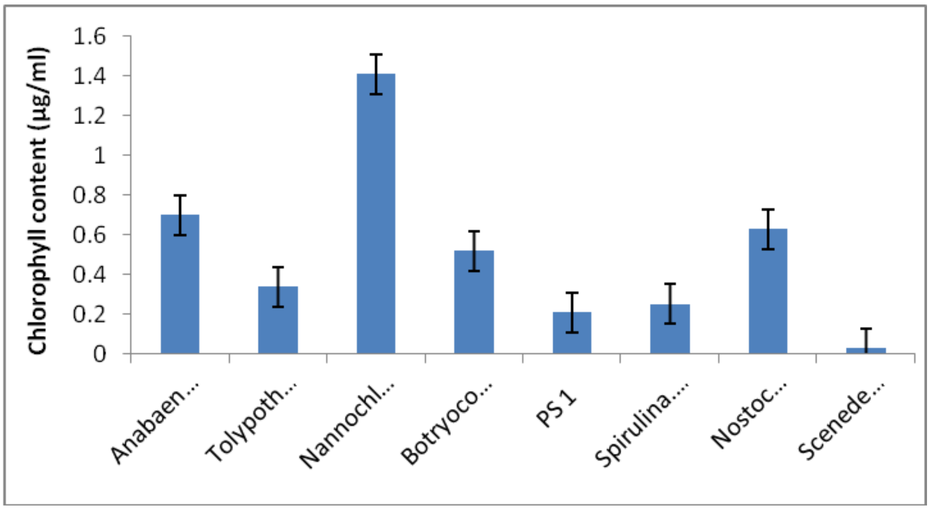


Fig 18: Chlorophyll estimation in algal cultures

4.5 Biomass estimation

Wet biomass estimation (Lab scale)

Growth of *Nannochloropsis oculata, Botryococcus braunii, Scenedesmus armatus, Nostoc muscorum* (ARM 442) and *Spirulina sp.*was measured at lab scale in terms of wet biomass in mg/ml. The peak growth up to 28 days was observed at weekly intervals for each culture. Maximum growth was observed in *Nostoc muscorum* (ARM 442) of 0.43 mg/ml followed by *Nannochloropsis oculata* i.e. 0.40mg/ml. The least growth was observed in *Scenedesmus armatus* of 0.21 mg/ml. The wet biomass of *Botryococcus braunii* and *Spirulina sp.* was observed to be 0.35

mg/ml and 0.34 mg/ml **(Table 10; Fig 19)**. The wet biomass of these microalgae shows the following trend in increasing order:

***Scenedesmus armatus* < *Spirulina sp.* < *Botryococcus braunii* < *Nannochloropsis oculata* < *Nostoc muscorum* (ARM 442).**

Wet biomass (mg/ml)					
Algal cultures	**0 day**	**7 days**	**14 days**	**21 days**	**28 days**
Nannochloropsis oculata	0.14±0.10	0.23±0.05	0.29±0.06	0.34±0.01	0.40±0.20
Botryococcus braunii	0.09±0.07	0.16±0.03	0.23±0.10	0.29±0.02	0.35±0.01
Scenedesmus armatus	0.02±0.50	0.09±0.10	0.11±0.20	0.13±0.60	0.21±0.10
Nostoc muscorum (ARM 442)	0.16±0.20	0.22±0.02	0.28±0.01	0.36±0.02	0.43±0.05
Spirulina sp.	0.12±0.20	0.18±0.01	0.21±0.02	0.24±0.002	0.34±0.70

Table 10: Wet biomass estimation at lab scale of different microalgae

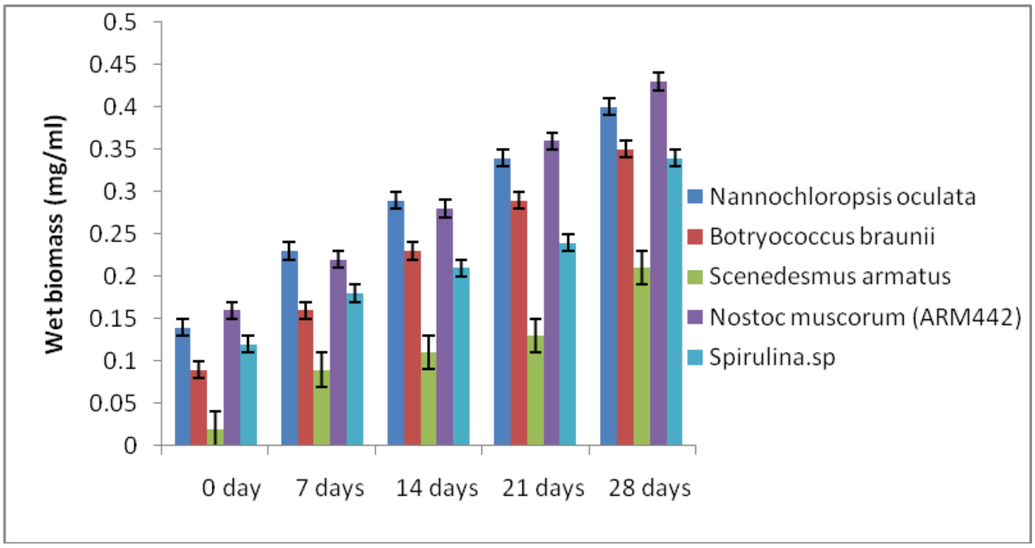


Fig 19: Wet biomass estimation at lab scale of different microalgae.

4.5.2 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 *Nostoc muscorum* (ARM 442) i.e. 0.15mg/ml whereas minimum growth was observed in *Scenedesmus armatus* i.e. 0.1mg/ml. The growth observed in *Nannochloropsis oculata* is 0.13 mg/ml followed by *Botryococcus braunii* (0.12 mg/ml) and finally *Spirulina sp.* (0.11 mg/ml) **(Table 11; Fig 20)**.

Dry biomass of different algae was in the order:

Scenedesmus armatus **<** ***Spirulina sp.*** **<** ***Botryococcus braunii*** **<** ***Nannochloropsis oculata*** **<** ***Nostoc muscorum*** **(ARM 442).**

Table 11: Dry biomass of different microalgae at lab scale

Dry biomass (mg/ml)					
Algal cultures	**0 day**	**7 days**	**14 days**	**21 days**	**28 days**
Nannochloropsis oculata	0.01±0.2	0.05±0.01	0.08±0.07	0.11±0.01	0.13±0.02
Botryococcus braunii	0.02±0.07	0.06±0.03	0.09±0.1	0.1±0.03	0.12±0.07
Scenedesmus armatus	0.009±0.1	0.01±0.2	0.03±0.5	0.09±0.1	0.10±0.01
Nostoc muscorum (ARM 442)	0.03±0.01	0.05±0.1	0.06±0.02	0.09±0.04	0.15±0.05
Spirulina sp.	0.02±0.03	0.04±0.01	0.05±0.07	0.07±0.04	0.11±0.06

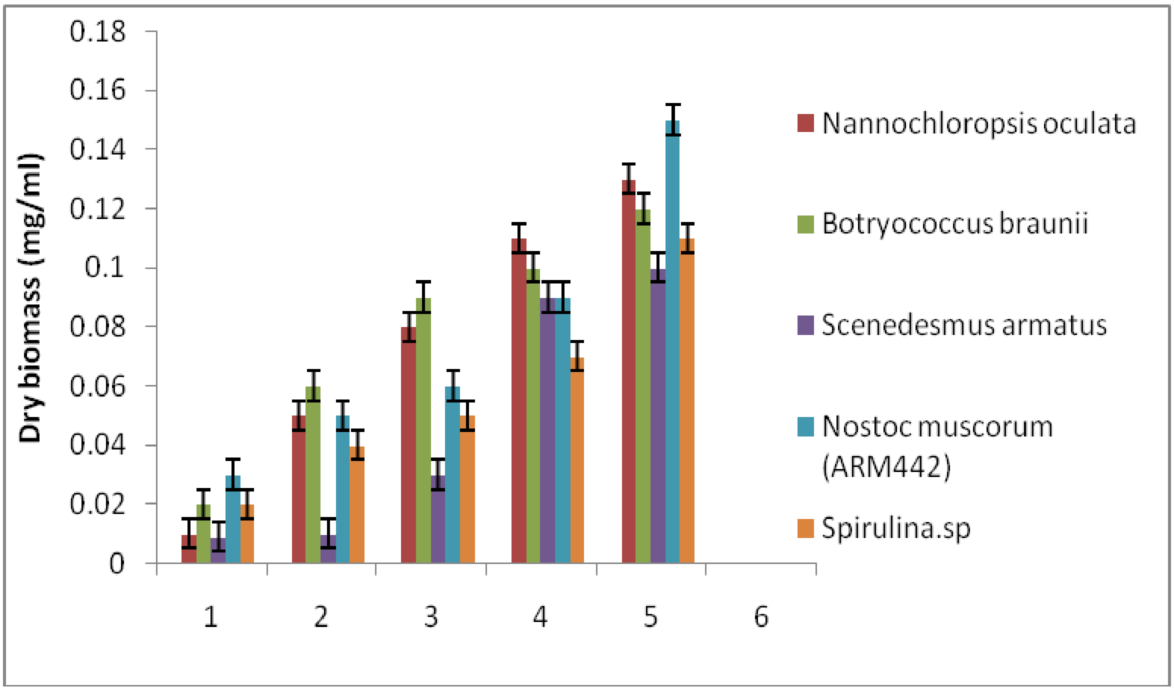


Fig 20: Dry biomass of different microalgae at lab scale

Table 12: Chlorophyll estimation at regular interval of time (lab conditions)

Algal cultures	**Chlorophyll (µg/ml)**				
	0 day	**7 days**	**14 days**	**21 days**	**28 days**
Nannochloropsis oculata	0.11±0.02	0.23±0.01	0.29±0.3	0.31±0.03	0.49±0.04
Botryococcus braunii	0.08±0.1	0.18±0.01	0.36±0.2	0.38±0.07	0.45±0.5
Nostoc muscorum (ARM 442)	0.17±0.03	0.23±0.04	0.49±0.06	0.77±0.3	1.06±0.1
Scenedesmus armatus	0.13±0.07	0.19±0.03	0.35±0.1	0.42±0.02	0.61±0.06
Spirulina sp.	0.22±0.01	0.36±0.2	0.67±0.04	0.79±0.03	1.47±0.02

At different intervals chlorophyll content of microalgae was measured. With time the chlorophyll content of microalgae increased as the biomass of microalgae was increased. Maximum chlorophyll content was found in *Spirulina sp.* (1.47µg/ml). The least chlorophyll content was observed in *Botryococcus braunii* (0.45µg/ml). The chlorophyll content in *Nannochloropsis oculata* was observed to be 0.49 µg/ml followed by *Scenedesmus armatus* (0.61 µg/ ml) and *Nostoc muscorum* (ARM 442) (1.06µg/ml) **(Table 12; Fig 21)**.

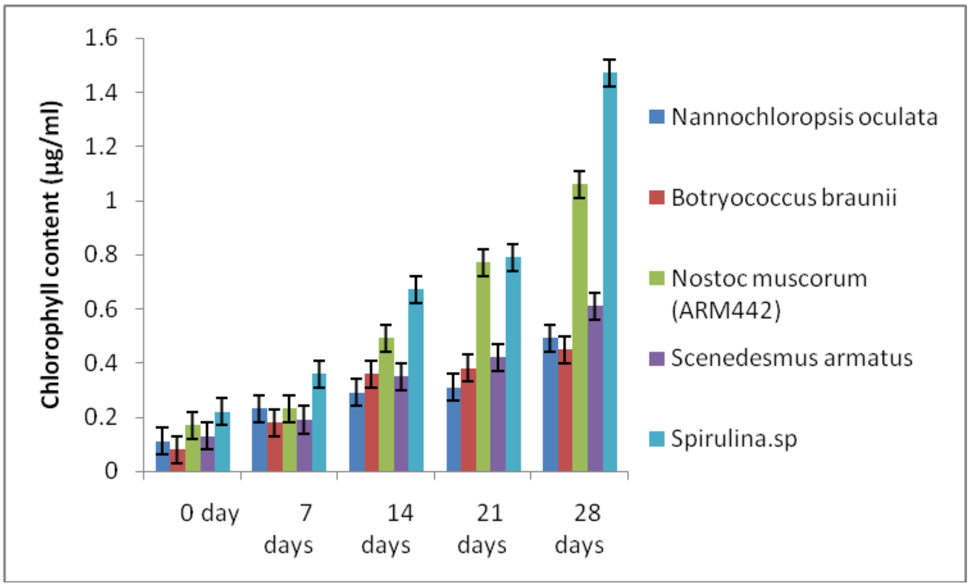


Fig 21: Chlorophyll estimation at regular interval of time (lab conditions)

4.6 Growth studies of microalgae in open conditions

It was noted that in tubs inoculated with *Botryococcus braunii* the pH ranged from 7.5-8.5, whereas in *Spirulina sp.* it was 8.5-9.0 and in *Nannochloropsis oculata* pH ranged from 7.0 – 7.5.The water temperature in each tub was in the range 20°C-31°C and water level was between 3-4 inches. It is generally recognized that microalgal cell sizes are inversely proportional to their growth rate, which increases with temperature in a certain range (Atkinson *et al*., 2003; Sayegh and Montagnes, 2011). Low temperatures usually reduce enzymatic activity, which causes a decrease in the growth rate (Davison, 1991) **(Table 13)**.

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 *Spirulina sp.* from 0.16 mg/ml on day 0 to 0.47 mg/ml on day 28. Similarly *Botryococcus braunii* showed 0.23 mg/ml on day 0 to 0.55 mg/ml on day 28 respectively. *Nannochloropsis oculata* showed 0.18 mg/ ml on day 0 to 0.43 mg/ml on day 28. The highest growth was observed in *Spirulina sp.* (0.55 mg/ml) and the least growth was observed in *Nannochloropsis oculata* (0.43 mg/ml) **(Table 14; Fig 22)**.

Variation of pH in tubs					
Algal cultures	**Incubation time in days**				
	0 day	**7 days**	**14 days**	**21 days**	**28 days**
Spirulina sp.	8.5	8.5	8.7	8.8	9.0
Botryococcus braunii	7.5	7.7	8.0	8.3	8.5
Nannochloropsis oculata	7.0	7.1	7.3	7.4	7.5
Variation of temperature (˚C) in tubs					
Spirulina sp.	20	22	24	26	27
Botryococcus braunii	26	26	29	30	31
Nannochloropsis oculata	25	27	28	29	30
Variation of water level (in inches) in tubs					
Spirulina sp.	4	3.9	3.7	3.5	3.2
Botryococcus braunii	4	3.7	3.5	3.3	3.0
Nannochloropsis oculata	4	3.8	3.6	3.4	3.3

Table 13: Observation of pH, temperature, water level at different interval of time in tubs

Algal cultures	Wet biomass (mg/ml)				
	0 day	7 days	14 days	21 days	28 days
Spirulina sp.	0.23±0.07	0.36±0.01	0.43±0.3	0.50±0.05	0.55±0.02
Botryococcusbraunii	0.16±0.07	0.35±0.03	0.39±0.02	0.41±0.02	0.47±0.01
Nannochloropsis oculata	0.18±0.02	0.26±0.01	0.33±0.05	0.39±0.06	0.43±0.2

Table 14: Wet biomass estimation in open conditions

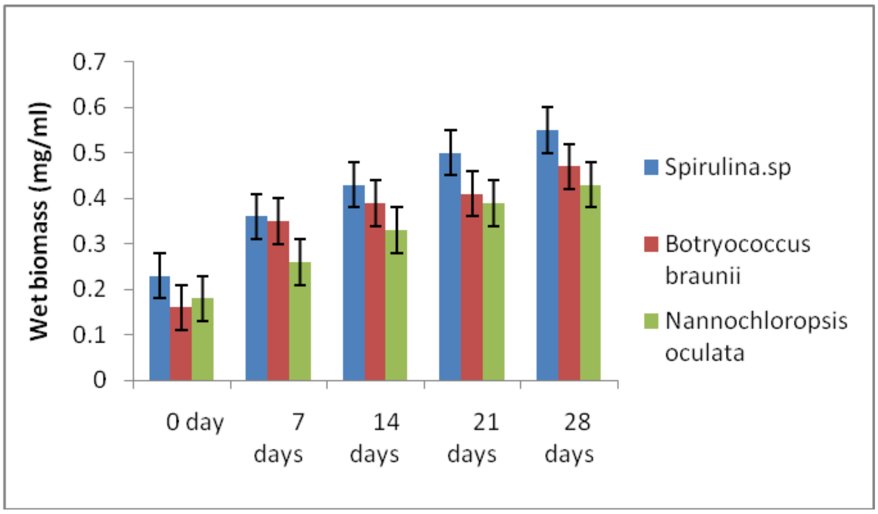


Figure 22: Wet biomass at regular interval in open conditions

Dry biomass estimation (open condition)

With the increase in the time interval of 7 days, dry biomass was observed in *Botryococcus braunii* from 0.03 mg/ml on day 0 to 0.14mg/ml on day 28. Mean biomass production was highest in tub containing *Spirulina sp.* i.e. 0.19 mg/ml harvested after 28 days of incubation. The biomass production in the tub containing *Nannochloropsis oculata* was the least (0.11 mg/ml). In *Nannochloropsis oculata* with increase in time there was a decrease in the biomass resulting in decrease in lipid content **(Table 15; Fig 23)**.

Algal cultures	**Dry biomass (mg/ml)**				
	0 day	**7 days**	**14 days**	**21 days**	**28 days**
Spirulina sp.	0.05±0.005	0.08±0.05	0.11±0.02	0.15±0.02	0.19±0.01
Botryococcus braunii	0.03±0.02	0.09±0.005	0.10±0.01	0.12±0.03	0.14±0.02
Nannochloropsis oculata	0.04±0.01	0.12±0.1	0.18±0.02	0.10±0.3	0.11±0.2

Table 15: Dry biomass estimation in open conditions

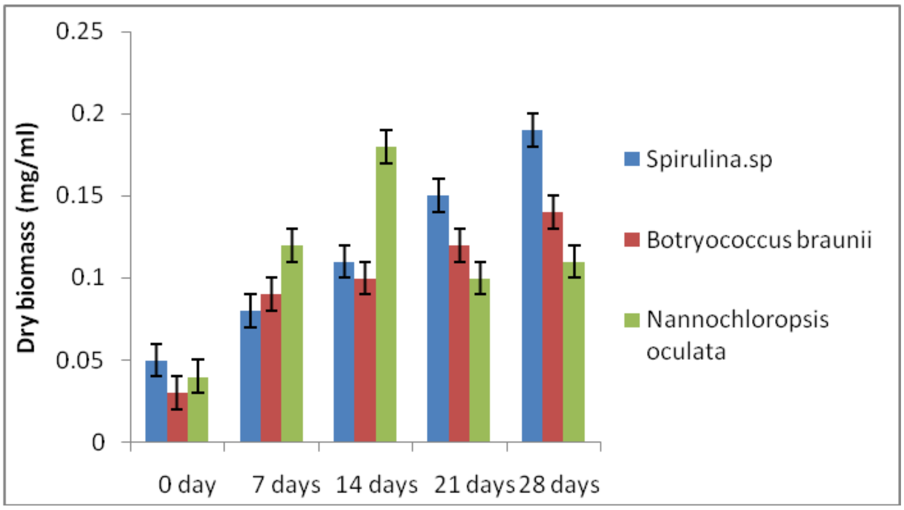


Figure 23: Dry biomass of algal cultures at regular intervals in open conditions

4.6.2 Chlorophyll estimation (Open conditions)

The chlorophyll content was measured at regular time intervals of 0, 7, 14, 21 and 28 days. The chlorophyll content of *Spirulina sp.* increased from 0.06 µg/ml on day 0 to 0.2 µg/ml on day 28 and the chlorophyll content of *Botryococcus braunii* increased from 0.01µg/ml on day 0 to 2.1µg/ml on day 28 **(Table 16; Fig 24)**.

	Chlorophyll (µg/ml)				
Algal cultures	**0 day**	**7 days**	**14 days**	**21 days**	**28 days**
Spirulina sp	0.01±0.005	0.02±0.01	0.03±0.01	0.04±0.01	0.5 ±0.05
Botryococcus braunii	0.06±0.02	0.10±0.02	0.13±0.01	0.19±0.05	0.25±0.06
Nannochloropsis oculata	0.03±0.02	0.08±0.1	0.12±0.03	0.24±0.01	0.20±0.3

Table 16: Chlorophyll estimation in algal cultures in open conditions

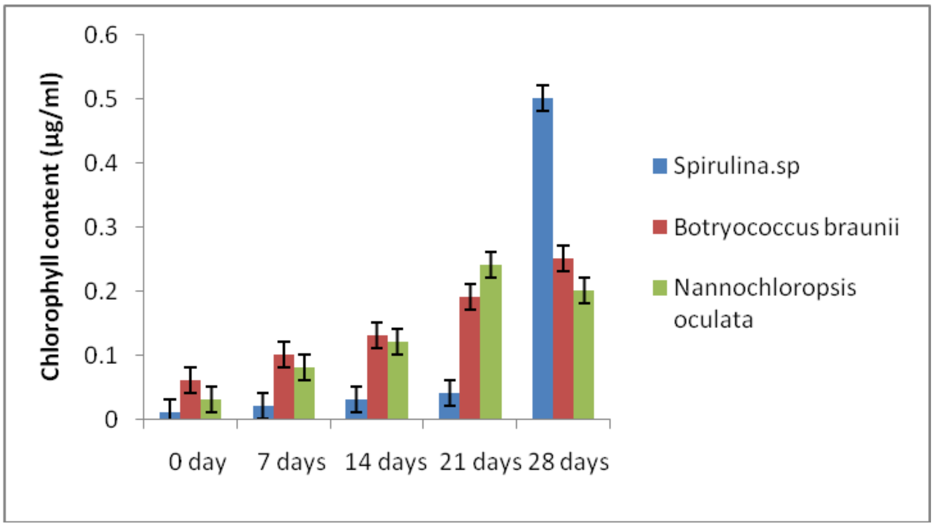


Figure24: Chlorophyll content in algal cultures at regular intervals in open conditions.

4.7 Lipid estimation of different algal cultures

Algal cultures	Lipid (mg/ml) in 0.1 ml of organic layer				
	0 day	**7 days**	**14 days**	**21 days**	**28 days**
Spirulina sp.	0.02±0.01	0.05±0.02	0.10±0.03	0.12±0.02	0.17±0.07
Botryococcus braunii	0.008±0.002	0.03±0.01	0.06±0.02	0.09±0.3	0.11±0.05
Nannochloropsis oculata	0.04±0.03	0.08±0.1	0.16±0.02	0.13±0.04	0.10±0.3
Lipid (mg/ml) in 0.2 ml of organic layer					
Spirulina sp.	0.05±0.03	0.25±0.02	0.3±0.03	0.31±0.05	0.35±0.04
Botryococcus braunii	0.04±0.03	0.07±0.03	0.09±0.02	0.13±0.01	0.16±0.02
Nannochloropsis oculata	0.09±0.02	0.12±0.3	0.18±0.01	0.15±0.04	0.13±0.1
	Lipid (mg/ml) in 0.5 ml of organic layer				
Spirulina sp.	0.12±0.02	0.28±0.03	0.40±0.01	0.44±0.03	0.57±0.04
Nannochloropsis oculata	0.16±0.01	0.23±0.2	0.47±0.03	0.34±0.1	0.20±0.04
Botryococcus braunii	0.09±0.02	0.11±0.04	0.15±0.01	0.16±0.03	0.22±0.02

Table 17: Lipid estimation at regular intervals (open condition)

With increasing amount of organic layer, the lipid content was also higher. Microalgal cultures (*Botryococcus braunii, Spirulina sp., Nannochloropsis oculata*) were grown in open conditions and lipid content was checked at regular intervals till 28 days of incubation. The lipid content of algal biomass increased with different interval of time. In 0.1 ml organic layer highest lipid content was found in *Spirulina sp.* i.e. 0.02 mg/ml, 0.05 mg/ml, 0.10 mg/ml, 0.12 mg/ml, 0.17 mg/ml on 0, 7, 14, 21 and 28 days respectively. Similarly lipid content was measured in 0.2ml organic layer at different time interval of 0, 7,14,21,28 days. The lipid content increased with the volume of organic layer. The maximum lipid content was found in *Spirulina sp.* of 0.05mg/ml, 0.25 mg/ml, 0.30 mg/ml 0.31 mg/ml ,0.35 mg/ml whereas lowest lipid content was found in *Botryococcus braunii* of 0.04 mg/ml, 0.07mg/ml, 0.09mg/ml, 0.13 mg/ml , 0.16 mg/ml on 0,7, 14,21,28 days respectively. The lipid content was measured in 0.5ml organic layer. The lipid content in both algal cultures were observed till 28 days of incubation and compared with biomass.

Lipid content in the different algal samples was observed at regular intervals of 7days for one month and was compared against biomass obtained at same rate interval of growth **(Table 17; Fig 25, 26, 27)**.

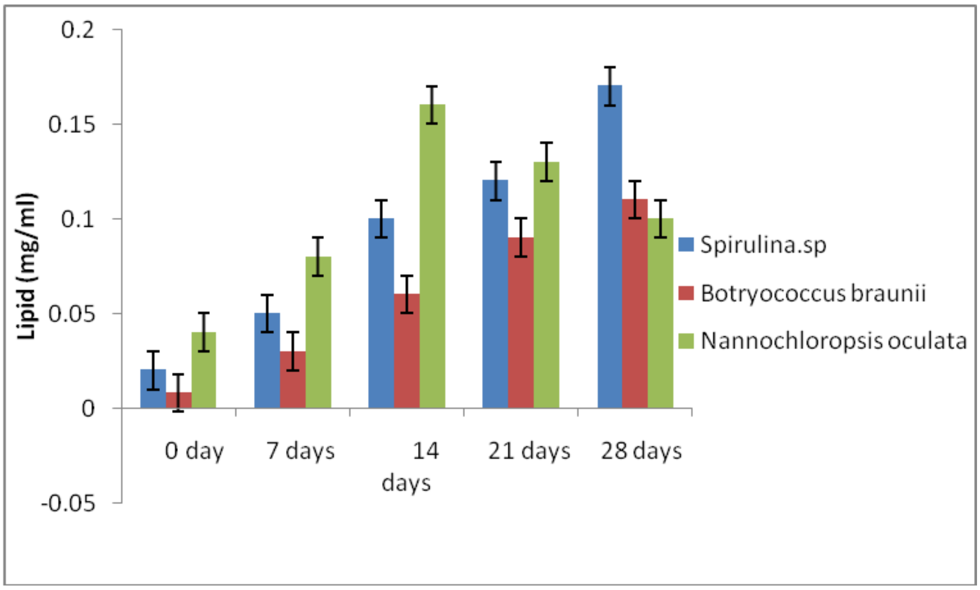


Fig 25: Estimation of lipid in 0.1 ml of organic layer in microalgae at regular intervals

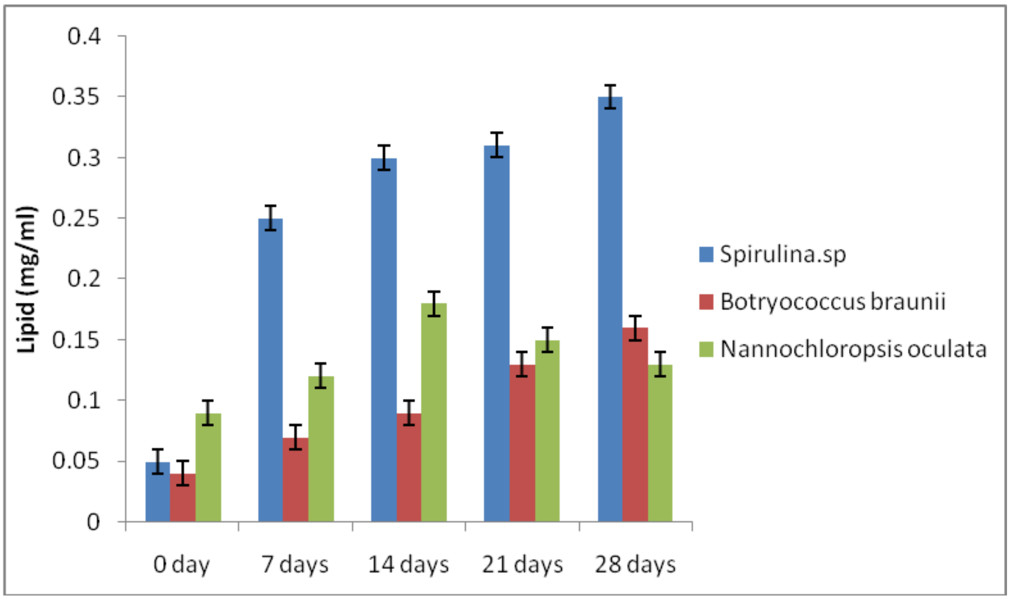


Fig 26: Estimation of lipid in 0.2 ml of organic layer in microalgae at regular intervals

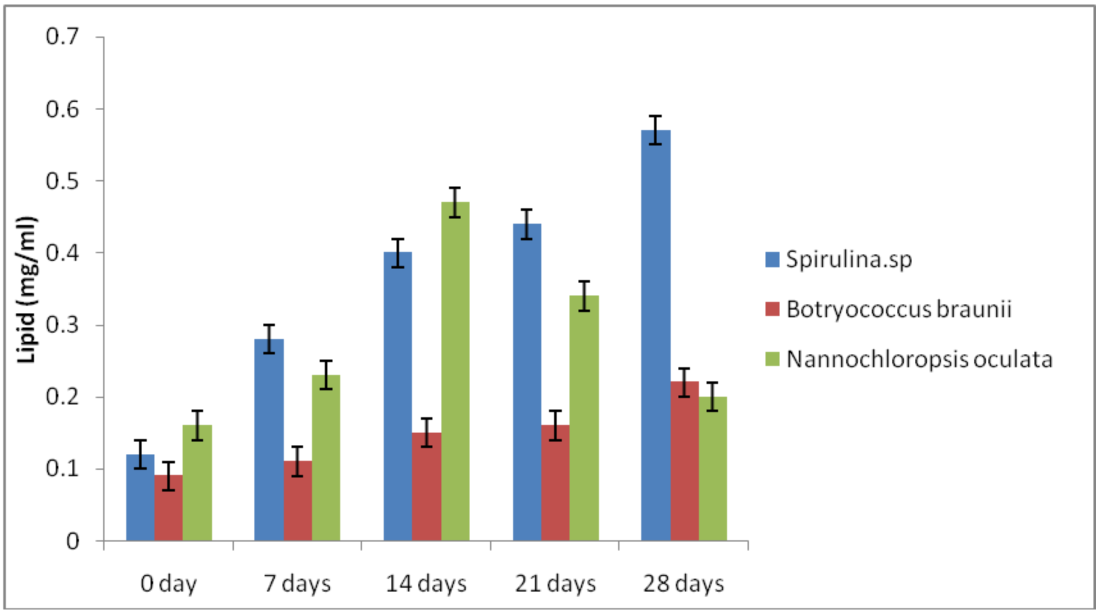


Fig 27: Estimation of lipid in 0.5 ml of organic layer in microalgae at regular intervals

4.8 Transesterification

In-situ transesterification was carried out for dry biomass of 30 days old culture of *Nannochloropsis oculata, Botryococcus braunii, Spirulina sp.* The three algal cultures were dried and the dry biomass was further transesterified and two phases were formed and the upper phase was further used for lipid estimation. The maximum lipid content was observed in *Spirulina sp.* (0.4 mg/ml) followed by *Nannochloropsis oculata* (0.36 mg/ml) and the minimum lipid content was observed in *Botryococcus braunii* (0.24 mg/ml) **(Table 18; Fig 28)**.

Algal cultures	**Lipid (mg/ml)**		
	0.1	**0.2**	**0.5**
Nannochloropsis oculata	0.03±0.02	0.27±0.05	0.36±0.03
Botryococcus braunii	0.08±0.03	0.13±0.03	0.24±0.03
Spirulina sp.	0.02±0.01	0.03±0.02	0.4±0.36

Table 18: Lipid estimation of transesterified algal cultures

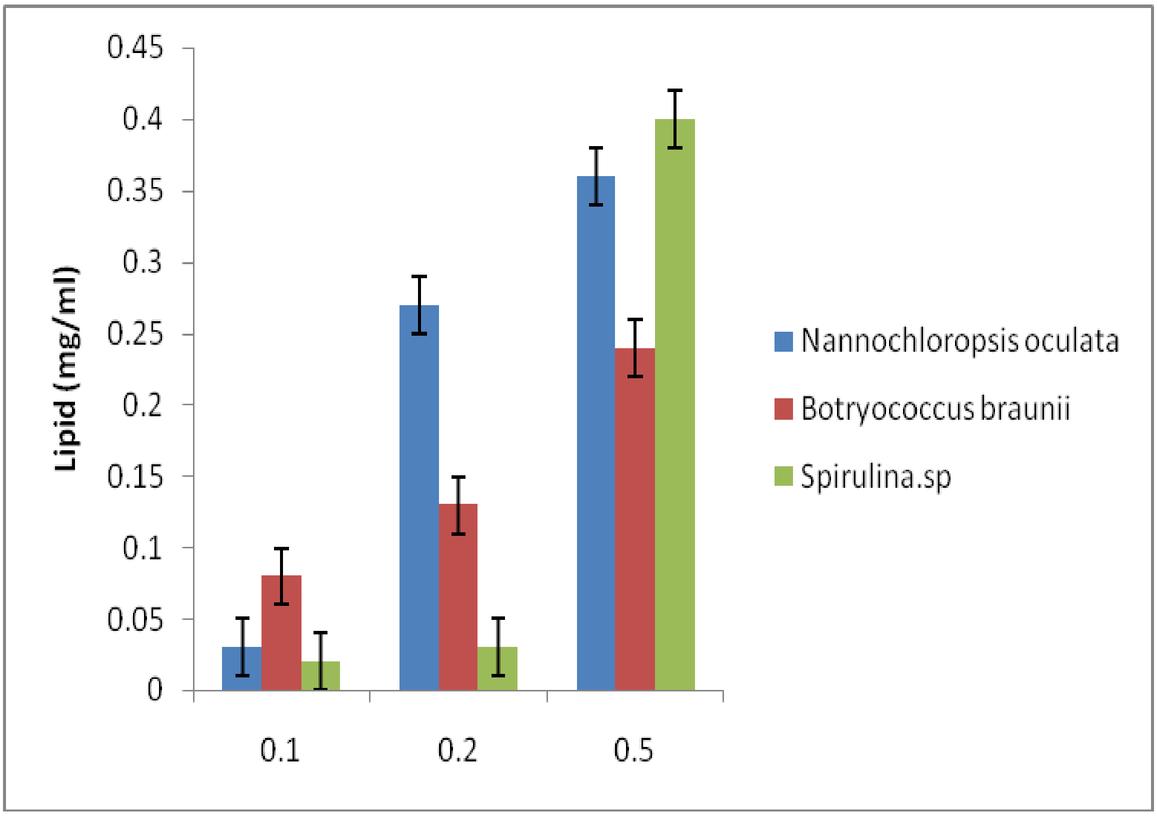


Fig 28: Lipid estimation of transesterified algal cultures

5. DISCUSSION

5.1 Lipid content in different algae

The highest lipid content was observed in *Nostoc muscorum* (ARM 442) (0.47 mg/ml) and the least lipid content was observed in *Anabaena variabilis* (ARM 441) (0.06 mg/ml). Average lipid content in microalgal cultures was found to be 0.33 mg/ml which is in confirmation with earlier studies where it had been reported 0.041 mg/ml lipid in *Nannochloropsis* species (Mata *et al.,* 2010). Three different methods were applied for disrupting the cell wall, tissue homogenization and ultrasonication and combination of the two methods. After disruption of the algal cells with a tissue homogeniser, the maximum lipid content was observed in *Nannochloropsis oculata* (0.15 mg/ml) and the minimum lipid content was observed in *Botryococcus braunii* (0.05 mg/ml). On treatment of algal cells with an ultrasonicator, the maximum lipid content was observed in *Nannochloropsis oculata* (0.19 g/ml) and minimum lipid content was observed in *Botryococcus braunii* (0.07 mg/ml). Ultrasonication is the use of sound to agitate particles, to disrupt algal cells. Though, such a method can only handle small amounts of biomass at a time, so it is not suitable for large scale extraction (Dunstan *et al*., 1992). Treatment of the algal cells with combination of ultrasonication and tissue homogenisation techniques resulted in greater release of lipids. *Nannochloropsis oculata* showed maximum lipid content when treated with tissue homogeniser, ultrasonicator and combination of both. *Spirulina sp.* showed maximum lipid content under open conditions (0.57 mg/ml). *Botryococcus braunii* showed lipid content of 0.22 mg/ml under open conditions (0.15 mg/ml).*Tolypothrix tenuis* (ARM 443), *Anabaena variabilis* (ARM 441), *Nostoc muscorum* (ARM 442), Isolate no. 5 *(PS 1), Scenedesmus armatus* showed 0.07, 0.06, 0.47, 0.25, 0.3 mg/ml lipid content under closed conditions.

5.2 Growth studies of microalgae

Oil content in microalgae can exceed 80% by weight of dry biomass (Metting, 1996; Spolaore *et al*., 2006). Growth studies in terms of biomass, chlorophyll showed that pH for *Spirulina sp.* ranged from 8.5 – 9.0, *Botryococcus braunii* ranged from 7.5 – 8.5, *Nannochloropsis oculata* ranged from 7 – 7.5. The water temperature in each tub was in the range of 20˚C – 31˚C. The maximum chlorophyll content was observed in *Nannochloropsis oculata* (1.41µg/ml) and minimum chlorophyll content was observed in Isolate no.5 (0.21µg/ml). The biomass estimation was observed for five different algae such as *Nannochloropsis oculata*, *Botryococcus braunii*, *Scenedesmus armatus*, *Nostoc muscorum* (ARM 442), *Spirulina sp.* at a regular time interval of 0, 7, 14,21and 28 days. *Nostoc muscorum* (ARM 442) (0.43mg/ml) showed maximum growth in terms of wet biomass and *Scenedesmus armatus* (0.21 mg/ml) showed minimum growth. The maximum growth in terms of dry biomass was observed in *Nostoc muscorum* (ARM 442) (0.15 mg/ml) and minimum growth was observed in *Scenedesmus armatus* (0.1 mg/ml). Overall with increase in time interval the biomass and chlorophyll increased in all the microalgae but maximum biomass increase was shown by *Nostoc muscorum* (ARM 442) and maximum chlorophyll content in *Nannochloropsis oculata.*

5.3 Mass cultivation of microalgae

The growth of three algal species *Botryococcus braunii*, *Spirulina sp.* and *Nannochloropsis oculata* was observed after inoculation in large tubs for parameters like biomass, chlorophyll and lipid content at regular time interval of 0, 7,14, 21, 28 days. The maximum growth was observed in *Spirulina sp.* in terms of wet biomass as well as dry biomass after 28 days of inoculation i.e. 0.55 mg/ml and 0.19 mg/ml and maximum chlorophyll content was also observed in *Spirulina sp.* (0.5µg/ml). 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 attained after 28 days of inoculation in all the three microalgal cultures (*Botryococcus braunii, Spirulina sp, Nannochloropsis oculata*). Overall in all the microalgae the lipid content increased with biomass at regular interval of 7 days.

5.4 *In – situ* transesterification

Direct (*In – situ*) transesterification is a process that blends the microalgae with an alcohol and a catalyst without prior extraction. A less polar solvent, like hexane or chloroform, can be added to increase the yield of biodiesel production (M. B. Johnson & Wen, 2009). The lipid content was estimated for the tranesterifed algal cultures of *Nannochloropsis oculata*, *Botryococcus braunii and Spirulina sp.* The maximum lipid content was observed in *Spirulina sp.* (0.4 mg/ml) and the least lipid content was observed in *Botryococcus braunii* (0.24 mg/ml). Overall with increase in biomass, there was an increase in lipid content of microalgae.

6. CONCLUSION

1. High lipid productivity of dominant, fast-growing algae is a major prerequisite for commercial production of microalgal oil-derived biodiesel. *Nostoc muscorum* (ARM 442) showed rapid growth and was found to contain maximum lipid under closed conditions.

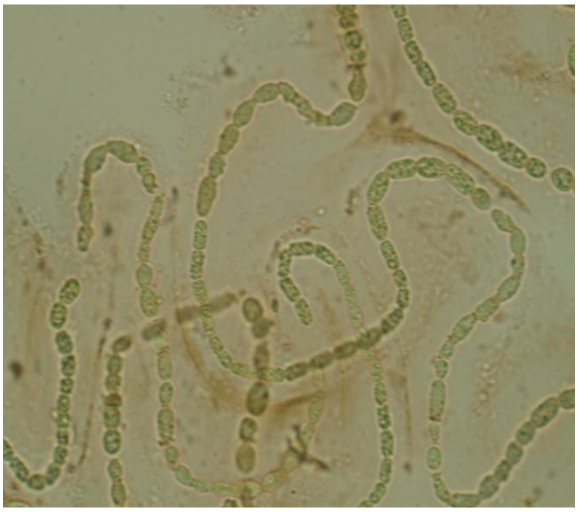
2. Disruption of algal cells with tissue homogeniser, ultrasonicator and combination of both techniques showed maximum lipid content in *Nannochloropsis oculata* (0.15, 0.19, 0.21 mg/ml). These disruption methods were useful in more lipid content extraction.

3. The maximum growth in terms of dry biomass as well as wet biomass was observed in *Nostoc muscorum* (ARM 442) in lab conditions (0.15 mg/ml and 0.43 mg/ml), and *Spirulina sp.* in open conditions (0.19 mg/ml and 0.55 mg/ml).

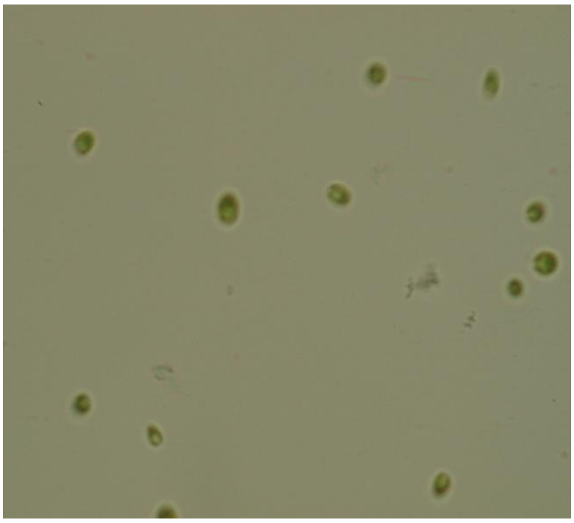
4. *In-situ* transesterification was also done using algal dry biomass directly. Layer of biodiesel appeared in all the species. After transesterification, the highest lipid content was observed in *Spirulina sp.* (0.4 mg/ml) and the least lipid was observed in *Botryococcus braunii* (0.24 mg/ml).

PHOTOMICROGRAPHS

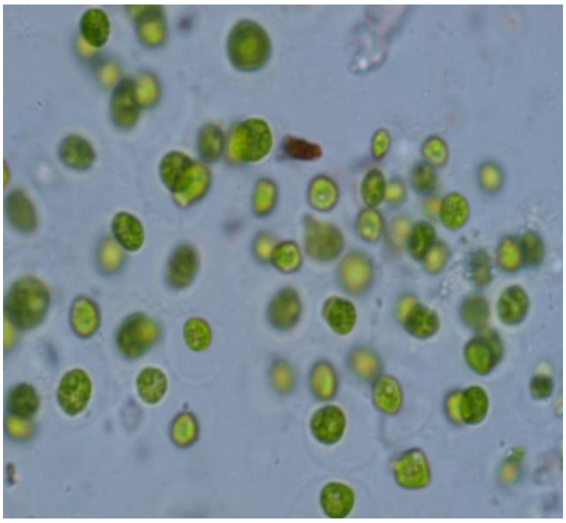
Nostoc muscorum **(ARM 442)**



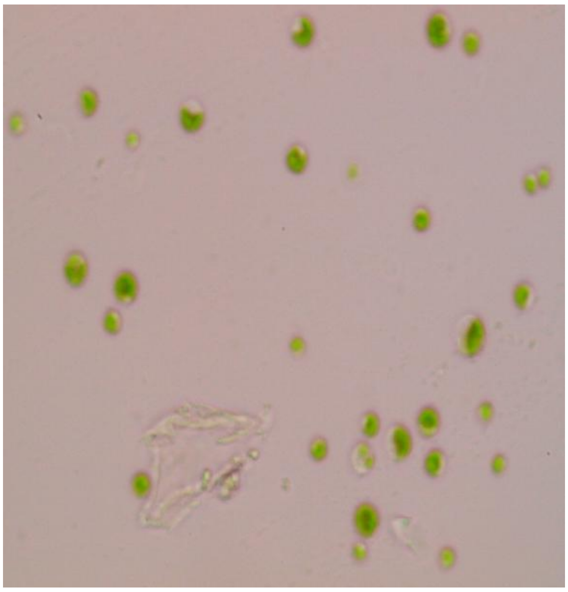
Anabaena variabilis **(ARM 441)**



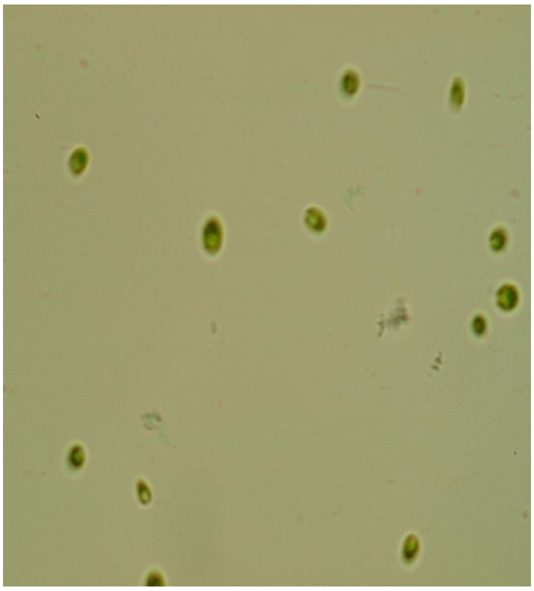
Botryococcus braunii



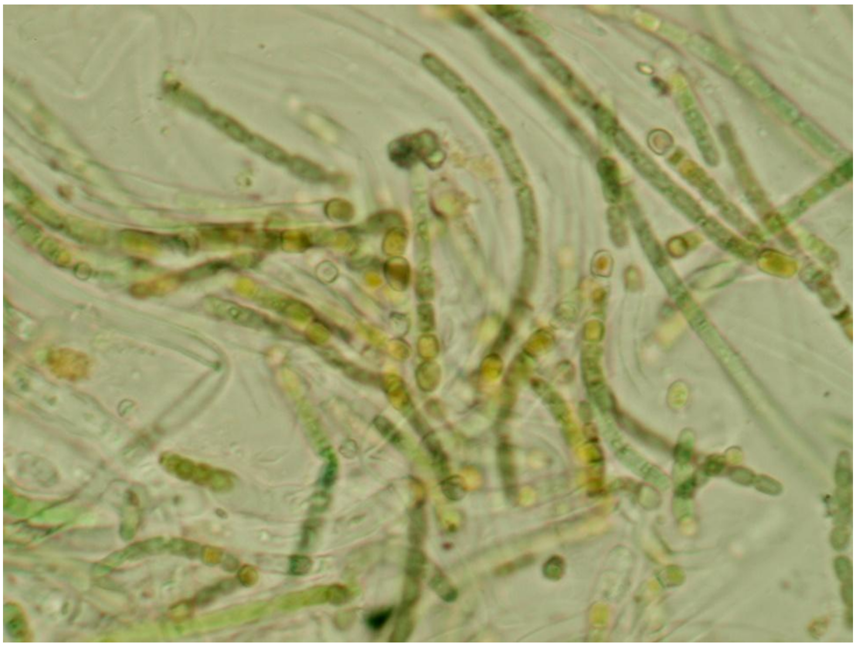
Nannochloropsis oculata



Ultrasonicated culture of *Scenedesmus*



Ultrasonicated culture of *Nannochloropsis oculata*



Ultrasonicated culture of Isolate no.5 *(PS 1)*

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