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Optimization of Growth and Lipid Production of the Chlorophyte Microalga *Chlorella Emersonii* as a Feedstock for Biodiesel Production

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Abstract : *Chlorella emersonii* has been isolated and tested for its growth, pigment, protein and free amino acid contents. The growth of *chlorella emersonii* was found to be 3.2 times more in alternate illumination than in continuous light condition. Similarly, chlorophyll content was found as 2.50% (Chl-a) and 0.62% (Chl-b) in alternate light period and 1.65% (Chl-a) and 0.50% (Chl-b) in continuous illumination. The total protein content was found to correlates with growth and chlorophyll content. Carotenoid and free amino acid content was higher in continuous light period than in alternate light illumination. In the present study, based on the results, it is identified that the alternate dark and light period is more suitable for the growth of *Chlorella emersonii* than continuous light illumination. Hence, the present research work is focused on the biodiesel production from the Chlorella emersonii. In this study, soxhlet extraction had been used to extract the algal oil which contains high percentage of fatty acids. The extracted algal oil was characterized using GC-MS spectral data. Based on the spectral data, totally five fatty acids were identified using NIST GC-MS library. Further, the crude algal oil was converted into biodiesel using enzyme-catalyzed trans-esterification reaction.
Keywords: *Chlorella emersonii*; Isolation; Growth; Pigment; Carotenoid; Proteins; Amino acid; enzyme catalysed trans-esterification**.**

Introduction

Biofuels production reduces GHG emissions, boosting the decarbonisation of transportation fuels and increasing the security of energy supply. It is renewable, biodegradable, and environmentally friendly fuel, biodiesel has received more attention in the past few years. Biodiesel refers to fatty acids methyl esters (FAMEs) from vegetable oils or animal fats with high stability; low water and volatiles content; a low amount of polymers containing sulfur and nitrogen elements[1-5]. In this emerging world, the use of fossil fuels as energy is now widely accepted as unsustainable due to depleting resources and accumulation of greenhouse gases in the environment. Global warming resulting from extensive CO_2 emissions due to human activities has become a major concern as an environmental issue. The microalgae also have certain advantages such as, a high growth rate, short growth time, high biomass production, and low land use to compare other corps[6].

Microalgae have been considered as one of the most promising feed stocks for biodiesel production due to their short cell cycle (within 24 hours), high oil content (20%– 50% normally and highest exceeding 80%), strong adaptive capacity to environment (high salinity, heavy metal ion, toxicants, high CO_2 concentration, etc.) and no occupation for cropping area[7]. *Chlorella species* has potential benefits and uses as food and feed supplement and also in health and cosmetic products. The growth of the algae depends up on pH, temperature, aeration, agitation, salt concentration and light illumination. Light plays a vital role as it straightly affects the

photosynthetic machinery. The current algae-based biodiesel is mainly produced by conventional route: extraction of the lipids from the microalgal biomass followed by its conversion to FAMEs and glycerol[7-9].

The algae growth is mainly depending up on by these factors like aminoacid, proteins, pigment and lipid, etc. *Prochlorococcus* species examined that, the effect of light on the cell growth were analysed with the growth in *Chlamydomonas geilteri*. Similarly, on the other hand, *Chlamydomonas ulvaensis*, *Pithophora kewensis*, *Cladophora flexuosa*, *Chaetomorpha melagonium* and *Rhizoclomium riparium* growth metabolism were studied, in the presence of light. The present study deals with the isolation, determination of growth, total protein content, chlorophyll content, carotenoid content and free aminoacid of Chlorella emersonii on alternate dark and light period and continuous illumination were analyzed. The present paper also describes about the biodiesel production from the *Chlorella emersonii.*

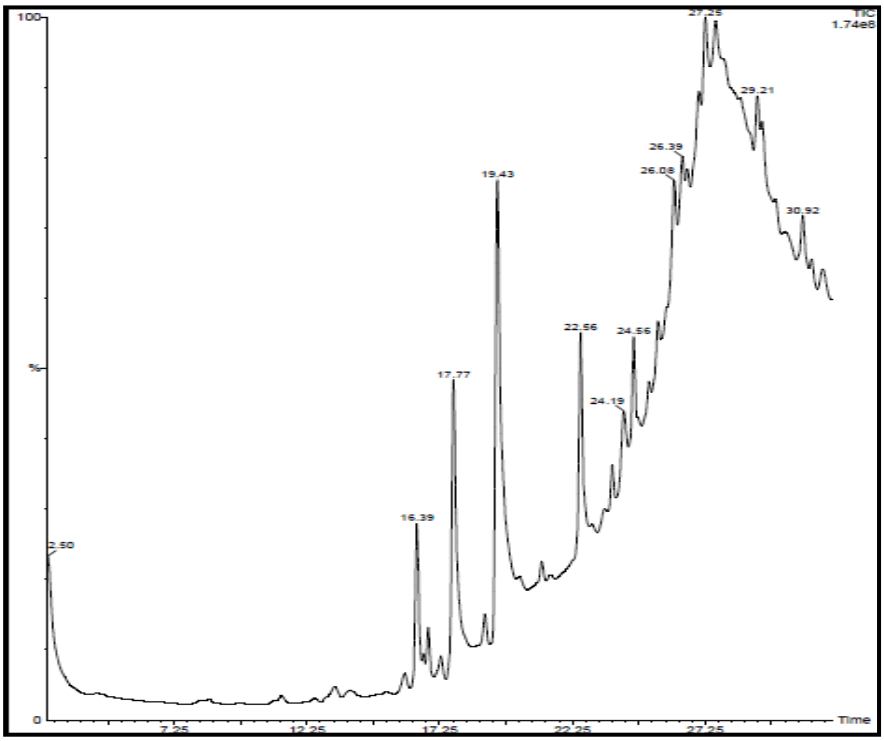

Fig. 1: represents the GC chromatogram of bio-oil

The major fatty acids present in *Chlorella emersonii* was Oleic acid, Linoleic acid, Hexadecenoic acid, Octacecadienoic acid, Cyclopropaneoctanic acid, Pentadecanoic acid, and Hexadecanoic acid respectively.

Materials and Methods

Collection of microalgae strain:

The water sample was collected from the pond in northern part of Tamil Nadu in Vellore district. The sample was taken and serial dilution was done by using Bristol medium agar plates. To this serially diluted culture, streak plates were done in MRS agar plates to obtain pure colonies. *Chlorella emersonii* was maintained in BBM medium in rotary shaker at 100rpm with a photoperiod of 12 hours /light/ 12 hours' dark, light intensity of 2000 lux at a temperature of at room temperature (28+2).

Growth Measurements:

Two microliter of algal culture was inoculated in 10 ml of the culture medium and incubated in continuous illumination and alternate light and dark light further analyzed for growth by measuring the optical density (OD), cell count (CC) and dry weight (DW) upto 5 weeks. Optical density was determined by measuring the absorbance in colorimeter at 670 nm and cell count was tested by measuring haemocytometer. The algal

culture was filtered in Whatman filter; the deposited sediment was washed thrice with distilled water and dried overnight in oven at 60 ◦C. The medium containing algae was subjected for the estimation of pigments, total protein and total free amino acids. All the experiments were done in triplicate. The experiment was performed for five weeks.

Protein estimation:

Extracellular protein content was determined by Lowry method[10] with bovine serum albumin (BSA) as standard.

Estimation of pigments:

The algal sample was extracted with 90% (v/v) acetone to determine the chlorophyll content. Carotenoid content was determined by extracting the samples with 80% (v/v) acetone. The sample was extracted with 100% methanol for chlorophyll estimation[11]. Based on cell concentration, the samples were diluted by 10–20 times. The sample containing vials were stored at 4°C for 30 min by wrapping in aluminum foil. Then the samples were centrifuged at 16,000g for 10 min. The obtained green supernatant was analyzed at 650 and 665 nm. Chlorophyll a (mg/L), chlorophyll b (mg/L), and total chlorophyll content (mg/L) were then calculated using the equations described by Hitkins and Baker. On 5th, 10th, 15th, 20th day the sample were analyzed for total protein content, Chlorophyll a, Chlorophyll b and Total Chlorophyll.

Estimation of amino acids:

Free amino acid was estimated by Lee and Takahashi method[12]. Alcoholic extract (1 ml) was mixed with ninhydrin reagent (5 ml) and heated for 20-25 minutes. After heating, the tubes were cooled and measured the absorbance at 570 nm with glycine as standard[13].

Statistical analysis:

All the experiments were performed with thrice with triplicate. The data was statistically analyzed in SPSS package 16.

Elicitation of Algal Oil using Soxhlet's extraction process:

The biomass of *Chlorella emersonii* harvested by centrifugation at 10,000 rpm and wet cell mass was dired at 60ºC at constant weight was obtained. Weight 100 grams of *Chlorella emersonii* powder material and was transferred into soxhlet's extraction tube. About 350 ml of n-hexane was poured into a round-bottomed flask and then, the Soxhlet's apparatus was connected with LB condenser. Finally, the organic solvent was heated using a heating mantle for about 48 hours at a temperature of 70°C. The rubber oil was collected from extraction process using distillation process.

Yield of oil in percentage:

After the algal oil was extraction by Soxhlet's apparatus was transferred into a measuring cylinder which was placed over water bath for 30 minutes at 70˚C, to ensure complete evaporation of solvent and volume of the oil was recorded. The percentage of oil was calculated by standard formula[13].

Fatty acid analysis:

The qualitative and quantitative analysis of fatty acids was analyzed using GC-MS spectrophotometer. Gas Chromatography: An Agilent 6890 gas chromatograph was equipped with a straight deactivated 2 mm direct injector liner and a 15m All tech EC-5 column (250µ I.D., 0.25µ film thickness). A split injection was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35ºC, hold for 2 minutes, then ramp at 20ºC per minute to 300ºCand hold for 5 minutes. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode). Mass Spectrometry: A JEOL GC mate II bench top double-focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-20001 software was used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 700 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra were acquired at a resolving power of 5000

(20% height definition) and scanning the magnet from m/z 65 to m/z 750 at 1 second per scan. Mass spectrometry library search: Identification of the components of the purified compound was matching their recorded spectra with the data bank mass spectra of NIST library V 11 provided by the instruments software.

Catalyst preparation:

Synthesis of magnetic nanoparticles coated with gum arabic: Magnetic Fe_3O_4 nanoparticles were prepared by co-precipitation method. Take the round bottom flask 2.33g of $FeCl_3 \cdot 6H_2O$ and 0.86 g of $FeCl_2 \cdot 4H_2O$ were dissolved in 100 ml deionized water under nitrogen and 10 ml 25% $NH_3 \cdot H_2O$ was added to the solution drop wise under vigorous stirring at 70^{o}C. After the colour bulk solution turned to black, and then the magnetite precipitates were washed several times with deionized water and once with ethanol and separated by magnetic decantation. For surface coating with gum arabic, magnetic nanoparticles were suspended in 25 ml distilled water containing about 100 mg of gum arabic powder, under mechanical stirring, and the mixture was ultra-sonicated for 30 minutes. This method allowed the gum arabic to mix thoroughly in the sample. After sonication, the sample was washed several times with water to remove untreated polymer. The solid product was isolated by magnet separation and dried under vacuum.

Lipase preparation:

Lipase was incubated with surfactants CTAB (cationic) which coat the hydrophobic pocket of the lipase and stabilize its open form. It is worth mentioning that the coexisting of surfactant molecules in the reaction surfactant solution does not affect enzymatic Trans-esterification in organic media. Surfactant lipase was prepared using cationic (CTAB). Lipase (1 g), suspended in 100 ml of 1mM phosphate buffer (pH 7), and 0.1% CTAB were added to reaction vessel and mixed thoroughly. Each sample mixture was sonicated in an ultrasonic bath for 20 min followed by incubation for 24h at 4^{o}C. After incubation, precipitates were collected by centrifugation at 250 rpm (4^{o}C), washed with 1mM phosphate buffer solution (pH-7) and dried under reduced pressure. A white powder was obtained and the yield was about 15%.

Lipase immobilization:

Gum Arabic coated magnetic nanoparticles (100 mg) were suspended in 2 ml of 25% (w/v) glutaraldehyde solution and were incubated at 20^{o}C for about 2 h. Particles were washed several time with water and dried. For lipase immobilization, 200 mg of surfactant coated lipase and 100 mg support material were mixed with 50 ml of 1mM phosphate buffer (pH-7). The mixture was incubated at 20^{o}C for about 3 h for immobilization. After reaction, immobilized lipase was recovered by a magnet and washed several time with distilled water. The immobilized lipase was dried overnight under vacuum by vacuum freeze drier.

Trans-esterification of Algal oil:

The trans-esterification of algal oil carried out using magnetic nanoparticles immobilized lipase enzyme as a biocatalyst. The Lipid and Methanol was mixed with a molar ratio of (1:10) and then the reaction was stirred at 90°C for about 5 hours. The completion of the reaction was monitored using TLC. Identification of the components of the purified compound was matching their recorded spectra with the data bank mass spectra of NIST library V 11 provided by the instruments software.

Results and Discussion

Biochemical production:

The growth of *Chlorella emersonii* was determined by measuring OD, CC, and DW up to 5 weeks. Alternate lights showed more growth compared to continuous illumination. OD value was increased up to 3.5 times and in continuous light, the OD was 3.0 times. Increased cell count and dry weight was seen in alternate light with 2.7 and 3.3 times respectively from the initial stage of the culture. In the case of continuous illumination, the concentration and DW was only 2.5 times. The result interprets that alternate light showed increased OD, DW, CC compared to continuous illumination[21,22].

Pigment Content:

Growth correlates with the pigment content. Chlorophyll a and b were found to be more in alternate light and dark illumination than in continuous light. The percentage of Chl-a and Chl-b pigment were 2.50 and 0.62 in alternate light period and 1.65 and 0.50 in continuous enlightenment respectively. The optical density (OD), cell count (CC) and dry weight (DW) were measured and found that the biomass concentration was more in natural day light. In alternate illumination, the Chl-a, Chl-b and total protein content was higher than in continuous illumination. But the carotenoids and free amino acid content was maximum in continuous illumination. Chlorella was found to react differently for changes in irradiances[14]. Continuous illumination was favorable for the growth of microalgae than alternate illumination during 1st and 2nd week. During 3rd week, growth and chlorophyll content were found to decrease and other biochemical production was identified to be higher. This may be due to photooxidation inside the microalgal cells, with greater light illumination the algae might synthesize the photosynthetic units very less might be to prevent the damage[15]. Fast nurturing microalgal cells display higher protein and lower carbohydrate content[16].

Estimation of Carotenoid content:

Carotenoid content was determined from the algal culture up to 5 weeks. Carotenoid content was found to be high (0.462 %) in continuous culture after 4 weeks while in alternate dark and light period the carotenoid content was more up to 3 weeks (0.370 %).

Determination of total protein:

Based on the result it is clear that the total protein content was correlates with the growth and chlorophyll content. Total protein content was 49.1% in alternate dark and light period and 43.3% in continuous illumination[20]

Free amino acid content:

Lee and Takahashi method have been used for the analysis of free amino acid. In the continuous illumination, the amount of free amino acid was found to be higher when compared to alternate illumination of light. Amount of aminoacid was identified as 622 µg/g fw and 597 µg/g fw in continuous and alternate illumination respectively. The optical density (OD), cell count (CC) and dry weight (DW) were measured and found that the biomass concentration was more in natural day light. In alternate illumination, the Chl-a, Chl-b and total protein content was higher than in continuous illumination. But the carotenoids and free amino acid content was maximum in continuous illumination. Chlorella was found to react differently for changes in irradiances[15]. Continuous illumination was favorable for the growth of microalgae than alternate illumination during 1st and 2nd week. During 3rd week, growth and chlorophyll content were found to decrease and other biochemical production was identified to be higher[18,19]. This may be due to photooxidation inside the microalgal cells, with greater light illumination the algae might synthesize the photosynthetic units very less might be to prevent the damage[17]. Fast nurturing microalgal cells display higher protein and lower carbohydrate content.

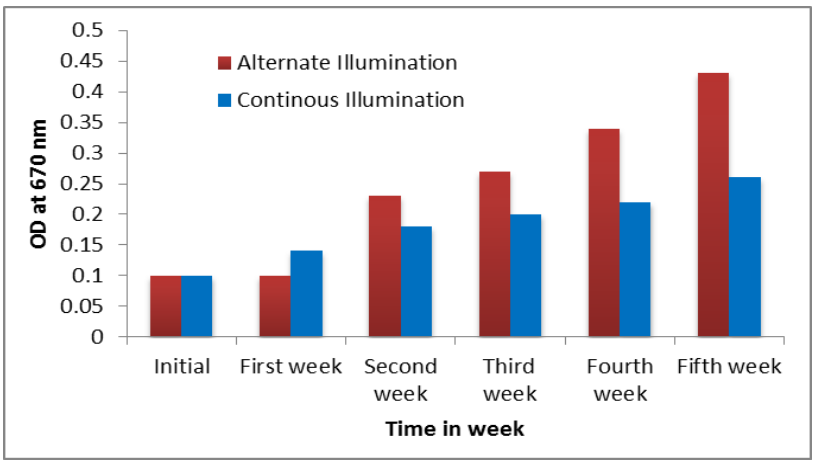

Figure 1: Growth of microalgae with reference to absorbance (OD) at 670 nm

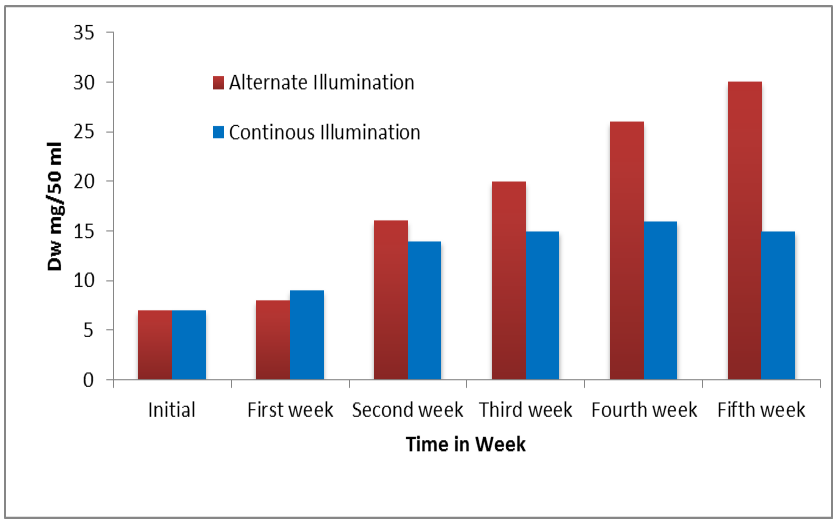

Figure 2: Growth of microalgae with reference to dry weight (DW)

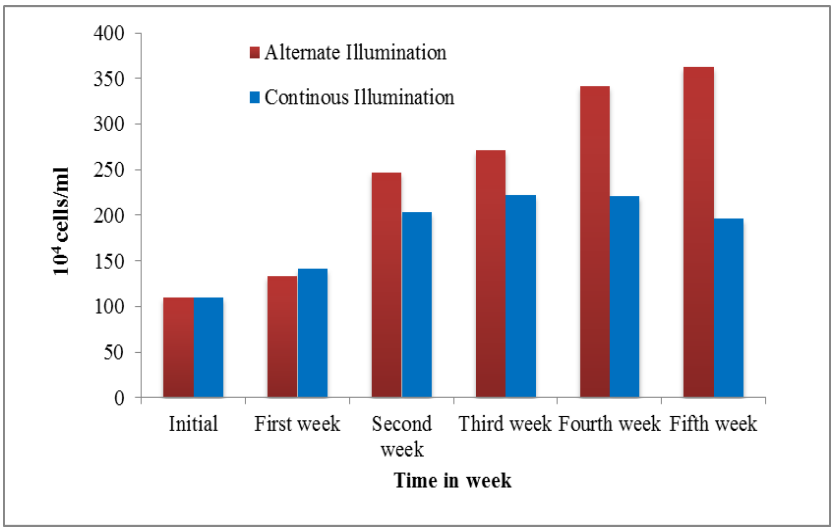

Figure 3: Growth of microalgae with reference to cell count (CC)

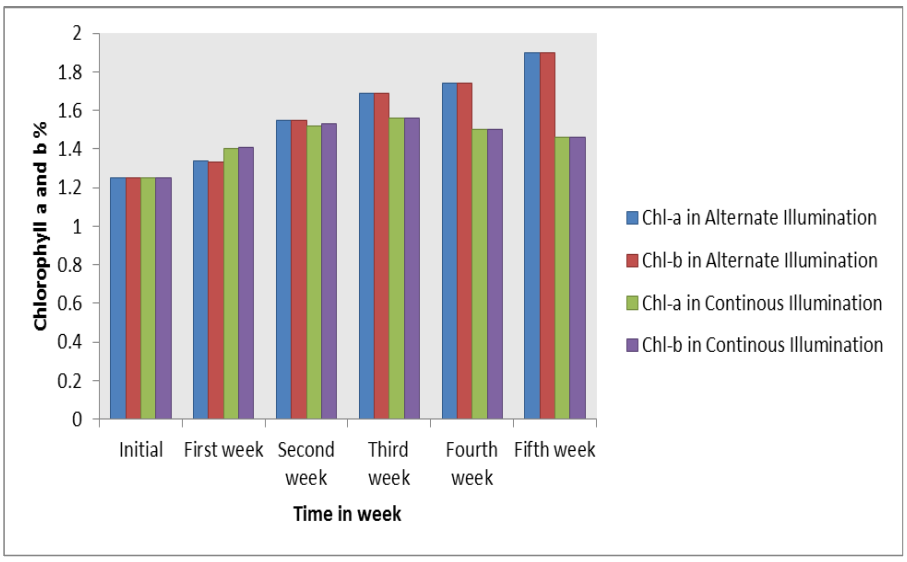

Figure 4: Concentration of Chlorophyll pigment a and b in alternate and continuous illumination

Algal oil extraction:

In the study the algal biomass was used as a raw material for the production of Biodiesel. The moisture content of the raw materials was calculated using standard protocol. Initially the algal biomass was dried using laboratory oven at 60°C at still constant weight was obtained. According to the *Chlorella sps* biomass production rate and oil/lipid concentration reached 176.6 mg/L/d and 50.5%, respectively. Therefore, using DSW-supplemented medium for microalgae growth has considerable potential for commercial microalgae cultivation. In this present study the *Chlorella emersonii* biomass production rate was 183mg/L/d and algal oil was extracted using soxhlet extraction. The maximum oil yield was achieved more than 84%, due to the high temperature and high process duration. Fatty acid analysis was done by GC-MS analysis: GC-MS analysis has been performed on the extracted bio-oil sample. The spectrum of the sample spread from 450 to 850 m/z values and the relative abundance of the resulted components varies from 10000000 to 110000000. The results revealed that the presence of fatty acids. The total compositions of the isolated components are under the peak reflection of different components. The mass spectroscopy of different obtained fatty acids and fatty acid methyl esters have been shown and their chemical structures are reported by Vila method[23]. Based on the spectra, we can find that the chemical and bonding structure of the fatty acids and fatty acid-methyl or ethyl esters. This shows that the obtained sample of *Chlorella emersonii* consist of plenty of fatty acids, which are the important features in producing the bio diesel. Based on the GC-MS spectrum of the different obtained constituents and their chemical structures were identified (Table -1).

Table -1: represent the fatty acid content present in the *Chlorella emersonii.*

Peak No.	RT (min).	Compound Name
1	16.39	Oleic acid
2	17.77	Linoleic acid
3	19.43	Hexadecenoic acid
4	22.56	Octadecanoic Acid
5	24.56	Cyclopropaneoctanic acid
6	26.39	Pentadecanoic acid
7	27.25	Hexadecanoic acid

Based on the GC-MS spectral data, the algal oil was converted into biodiesel using enzymatic trans-esterification process. The completion of the reaction was monitored by TLC. The produced biodiesel sample was also analysed using GC-MS spectrophotometer. The results clearly confirm the conversion of algal oil into biodiesel, with an evidence stated that the fatty acid content present in the algal oil were all converted into fatty acid methyl esters. The fatty acid methyl ester compositions present in the biodiesel were shown in the table-2.

Table -1: represent the fatty acid methyl ester content present in the produced biodiesel.

Peak No.	RT (min).	Compound Name
1	15.87	Oleic acid methyl ester
2	16.49	Linoleic acid methyl ester
3	18.17	Hexadecenoic acid methyl ester
4	21.96	Octadecanoic Acid methyl ester
5	23.32	Cyclopropaneoctanic acid methyl ester
6	25.42	Pentadecanoic acid methyl ester
7	26.28	Hexadecanoic acid methyl ester

Conclusion

A new species *Chlorella emersonii* was isolated from the fresh pond water bodies and tested for its growth, pigment, protein and free amino acid contents. The growth of chlorella emersonii was found to be 3.1 times more in alternate illumination than in continuous light condition. Similarly, chlorophyll content was found as 2.50% (Chl-a) and 0.62% (Chl-b) in alternate light period and 1.65% (Chl-a) and 0.50% (Chl-b) in continuous illumination. The total protein content was correlated with growth and chlorophyll content. Carotenoid and free amino acid content was higher in continuous light period than in alternate light illumination. It is concluded that, the alternate dark and light period is more suitable for the growth of *Chlorella emersonii* than continuous light illumination. Fatty acid identified in GC-MS spectral data is suitable for the biodiesel production the percentage yield efficiency was calculated for the produced biodiesel, it is above 85%. This can be achieved using a novel catalyst lipase immobilized magnetic nanoparticle.

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