



Growth, Biochemical Sketch and Hydrocarbon Derivative Production from a Newly Isolated *Chlorella Vulgaris*

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ABSTRACT

Chlorella vulgaris has been isolated and tested for its growth, pigment, protein and free amino acid contents. Growth was found to be 3.6 times more in alternate illumination than in continuous light condition. Chlorophyll content was found as 2.501% (Chl-a) and 0.63% (Chl-b) in alternate light period and 1.68% (Chl-a) and 0.51% (Chl-b) in continuous illumination. 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. Based on the results from the present study, it is identified that alternate dark and light period is more suitable for the growth of *Chlorella vulgaris* than continuous light illumination. The present paper describes about the biodiesel production from the *Chlorella vulgaris*. 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 acid-catalyzed trans-esterification reaction.

Keywords: *Chlorella vulgaris*; Isolation; Growth; Pigment; Carotenoid; Proteins; Amino acids; trans-esterification.

INTRODUCTION

Global warming and the exhaustion of fossil fuels are the major problems in world-wide. Thus, the various sources such as plants, microalgae, and animal fat, for the production of biodiesel, have been attempted as an alternative energy source¹. The production of biodiesel has two major advantages, mitigation of carbon dioxide and as a substitute for petroleum². 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 CO₂ 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 crops³.

Microalgae have important environmental benefits, reduces CO₂ through photosynthesis and involves bioremediation to remove the heavy metals, such as nitrogen and phosphorous in wastewater⁴. *Chlorella* has potential benefits and uses as food and feed supplement and also in health and cosmetic products. Growth of the algae depends on pH, temperature, aeration, agitation, salt concentration and light illumination. Light plays a vital role as it straightly affects the photosynthetic machinery. Phytoplankton photosynthesis is determined by temperature and irradiance. The nutritional value is affected by these factors like amino acid, proteins, pigment and lipid Previous works in *Prochlorococcus* sp examined the effect of light on cell growth, analyzed growth in *Chlamydomonas gellteri* at different temperature effect of light and temperature on

polysaccharide content in *Chlamydomonas ulvaensis* influence of light on growth of *Pithophora kewensis*, *Cladophora flexuosa*, *Chaetomorpha melagonium* and *Rhizoclonium riparium* were studied. The present study deals with the isolation, determination of growth, total protein content, chlorophyll content, carotenoid content and free amino acid of *Chlorella vulgaris* on alternate dark and light period and continuous illumination were analyzed.

The present paper also describes about the biodiesel production from the *Chlorella vulgaris*.

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 vulgaris* 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 analysed 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 method⁵ 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 estimation⁶. 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 aluminium 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 Baker⁶, on 5th, 10th, 15th, 20th day the sample were analysed for total protein content, Chlorophyll a, Chlorophyll b and Total Chlorophyll.

Estimation of Amino Acids

Free amino acid was estimated by Lee and Takahashi method⁷. 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.

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 vulgaris* harvested by centrifugation at 10,000 rpm and wet cell mass was dried at 60°C at constant weight was obtained. Weight 100 grams of *Chlorella vulgaris* 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 formula.⁸

$$\text{oil content (\%)} = \frac{\text{weight of the oil}}{\text{weight of the sample}} \times 100$$

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 35°C, hold for 2 minutes, then ramp at 20°C per minute to 300°C and 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.

Transesterification of Algal Oil

The transesterification of algal oil carried out by using 5% H₂SO₄. Take the Lipid/ Methanol in the molecular ratio 1:10 ratio and reaction was performed at 70°C under constant stirring at 6000 rpm for 5 hours. After the reaction it was transfer in separating funnel allow for 2 hours for settle down to form a two layer. 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

Growth of *Chlorella vulgaris* was determined by measuring OD, CC, and DW upto 5 weeks. Alternate lights showed more growth compared to continuous illumination. OD value was increased upto 3.6 times and in continuous light, the OD was 3.1 times. Increased cell count and dry weight was seen in alternate light with 2.8 and 3.4 times respectively from the initial stage of the culture. But in continuous illumination the concentration and DW was only 2.6 times. The result interprets that alternate light showed increased OD, DW, CC compared to continuous illumination.



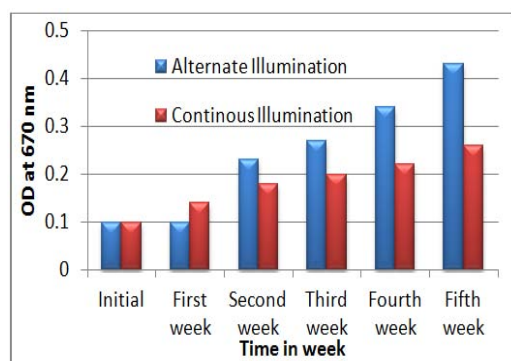


Figure 1: Growth of microalgae with reference to absorbance (OD) at 670 nm

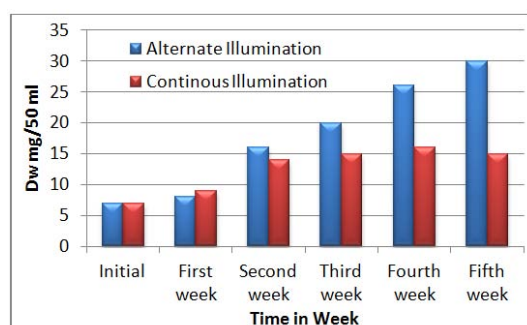


Figure 2: Growth of microalgae with reference to dry weight (DW)

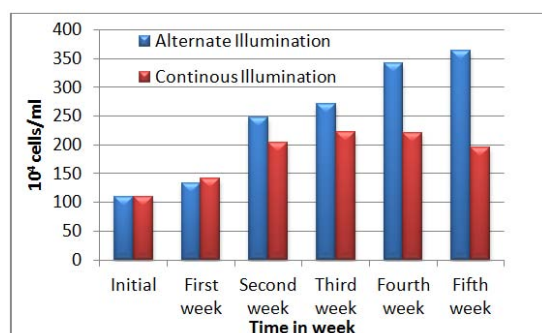


Figure 3: Growth of microalgae with reference to cell count (CC)

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 2.501 and 0.63 in alternate light period and 1.68 and 0.51 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 irradiances⁹. Continuous illumination was favourable for the growth of microalgae than alternate illumination.¹⁰ 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 photo oxidation inside the microalgal cells, with greater light illumination the algae might synthesize the photosynthetic units very less might be to prevent the damage¹¹. Fast nurturing microalgal cells display higher protein and lower carbohydrate content¹² similar study showed.

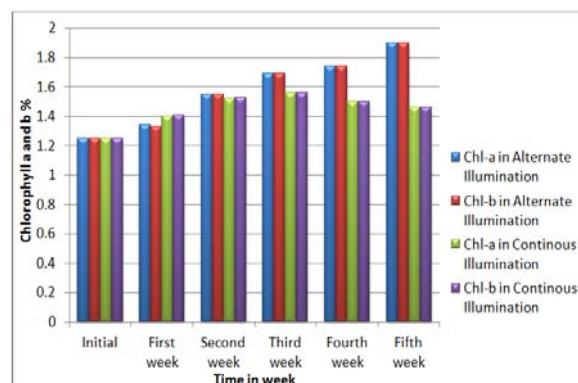


Figure 4: Concentration of Chlorophyll pigment a and b in alternate and continuous illumination

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 (0.371 %).

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 49.3% in alternate dark and light period and 43.9% in continuous illumination.

Free Amino Acid Content

Lee and Takahashi method⁷ 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 amino acid was identified as 623 $\mu\text{g/g}$ fw and 598 $\mu\text{g/g}$ 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 irradiances⁹. Continuous illumination was favourable for the growth of microalgae than alternate illumination¹⁰ 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 photo

oxidation inside the micro algal cells, with greater light illumination the algae might synthesize the photosynthetic units very less might be to prevent the damage¹¹. Fast nurturing micro algal cells display higher protein and lower carbohydrate content showed¹² by similar studies.

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 were 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 51.7%, respectively¹³. Therefore, using DSW-supplemented medium for microalgae growth has considerable potential for commercial microalgae cultivation and in this present study the *Chlorella vulgaris* biomass production rate was 185mg/L/d and algal oil was extracted using n-hexane as an organic solvent at temperature 70°C. The maximum oil yield was achieved more than 85% due to the high temperature and high process duration.

Fatty Acid Analysis by Gas Chromatography and Mass Spectrometry (GCMS) Analysis

Gas Chromatography and Mass Spectrometry (GCMS) 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 Vila¹⁴. 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 vulgaris* 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: Represents the isolated fatty acids from the *Chlorella vulgaris*

Peak No.	RT (min)	Compound Name
1	14.72	9-Hexadecenoic acid
2	15.26	12,15-Octacecadienoic acid
3	15.65	Cyclopropaneoctanic acid
4	16.15	Pentadecanoic acid
5	17.13	Hexadecanoic acid

The major fatty acids present in *Chlorella vulgaris* were 9-Hexadecenoic acid (37%), 12,15-Octacecadienoic acid

(36%), Cyclopropaneoctanic acid (10%), Pentadecanoic acid (17%), and Hexadecanoic acid (12%) respectively. Fatty acid compositions of the green algae on the 44th day of growth are represented in Table 1.

Trans-esterification Process

In the present study, the algal oil was used for the biodiesel production by trans-esterification process using the H₂SO₄ as a catalyst. The maximum biodiesel yield was achieved more than 85% by using *bio*-catalyst at 70°C for 5 hours (Table 2).

Table 2: Represents the presence of fatty acid methyl esters in the biodiesel samples from the *Chlorella vulgaris*

Peak No.	RT (min)	Compound Name
1	13.76	9-Hexadecenoic methyl ester
2	14.28	12,15-Octacecadienoic methyl ester
3	17.63	Cyclopropaneoctanic methyl ester
4	18.16	Pentadecanoic methyl ester
5	19.18	Hexadecanoic methyl ester

CONCLUSION

In this study, *Chlorella vulgaris* has been isolated and tested for its growth, pigment, protein and free amino acid contents. Growth was found to be 3.6 times more in alternate illumination than in continuous light condition.

The algal oil was converted into biodiesel *via* trans-esterification process. The algal oil was extracted from using Soxhlet extraction.

The produced algal oil was converted into bio-diesel using acid catalyst and maximum yield was achieved (85%). 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 acid-catalyzed trans-esterification reaction. Finally, the produced biodiesel was analyzed for their physicochemical properties using ASTM Standard.

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