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Effect of different culture media formulations on growth and biodiesel production potential of *Chlorella pyrenoidosa*

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Abstract

In the present research study, photobioreactor was used for the cultivation of algae. *Chlorella .pyrenoidosa* was grown in six different media and modified CHU-11 media (containing urea) was found to be the best on the basis of optical density and pigment content in algal cells. Algal biomass was characterized to assess the growth, biochemical content and lipid yield. The % oil yield was assessed and found to increase during the eight week period. The extracted algal oil was transesterified to produce biodiesel (FAME). The biodiesel production was done by the transesterification process. FTIR analysis of the produced biodiesel was also done to assess the functional groups. The rich diversity and lipid content of algae makes it a potential feedstock for biodiesel generation to meet the energy demands of the world, also the use of biofuels controls environmental pollution.

Key words: *Algal biomass, FAME, feedstock, biofuels, lipid content.*

1. INTRODUCTION

Biomass feedstocks are energy sources derived from plants, microbial cells, and the wastes and residues associated with their processing (e.g. agricultural residues, forestry and municipal wastes). These energy sources are amongst the most promising, most hyped and most heavily subsidized renewable energy sources [Phukan *et al.*,2011]. Biomass feedstocks can be categorized into three generations viz. first generation, second generation and third generation feedstocks. First generation feedstocks such as corn, barley etc have several economic and environmental limitations, use of such crops leads to competition with agriculture for arable land used for food production and can lead to severe food shortages. In addition, intensive use of fertilizers and pesticides application and water use can also lead to environmental problems [Schnek *et al.*,2008]. Second generation biofuels derived from lignocellulosic biomass requires costly technologies involving pre-treatment with special enzymes for conversion of woody biomass into fermentable sugars [Brennan & Owende 2010]. Third generation biofuels derived from microalgae are considered as most promising and viable alternative energy resource, devoid of the major drawbacks associated with first and second generation feedstocks. Microalgae are photosynthetic eukaryotic organisms which can produce high-added-value compounds such as hydrocarbons, pigments, carbohydrates, proteins and lipids [Banerjee *et al.*,2002, Chisti 2007, Tran *et al.*,2009, Hidalgo *et al.*,2013]. These microorganisms can accumulate important quantities of lipids [Hindalgo *et al.*,2013, Balat & Balat 2010] and additionally, they present a fast growth and high productivity compared to agricultural crops [Chisti 2007, Hindalgo *et al.*,2013, Mata *et al.*,2010].

In the present study biodiesel has been produced from the microalgae *Chlorella pyrenoidosa*. ***Chlorella pyrenoidosa*** is a fresh water alga. It is a single celled green algae belonging to the phylum Chlorophyta. For the present research the algal strain was purchased from National Collection of Industrial Micro-organisms, National Chemical Laboratory, Pune, Maharastra, India. A suitable media was screened, for the cultivation of the algae in a photobioreactor. The protein, carbohydrate chlorophyll content and % oil yield was estimated on a weekly basis. After eight weeks the algal biomass was harvested and algal oil was extracted. The extracted algal oil was transesterified. FTIR of the produced biodiesel was also carried out.

2. Materials and methods

A suitable media was screened, for the cultivation of the algae in a photobioreactor by growing the algae in six different media having different composition. The composition of the six media used is given in the table 1 [Mandalam & Palsson 1998, Vonshak & Richmond 1986]. Each media was prepared in two sets *viz.* one without urea (C) and the other with urea (C+U). The growth of the algal culture in different media was determined in terms of optical density, chlorophyll and

caroteinoid content of the algae at an interval of 12 hours. The algal biomass was analyzed for the following biochemical parameters.

Table 1: Composition of different media

COMPONENTS (g/l)	**CM**	**BGM**	**RM**	**CHU-11**	**BBM**	**N8**
KNO_3	2		0.3			0.1
$NaNO_3$		1.5		1.5	25	
K_2HPO_4	0.1	0.04	0.08	0.04	7.5	
KH_2PO_4			0.02		17.5	0.74
$MgSO_4.7H_2O$	0.1	0.075	0.01	0.08	7.5	0.05
$CaCl_2.2H_2O$		0.036	0.058	0.04	2.5	0.013
Citric acid		0.006		0.006		
$NaCO_3$		0.02		0.02		
NaCl			0.02		2.5	
H_3BO_3	0.00286	0.00286	0.003		11.4	
$MnCl_2.4H_2O$	0.00181	0.00181				
$ZnSO_4.7H_2O$	0.00022	0.00022	0.001			
$Na_2HPO_4.2H_2O$						0.26
$NaMoO_4.2H_2O$	0.0004	0.00039				
$CuSO_4.5H_2O$	0.00008	0.00008	0.00008			
$Co(NO3)_2.6H_2O$		0.00005	0.0026			
$(NH_4)_6Mo7O_{24}.4H_2O$		0.003	0.0001			
$MnSO_4$			0.015			
$FeSO_4.7H_2O$	0.00557					
Na_2EDTA	0.00745	0.00001				
Fe EDTA						0.001
EDTA			0.00007	0.001	1 ml	
Ferric ammonium citrate				0.006		

Fe solution stock ($FeSO_4.7H_2O$= 4.28g/l) Conc. H_2SO_4= 1 ml/l)					1 ml	
Trace metal solution components (g/l)				1 ml	1 ml	
1.H_3BO_3				2.86		
2. $MnCl_2.4H_2O$				1.81	1.44	12.98
3. $Na_2MoO4.H_2O$				0.39		
4. $ZnSO_4.7H_2O$				0.222	8.82	3.2
5. $CuSO_4.H_2O$				0.079	1.57	1.83
6. $Co(NO_3)_2.6H_2O$				0.049	0.49	
7. MoO_3					0.71	
8. EDTA stock(g/100 ml)						
$EDTANa_2$					5	
KOH					3.1	
9. $Al2(SO4)_3.18H2O$						3.58
Urea	1 mL.	1 mL	1ml	1 ml	1ml	1ml.

Total Carbohydrate [Dubois *et al.*,1956], Protein content [Lowry *et al.*,1951], Chlorophyll content in algal cells was calculated by Arnon's method [Arnon 1949]. % Oil yield from algal biomass was also calculated [Dutta *et al.*,2014]. A reactor was designed and developed for the cultivation of the algae. A rectangular glass reactor of five litre capacity was developed which consisted of four ports (i.e. a. Temperature, b. pH, c. media supply and d. aeration). The dimensions for the reactor were 12x6x9 inches. The reactor also consisted of a sampling port for the collection of sample. Cultures were illuminated with the cool white daylight with the help of 40 watt fluorescent lamp. 5 litres of sterile modified CHU-11 media was filled in the reactor. 100mL algal culture was used to inoculate the reactor. pH value was maintained at seven for the growth of the algae. Optical density, temperature and pH were recorded daily. Optical density of the cultures was measured at 680 nm wavelength. Harvesting of algal biomass was done by centrifugation method. Centrifuge (Eltek TC8100F) was used for centrifugation. The culture was taken and centrifuged at 5000 rpm for 10 minutes. The settled biomass was collected from the base of the centrifuge tube. The settled biomass was collected from the bottom of the centrifuge tubes and kept in a petriplate for overnight drying in an oven at 80°C. Extraction of algal oil was done by the use of Soxhlet apparatus. Extraction was performed by the use of organic solvents (Chloroform: methanol; 2:1), at 60°C for 18 hours with a siphoning rate of 4-5 cycles per hour. 5 gm dried algal biomass was filled in 25x100 mm cellulose thimble. The left over extract containing the algal oil was collected in a vial and was used for the

transesterification reaction. Transesterification of algal oil was done by the method proposed by Hossain*et al.*,[16]. The transesterified algal oil was analysed by FTIR, for the presence of Fatty Acid Methyl Esters through Gujarat Laboratory, Ahmedabad, Gujarat.

3. Results

Screened suitable media for the growth of *Chlorella pyrenoidosa* showed best results in modified CHU -11(C+U) media. The highest optical density of *Chlorella pyrenoidosa* in different media without urea was recorded in the CM media whereas the lowest OD value (1.4) were recorded in RM media The best media was evaluated on the basis of increase in the optical density of algal culture at every 12 hour and growth of pigment in the algal cells(Fig. 1,2). The optical density of the algal culture in the media containing urea (C+U); urea served as an additional source of nitrogen, carbon and showed best growth of algae as compared to the media without urea (C); both optical density (5.7) and the pigment content (60.76 mg/g f.w.) was greater in the enhanced (containing urea) media..

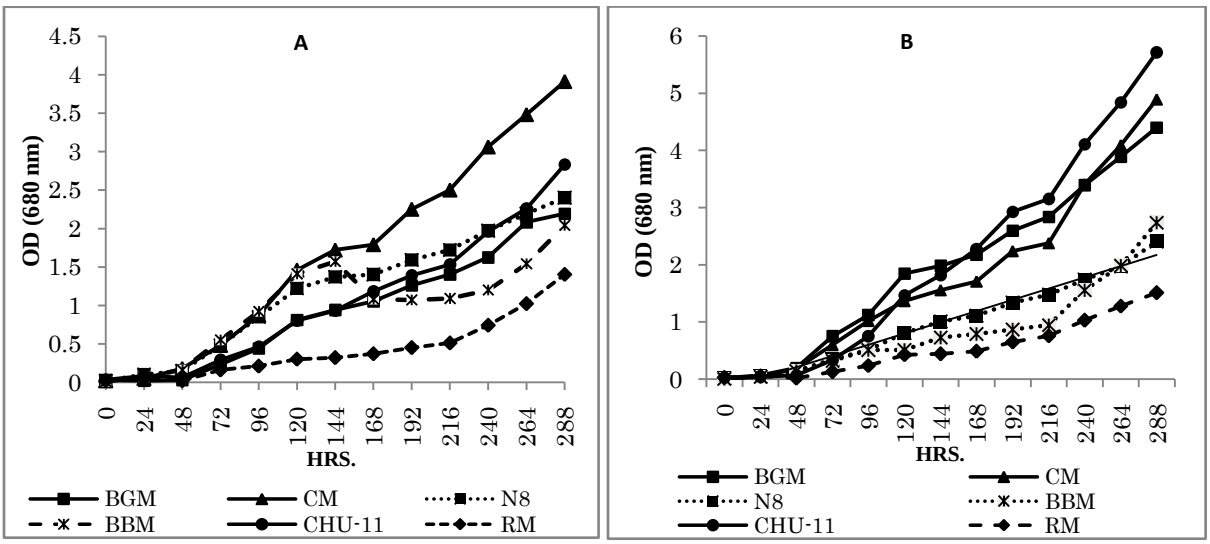

Fig.1 Growth of *Chlorella pyrenoidosa* in different media (A) media without urea, (B) media with urea

Chlorophyll content of an algal culture is an indicator of its photosynthetic activity and growth. The modified CHU-11 media containing urea showed the highest chlorophyll content (66.66 mg/g f.w.) in the algal cell (Fig. 2B), whereas the lowest values (2.7 mg/g f.w.) were recorded in RM media. On the other hand highest chlorophyll content in medias without urea CM media showed the highest chlorophyll content (30.1 mg/g f.w.) and RM showed the lowest chlorophyll content (2.3 mg/g f.w.)(Fig. 2A)

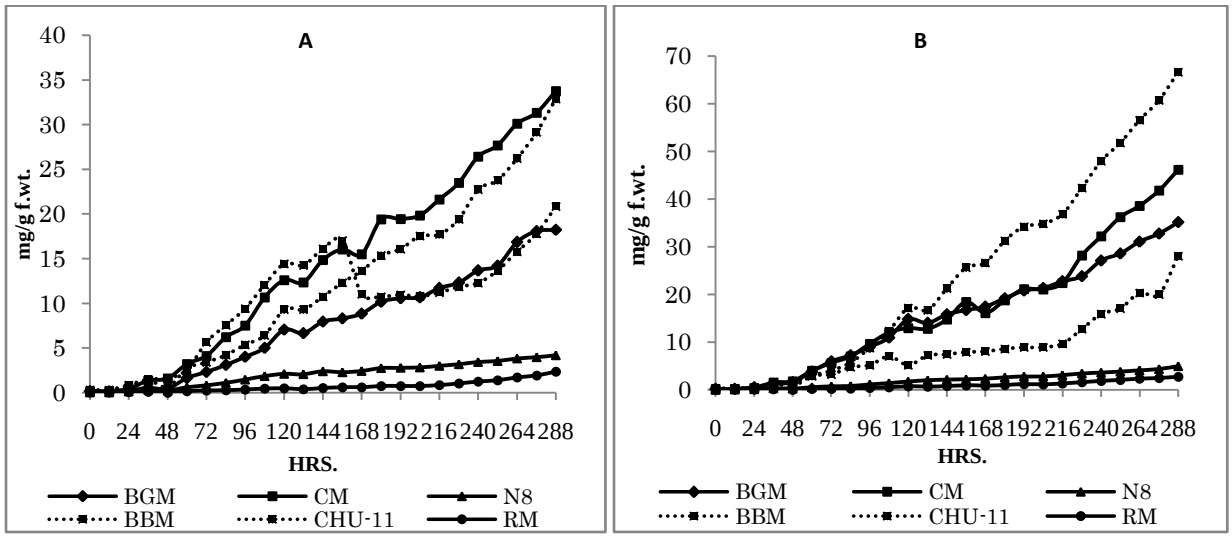

Fig.2 Total chlorophyll content in *Chlorella pyrenoidosa* in different media, (A) without urea, (B) with urea

The optical density of the culture in the photobioreactor was monitored daily upto 56 days along with the pH and temperature. pH of the reactor media was adjusted at 7 and the temperature during the cultivation period was in the range of 19°C to 24°C. Up to the 9th day of the experiment, algae grew at a slow rate and no significant rise in the optical density was noticed (Fig. 3); however after the 9th day there was a noticeable increase in the optical density (0.048) of the algal culture. The increase in the optical density continued up to 35 day, after which the OD value started to decrease. At the end of the experiment the OD of the culture was 0.71.

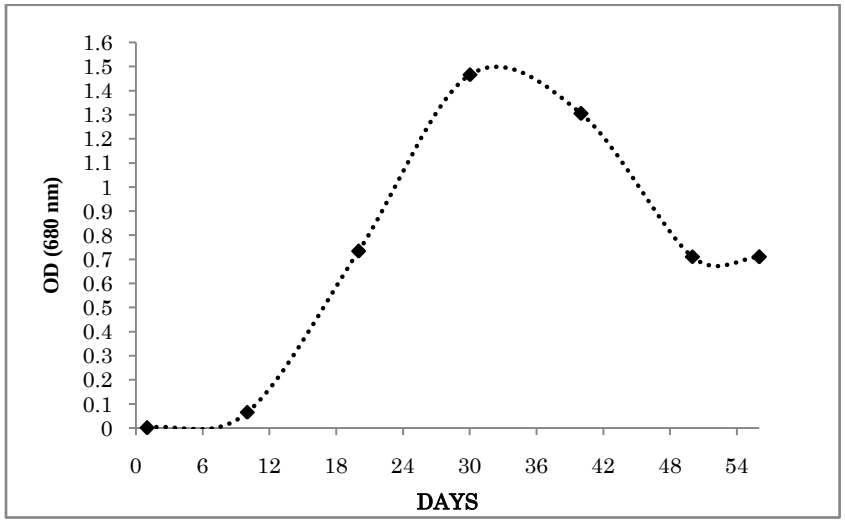

Fig. 3 Optical density of *Chlorella pyrenoidosa* in the reactor

Protein, carbohydrate and pigment content of the algae were estimated regularly at an interval of 1 week. The total time period for the experiment was 56 days (8 weeks). The biochemical analysis of *Chlorella pyrenoidosa* showed higher amount of protein content as compared to the carbohydrate. Protein and carbohydrate both increased with the increase in incubation period. The maximum amount of carbohydrate and protein was recorded in the seventh week 15.74 % and 46.69 % respectively, which started to decrease at the end of eight week (14.50 % and 44.17 %) respectively.

Protein content ranged from 11.89 - 46.69 (%) in algae which was higher than the carbohydrate content (range 3.67 - 15.74 %)(Fig. 4 A, B). The chlorophyll content of *Chlorella pyrenoidosa* varied from lowest value of 0.18 mg/g f.wt. to the

maximum value of 1.28 mg/g fw (Fig. 4C,D). The chlorophyll content increased proportionally to the increasing optical density of *Chlorella pyrenoidosa*. The percent oil yield depends on the accumulation of lipid in the algal cells. The oil yield increased with the growth of algae. The highest oil yield (46.5%) was obtained in the end phase of the cultivation period (Fig. 4D).

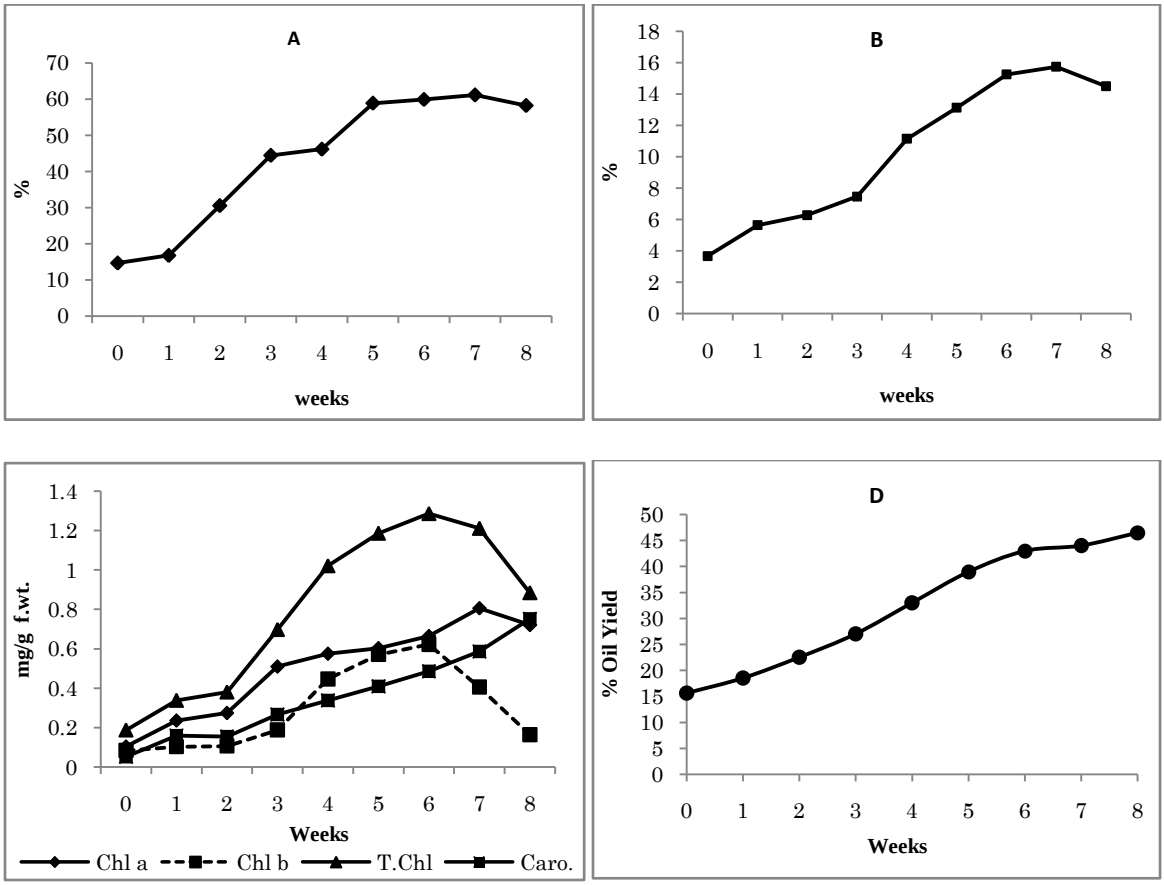

Fig.4 (A) Protein, (B) Carbohydrate, (C) Chlorophyll content in *Chlorella pyrenoidosa* during biomass production, (D) % Oil Yield from algal biomass

After the transesterification of algal oil the produced biodiesel was qualitatively analysed by FTIR for the presence of FAMEs. The FTIR spectra were analyzed for the analysis of methyl esters based on the peaks and chemical groups in the region 600-4000 cm^{-1}. The integration of the peaks in the region of 1777- 1576 cm^{-1} may be assigned to the presence of un-conjugated alkyl aldehydes, alkyl estersand carbonyl group (1800-1500 cm^{-1}) and around 1743 and 1784, 1026-1308 cm^{-1} (C-O vibrations) are clear and could be attributed to vibrational modes corresponding to individual functional groups.

4. Discusssion

Among the six media modified CHU- 11 media gave the best results, which may be attributed to its rich nutrient composition (micro- nutrients and trace elements). Nitrogen is a micronutrient for plants and plays a vital role in the formation of chlorophyll and protein contents. Algal cells grown under nitrogen stress show low optical density, protein and chlorophyll content. The cell size of *A. falcatus* changed under nitrogen limitation, the nitrogen limited cells were found to be less dense and protein content in the algae; [Rhees 1978, Harrison *et al.*,1990] also found lower protein content in a variety of nitrogen limited algae than in non limited control cells [Harrison *et al.*,1990]. Presence of K_2HPO_4 and Mg have a positive effect on algal growth [Turpin 1986, Ilavarasi *et al.*,2011], suggested that K_2HPO_4 enhance dark reaction in *Selenastrum sp*. that leads to its rapid growth, Mg is required by the cells for the chlorophyll synthesis [Mandalam & Palsson 1988]. Magnesium occupies the central position in the chlorophyll molecule and all algal species have an absolute requirement of this element. Mg also has a key role in aggregation of ribosomes into functional units and for the formulation of catalase [Kulshreshtha& Singh 2013]. Magnesium deficiency interrupted cell division in *Chlorella* which results in abnormally large cell formation [Round 1966, Sharma *et*

al.,2012]. Metals such as Iron (Fe), Manganese (Mn), Cobalt (Co), Zinc (Zn), Copper (Cu) and Nickel (Ni) are the six most important trace metals required by algae for various metabolic functions [Brutland *et al.*,1991]. The trace metals requirement of algae was fulfilled by the use of trace metals solution. The results of increased growth of algal cultures in enhanced media agreed with the study reported by X. Zhou *et al.*,2013, according to which high lipid productivity was achieved with large outdoor photobioreactors using the optimized concentrations of key nutrients such as urea and monopotassium phosphate.

Estimation of microalgae growth is generally expressed in: increase in optical density, protein, pigment and carbohydrate contents over a period of time [Becker 1994, Junior *et al.*,2007]. The results of culture maintenance for a longer period agreed with the study by Donald E. Leone [Donald 1963]; according to which algal cultures could be maintained for periods of several weeks by supplementing the nutrient containing minimal amount of certain salts. In the initial stage more and more available nutrients are utilized for the formation of cell structure and reserved food material, as the cells are increasing in number. Rapidly growing microalgal cultures exhibit higher protein and low carbohydrate content [Sayegh & Montagnes 2011]. The results of the biochemical analysis of the algae are similar to the observations found in *Ankistrodesmusfusiformis* [Sharma *et al.*,2012, Singh & Srivastava 1991], where the *Ankistrodesmusfusiformis* contained higher protein content as compared to the carbohydrate. Similar results for protein content were reported by Whyte 1987, Brown 1991, Herrero *et al.*,1991 and Valenzuela *et al.*,2002. The pigment content in *Chlorella pyrenoidosa* increased with the increase in its optical density. The results of the chlorophyll content are in favour of the previous study done be Enrique *et al.*,2002 which showed exponential growth of chlorophyll content with the algae growth.

Oil yield (%) is based on the amount of lipid accumulated in the algal cells. In the present study the percent oil yield showed an increasing trend, similar trend of lipid accumulation was observed by Brown *& Zeiler 1993* in stationary phase cultures of *Isochrysis sp*. (clone T- Iso). The increase in the high lipid content may be due to the depletion of nutrient from the media which enhances the lipid accumulation in the algal cells in the final week of the study.

Methyl esters are noted as fairly strong absorbers in the infrared region of the electromagnetic spectrum. The FTIR spectra were analyzed based on the peaks and chemical groups through a range of 600-4000 cm^{-1}cm. The integration of the sides 1743- 1784 cm^{-1} and 1576-1717 cm^{-1} may be assigned to the presence of un-conjugated alkyl aldehydes, alkyl estersand carbonyl group (1800-1500 cm^{-1}) and around 1026- 1308 cm^{-1} (C-O vibrations) are clear and could be attributed to vibrational modes corresponding to individual functional groups [Dutta *et al.*,2014, Derrick *et al.*,1999, Kothari *et al.*,2013]. The carboxylic groups appear around the same frequency for methyl esters and triglycerides; for long chain Fatty Acids it appears at around 1700 cm^{-1} [Da Ros *et al.*,2013]. The position of carbonyl band in FTIR is suggested to be sensitive to substitution effects and change in the structure of molecule [Kothari *et al.*,2013, Pasto *et al.*,1992].

5. Conclusion

The present study illustrates the use of microalgae *Chlorella pyrenoidosa*as a potential feedstock for biodiesel generation. The screened modified CHU-11 media proved suitable for the cultivation of this algal strain in closed photobioreactors. Biodiesel from the algal biomass was generated through transesterification reaction. FTIR analysis of the transesterified algal oil was done for the presence of FAMEs. Apart from biodiesel other forms of bioenergy (biogas, bioethanol, biobutanol etc.) can also be obtained from algae. The rich diversity and lipid content of algae makes it a potential feedstock for biodiesel generation to meet the energy demands of the world, also the use of biofuels controls environmental pollution and helps in the maintenance of cleaner environment.

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