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Chlorococcum humicola (Nageli) Rabenhorst as a Renewable Source of Bioproducts and Biofuel

Santhoshkumar K.[1], Prasanthkumar S.[1] & J. G. Ray[1]

[1] Laboratory of Ecology & Ecotechnology, School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India

Correspondence: J. G. Ray, Laboratory of Ecology & Ecotechnology, School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India. E-mail: jgray@mgu.ac.in

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Abstract

Among the diverse new generation biomass yielding species, green algae are the most promising organisms. Compared to biomass production of other organisms, production of algae is less laborious, quite fast, and more economical. Moreover, eutrophicated waters get naturally purified in the cultivation process of algae. Algal biomass from monoculture of specific species, which are rich in carbohydrates, proteins and lipids, is considered a good source of diverse bio-products and feed-stock for food, feeds and bio-fuels. Quantity and quality of algal biomass for specific products depend on the species and strains as well as environmental conditions of cultivation. In this connection, biomass productivity and oil-yield of a local strain of *Chlorococcum humicola* (Nageli) Rabenhorst was assessed in Bold's Basal Medium. Long-term storage capacity of the alga was tried by entrapping the algal cells in sodium alginate beads, which showed viability up to 14 months. Estimation of total carbohydrate, protein, lipid and chemical characterization of oil as well as the feasibility of its conversion to biodiesel revealed the industrial potential of this local strain as a source of food and biofuel. Fatty acid profiling of the extracted oil showed that 70% are mono-saturated and 12.2 % are nutritionally important polyunsaturated fatty acids. The oil could be effectively trans-esterified to methyl esters and the conversion was confirmed by FTIR spectroscopy. Further standardization of the mass production of the alga in natural environmental conditions for biomass and oil is progressing to optimize its value as globally competent food, nutraceutical and biofuel resource.

Keywords: *Chlorococcum humicola*, algal biomass, algal biodiesel, algal bioproducts, synthetic seed

1. Introduction

Green algae are significant new biomass resource for the production of natural bioactive compounds and renewable energy. They have unique nutritional quality (Becker, 2007) that can add value to conventional food preparations for humans and animals. Because of the high protein content, biomass from many species of microalgae is generally considered as the potential source of proteins (Spolaore, Joannis-Cassan, Duran, & Isambert, 2006) for the future. In addition to proteins, green algae are good source of carbohydrates and lipids for food and fuels. Among the known commercially and industrially amenable green resources, algae can provide the highest and cheapest biomass per unit light and area. Simultaneously they can remediate nutrient load or degrade other toxic pollutants in water (Chiu et al., 2015) and reducing atmospheric CO_2 level through carbon fixation process (Morais & Costa, 2007). Moreover, several pharmaceutical products are derived from algal biomass (Yamaguchi, 1997), especially from that of Chlorophytes.

Biopharmaceutical industries are in search of low cost biomaterials for production of therapeutics in a sustainable manner (Johnson, 2008). *Chlorococcum humicola* (Nageli) Rabenhorst (Figure 1) is a freshwater unicellular green alga coming under the class Chlorophyta. Even though *C. humicola* is proved to be a rich source of structurally novel and biologically active metabolites (Bhagavathy, Sumathi & Jancy Sherene Bell , 2011), biomass-productivity of its specific strains are not well known.

Long-term storage of algal stock in sodium alginate beads in pure culture form is useful for stock culture management (Gaudin, Lebeau, & Robert , 2006) as well as long term storage of the algal seeds (Faafeng, Donk, & Källqvist, 1994) for biomass production and production of secondary metabolites (Moreno-Garrido, 2008). Immobilized algae can also be used in wastewater treatment (Travieso et al., 1996) and removal of heavy metals

from waste water (Becker, 1994; Murugesan, Maheswari, & Bagirath, 2008). However, specific methods to develop a seed material of this alga for convenient mass-cultivation remain quite unexplored.

Protein content of green algae varies depending upon the species and strains (Fleurence, 1999; Gatenby et al., 2003). In addition to proteins, carbohydrates present in algae are also variable that have important value as food and fuels. Algal carbohydrates are easily digestible compounds that have high demand in the preparation of conventional foods, pharmaceutical and nutraceutical compounds (Becker, 2007). Moreover, the residual biomass rich in carbohydrates after the extraction of lipids or proteins is used for the production of ethanol (Gao, Shimamura, Ishida, & Takahashi, 2012). Some of the algal lipids such as omega-3 fatty acid and DHA are nutritionally valuable (Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). Polyunsaturated fatty acids, especially omega-3 and omega-6 in algal oils are used as medicines for health of heart and brain (Ignarro, Balestrieri, & Napoli, 2007). Essential fatty acids present in algae are used as dietary supplements in many pharmaceutical products (Benatti, Peluso, Nicolai, & Calvani, 2004). Algal biomass rich in oils is also used as a natural source of bio-fuels, which is highly cost effective, environmental-friendly and renewable source of liquid fuel (Scott & Bryner, 2006). However, magnitude of oil and biomass production of an alga depends on the cultural conditions and biomass productivity (Olofsson et al., 2012). Naturally, chemical characterization of the bio-oil as well as the general biomass of hitherto unexplored species and local strains of green algae becomes quite meaningful. Such kinds of data are universally significant to assess the industrial potential of new species or strains of algae.

Assessment of biomass productivity, total proteins, carbohydrates and lipid content as well as lipid characterization of a local strain of *C. humicola* was the major objective of the current investigation. Since trials of long-term storage are essential to ensure continuous industrial production of algae, synthetic seed preparation and its viability in the 'seed form' became another objective. Overall, the present investigation point to the significance of a local strain of C. *humicola* as a potential feed-stock for food and fuel. Synthetic seeds of this alga could be preserved for more than a year.

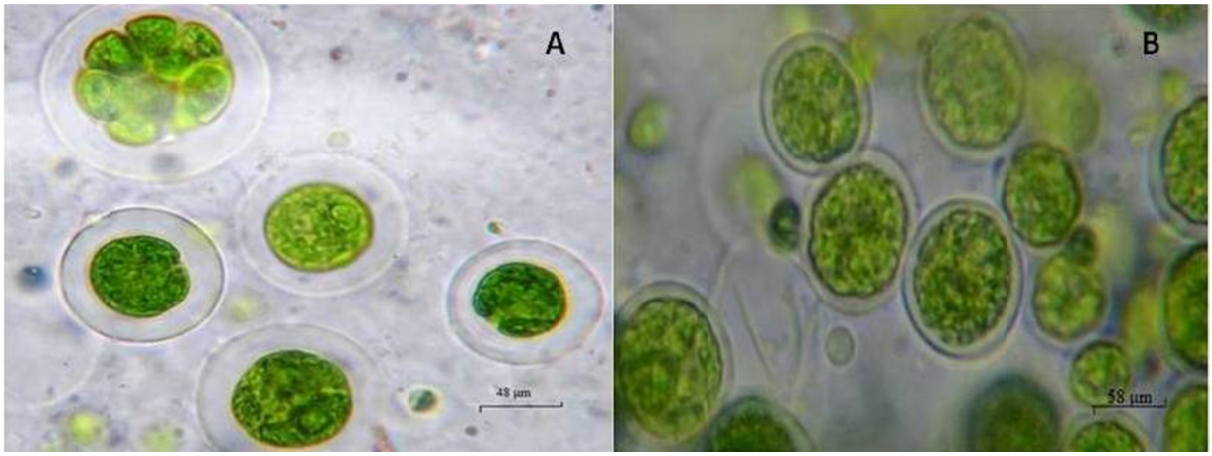

Figure 1. *Chlorococcum humicola* showing solitary cells (A) and colonial cells (B)

2. Materials and methods

2.1 In vitro Culture of Algae

C. humicola is unicellular, non-motile, spherical cells having smooth cell walls. Cells are seen in colonies or solitary and are varied in sizes (48 µm- 58 µm) having a 'hollow sphere like chloroplast', completely filling each cell, with a lateral notch and a single pyrenoid (Phillipose, 1967). In the current investigation, viable cells of a local strain of *C. humicola* were isolated from a fresh water temple pond (9°45'02.9"N 76°23'45.6"E) of Kottayam District of Kerala, India. Pure culture of the strain in BBM is maintained in the algal culture facility centre in the Ecotechnology Laboratory, School of Biosciences, Mahatma Gandhi University.

Temperature and pH of the collected water samples were measured. 100 mg of fresh biomass after centrifugation of the pure culture was inoculated and cultured in one litre flasks using Bold's Basal Medium (BBM) (Andersen, 2005) in triplicate (Figure 2). All the culture vessels were incubated under controlled conditions of light (8000 Lux), temperature (24 ± 2^0C) and pH (7.30). Productivity was measured on completion of 30 days of growth. On

completion of the incubation period, biomass was collected by centrifugation and the solid biomass was further air dried. Percentage increase of biomass per day per litre was calculated as productivity of algae as per the formula:

$$\%\ \text{of Biomass productivity of algae } /\text{L/day} = \frac{\text{Final dry weight}}{\text{Initial dry weight x total no. of culturing days x Vol.}} \times 100\%$$

Figure 2. Culture of *C. humicola* for biomass production of alga in BBM – intensity of colour reveals growth intensity

2.2 Synthetic Seed Preparation and Test for Long Term Storage Viability

Synthetic seeds of algae were prepared by using sodium alginate. Sodium alginate (4%) were prepared in sterilized BBM media and stirred continuously at 60^0C in a water bath for one hour. One gram of uniculture of algae sample was washed out with sterile water and mixed with prepared sodium alginate slurry in the ratio of 1 volume of cell inoculums: 2 volume of sodium alginate. It was then dropped into 0.2 M calcium chloride solution using a pipette to form calcium alginate beads with algal cells entrapped. The beads were kept for 30 minutes to solidify. They were then washed 3 to 4 times with distilled water. Algal seeds were stored under dark condition in a refrigerator at 4 degree Celsius. At every three month interval, viability of the beads was tested by culture (5 beads for 100 mL culture medium) in BBM to assess their viability.

2.3 Quantitative Estimation of Carbohydrates

Carbohydrate content was determined as per the method of Dubois et al. (1956), using glucose as standard; 100 mg of the lyophilized algal sample was hydrolyzed by keeping it in boiling water bath for three hours with 5 mL of 2.5 N HCl and cooled to room temperature. The total volume was made up to 100 mL by using double distilled water and centrifuged at 2723g for 5 minutes at 4^0C. The supernatant was used for carbohydrate estimation.

2.4 Quantitative Estimation of Total Proteins

Total protein content was determined as per the method of Lowry (Lowry, Rosebrough, Lewis, & Randall, 1951), using bovine serum albumin as standard; 5 mg of freeze dried algal sample was mixed with 5 mL of 80% acetone (Rotek vortex mixture: 1331) for 1 minute and centrifuged at 5000 rpm for 5 minutes at 4^0C and the pellet was homogenized with 0.2 mL of 24% TCA (w/v) and centrifuged at 7000 rpm for 5 minutes at 4^0C. The homogenate was incubated at 95 ^{0}C for 15 minute in a water bath and cooled to room temperature. 600µL of ultra pure water was added and centrifuged at 15000 rpm at 4^0C for 2 minutes. The pellet was collected and re-suspended in 0.5 mL Lowry D reagent and it was then incubated at 55^0C for 3 hours. The sample was cooled to room temperature (27^0C – 30^0C) and centrifuged at 15000 rpm for 20 minutes at 4^0C. The supernatant was collected and used for estimation of total proteins.

2.5 Extraction of Algal Oil

Total lipids were extracted as per the method of Bligh and Dyer (1959) using Soxhlet; 10 gm of freeze dried biomass was taken into a round bottom flask and added 100 mL of chloroform: methanol (2:1 v/v) mixture into the biomass. The biomass was then kept soaked in the organic solvents for 4 hrs under continuous shaking in a rotary shaker at 750 rpm; afterwards the mixture was centrifuged at 6000 rpm for 5 minutes at room temperature (27^0C - 30^0C). Residual biomass was separated from the extract and then the oil along with the solvent was transferred in to a separating funnel. About 40 mL of distilled water was added to this mixture to separate the oil from the solvent. The oil got separated as an organic phase in bottom layers; this was then collected into a bottle. The separated biomass and the oil were made free of the solvent by using rotary evaporator.

The air dried residual biomass free of the solvent was further subjected to hot method of extraction for collection of the remaining neutral lipids. The biomass was taken in to Soxhlet extractor with 75 mL of hexane, refluxed under 70^0 C for 2 hours. The extracted oil components were collected and the oil was made-free of the solvent by using rotary evaporator. Finally, the two extracted oil samples were mixed together to get the total oil.

2.6 Chemical Characterization of Algal Oil

Chemical characterization of the oil was carried out using the advanced Government of India analytical facility at CARE Kerala, Chalakkudy. Exactly 50 mg of algal oil was saponified with 1 mL of saturated KOH-CH_3OH solution at 50^0 C for 10 minutes and then followed by methanolysis with 5% HCl in methanol at 60^0C for another 10 minutes in screw capped test tubes. The methyl fatty acids were separated by adding 2 mL of water into it and fatty acid phase was recovered. GC-MS (Agilent make 7890A- 5975C) instrument was used for the fatty acid profiling. 1 mL of methyl fatty acid sample was injected to the GC column. Helium was used as carrier gas at flow rate of 54 mL/min. Chromatographic data was recorded and compared using Agilent data analysis software.

2.7 Transesterification of Algal Oil and the Production of Biodiesel

400 mg of algal oil extracted were taken into a round bottom flask and mixed with 15 mL of methanolic sulphuric acid containing 2% sulphuric acid in methanol (v/v) and refluxed at 60^0 C for 4 hours with continuous shaking. The reaction was monitored by thin layer chromatography (TLC) with the solvent system, Hexane: Ethyl acetate/ hexane: Toluene at the ratio of 9:1. The reaction was continued till the oil spot was disappeared on TLC plate. After the completion of reaction (2-4 hr), the contents were transferred to separating funnel and 25 mL water was added to it. The aqueous layer was extracted twice with ethyl acetate (25 mL each) and pooled the ethyl acetate layer. The extract was dried over anhydrous Na_2SO_4 and concentrated under vacuum.

2.8 FTIR Analysis of Algal biodiesel

FTIR characterization for 'biodiesel' samples produced was carried out (IS10 FTIR, Thermo Scientific) in transmission mode in 400-4000 cm^{-1} wave number range.

Fatty acid composition of algal oil

Percentage of oil in algae (%) was calculated using the formula (Abubakar, Mutie, & Muhoho, 2012)

$$= \frac{\text{Weight of oil (g)}}{\text{Dry weight of sample (g)}} \times 100$$

3. Results and Discussion

3.1 Biomass Productivity of Alga in Vitro Culture Media

Since BBM is known to enhance maximum production of protein and chlorophyll in green algae (Sankar & Ramasubramanian, 2012), the same medium was used for the assessment of biomass productivity of *C. humicola* (Table 1) in the current experimentation. Productivity of 73.8% mg/ L/ day obtained suggests the alga to be a suitable candidate for high yield of biomass and other derivatives, easily amenable to industrial trials.

Table 1. Biomass productivity, carbohydrates, proteins and lipids contents in the biomass of *C. humicola*

Cultural conditions	Medium (BBM)
Quantity of the medium	1 L
pH	7.30
Temp in ^{0}C	24±2
Light intensity (Lux)	8000
Duration of days	30
Fresh Weight of inoculums (mg)	100
Dry weight of inoculums (mg)	19.3
Dry weight of biomass after 30 days (mg)	427.57 ± 4
Growth of alga mg/L/day	73.8%
Carbohydrates mg/gm of biomass	22.4%
Proteins in mg/gm of biomass	25.5%
Lipids in mg/gm of biomass	13%

3.2 Experimentation on Long Term Storage and Viability

In general, encapsulation of micro algae in alginate as 'synthetic-seed-material' (SSM) can be considered a profitable method, to reduce the cost of long-term storage of pure culture or stock maintenance. Synthetic seed material of algae has several other industrial applications such as phycoremediation (Rai & Mallick, 1992) hydrogen production (Das, 2001) and maintenance of aseptic specimens of algae during culture transportation. In the present experimentation, successful preparation of SSM of *C. humicola* and its long-term maintenance is achieved (Figure 3). The SSM of *C. humicola* was stored at 4^0C in a usual laboratory refrigerator for about 14 months, and the same was successfully cultured in BBM at every three-month intervals with quite same viability till the 14^{th} month (Figure 4; Table 2). This fact is evidential to viability of the SSM for further duration. Even though, it is well known that alginate encapsulation method of algae maintains ultra structural integrity and normal physiological activities (Corrêa et al., 2009; Dainty, Goulding, Robinson, Simpkins, & Trevan, 1986) during sufficiently long period of time, this is the first demonstration of retention of green-algae in alginate beads over a year with quite good viability.

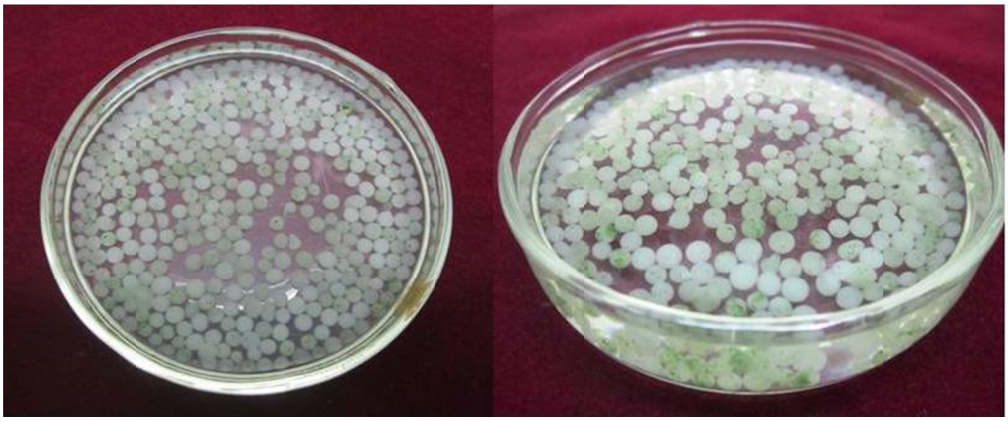

Figure 3. Synthetic seed materials (SSM) of Algal cells after preparation

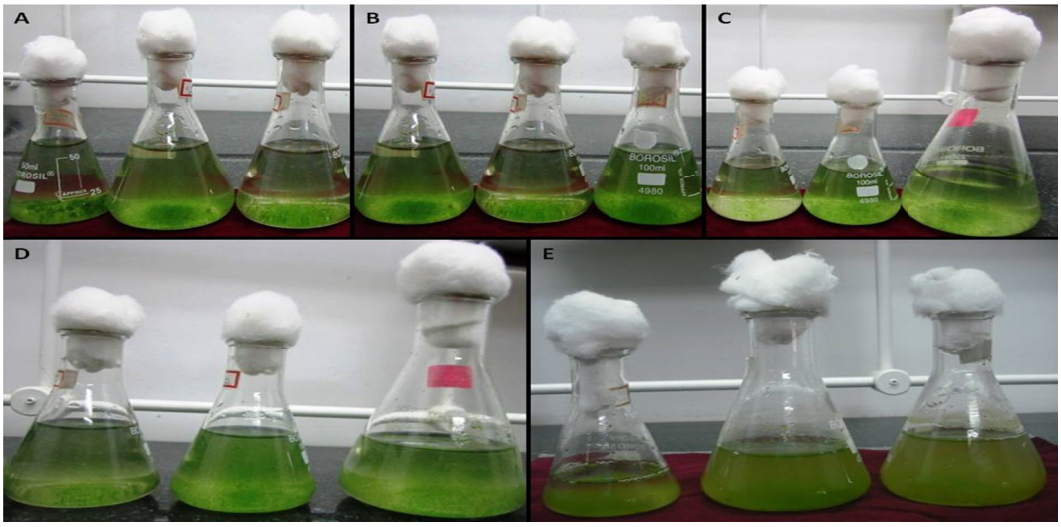

Figure 4. Viability testing by culturing of *C. humicola* in Bold's basal medium: A. after 3 months; B. after 6 months; C. after 9 months; D. after 12 months; E. after 14 months – intensity of the colour reveals equal viability

Table 2. Growth rate of *C. humicola* in terms of number of cells/100 mL after culture of SSM in BBM at different intervals

No. of cells in one seeds/ mL of inoculum	**After 3 month cells / mL**	**After 6 month cells / mL**	**After 9 month cells / mL**	**After 12 month cells / mL**	**After 14 month cells / mL**
$1.84\pm0.039 \times 10^6$	$13.3\pm0.07\times10^7$	$13.3\pm0.04\times10^7$	$13.4\pm0.02\times10^7$	$13.4\pm0.02\times10^7$	$13.3\pm0.02\times10^7$

3.3 Total Proteins, Carbohydrates and Lipids

Chemical characterization of the biomass of the strain showed 22.4 % carbohydrates, 25.5 % proteins and 13 % of lipids (Table 1). Uma et al. (2015) reported more or less similar protein content for *C. humicola* cultured in outdoor environments in CFTRI medium enriched with NPK fertilizer; even higher protein content is known for *Chlorella sp.* cultured in BBM (Sankar & Ramasubramanian, 2012). But the lipid content observed in the present experimentation using the local strain of *C. humicola* is found to be higher than that of the previous reports. Since protein and lipid content of algae depend not only on the species, but also on diverse environmental conditions (Morris , Smith & Glover,1981), further standardization for optimum yield of proteins and lipids is essential in the assessment of C. *humicola* as a protein or lipid-rich algal resource.

3.4 Transesterification of Oil and FTIR Confirmation of Biodiesel

Chemical profile of the oil from this alga (Table 3) has shown 70 % monosaturated fatty acids, 17.4 % monounsaturated fatty acids and 12.2 % polyunsaturated fatty acids. Commonly used lipids for biodiesel productions have C16:0 and C18:1fatty acids (Knothe, 2005). Usually algal oil with saturated and poly unsaturated fatty acids containing 14-18 carbon molecules such as C14:0, C16:0, C16:1, C18:1, C18:2, C18:3 are used as the feed-stock for biodiesel productions (Duong , Li , Nowak & Schenk, 2012; Stansell, Gray & Sym, 2012). Chemical characterization of the oil extracted from C. *humicola* obtained in the current investigation revealed that 95.4% of it is C14-18 fatty acids.

According to the American Society for Testing and Materials (ASTM) D6751 and European EN 14214 standards, monosaturated fatty acids are given preferences for the production of good quality biodiesels (Knothe, 2005). Moreover, algal oil containing fatty acids such as palmitic, stearic, oleic and Linoleic acids are considered good for biodiesel (Knothe, 2008). Therefore, the oil extracted from C. *humicola* was subjected to transesterification trials. The IR spectra (Figure 5) peak $1741.72cm^{-1}$ of transesterified algal oil confirmed the formation of biodiesel. Since the quality of the oil remains the same irrespective of the medium used (Mahmah, Chetehouna, & Mignolet, (2011), this alga may be considered as a good oil resource for biodiesel production; however, further standardization of environmental conditions and media is required to assure the optimum oil yield.

Observation of 12.2 % of the total fatty acids to be of the two important essential fatty acids in this local strain, it

may be considered a nutraceutically valuable alga. Only a very low concentrations (1.41- 4.04 %) of Linoleic acid and Linolenic acid (0.19- 0.67%) is known for Chlorella sp (Hempel, Petrick, & Behrendt, 2012). Since, *Chlorella* species are the major group of micro algae used for industrial production of essential fatty acids (Pulz & Gross 2004), this local strain of *C. humicola* with significant amount of essential fatty acids may be considered one of the best green algal resources for essential fatty acids.

Table 3. GCMS fatty acid profile of *Chlorococcum humicola*: *monosaturated fatty acids (70%), ** monounsaturated fatty acids (17.4%), *** polyunsaturated fatty acids (12.2%)

Fatty acid profile	Result in 1 ml (ppm)	% of fatty acids
Caproicacid (C6:0) *	66.04	11.2
Undecanoicacid (C11:0)*	36.51	6.2
Lauricacid (C12:0)*	68.42	11.6
Myristicacid (C14:0)*	66.59	11.3
Pentadecanoicacid (C15:0)*	33.17	5.6
Palmiticacid (C16:0)*	80.29	13.6
Palmitoleic acid (C16:1 cis)**	34.46	5.8
Stearicacid (C18:0)*	64.86	10.9
Oleic acid (C18:1 cis)**	68.37	11.6
Linoleic acid (C18:2 cis)***	34.47	5.8
Linolenic acid (C18:3 cis)***	37.79	6.4

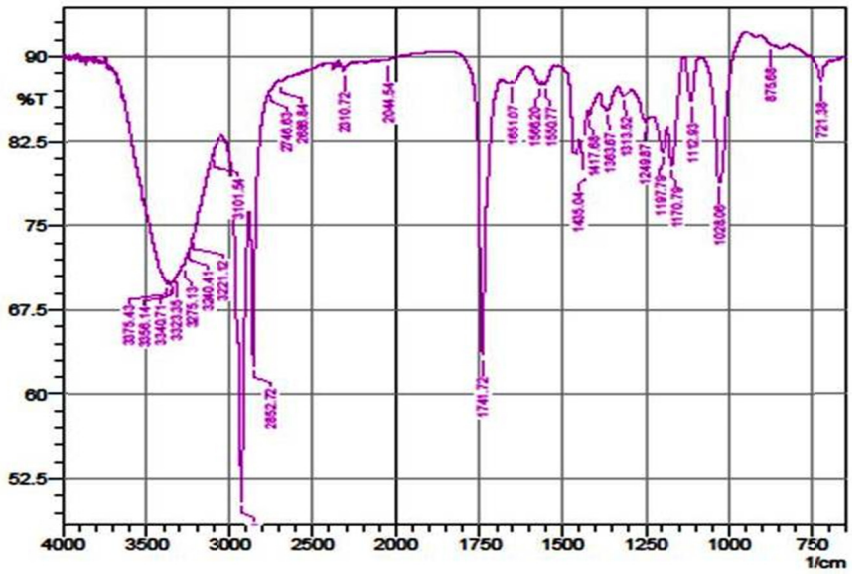

Figure 5. FTIR spectrum of algal biodiesel – peak at 1741.72cm^{-1} confirm the formation of biodiesel

4. Conclusion

Chemical analysis of the biomass of *C. humicola* has shown that it is rich in carbohydrate, protein and lipids, especially of nutraceutically significant compounds in its oil, which indicate industrial value of this alga. High percentage of monosaturated fatty acids in its oil indicates this local strain of *C. humicola* as a good candidate for biofuel feedstock. Sodium alginate encapsulation method is found quite feasible for maintaining a stock culture of this alga for sufficiently long period of 14 months. Further explorations on lipid production potential of this alga in different media under varied levels of diverse nutrients and heterotrophic conditions are essential for ensuring its industrial applications.

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