FATTY ACID PROFILE ANALYSIS OF NOVEL MICROALGAE ISOLATED FROM NORTH EASTERN ESTUARIES OF TAMILNADU

INTRODUCTION

The eastern coast of Tamil Nadu is a rich resource for research. The microbial load of the marine and estuaries have paved way for enormous research. Estuaries are a unique environment, which acts as an interface between the rivers and oceans. Estuary is a natural laboratory in which the interactions and adaptations of the microorganism are observed to a wide range of environmental conditions (Nedumaran et al. 2011). Estuarine microalgae are found to adapt to environmental conditions which are not experienced by marine microalgae. The salinity change, turbidity and tidal waves are found to be the extremity, which are usually adapted (Joint 1981). Microalgae are advantageous, as they can duplicate by division, which can reduce processes that take years in crop plant growth to few months in algae (Hannon et al. 2010).

Microalgae are eukaryotic, and in evolutionary terms, they can be either ancient species or recent ones. This diversity creates the capability for microalgae to be a valuable source of a multitude of products that, at some level, support the food, cosmetics, pharmaceutical and fuel industries. Microalgae have much more oil than macroalgae and are much faster and easier to grow. Although a number of algal species belonging to *Xanthophyta*, *Pheophyta*, and *Chrysophyta* are known to accumulate the storage products in the form of fats and lipid, still the microalgae particularly belonging to chlorophyta (*Botrycoccus*, *Dunaliela*, *Chlorella*, *Scenedesmus*, etc.) are

being commercially exploited because they are able to double their biomass within 24 hours (Song et al. 2008).

The production of algal biomass, as well as the cultivation of all the other energy crops, requires input such as electricity, fertilizers, water and raw materials, which means a more or less direct consumption of energy, water and a release of substances with a potential pollution impact (CO_2, NO, SO, etc.). The biochemical composition of the algal biomass is kept in mind to study the ability of the microalgae to produce lipids. Some strains can accumulate up to 60% of lipid, mainly triglyceride (TAG), the fraction suitable for the production of biodiesel by transesterification. The enormous expectation placed on microalgae has made researchers to work on it and screen the potentiality of these microorganisms (Chisti 2007).

In the present study, microalgae were isolated from estuarine samples obtained from the northeastern estuaries of Tamil Nadu. The microalgae were grown and its dry biomass, chlorophyll, carotenoid, protein, carbohydrate and lipid were analyzed. The lipid was transesterified and the FAME was analyzed by GC–MS and FTIR. From the transesterified samples, oil yield and biodiesel yield were studied and the microalgae were screened for their higher percentage yield of biodiesel, which was then used for the further studies.

MATERIALS AND METHODS

Most of the experiments in this thesis were done in triplicates and their mean value was calculated, from which the standard deviation was deduced and the graphical representations were made with the standard error bars.

3.2.1 SAMPLE COLLECTION

The water samples from northeastern estuaries of Tamil Nadu, i.e., Muttukadu, Chennai (12°48′36.144″N 80°14′53.9376″E), Adyar Estuary, Chennai (13°0′12.1932″N 80°15′18.1548″E), Thengaithittu Estuary, Pondicherry (11°54′54.2052″N 79°49′50.808″E), and Pichavarm, Chidambaram (11°25′57.09″N 79 °46′54.0444″E) were collected in sterile sample collectors. The collected samples were brought to the laboratory for further analysis.

DETERMINATION OF pH

pH is a measure of acidity or alkalinity of the solution. pH is represented as the negative logarithm of hydrogen ion concentration of the solution

$$pH = -\log_{10}[H^+].$$

pH denotes the power of hydrogen ion activity in mole per liter. pH of the water samples were estimated by using pH meter (ELICO).

ESTIMATION OF BIOLOGICAL OXYGEN DEMAND (BOD)

Biological oxygen demand is used to determine the relative oxygen requirement of river water, sea water, effluents and polluted waters. The BOD was determined by Wrinkler's iodimetric method. Fifty milliliters of the collected water sample was added to 1 liter distilled water. The BOD bottles were rinsed with water. The pH of the sample was noted and neutralized using alkali or acid. Two BOD bottles were filled with the sample. Air bubbles were avoided. Two milliliters of manganese sulfate and 2 ml of alkaline iodide azide solution were added. The bottle was closed and observed for formation of brown precipitate and allowed to settle halfway. Two to

three drops of sulfuric acid was added to it. The acidified sample was titrated against sodium thiosulfate solution. A pale yellow coloration occurred. Two drops of starch indicator was added to the sample. The sample was again titrated till the disappearance of blue color. It is stated as the initial oxygen ($D1$). The bottle with the remains was incubated for 3–5 days. The oxygen concentration was estimated after 5 days and it is termed as final concentration ($D2$)

$$\text{BOD} = D1 - D2 \text{ mg l}^{-1}.$$

ESTIMATION OF CHEMICAL OXYGEN DEMAND (COD)

Chemical oxygen demand was determined by Reflux titration method. The COD is used as a measure of the oxygen equivalent of the organic matter content of the sample that is susceptible to oxidation by a strong chemical oxidant. To 20 ml of the sample taken in a conical flask, 10 ml of the potassium dichromate solution, a pinch of each silver sulfate and mercuric sulfate and 30 ml of sulphuric acid were added. The contents were refluxed for 2 hours. The flask was cooled and the contents were diluted to about 150 ml with addition of distilled water. Two to three drops of ferroin indicator solution was added and titrated against ferrous ammonium sulfate solution. The end point was determined by change of blue green color to reddish blue. Distilled water was considered as blank and the titration was carried out.

$$\text{Chemical oxygen demand (mg l}^{-1}) = \frac{(B-T)\times N \times E \times 1000}{\text{Volume of sample (ml)}},$$

where

T = volume of the titrant (FAS) used against sample (ml)

B = volume of the titrant used against blank (ml)

N = normality of ferrous ammonium sulphate

E = equivalent weight of oxygen (8).

ISOLATION AND IDENTIFICATION OF THE MICROALGAE

The collected water sample was inoculated in BG11 medium for isolation of microalgal cultures and incubated in a growth chamber with light flux of 16:8 hours.

The purity of culture was ensured by repeated inoculation and identification was accomplished by determining cellular morphology observed by using light microscope.

MASS CULTIVATION OF THE MICROALGAE

The BBM (Bold's Basal Medium) was prepared and purified form of the microalgal cultures were inoculated (5% of 15 days old culture was used as seed inoculum) and mass cultivated. At a duration of 2 days, 5 ml of fresh sterile BBM was added to the flask placed for mass cultivation at room temperature in a growth chamber with light flux of 16:8 hours.

ESTIMATION OF BIOMASS BY DRY WEIGHT METHOD

At the specified incubation timing, the growth of algal sample was calculated. The cultivated algal sample was centrifuged for 15 min at 10,000 rpm and the pellet was collected. Then the algal cells was dried at 80 °C for 30 min and weighted to determine the dry weight.

SPECIFIC GROWTH RATE DETERMINATION

The microalgal cultures were inoculated in BG11 medium and its optical density was observed every 24 hours once for about 20 days and plotted as a growth

curve graph. From the optical density observed its specific growth rate was calculated using the following equation (Clesceri et al. 1989):

$$SGR\,(\text{mg l}^{-1}) = \frac{\ln(x_1 - x_2)}{t_2 - t_1},$$

where

X_1 = biomass concentration at the end of selected time interval,

X_2 = biomass concentration at the beginning of selected time interval, and

$t_2 - t_1$ = elapsed time between selected time.

ESTIMATION OF CHLOROPHYLL (HISCOX AND ISRAELSTAM 1979)

At the specified incubation timing, the chlorophyll of algal sample was calculated. The culture was centrifuged at 5000 rpm for 10 min and the pellet was homogenized in motor and pestle with 5 ml of methanol and kept in room temperature for 30 min. The absorbance of the pooled extract was measured spectrophotometrically at 645 nm and 663 nm wavelength.

$$\text{Total chlorophyll C} = 20.2(A_{645}) + 8.02(A_{663}),$$

where

V = Volume of sample and

W = Weight of sample

ESTIMATION OF CAROTENOIDS (DE CARVALHO ET AL. 2012)

At the specified incubation timing, the carotenoid of algal sample was calculated. The algal dry biomass was homogenized in a mortar and pestle and

extracted repeatedly with acetone. The absorbance was measured at 470 nm wavelength and the concentration of carotenoids was determined by using the following formula:

$$\text{Total carotenoids C} = \frac{A_{470} \times V \times 10^4}{A^{1\%}_{1\,\text{cm}} \times \text{DCW}} \mu\text{g ml}^{-1},$$

where

V = volume of extract,

DCW = dry cell weight of sample and

$A^{1\%}_{1\,\text{cm}}$ = β-carotene extinction coefficient in petroleum ether (2592).

ESTIMATION OF CARBOHYDRATE (DUBOIS ET AL. 1956)

At the specified incubation timing, the carbohydrate of algal sample was calculated. The algal sample was harvested by centrifugation at 10,000 rpm for 5 min and was homogenized in a mortar and pestle and extracted repeatedly with water. The cell free extract was analyzed for total carbohydrate by phenol–sulfuric acid. 0.5 ml of the extracted algal sample was taken in a clean dry test tube. 0.5 ml of 5% phenol in 0.1 ml HCl was added to the test tube. Then, 2.5 ml of concentrated sulfuric acid was added to it. The mixture was vortexed. It was left to cool to room temperature and the absorbance was read at 490 nm and plotted against the standard graph to determine the total carbohydrate concentration.

ESTIMATION OF PROTEIN (LOWRY ET AL. 1951)

At the specified incubation timing, the protein of the algal sample was calculated. The algal sample was centrifuged at 6000 rpm for 10 min. The pellet was resuspended in 5 ml of 1 M NaOH and boiled for 10 min. The cell free extract was

analyzed for protein by the method of Lowry et al. Protein reacts with the Folin–Ciocalteau reagent to form a colored complex. Algal sample was taken and 0.5 ml of reagent C was added and allowed to stand at room temperature for 10–15 min. Then, 0.5 ml of reagent D was added and the tubes were placed in dark condition for 30 min for formation of blue colour. The suspension was read at 650 nm wavelength in UV spectrophotometer. Then, it was plotted against the standard graph to determine the protein in the sample.

LIPID EXTRACTION (BLIGH AND DYER 1959)

At the specified incubation timing, the lipid of algal sample was calculated. The cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The cells were washed once with distilled water. The pellet was dried in an oven for 2 hours at 80°C. For 1 g of algal biomass, 2 ml of methanol and 1 ml of chloroform was added and kept for 18 hours at 25 °C.

The mixture was agitated in vortex for 2 min. One milliliter of chloroform was again added and the mixture was shaken vigorously for 1 min. After that, 1 ml of distilled water was added and the mixture was agitated in a vortex again for 2 min. The layers were separated by centrifugation for 10 min at 2000 rpm. The lower layer was separated and the procedure was again repeated with the pellet. The two supernatants collected were allowed to stand for 2 hours. Lower organic layer with the lipids was transferred to a clean pre-weighed vial ($W1$). Evaporation was carried out in hot air oven at 80 °C for 50 min. The weight of the vial was again recorded ($W2$). Lipid content was calculated by subtracting $W1$ from $W2$ and expressed as % dry cell weight.

$$P_{\text{Lipid}}\ (\text{g l}^{-1}\text{day}^{-1}) = (C_{\text{Lipid}}\ (\text{g g}^{-1}) \times \text{DCW}\ (\text{g l}^{-1})) / \text{Time (day)}$$

where

P_{Lipid} = lipid productivity (g L^{-1} day^{-1}),

C_{Lipid} = lipid content of the cells (g g^{-1}),

DCW = dry cell weight (g L^{-1}) and

Time = cultivation periods (day).

The FAME samples were mixed with KBR powder and ground well to a fine mixture. It was preserved to a disc using a hydraulic press into tablets. The disc was subjected to FTIR spectral measuring within the frequency varies of 4000 to 400 cm^{-1}. The algal powder was characterized under victimization Fourier rework infrared spectrophotometer.

The FAME samples were analyzed with GC–MS. The GC–MS analysis was carried out using ThermoGC-Trace Ultra version: 5.0, Thermo MS DSQ II. The column used was ZB-5-MS capillary standard non-polar, measuring 30 m × 0.25 mm with a film thickness of 0.25 μm composed of 5% phenyl arylene and 95% dimethylpolysiloxane. The carrier gas used was helium (flow rate is 1.0 ml/min). One microlitre sample injection was utilized. The oven temperature was programmed initially at 70 °C for 6 min, then increased to 260 °C. And then programmed to increase to 280°C ending in a 5 min. Total retention time was 90 min. The MS transfer line was maintained at 180 °C. GC–MS was analyzed using electron impact ionization 70 eV and data was evaluated using total ion count for compound identification and quantification. The spectrums of the components were compared with the database of

spectrum of known components stored in the GC–MS library. Measurement of peak areas and data processing were carried out by Thermo-MS DSQ II SPL software.

BIODIESEL YIELD EVALUATION

The yield of biodiesel produced from transesterification was estimated as its weight relative to (1) the weight of total lipid/oil present in the biomass and (2) the weight of algal biomass.

The yield of the biodiesel was calculated by the following equation:

$$\text{Percentage yield} = \left(\frac{\text{Weight of biodiesel}}{\text{Weight of oil}} \right) \times 100.$$

The percentage yield of biodiesel was used as a screening process from the isolated microalgae, screened for its potency, and the selected microalgae were used for the further studies.

MOLECULAR CHARACTERIZATION OF ISOLATES

The potential microalgae, M24 (*Scenedesmus* sp.) was characterized by molecular characterization.

ISOLATION OF GENOMIC DNA

Molecular phylogenetic analysis of the microalgae was done. Fifty milliliters of microalgal culture was centrifuged at 7000 rpm for 10 min. The pellet was crushed in a mortar and pestle in cold condition. Subsequently microalgal DNA was isolated by using the GeNei™ Genomic DNA Extraction Kit, following the manufacturer's instruction. The quality of DNA was checked by visualization of genomic DNA after gel electrophoresis in 0.8% agarose in gel doc system.

AMPLIFICATION OF 18S rRNA

Amplification of extracted DNA was carried out in a gradient thermal cycler (GeNei); the common forward and reverse primers were used for the sequence of 18s rRNA for *Scenedesmus* sp. Amplification was performed for 25 cycles, 1.0 µl of extracted DNA each 2 µl of forward and reverse prime, 10 µl of PCR amplicon (master mix) and 5.0 µl of double distilled water. The amplification conditions were as follows: denaturation at 94 °C for 1 min followed by annealing at 50 °C for 5 s, extension at 60 °C for 4 min and a final extension at 72 °C for 3 min. PCR products (~1500 bp fragment) were confirmed using 0.8% agarose gel electrophoresis.

PURIFICATION AND NUCLEOTIDE SEQUENCE ANALYSIS

PCR products were purified with QIA quick spin columns (Qiagen, Inc., Chatwsorth, CA). Nucleotide sequence analysis was carried out in ABI 3130 genetic analyzer combined with Big Dye terminator version 3% cycle sequencing kit. The sequence was analyzed at BLAST in NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). The sequences, which have maximum identity, were selected and aligned on clustalW multiple alignment software. The strains' phylogenetic relationship was assessed by the maximum likelihood method with 1000 replicons. The evolutionary distances were computed using the kimure-2-parameter method. Evolutionary analyses were conducted in MEGA 7.

RESULTS AND DISCUSSION

Water samples from various estuaries such as Adayar Estuary, Chennai; Thengaithittu Estuary, Pondicherry; Pichavaram, Chidambaram and Muttukadu, Chennai were collected in sterile sample collectors (Figure 3.1 and 3.1a).

The samples were brought to the laboratory and the physicochemical parameters of the water samples were analyzed. Parameters such as pH, BOD and COD were analyzed. Table 3.1 showed the results, i.e., the water sample from the Muttukadu estuarine was observed to have the highest pH, 7.5 ± 0.3. The BOD observed in the estuaries of Adayar, Thengaithittu and Pichavaram measured was 3.6 ± 0.15 mg L^{-1}. The highest COD level was observed in Pichavaram, 252 ± 0.2 mg L^{-1}. Microalgae are capable of fixing carbon dioxide by photosynthesis and excess nutrients can be fixed (Omprakash 2014). Photosynthetically produced oxygen relieves BOD in the waste water. Microalgae have a higher ability to remediate a wide range of toxins and wastes (Lalibert et al. 1994).

Figure 3.1 Study site of northeastern Estuary of Tamil Nadu

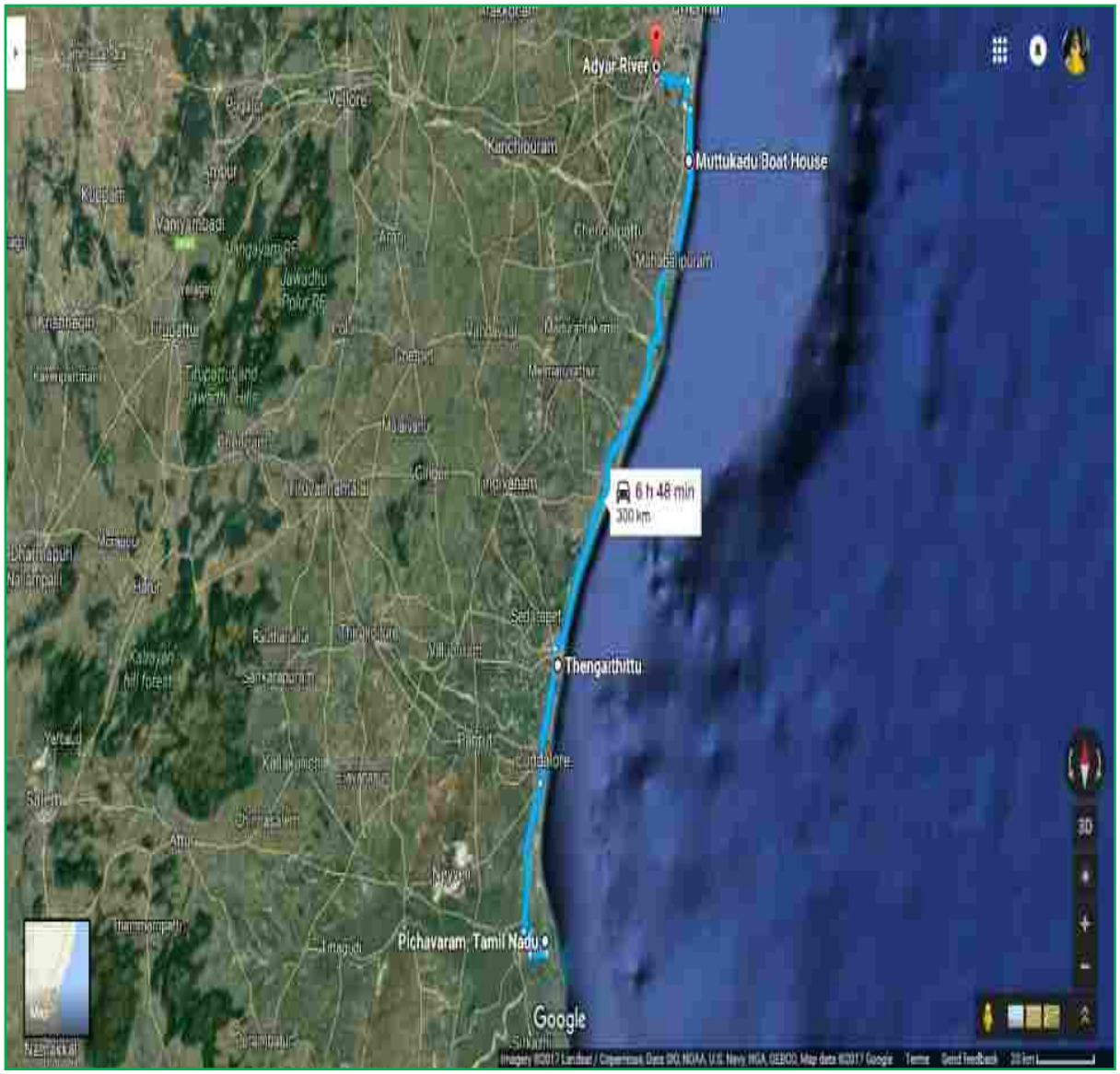


Figure 3.1a Various stations of estuaries in northeastern Tamil Nadu

Pichavaram

Thengaithittu

Muttukadu

Adyar

Table 3.1 Physio-chemical analysis of the estuarine samples

S.No.	Name of the place	pH	BOD (mg L^{-1})	COD (mg L^{-1})
1	Pichavaram	6.0 ± 0.2	3.6 ± 0.15	252 ± 0.20
2	Thengaithittu	6.0 ± 0.2	3.6 ± 0.15	84 ± 0.40
3	Muttukadu	7.5 ± 0.3	3.2 ± 0.2	84 ± 0.4
4	Adayar Estuary	7.0 ± 0.2	3.6 ± 0.15	168 ± 0.35

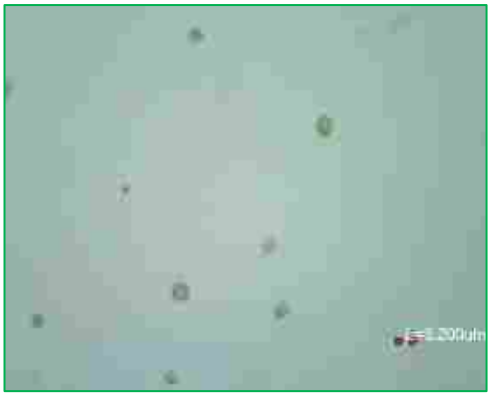
Figure 3.2 Isolation of microalgal colonies in BG11 medium

Microalgae were isolated from the water samples and identified microscopically. They were tooth picked and transferred to BG11 medium and grown. The microalgal cultures were repeatedly reincoulated to obtained pure cultures. The microalgal microscopic observation (Figure 3.3) were analyzed with images of the Algaebase for identification (www.algaebase.org). The isolated pure microalgal cultures were noted as A42 (*Oocystis* sp.), A43 (*Nannochloropsis* spp.), A51 (*Nannochloropsis* spp.), M21 (*Bracteacococcus* sp.), M24 (*Scenedesmus* sp.), P38 (*Pseudokrichneriella* sp.), P39 (*Cryptomonas* sp.), M30 (*Chlorella* sp.) and T11 (*Chlorella* sp.). The identified microalgae were mass cultivated in the BBM. Geldenhuys et al. (1988) have reported that BBM is a nutritionally rich medium which would help in the mass cultivation of microalgae.

Figure 3.3 Microscopic observation of the microalgae involved in the study (under light microscope of 100x magnification)

3.3a Culture – M21

Bracteacococcus sp.

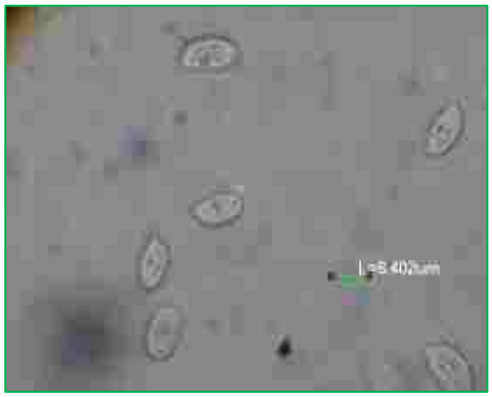


3.3b Culture – M24

Scenedesmus sp.

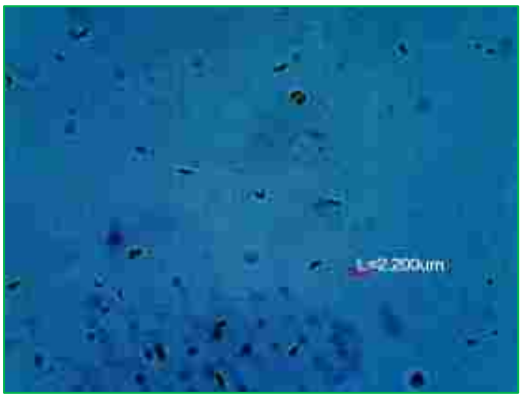
3.3c Culture – P39

Cryptomonas sp.



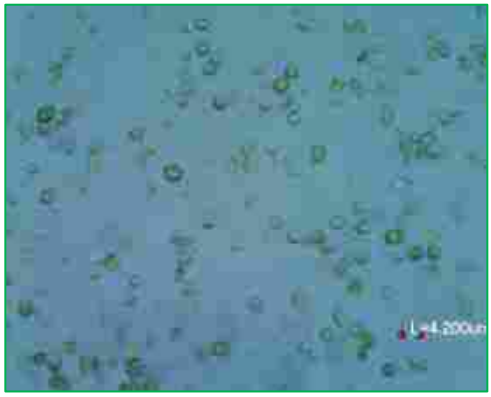
3.3d Culture – P38

Pseudokrichneriella sp.



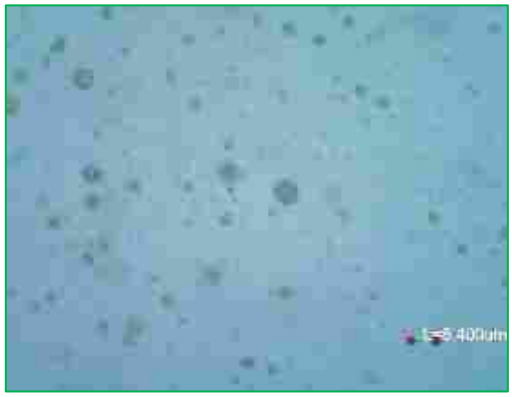
3.3e Culture – A42

Oocystis sp.



3.3f Culture – M30

Chlorella sp.

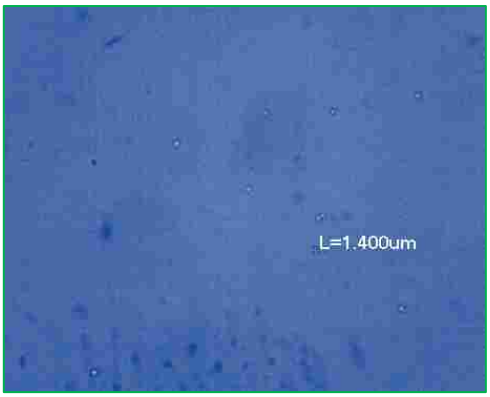


3.3g Culture – A43

Nannochloropsis spp.

3.3h Culture – A51

Nannochloropsis sp.



3.3i Culture – T11

Chlorella sp.

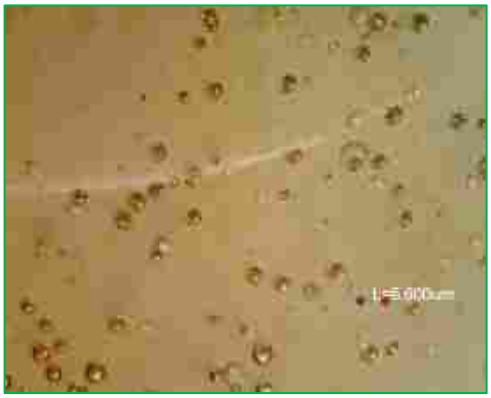


Figure 3.4 Mass cultivation of the microalgae in BBM

Figure 3.5 Growth of the microalgae in BBM

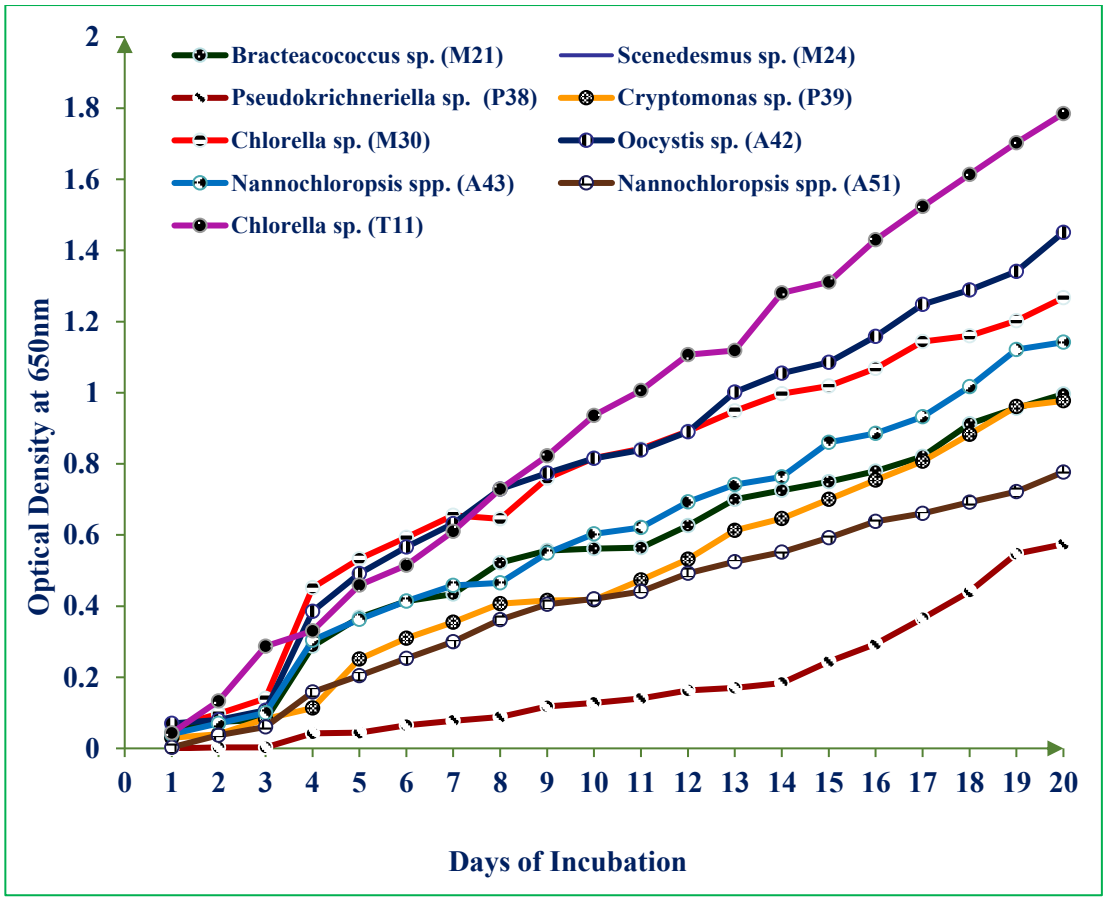


Table 3.2 Specific growth rate for the microalgae grown in BBM

S.No.	Culture	Specific growth rate (day^{-1})
1	*Bracteacococcus* sp. (M21)	0.053
2	*Scenedesmus* sp. (M24)	0.090
3	*Cryptomonas* sp. (P39)	0.051
4	*Chlorella* sp. (M30)	0.189
5	*Pseudokrichneriella* sp. (P38)	0.040
6	*Oocystis* sp. (A42)	0.069
7	*Nannochloropsis* spp. (A43)	0.087
8	*Nannochloropsis* spp. (A51)	0.046
9	*Chlorella* sp. (T11)	0.057

Nannochloropsis are marine algae, which are also found in fresh and brackish water. They belong to the family *Eustigmataceae*. They are small and found usually in about 2 to 3 micrometer in size. They have higher concentration of pigments such as astaxanthin, zeaxanthin and canthaxanthin. They are recently known for their industrial application due to its ability in accumulating PUFA (Boussiba et a. 1987).

Oocystis is a planktonic genus, a freshwater green algae. It is at times abundantly found in sewage ponds. They belong to the family *Oocystaceae*. They have disc shaped chloroplast and the golgi bodies are found around the nucleus. *Oocystis* are usually used in the tertiary treatment of waste water (Aragon et al. 1992).

Pseudokrichneriella is a sickle shaped green algae belonging to the family *Selenastraceae*, which are commonly found in freshwater. Due to its high growth rate and sensitivity to toxicity, it is used as ecotoxoicological bioindicator. It has been reported that *Pseudokrichneriella* sp forms autospores through multiple fission after two nuclear divisions (Yamagishi et al. 2017).

Scenedesmus sp belongs to the class *Chlorophyceae*, family *Scenedesmaceae*. They are non-motile. They are diversely found in freshwater and marine water. They can exist as unicells or in coenobia of four or eight cell (Miquel 1999). Scenedesmus has a high biomass productivity and it has been attracting researchers in biodiesel production recently (Christenson and Ronald 2011).

Bracteacoccus is a genus of the family *Chlorococcaceae*. They are found in fresh water and soil sites. The average cell size is 7.3 μm. They have larger quantity of chlorophyll a and b. They have the ability to growth in the dark even for two days with the carbon substitutes in the medium of growth (Lynch 1977).

Cryptomonas is a phytoplankton, found in fresh water systems of the family *Cryptomonadaceae*. They are brownish or greenish in color. They produce toxins and used to feed small zooplankton. They have a purple pigment namely, phycoerythrin, which is responsible for the brown coloration (Choi et al. 2013).

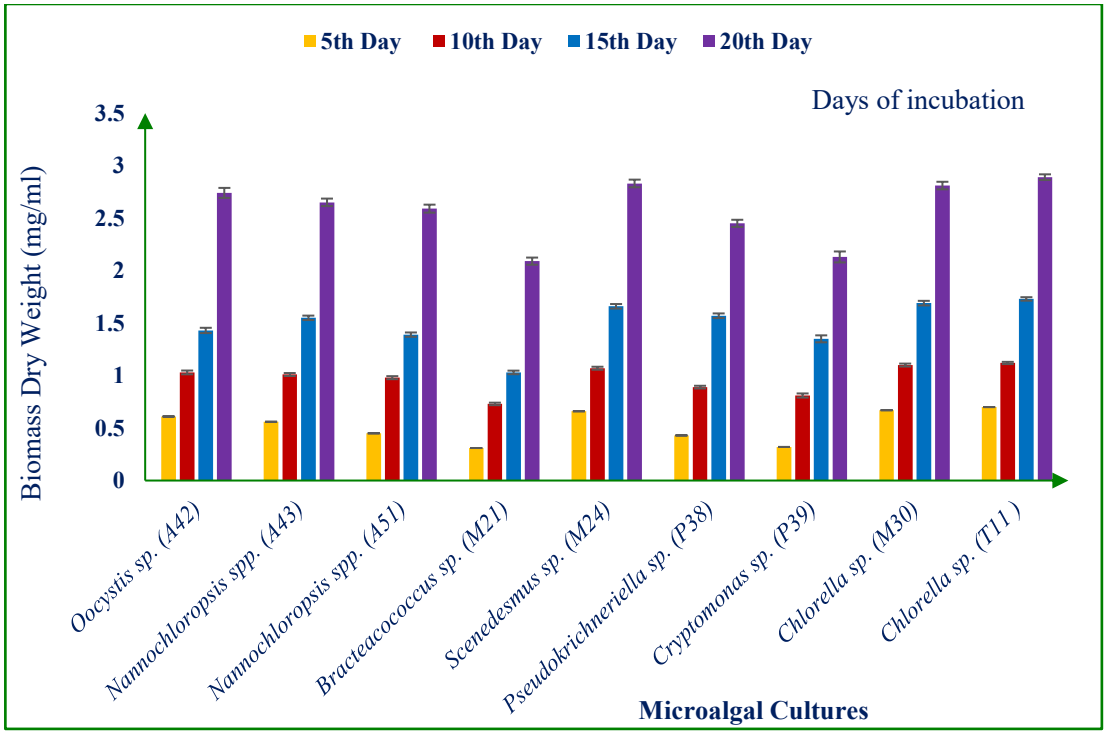
Chlorella is a green algae, with high photosynthetic activity. They are used as fodder and also have an industrial value. It is recently studied as an ideal feedstock for biofuel production, as they can fix carbondioxide and can remove greenhouse gas through biomitigation (Liu and Chen 2014).

The growth curve and specific growth rate were calculated, which are shown in Figure 3.5 and Table 3.2, respectively. Microalgal growth rate is measured and many researchers have used it to determine the productivity of the water sample, as microalgae can be used as indicator organisms. They can be sensitive to physical, chemical and biological factors i.e., nutrient supply, temperature and light which play a vital role in the growth (Maqsood 1974). The specific growth curve is determined by the classical Beer Lambert law application as shown in Figure 3.5. Microalgae and cyanobacteria are unicellular phototrophs which can multiply fast and generate biomass at a higher rate. Microalgae do not need to grow leaves, stalk and flowers. Their growth rate is high as their duplication time is short. The results inferred that M30 (*Chlorella* sp.) has the highest growth rate of 0.189 per day and M24 (*Scenedesmus* sp.) has 0.090 per day as stated in Table 3.2.

The microalgae was mass cultivated and the biomass, chlorophyll, carotenoid, protein, carbohydrate and lipid were estimated every 5 days once for 20 days. The mass cultivated microalgae were analyzed for the biomass by dry weight method.

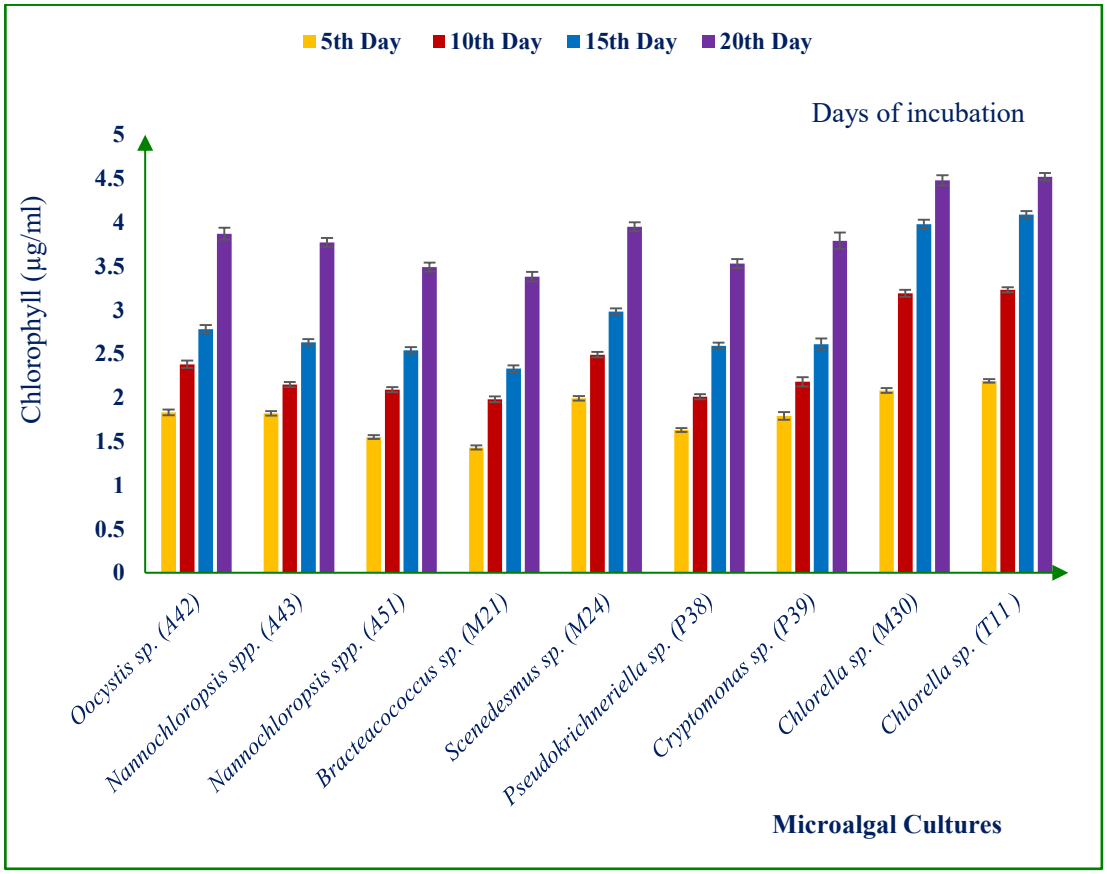
From the results, it was observed that T11 (*Chlorella* sp.) and M24 (*Scenedesmus* sp.) has the highest biomass concentration measuring 2.89 ± 0.027 mg/ml and 2.83 ± 0.037 mg/ml, respectively, during the 20th day estimation. And M21 (*Bracteacococcus* sp.) has the lowest biomass concentration measuring 2.09 ± 0.034 mg/ml and it was represented in Figure 3.6.

Figure 3.6 Analysis of dry biomass (mg/ml) of the microalgal cultures



Chlorophyll is one of the valuable bioactive compounds that can be extracted from microalgal biomass (Hosikian et al. 2010). The chlorophyll concentration was estimated by Hiscox and Istaelstam (1979) method and T11 (*Chlorella* sp.) had the highest chlorophyll concentration, whereas M21 (*Bracteacococcus* sp.) had the lowest concentration measuring of about 4.52 ± 0.042 µg/ml and 3.38 ± 0.055 µg/ml, respectively, at the 20th day estimation (Figure 3.7).

Figure 3.7 Analysis of chlorophyll (µg/ml) in the isolated microalgal cultures



The highest carotenoid concentration was observed which measured about 3.63 ± 0.024 µg/ml in T11 (*Chlorella* sp.) and the lowest concentration was observed in the M21 (*Bracteacococcus* sp.) measuring about 1.35 ± 0.022 µg/ml. Carotenoids are formed in secondary metabolism. Lipids and carotenoids share a common precursor, acetyl-CoA, and carotenoid production is not growth associated (Braunwald et al. 2013). The isolated microalgae show almost 10% to 20% of carotenoid from its biomass, which is shown in Figure 3.8.

Figure 3.8 Analysis of carotenoid (µg/ml) from the isolated microalgal cultures

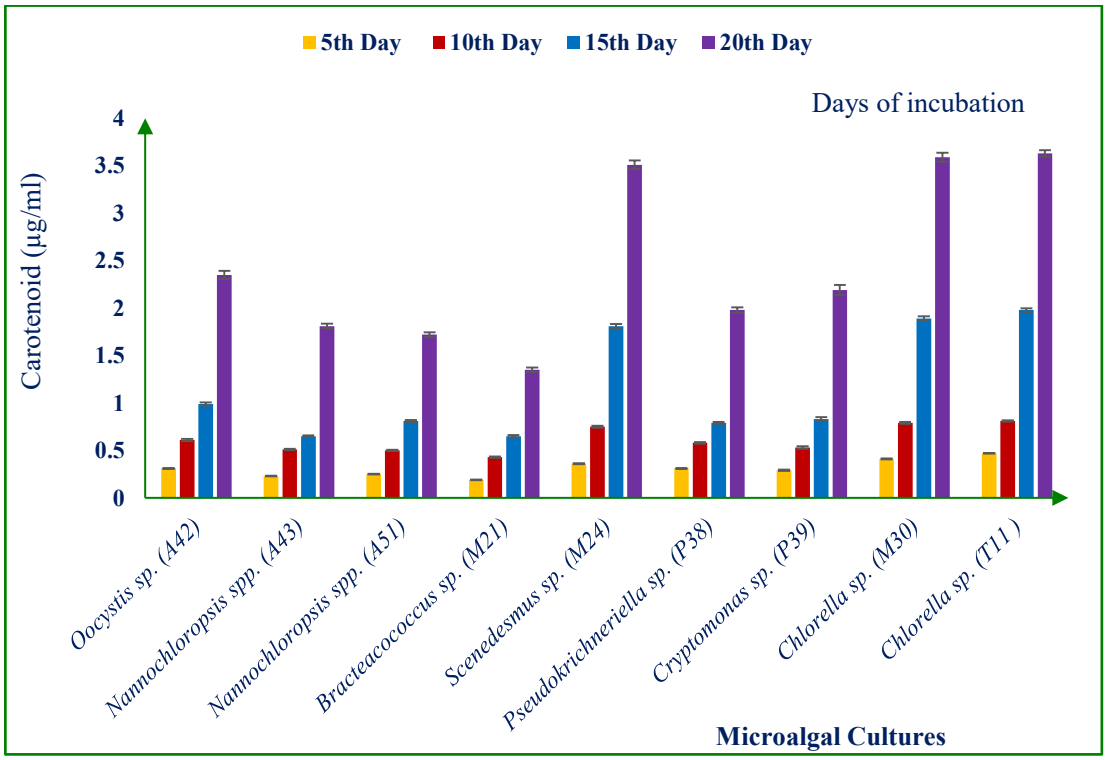
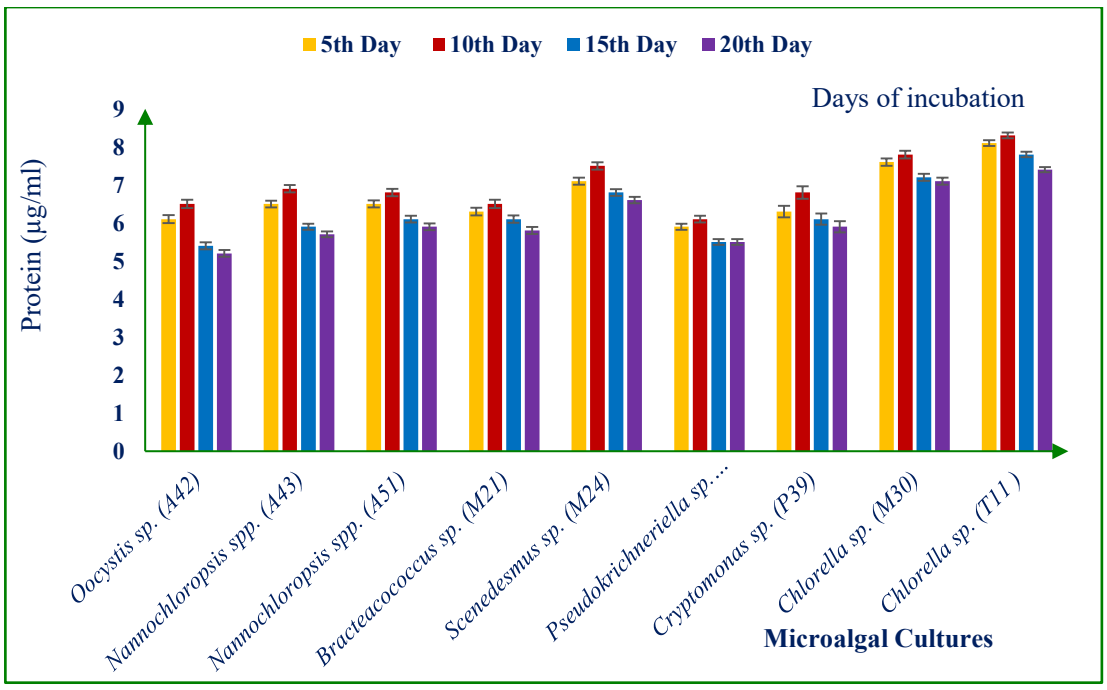


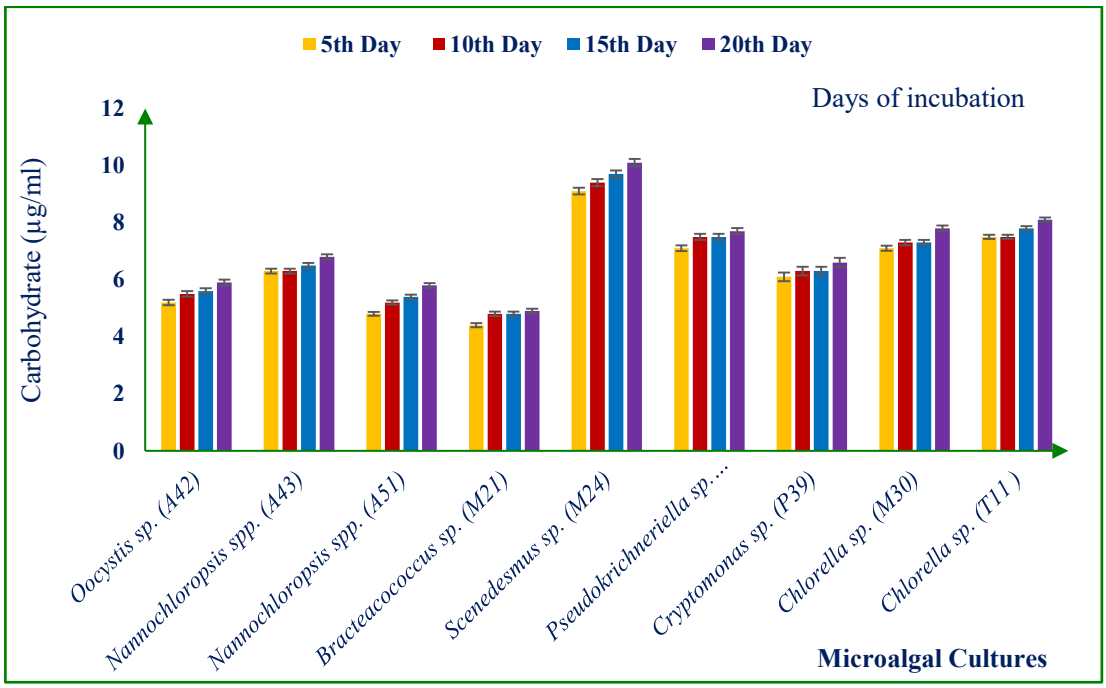
Figure 3.9 Analysis of protein (µg/ml) of the isolated microalgal cultures



Normally microalgae contain three kinds of organic substance: protein, carbohydrate and natural lipid (William 2003). The protein concentration was observed as 7.4 ± 0.069 µg/ml in T11 (*Chlorella* sp.) and 5.2 ± 0.091 µg/ml in A42 (*Oocystis* sp.), which where the highest and lowest, respectively. There was a gradual decrease in the protein concentration (Figure 3.9). This could have been due to the carbohydrate accumulation which would have decreased the protein concentration. Generally algal cultures in the exponential growth phase contain more protein, when cultures in the stationary phase have more carbohydrates and glycogen (De Pauw et al. 1984). Microalgae are usually focused due to its efficacy in biomass production per hectare, which is approximately 5 to 30 times higher than crop plants (Sheehan et al. 1998). And they have high lipid and carbohydrate content which makes it ideal for biofuel production.

The highest concentration of carbohydrate was observed in the 20th day estimation of M24 (*Scenedesmus* sp.) and the lowest concentration was estimated in the M21 (*Bracteacococcus* sp.) which measured about 10.1 ± 0.131 µg/ml and 4.9 ± 0.08 µg/ml, respectively (Figure 3.10). Due to microalgae's high photosynthesis efficacy, which plays a vital role in CO_2 fixation, lipids and carbohydrates accumulate (Subhadra and Edwards 2010). They are a promising feedstock, as they contain high content of lipid and carbohydrate, which are energy rich compounds (Ho et al. 2012). There are variations observed in the accumulation of neutral lipids and carbohydrate as a carbon in the day and night. They act as energetic reserves during the day and these reserves are used for the respiration path and mobilized to produce functional carbon during the night (Suhenik and Carmeli 1990, Cuhel et al. 1984).

Figure 3.10 Analysis of carbohydrate (µg/ml) of the isolated microalgal cultures



The lipid concentration was estimated by the Bligh and Dyer method (1959) and the highest lipid concentration was observed as 39.8 ± 0.373% in T11 (*Chlorella* sp.), followed by M24 (*Scenedesmus* sp.) as 39.1 ± 0.505% and the lowest was observed as 34.2 ± 0.481 % in P38 (*Pseudokrichneriella* sp.) (Figure 3.11). Microalgae have the ability to accumulate lipids and about 3% to 8% of solar energy can be converted to biomass, whereas observed yields for terrestrial plants are about 0.5%. Many microalgae species can be induced to accumulate substantial quantities of lipids thus contributing to a high oil yield. The average lipid content varies between 1% and 70%, but under certain conditions, some species can reach 90% of dry weight (Li et al. 2008, Sheehan et al. 1998).

The lipids include neutral lipids, polar lipids, wax esters, sterols and hydrocarbon. Many algal species grow rapidly and produce extensive amount of TAG

or oil, which is referred to as oleaginous algae; it is being stated as the cell factories which produce oils and other lipids for biofuels (Benemann 1980).

Figure 3.11 Analysis of lipid (%) of the isolated microalgal cultures

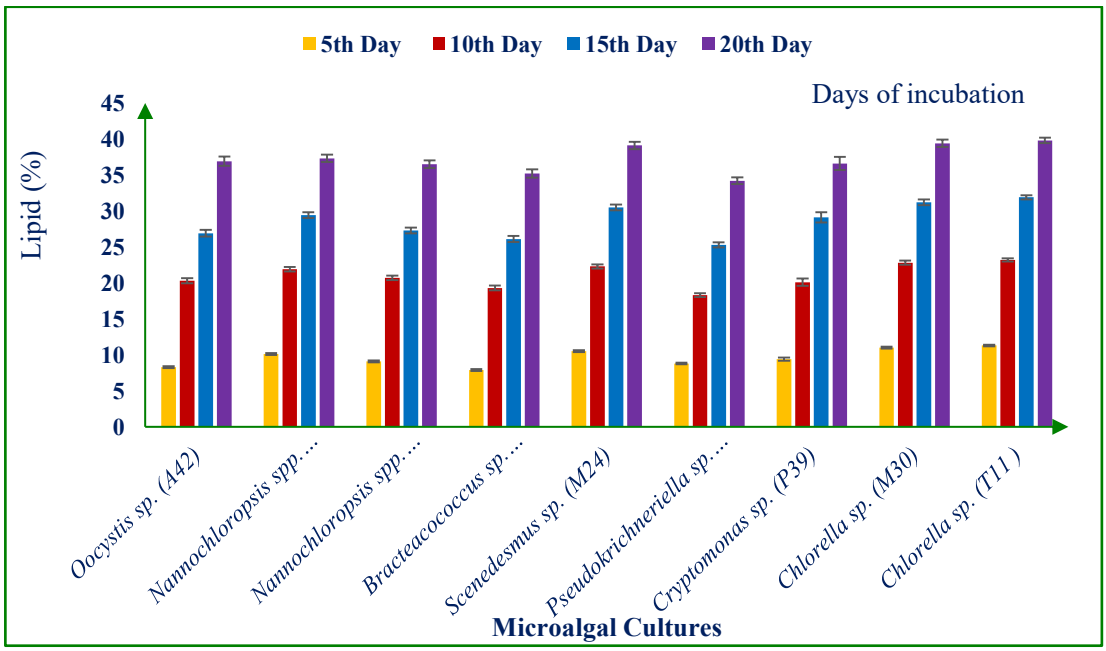


Figure 3.12 Extraction of lipid from microalgae

The lipid obtained from the microalgal cultures were transesterified and the FAME was analyzed by FTIR and GC–MS analysis. Many researchers reported that FTIR is applied to analyze biodiesel. FTIR is used to identify the chemical's property: (1) organic or inorganic and (2) gas, liquid or solids. Analyses of the transesterified

samples were represented in Figures 3.13a–3.13j. The IR spectrum shows main absorption bands, which characterize the different bonds, C=O carbonylic compounds, which show a strong C=O stretching absorption in the region 1870 to 1540 cm^{-1}. Esters appear in 1750 to 1735 cm^{-1} as a stretch absorption region and in 1300 to 1000 cm^{-1} showing a stretch of two or more bands. H_2O absorption bands can be observed in 1800 to 1200 cm^{-1}. The FTIR analysis of the nine microalgal cultures showed the presence of ester groups, i.e., wavenumbers were observed from 1200 to 900 cm^{-1} in all the cultures. It showed the presence of esters with stretch of two or more bands. Strong ester stretches were observed in T11 (*Chlorella* sp.), M24 (*Scenedesmus* sp.), A43 (*Nannochloropsis* spp.), A42 (*Oocystis* sp.) and P38 (*Pseudokrichneriella* sp.), i.e., 1732, 1745, 1741, 1743 and 1730 cm^{-1}.

Figure 3.13a FTIR analysis of FAME transesterified in T11 (*Chlorella* sp)

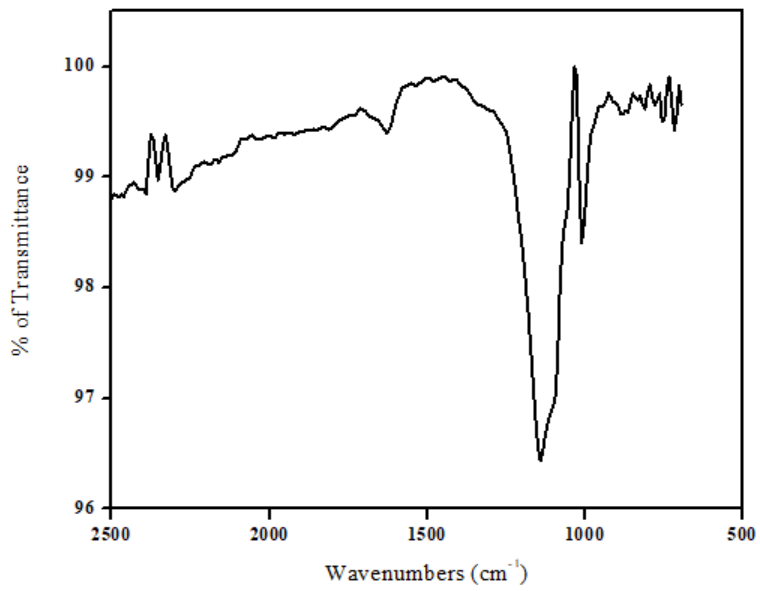


Figure 3.13b FTIR analysis of FAME transesterified in A43 (*Nannochloropsis* spp)

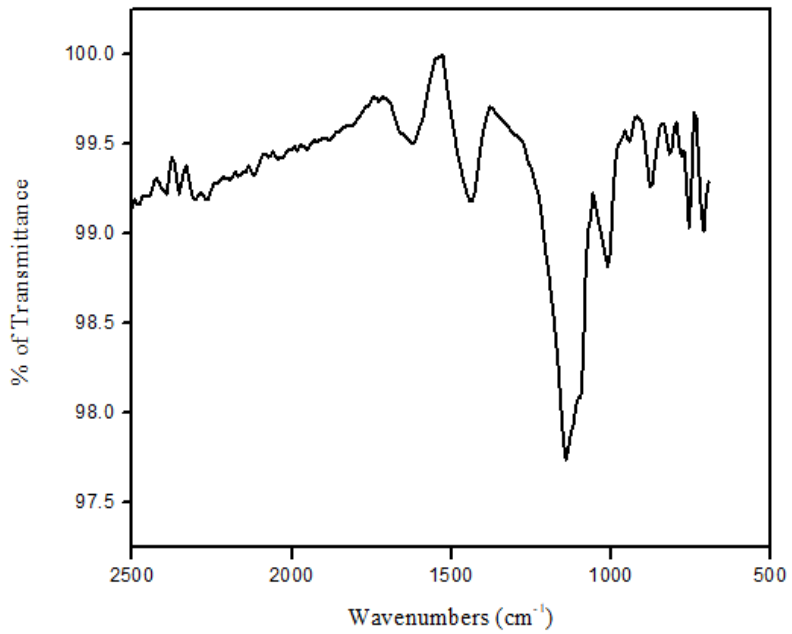


Figure 3.13c FTIR analysis of FAME transesterified in A51 (*Nannochloropsis* sp)

Figure 3.13d FTIR analysis of FAME transesterified in A42 (*Oocystis* sp)

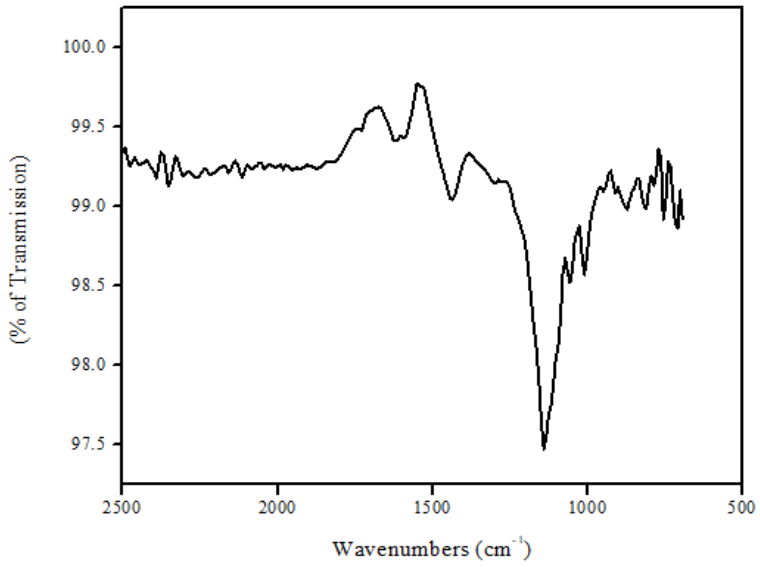


Figure 3.13e FTIR analysis of FAME transesterified in M21 (*Bracteacoccus* sp)

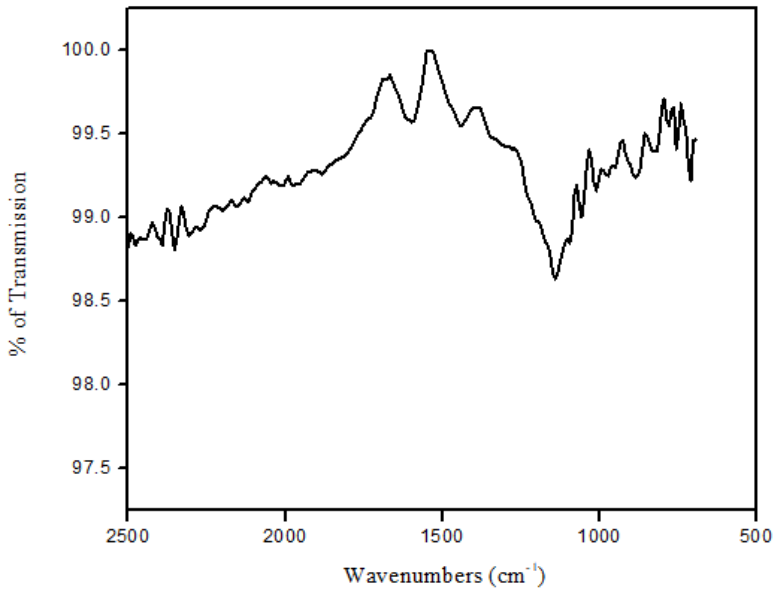


Figure 3.13f FTIR analysis of FAME transesterified in M24 (*Scenedesmus* sp)

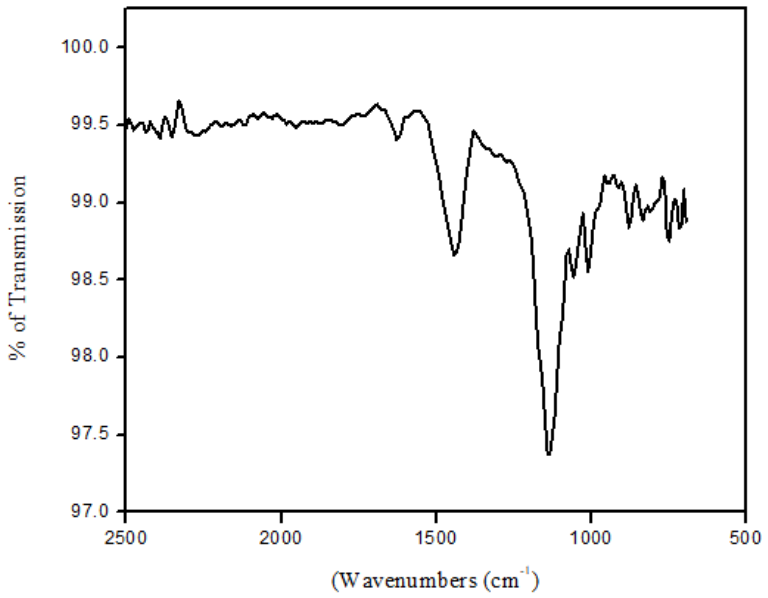


Figure 3.13g FTIR analysis of FAME transesterified in P38 (*Pseudokrichneriella* sp.)

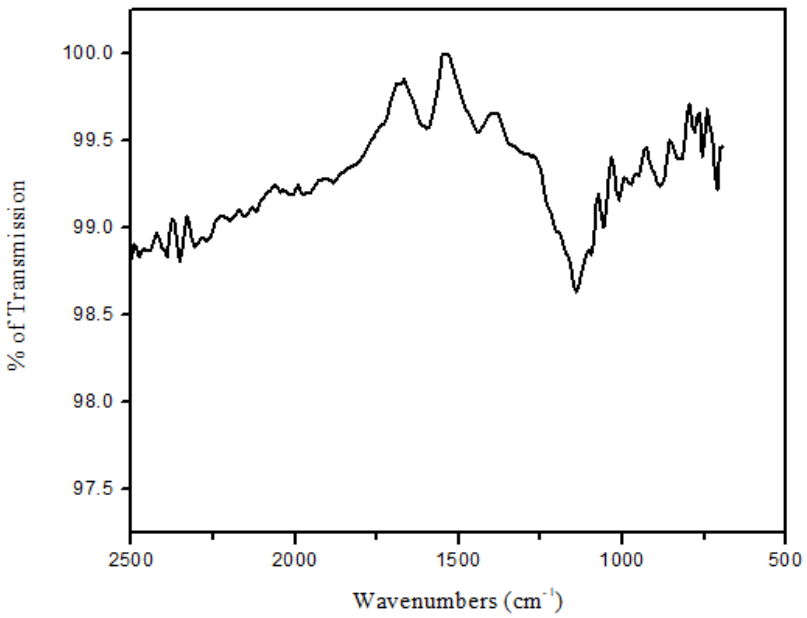


Figure 3.13h FTIR analysis of FAME transesterified in P39 (*Cryptomonas* sp.)

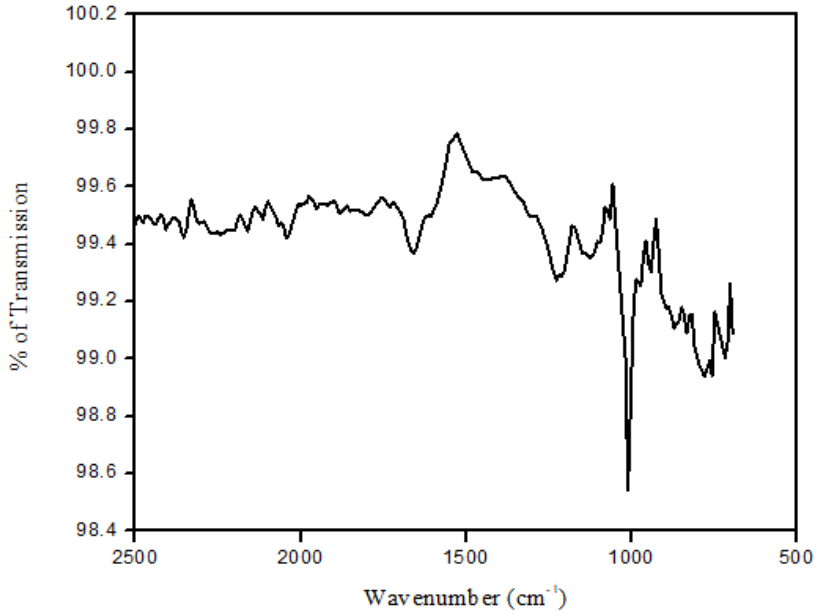
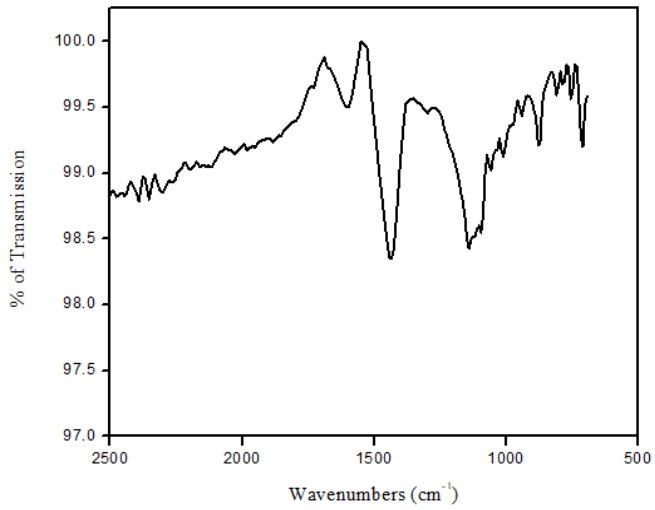


Figure 3.13i FTIR analysis of FAME transesterified in M30 (*Chlorella* sp.)



The gas chromatographical analysis was done for the lipid produced by the microalgae, A42 (*Oocystis* sp.), A43 (*Nannochloropsis* spp.), A51 (*Nannochloropsis* spp.), M21 (*Bracteacococcus* sp.), M24 (*Scenedesmus* sp.), P38 (*Pseudokrichneriella* sp.), P39 (*Cryptomonas* sp.), M30 (*Chlorella* sp.) and T11 (*Chlorella* sp.) and represented in table 3.3. A43 (*Nannochloropsis* spp.) showed the presence of C16:0, C18:1, C12:1 and C14:0 as a mass percentage of 23.9, 20.6, 12.1 and 3.4, respectively. In M24 (*Scenedesmus* sp.), C14:0, C16:0, C16:1, C18:1 and C18:3 was observed as 4.46, 26.4, 0.73, 23.2, and 17.3, respectively. In T11 (*Chlorella* sp.) C16:0, C16:1, C18:1 and C20:0 was observed as 3.4, 27.36, 26.69 and 0.92, respectively. A good quality of biodiesel should have 5:4:1 mass fatty acid ratio of C16:1, C18:1 and C10:0 (Schenk et al. 2008). And this ratio was approximately observed in M24 (*Scenedesmus* sp.), A43 (*Nannochloropsis* spp.), M30 (*Chlorella* sp.), and T11 (*Chlorella* sp.). Microalgal lipids are usually composed of unsaturated fatty acids of about 60% to 80% and it seemingly has 19.5% to 30.3% of palimitic acid (C16:0) (Gouveia and Oliveira 2009). Olofsson et al. (2012) has stated that stearic acid, palmitic acid and oleic acid are the fatty acids that have good potential to be converted to biodiesel. And the high content of C18:0 and C18:2 is a prerequisite for biodiesel production, as the oxidative stability and adaptability potency is significant in industrial scale production. The characteristics of biodiesel depends on the proportion of the long chain and short chain fatty acids and also the presence of one or more double bonds.

Table 3.3 Composition of the methyl and ethyl esters in the microalgae

S. No	Fatty acid	Mass percentage								
		***Oocystis* sp. (A42)**	***Nannochloropsis* spp. (A43)**	***Nannochloropsis* spp. (A51)**	***Bracteacococcus* sp. (M21)**	***Scenedesmus* sp. (M24)**	***Pseudokrichneriella* sp. (P38)**	***Cryptomonas* sp. (P39)**	***Chlorella* sp. (M30)**	**`*Chlorella* sp. (T11)**
1.	Caprate (C10:0)	—	—	—	—	—	—	—	—	—
2.	Myristate (C14:0)	—	3.4	—	—	4.46	—	—	3.2	—
3.	Palmitate (C16:0)	—	23.9	23.54	3.7	26.4	20.4	——	18.6	3.4
4.	Palmitoleate (C16:1)	1.6	—	—	21.0	0.73	—	—	1.4	27.36
5.	Stearate (C18:0)	6.3	—	38.60	6.78	—	—	—	—	—
6.	Oelate (C18:1)	—	20.6	—	3.4	23.2	—	—	—	26.69
7.	Linoleate (C18:2)	—	12.1	—	—	—	—	—	15.2	—
8.	Linolenate (C18:3)	—	—	—	6.78	17.3	16.86	6.78	24.3	—
9.	Arachidate (C20:0)	—	—	1.16	—	—	—	—	—	0.92
10.	Behenate (C22:0)	12.5	—	—	—	—	—	—	—	—

Note: "—" denotes 'not detected'.

Table 3.4 Analysis of the biodiesel produced by microalgal cultures

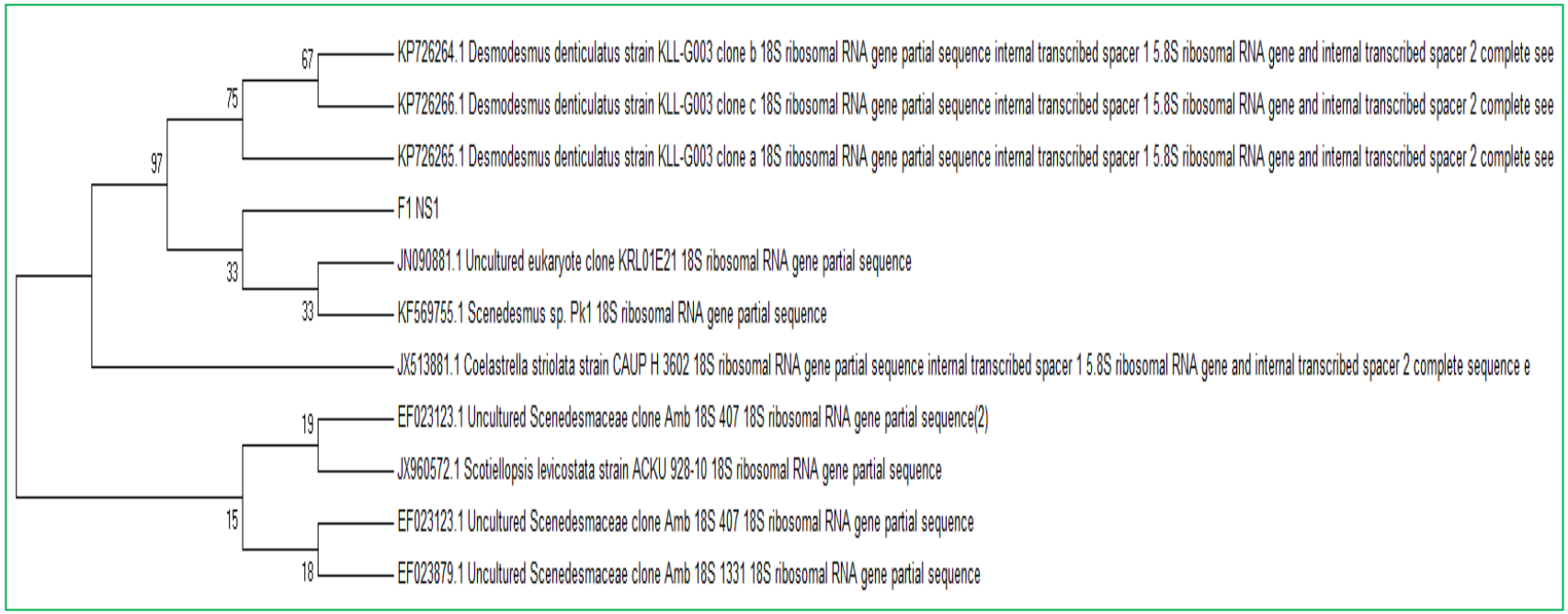
S.No	Microalgal cultures	Oil yield (mg/g)	Biodiesel yield (mg/g)	Percentage of yield
1	*Bracteacococcus* sp. (M21)	4.918	1.941	39.47
2	*Scenedesmus* sp. (M24)	3.964	2.218	55.95
3	*Pseudokrichneriella* sp. (P38)	5.302	2.670	50.36
4	*Cryptomonas* sp. (P39)	5.403	2.445	45.25
5	*Chlorella* sp. (M30)	6.713	2.796	41.65
6	*Oocystis* sp. (A42)	5.597	2.393	42.76
7	*Nannochloropsis* spp. (A43)	5.712	2.810	49.19
8	*Nannochloropsis* spp. (A51)	6.514	2.376	36.48
9	*Chlorella* sp. (T11)	6.008	3.322	55.29

The transesterified samples were studied for their oil yield and biodiesel yield which is represented in table 3.4. The percentage of the yield was calculated and it was found that M24 (*Scenedesmus* sp.) had the highest of 55.94% and A51 (*Nannochloropsis* spp.) had the lowest of 36.48%. Fatty acids are medium chain (C10–C14), long chain (C16–C18) and very long chain (≥C20). The fatty acid composition of microalgae is usually the same as plant oils which are used for biodiesel production (Ohlrogge and Browse 1995). But microalgal fatty acids shows many variations when compared to higher plants. They have medium chain fatty acids (e.g. C10, C12, and C14) and many species have very long chain fatty acids (≥C20) (Hu et al. 2008). The biodiesel characteristics are affected by the proportion of the long chain and short chain

fatty acids and also with the number of double bonds. This percentage of yield was used as a screening process to identify the potential microalgae. A43 (*Nannochloropsis* spp.), M24 (*Scenedesmus* sp.), P38 (*Pseudokrichneriella* sp.) and T11 (*Chlorella* sp.) were taken forward for further analysis.

The potential microalgae M24 (*Scenedesmus* sp.) was characterized for its molecular identification and confirmation. Maximum Likelihood method was used to infer the evolutionary history based on the Tamura-Nei model (Tamura and Nei 1993). The bootstrap consensus tree can be inferred from 1000 replicates and it was represented and analyzed by the evolutionary history of the taxa (Felsenstein 1985). The sequences of 18s rRNA was compared with the identified microalgal sequences acquired from the site of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). All the sequences were aligned through CulstalW of MEGA 7. The Phylogenetic tree (Figure 3.14) was constructed (Kumar et al. 2016).

Figure 3.14 Phylogenetic tree of the microalgal isolate based on the 18srRNA gene sequence



CONCLUSION

Microalgal cultures were isolated from the water samples from northeastern estuaries of Tamil Nadu. Estuarine microalgae are stated to have a higher adaptation condition due to its linkage characters of both the river and sea. Nine microalgal cultures were isolated from various study stations. They are A42 (*Oocystis* sp.), A43 (*Nannochloropsis* spp.), A51 (*Nannochloropsis* spp.), M21 (*Bracteacococcus* sp.), M24 (*Scenedesmus* sp.), P38 (*Pseudokrichneriella* sp.), P39 (*Cryptomonas* sp.), M30 (*Chlorella* sp.) and T11 (*Chlorella* sp.). The species were studied for its growth by optical density and the specific growth rate was determined. The growth characters, i.e., dry biomass weight, chlorophyll, carotenoid, protein, carbohydrate and lipid were studied. The lipid was extracted and transesterified. FAME was analyzed using FTIR and GC–MS. In FTIR spectrum, peaks of ester stretch and stretch of two or more bands were observed in M24 (*Scenedesmus* sp.), A42 (*Oocystis* sp.), A43 (*Nannochloropsis* spp.) and T11 (*Chlorella* sp.). The GC–MS results revealed the presence of palmitic acid, linolenic acid, linoleic acid and myristic acid in M21 (*Bracteacococcus* sp.), M24 (*Scenedesmus* sp.), M30 (*Chlorella* sp.), and T11 (*Chlorella* sp.). The oil yield and the biodiesel yield were studied and used as a method to screen the potential microalgae. From the percentage of yield of biodiesel, A43 (*Nannochloropsis* spp.), M24 (*Scenedesmus* sp.), P38 (*Pseudokrichneriella* sp.), and T11 (*Chlorella* sp.) were screened to be potential and were taken forward for further analysis.