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Mass cultivation of micro alga *Chlorococcum humicola* in open raceway pond and assessment of bio-fuel potential

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Abstract

The biomass production of *Chlorococcum humicola* in open raceway pond using improved CFTRI medium was investigated. The cell count showed a good division rate up to day 8th. The pH and conductivity showed some remarkable changes in relation with the cell division. The pigment analysis showed high percentage of β-carotene compared to that of chlorophyll a and b. Biochemical analysis revealed the lipid contents were up to 10 fold the percentage of proteins and carbohydrates. Transesterification of lipid was made for FAME (fatty acid methyl ester) analysis using GCMS. The results revealed a good percentage of saturated and unsaturated long chain fatty acids.

Key words: *Chlococcoccum humicola*, raceway pond, bio-fuel potential

Introduction

Microalgae have long been recognized as potentially good sources for biofuel production because of their high oil content and rapid biomass production. In recent years, use of microalgae as an alternative biodiesel feedstock has gained renewed interest from researchers, entrepreneurs, and the general public. (Zhiyou Wen. 2009). Algal biomass contains three main components: carbohydrates, proteins, and lipids/natural oils. Because the bulk of the natural oil made by microalgae is in the form of TAGs (triglycerols) which is the right kind of oil for producing biodiesel-microalgae are the exclusive focus in the algae-to-biofuel arena. Microalgae grow very quickly compared to terrestrial crops. They commonly double in size every 24 hours. During the peak growth phase, some microalgae can double every 3.5 hours (Chisti 2007).

Plants and algae are good candidates, as alternative energy sources, as they obtain their energy from the sunlight and build up their biomass by removing carbon dioxide from atmosphere through photosynthesis. In this way, any time a fuel originated from plants or algae is burnt, the carbon dioxide emitted is the very same that was previously removed by those organisms. However, differently from plants, algae cultivation does not compete for land crop occupation. Microalgae are particularly attractive as they are photoautotrophic organisms that grow in simple inorganic medium and contrary to higher plants, each cell is photosynthetically competent so that the amount of carbon dioxide fixed is much higher in a biomass base [John, et al, 2011]. The present investigation deals with mass cultivation of *Chlorococcum humicola* in an open raceway pond (1 KL) and assessment of the bio-fuel potential of the biomass.

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Materials and Methods

Micro Algae Used for Mass Cultivation

A green algae, *Chlorococcum humicola* was used for mass cultivation in an open raceway pond, obtained from the Culture Collection of Vivekananda Institute of Algal Technology (VIAT).

Preparation of Algal Inoculum

The inoculums of the algal cultures to be used for the outdoor mass cultivation was prepared under laboratory condition. *Chlorococcum humicola*, was grown in CFTRI medium (Venkataraman, 1985). The cultures were grown at 24 ± 1^0C in a thermo-statically controlled room and illuminated with cool white inflorescence lamps (Philips 40W, Cool daylight 6500K) at an intensity of 2500 lux in a 12 hr: 12 hr light and dark regime.

Out-Door Cultivation of Micro-Algae

Improvised CFTRI medium (Venkataraman, 1985) was used for outdoor cultivation of micro alga. An outdoor algal raceway pond was constructed with the wall thickness of 25cm. The inner dimensions of the pond were such that the length was 3.65 m and the width was 2.3 m. The depth of the pond was kept at 0.44 m keeping in mind the sufficient light penetration for the growth of algae. A partition wall was constructed in the middle of the pond with a length of 2.46 m and width of 11cm. The floor was constructed with a slight slope on either side of the partition in the opposite direction to enable proper mixing of the culture. The inside of the pond was covered with ceramic tiles lining to avoid seepage of the medium into the wall. The tank was provided with a tap water connection for preparation of the medium and two outlets were provided so as to enable to clean the tank.

Algal Cultivation

Chlorococcum humicola was grown in improvised CFTRI medium with the incubation temperatures ranging from 30 to 40 degrees Celsius with natural day night cycle (45 to 60 Klux). 1 KL of the medium was prepared using tap water and care was taken that the depth of the medium did not exceed 15 cm. The algal inoculums was added into the medium and grown with daily stirring and sample was harvested. Light intensity during the trials was measured using lux meter (lutron LX –101A).

Microscopic Examination and Growth Measurement

The micro algal cultures were microscopically examined using Olympus (HB) microscope. Growth was measured by counting cells using a haemocytometer (Neubauer, improved) and the results were plotted in a semi-logarithmic graph. For dry weight method, the algal cultures were pelleted by centrifugation at 7500 rpm (Remi cooling microfuge) for 15 minutes. Cells were washed with distilled water, again centrifuged and dried in an oven for 24 hours or until constant weight.

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pH and Conductivity

For all the trials, pH was measured using Digital pH meter (Elico L ± 120) and conductivity using digital conductivity meter (Equiptronics EQ-660A) at 25 degrees Celsius.

Extraction and Estimation of Pigments

Chlorophyll 'a' and 'b' was estimated from the extinction coefficients given by Jeffery and Humphrey (1975). Extraction and quantification of □-carotene was done as described by Shaish *et al., (*1992)

Extraction and Estimation of Biochemicals

Extraction and quantification of total carbohydrates was done as described by Pons et al., (1981). Extraction and quantification of total protein was done as described by Lowry *et al., (1951).* Lipid extraction and quantification of cyanobacterial homogenate was done as described by Bligh and Dyer, (1959)

Assessment of biofuel potential

Transesterification

The entire process of FAME preparation was done following the protocol of Rasoul-Amini *et al.*, (2009). Then, the oily substance was further subjected to Gas Chromatography analysis.

GC-MS Analysis

For GC/MS a GC-MS-QP 2010 [SHIMADZU] gas chromatograph linked to mass spectrometer system equipped with a capillary column DB-5ms (30.0m x 0.25mm, 0.25µm film thickness) was used. The GC column oven temperature was programmed from 70°C to 300°C. The initial temperature was 70°C (hold time 2min) and rose to 300°C (hold time 7min) at the rate of 10^0C min^{-1}. The total run time was 34.0 min. Helium with 99.9995% purity was used as carrier gas with a constant flow of 1.51 ml/min. The GC-MS interface temperature was at 280°C. Injector and detector temperatures were set at 200°C. 1µl of each sample was injected in split ratio of 1:10. The MS scan range was set from 40-1000 Dalton. Compound identification was obtained by comparing the retention times with those of authentic compounds and the spectral data obtained from library data of the corresponding compounds. Quantities of the compounds are presented as relative area percentage as derived from the integrator.

Results and Discussion

1KL of improvised CFTRI was prepared in the experimental algal pond and 1 L of actively grown culture of *Chlorococcum humicola* were inoculated. It was cultured for 30 days with frequent samplings. The culture was sampled at frequent intervals and the cell numbers were measured using a haemocytometer (Neubauer improved). The growth pattern showed gradual increase in the cell numbers up to day 3 and thereafter steep increase from day 4 to 7. Then from day 8 cell

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numbers got gradually decreased. The growth rate of the outdoor culture had a slight edge with (0.19 divisions/day) over that of the indoor culture with (0.17 divisions/day) (indoor culture results not discussed). The results are given in Table 1 and fig 1.

Both pH and Conductivity were measured daily and the results indicate that the pH level rose and electrical conductivity gradually decreased in accordance with cell division from day to day. The initial day pH is 7.6 and in final day the pH is 9.42. The electrical conductivity was ranging between 1.6 to 3.6 mmhos/cm. The results are given in Table 1 and fig 2.

Chlorococcum humicola sampled on day 8 (the day on which the culture attained maximum growth) were pelleted by centrifugation. Both Chlorophyll and β-Carotene were analyzed by Spectophotometric method. Among the pigments β-Carotene was the highest with 0.1446 µg/10^6cells followed by Chlorophyll a with 0.0301 µg/10^6cells and Chlorophyll b with 0.0095 µg/10^6cells. The results are given in Table 2 and fig 3.

Culture samples were collected from the algal pond on day 8 for the analysis of protein, carbohydrate and lipid. The results show that the protein content was 0.4270 µg/10^6 cells and the total carbohydrate was 0.5053µg/10^6cells on day 8. Among all the three bio-molecules, lipid content was the highest with 13.023 µg/10^6 cells on day 8 and thereafter, there was a slight reduction in the lipid content. The results are given in Table 3 and fig 4.

Table 1: Growth pattern, pH and electrical conductivity of *Chlorococcum humicola* grown in open raceway pond

S.No	Days	Cell Count (x 10^4)	pH	Electrical Conductivity (µmhos/cm)
1	01	30	7.6	3.6
2	02	42	7.68	3.4
3	03	52	7.82	2.5
4	04	88	7.88	2.2
5	05	120	8.02	1.9
6	06	180	8.84	1.6
7	07	220	9.07	1.7
8	08	224	9.33	1.7
9	09	198	9.38	1.6
10	10	190	9.42	1.3

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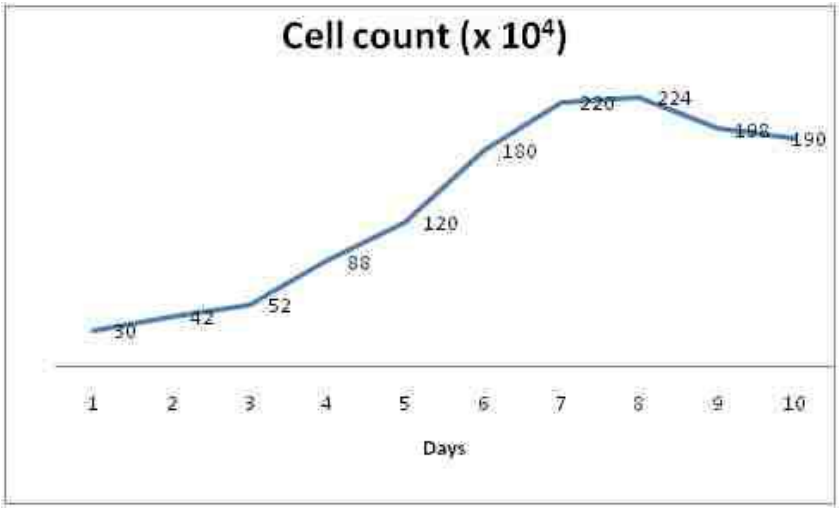

Fig 1: Growth pattern of *Chlorococcum humicola* grown in open raceway pond

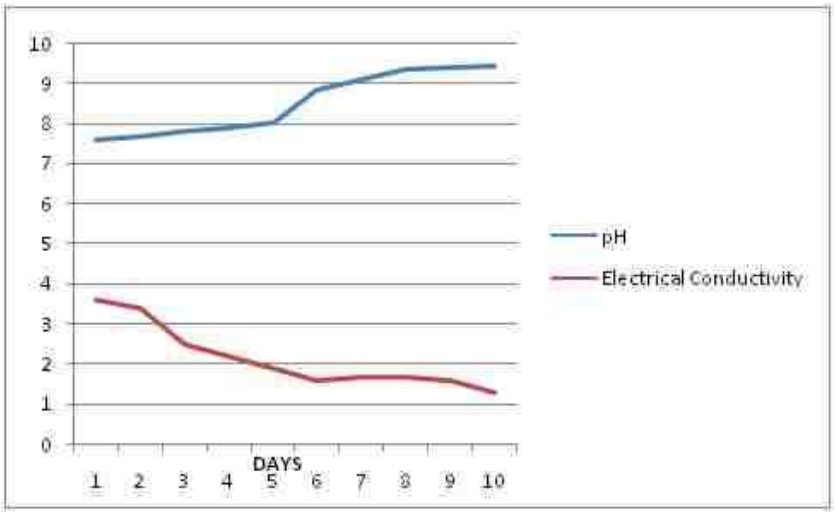

Fig 2: Changes in pH and Conductivity of culture of *Chlorococcum humicola* grown in open raceway pond

Table 2. Chlorophyll & Carotenoid levels of *Chlorococcum humicola* grown in open raceway pond

S.No	**Pigments**	**(µg/10⁶cells)**
1	Chlorophyll a	0.0301
2	Chlorophyll b	0.0095
3	□-Carotene	0.1446

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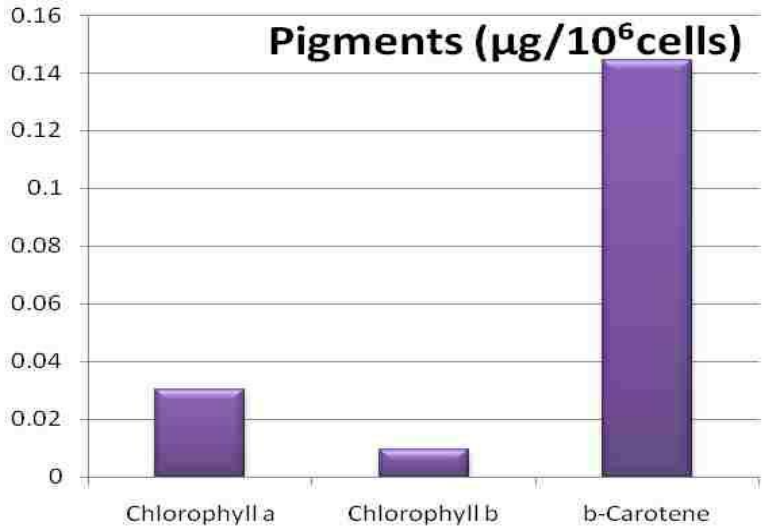

Fig 3. Chlorophyll & β-Carotene levels of *Chlorococcum humicola* grown in open raceway pond

Table 3 . Biochemical composition of *Chlorococcum humicola* grown in open raceway pond

S.No	Biochemicals	(µg/10⁶cells)
1	Carbohydrate	0.5053
2	Protein	0.6409
3	Lipid	13.0230

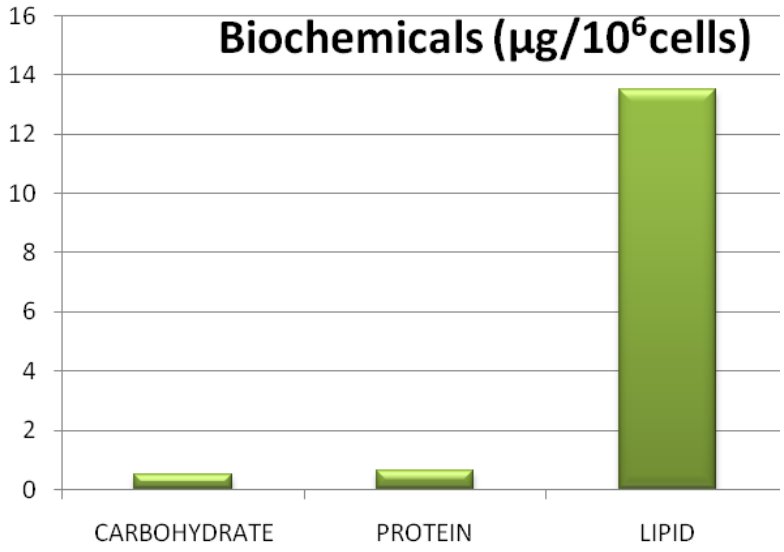

Fig 4. Biochemical composition of *Chlorococcum humicola* grown in open raceway pond

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Biofuel Assessment of *Chlorococcum humicola*

Table 4: FAME analysis (GCMS) of *Chlorococcum* humicola

S.No.	Compound Names	FAME Formula (acronym)	RT (min)	Amount (ppm)	Area %
1	Methyl caproate	C6:0	7.274	72.2925	4.72
2	Methyl caprylate	C8:0	10.107	66.7318	5.36
3	Methyl undecanoate	C11:0	11.736	36.4217	2.43
4	Methyl laurate	C12:0	13.637	70.5096	6.40
5	Methyl tridecanoate	C13:0	15.795	34.5737	3.67
6	Methyl myristoleate	C14:1	17.737	31.9377	1.16
7	Methyl myristate	C14:0	18.044	66.6931	7.91
8	Methyl pentadecenoate (cis-10)	C15:1	20.076	35.312	1.42
9	Pentadecanoic acid, methyl ester	C15:0	20.371	33.9732	3.78
10	Methyl palmitoleate	C16:1	22.184	35.256	1.14
11	Methyl palmitate	C16:0	22.664	81.5529	9.78
12	Heptadecenoic acid, methyl ester (cis-10)	C17:1	24.477	35.7428	1.21
13	Heptadecanoic acid, methyl ester	C17:0	24.968	34.1087	3.57
14	Gamma-Linolenic acid, methyl ester	C18:3n6	26.081	38.6343	1.13
15	Methyl linoleate	C18:2	26.438	35.7844	1.30
16	Cis-Methyl oleate	C18:1	26.59	68.0282	3.24
17	Trans-Methyl elaidate	C18:1	26.745	31.7552	1.23
18	Methyl stearate	C18:0	27.191	65.2634	7.23
19	Arachidonic acid methyl ester	C20:4n6	29.937	85.2693	1.05
20	Eicosapentaenoic acid, methyl ester (cis-5, 8, 11, 14, 17)	C20:5n3	30.073	41.8849	1.29
21	Eicosatrienoic acid, methyl ester (cis-8,11,14)	C20:3n6	30.333	39.77	1.11

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22	Eicosadienoic acid, methyl ester (cis-11,14)	C20:2	30.738	38.3799	1.25
23	Eicosenoic acid, methyl ester (cis-11)	C20:1	30.864	34.6127	1.99
24	Eicosanoic acid, methyl ester	C20:0	31.419	33.4144	6.79
25	Heneicosanoic acid, methyl ester	C21:0	33.421	36.0645	2.95
26	Docosahexaenoic acid, methyl ester (cis-4,7,10,13,16,19)	C22:6n3	33.831	56.4491	0.92
27	Docosadienoic acid, methyl ester (cis-13,16)	C22:2	34.851	16.841	0.39
28	Methyl erucate	C22:1	34.846	35.7636	1.25
29	Methyl behenate	C22:0	35.359	66.6176	5.62
30	Tricosanoic acid, methyl ester	C23:0	37.23	40.0919	2.56
31	Methyl nervonate	C24:1	38.578	48.5165	1.00
32	Methyl lignocerate	C24:0	39.037	85.7198	5.16

A detailed fatty acid methyl ester analysis of *Chlorococcum humicola* grown in open tank using a transesterification method and GC-MS revealed 32 different fatty acids from C6:0 to C24:1 chain length, with odd and even carbon numbers, different degrees of unsaturation, and methyl- substitutions. The fatty acid methyl ester profile is one of the key factors that determine the suitability or otherwise of any feedstock for use in biodiesel fuel production (Knothe, 2009). The compounds namely Methyl myristate, Methyl palmitate, Methyl myristate, Methyl stearate, Eicosanoic acid, methyl ester, Methyl behenate, Methyl lignocerate were found in high percentage. Interpreting the results (Table 4) indicates saturated FAME to be close to 80%, which otherwise exhibits much higher degree of oxidative and thermal stability. The compounds namely cis-4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, Cis-13,16-Docasadienoic acid, methyl ester, Methyl nervonate etc., were found in low percentage in *Chlorococcum humicola*.

Conclusion

The present study deals with growing *Chlorococcum humicola* in open raceway pond and utilizing the biomass feedstock for biofuel assessment. In addition extraction and estimation of various biochemicals and pigment analyses were made. The GCMS analysis of FAME showed the presence of 32 different compounds of fatty acids. The methyl esters appears to be in significant proportion of FAME indicates *Chlorococcum humicola* as a better biofuel candidate. Based on the results obtained, further trials have to be carried out for studying economic feasibility during the scaling up process.

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