Int J Pharm Bio Sci 2015 July ; 6(3): (B) 251 - 262

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

SCREENİNG AND OPTİMİZATİON OF CULTURE MEDİA FOR *CHLORELLA SP.* AS A RAW MATERIAL FOR BİODİESEL PRODUCTİON

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ABSTRACT

Alga biomass based biofuels seems to be the most promising renewable fuels for the future. However, large scale algae cultivation is mandate for industrial biofuel production. Before going to large scale, screening and media optimization is necessary for low-cost microalgae biofuels, as media composition significantly affect the microalgal growth, lipid yield, and microalgae biodiesel cost. This investigation studied the effect of different media i.e Blue green-11(BG-11), Bold basal medium, Fog's medium and Basal medium on microalgae growth and lipid productivity. Out of them, BG-11 medium was observed best for biodiesel production with higher lipid yield. Furthermore, algae cultivation in one liter polybag and 7 liter capacity photobioreactor under outdoor condition was also carried out using BG-11 as a grwoth medium. Additinally,.different lipid extraction method tested with biomass of *Chlorella sp.* using ethanol, methanol, hexane and a mixture of chloroform: methanol. Among the tested methods, the mixture of chloroform:methanol (2:1) was most effective, extracting an average of 15% of total lipids. Lipid profiles of *Chlorella sp.* were compared with Jatropha biodiesel in the percentage of saturated and unsaturated fatty acids; however, *Chlorella sp.* have more poly-unsaturated fatty acids such as linoleic (C18:2) and linolenic (C18:3) acids than Jatropha oil. Biodiesel produced by transesterification of *Chlorella sp.* satisfies international Physico-chemical standard parameters such as Cetane number, Saponification value, Iodine value and Degree of unsaturation.

KEYWORDS: **Microalgae, Screening, Bioenergy, Lipid, Biodiesel production.**

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1. INTRODUCTION

Due to increasing pollution, shortage of fossil fuels and the urgency of CO_2 emission reduction in recent years, the industrialization of biofuels from microalgae has been getting more attention owing to the potential for biofuels production and CO_2 emission reduction. Microalgae are the sunlight-driven cell factories that convert carbon dioxide to several different types of renewable bio fuels, while the photosynthesis mechanism in microalgae is similar to other plant[1]. Microalgae are categorized into four major classes in terms of their abundance: diatoms, green algae, blue-green algae, and golden algae. Microalgae can either be autotrophic or heterotrophic; the former require only inorganic compounds such as CO_2, salts and a light energy source for growth; while the latter are non-photosynthetic therefore require an external source of organic compounds like glucose, glycerol as well as nutrients as an energy source. Some photosynthetic algae are mixotrophic, i.e. they have the ability to both perform photosynthesis and acquire exogenous organic nutrient. Microalgae are considered a potential source of biodiesel because of a number of advantages, such as their relatively simple cellular structure, high lipid content and photosynthetic efficiency, and, consequently, their higher biomass and lipid yields when compared to other energy crops[1-3]. Microalgae can be cultivated in waste or blackish water, avoiding competition with food/feed crops for agricultural land and freshwater[4, 5]. The screening and optimization of medium is vital necessities for high lipid productivity of microalgae photoautotrophic cultivation. The quality of medium used for their cultivation greatly affects the growth performance of microalgae[6-8]. The cell growth and lipid accumulation is mostly affected by the composition of medium such as carbon, nitrogen, phosphorus and some trace elements[9]. Huerlimann et al. cultured four algae (*Isochrysis sp.*, *Nannochloropsis sp.*, *Tetraselmis sp.*, and *Rhodomonas sp.*) in three different nutrient media (L1, f/2, and K-medium) and observe significant differences in biomass yield, lipid content and lipid productivity existed due to both species and medium differences[10]. Dayananda et al. also grew *Botryococcus braunii* in BG-11, BBM and BBM nutrient media and analysis the effect of culture media composition on biomass and lipid productivity[11]. Sharma et al cultured *C. vulgaris* in Juller's medium, Bold's basal medium, modified Chu-10 medium, N-8 medium, and Kuhl's medium, to observe effect on the growth, morphology and pigments and found modified chu10 medium as best growth medium[12]. Some researcher found that macronutrients including nitrogen, phosphorus and their ratios had major effects on the contents of protein, carbohydrate, lipid, fatty acids, chlorophyll-a and carotenoids of microalgae[13]. However very few reports are available, which describes the effect of culture media on *Chlorella sp.* The objective of the present study was to study the influence of four enriched culture media on biomass productivity, lipid content and yield of microalga. The feasibility of microalgae culture under outdoor condition in closed photobioreactor was also examined.

2. MATERIALS AND METHODS

2.1 Isolation and identification of microalgae species

Microalgae was collected from Dehradun Uttrakhand, India. Microalgae samples (about 5 ml) were inoculated into 5-ml autoclaved BBM medium [14] in 20-ml test tubes and cultured at 24^{O}C for 2 week with cool white fluorescent light. The light intensity was approximately 2500 lux and the diurnal cycle was 8 h dark/16 h light. The pre-cultured samples were streaked on BBM medium-enriched agar plates and cultured for another 15 days with same culture condition. The single colonies on agar were picked up and cultured in liquid BBM medium, and the streaking and inoculation procedure was repeated until pure cultures were obtained. The morphology of pure strains was regularly examined under an optical microscope. The strains were identified morphologically. The stained sections were observed under 100× and photographed by a Leica DM2000 LED fluorescent microscop (serial no. 372671) and identification was done using botanical approaches [15].

2.2 *Medium screening and optimisation*

Four media that have been reported for photoautotrophic culture and lipid production of *Chlorella sp.* were chosen as the candidates include BBM, BG-11, Fog' s medium and Bold basal medium.

Bold's Basal Medium [16]

$NaNO_3$ 250 mg l^{-1}, K_2HPO_4 75 mg l^{-1}, $MgSO_4$-7H2O 75mg l^{-1}, $CaCl_2$-2H2O 25mg l l^{-1},KH_2PO_4 175 mg l^{-1},NaCl 25 mg l^{-1}Alkaline EDTA solution 1ml l^{-1}(Alkaline EDTA solution: 5g Na_2-EDTA and 3.1 g KOH in 100 ml distilled water), Acidified Iron solution 1ml l^{-1}(Acidified Iron solution $FeSO_4$-7H2O .498g and 0.1 ml H_2SO_4 in 100 ml distilled water) Trace metal solution 1 ml l^{-1}(Trace metal solution: $MnCl_2$-4H2O 1.44 g l^{-1}, $ZnSO_4$-7H2O 8.82 g l^{-1}, $(NH_4)_6$ Mo_7O_{24}-2H2O 0.88 g l^{-1}, $Co(NO_3)_2$-6H2O 0.49 g l^{-1}, $CuSO_4$-5 H_2O 1.57 g l^{-1}).

BG-11 medium[17]

$NaNO_3$ 1500 mg l^{-1}, K_2HPO_4 40 mg $l^{-1}MgSO_4$-7H2O 75mg l^{-1}, $CaCl_2$-2H_2O 36mg l^{-1},Citric acid 6 mg l^{-1}, Trace metal solution 1 ml l^{-1}(Trace metal solution: $FeC_6H_5O_7$-NH_4OH 6 g l^{-1}, Na_2EDTA 1 g l^{-1}, $MnCl_2$-4H_2O1.81 g l^{-1}, $ZnSO_4$-7H_2O 0.222 g l^{-1}, Na_2 MoO_4-2H2O, 0.39 g l^{-1},$CuSO_4$-5H_2O 0.08 g l^{-1},H_3BO_3 2.86 g l^{-1}.

Fog medium [18]

KNO_3 2000 mg l^{-1}, K_2HPO_4 200 mg l^{-1}, $MgSO_4$-7H2O 200mg l^{-1}, $CaCl_2$-2H_2O 100mg l^{-1},Fe-EDTA solution 5ml l^{-1}(Fe-EDTA solution: 745 mg Na_2EDTA and 557mg $FeSO_4$-7H2O in 100 ml distilled water) , Trace metal solution 1 ml l^{-1}(Trace metal solution:, $MnCl_2$-4H_2O 1.81 g l^{-1}, $ZnSO_4$-7H_2O 0.222 g l^{-1}, Na_2 MoO_4-2H2O, 0.39 g l^{-1},$CuSO_4$-5H_2O 0.08 g l^{-1},H_3BO_3 2.86 g l^{-1}).

M_4N medium[17]

KNO_3 5000 mg l^{-1}, K_2HPO_4 1250 mg l^{-1}, $MgSO_4$-7H_2O 25000 mg l^{-1}, $CaCl_2$-2H_2O 100mg l^{-1}, $FeSO_4$-7H_2O 30 mg l^{-1}in 100 ml distilled water) , Trace metal solution 1 ml l^{-1}(Trace metal solution:, $MnCl_2$-4H_2O 1.81 g l^{-1}, $ZnSO_4$-7H_2O 0.222 g l^{-1}, Na_2 MoO_4-2H2O, 0.39 g l^{-1},$CuSO_4$-5H_2O 0.08 g l^{-1},H_3BO_3 2.86 g l^{-1}).

2.3 Algal growth and biomass estimation

Chlorella sp. was cultured in 250 ml Erlenmeyer flasks containing 125 ml liquid using above four nutrient media composition under cool florescence light (~2500 lux) at 24°C (±1°C) and 16:8 Light in a Photobioreactor. At stationary phase, *Chlorella sp.* was harvested using centrifuge and dried at 60°C in oven to get dry biomass for lipid extraction. *Chlorella sp.* was also cultured in one litter polybag and 7 L capacity photobioreactor made up of aquarium glass (75 cm height × 20 cm diameter). The agitation in the culture medium was carried out by sparging filtered air. The growth of *Chlorella sp.* was observed by measuring the optical density at 680 nm (OD_{680}) using UV-visible spectrophotometer (Thermo Scientific) daily and related to algal biomass (g/l).

The maximum specific growth rate (μ_{max} day^{-1}) at exponential stage was calculated as follows

$$_{max}\ (day^{-1}) = (\ln X_2 - \ln X_1)/ (t_2 - t_1) \qquad (1)$$

Where X_1 and X_2 were the dry biomass weight (g/l) at time t_1 and t_2 respectively.

The doubling time (T_D,days) was calculated as follows

$$T_D(days) = \ln(2)/ \mu_{max} \qquad (2)$$

The rate of biomass production (P, mg $L^{-1}day^{-1}$) was calculated by following equation

$$P\ (\text{mg } L^{-1}day^{-1}) = (X_2 - X_1)/t_x \qquad (3)$$

Where X_1 and2_2 were the dry biomass weight (g/l) at time t_x
Lipid was extracted by applying folch extraction method [19].

Lipid was also extracted using ethanol, methanol, hexane and a mixture of chloroform: methanol in ratios 1:2 and 2:1.

2.4 *Fatty acid analysis*

Fatty acid is converted in to their methyl esters by transesterification of lipid using 1 ml of 1% NaOH in MeOH followed by heating for 15 minute at 55 ^{o}C, adding 2 ml of 5% methanolic HCl and again heated for 15 minute at 55^{o}C, washed by 1ml distilled water. FAME is extracted with hexane (3×1) and evaporated to dryness[20]. The fame was re-dissolved in 200µl hexane and analysed using a GC (gas chromatograph) Nucon 5765 series with EOX column (serial no 5061; 30 m length, 0.25 mm ID and 0.25 mm outer dia). Pure Nitrogen (99.9%) used as carrier gas with a flow rate of 1 ml/min and pre-column pressure of 49.7kPa. The initial temperature was set to be 120º C for 2 min, followed by a 4º C/min ramp up to 240º C and maintained for 30 min. the injector and FID detector temperature was set 240^{o}C and 230^{o}C respectively. Fame peaks are identified by comparison of their retention time with authentic standard by GC and quantified by normalization.

2.5 *Analysis of biodiesel quality*

The key qualities of biodiesel the Saponification value (SV), Iodine value (IV), Cetane number (CN), (DU) degree of unsaturation, Cold filter Plugging point (CFPP) and oxidation values[21] were analysed by using empirical Eq. 3-10.

$$SV = \Sigma (560 \times N) / MW \quad (4)$$

$$IV = \Sigma (254 \times N \times D) / MW \quad (5)$$

$$CN = (46.3 + 5458 / SV) - (0.225 \times IV) \quad (6)$$

$$DU = (MUFA, wt\ \%) + (2 \times PUFA, wt\ \%) \quad (7)$$

Long chain saturated Factor (LCSF)

$$= (0.1\times 16.0) +(0.5\times 18.0) +(1\times 20.0) + (2\times 24.0) \quad (8)$$

$$\text{Cold filter Plugging point (CFPP)} = (3.1417\times LCSF)-16.4777 \quad (9)$$

$$\text{Oxidation stability } Y= (117.9295/x) + 2.5905 \ (0<100) \quad (10)$$

Where N is the percentage of each fatty acid, MW is the molecular mass of fatty acid, D is the number of double bonds, MUFA is monounsaturated fatty acids and PUFA is polyunsaturated fatty acid by wt. %. X is the content of linoleic acid (wt %) During the entire experiment, the measurements of the values were done in triplicates and the mean and ± standard deviation (SD) was calculated using Graph Pad Prism 6 statistical software.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of microalgae species

Figure 1 shows the pictures of the green algal strains taken by an optical microscope and identified using botanical approaches. The aliquot of the sample was placed in the centre of the clean glass slide and covered with a thin cover slips and then exmined by microscope. Microscopic observation of microalalgal isolates revealed that they were unicellular small green cells, spherical or ellipsoidal shape with 3-10µm size. Microscopic analysis of the samples allowed preliminary identification of isolates as *Chlorella sp.*

3.2. Screening and optimization of media

Chlorella sp. was cultured in four media i.e BG-11, BBM, Fog's and M_4N medium in 250

ml Erlenmeyer flasks containing 125 ml media. Theses conical flask was kept in a photobioreactor having culture condition: light intensity-2500 lux, light: dark duration-16:8 and culture temperature of 24^{o}C. The results revealed that *Chlorella sp.* had approximately 2 d lag period and reached the exponential phase within 4–6 d in all media. Within 15 days microalgae cells achieved stationary phase and after that cells growth was very slow as shown in figure 2. As microalgae shows different biochemical composition (lipid, protein, carbohydrate, chlorophyll) in different media[13] which reflected by different OD of sample, so it is very difficult to correlate microalgae growth to optical density (OD). To avoid this error actual biomass concentration DW (dry weight) was used to study microalgae growth in different media (shown in figure 3). It was examined that highest biomass concentration of *Chlorella sp.* was in BG-11 (1.39±0.04 g/l), followed by BBM (1.21 ±0.05 g/l), fog's medium (1.25±0.04g/l) and M_4N (1.23±.02 g/l). This can be explained by the fact that higher nitrogen concentration is favorable for increasing biomass growth[22]. However, M_4N (having maximum nitrogen concentration) shows minimum biomass concentration which is due to limitation or excess of some micro and macronutrient available in media composition. Trends of extracted lipid content of *Chlorella sp.* grown in different media was shown in figure 4. Maximum lipid content was observed in BBM media (15.87±0.77 %), followed by BG-11 (13.76 ± 0.40%), fog's medium (13.01± 1.19%) and M_4N (12.55±.53%). Microalgae grown in BBM showed maximum lipid as it contain minimum nitrogen concentration. These data supported by the fact that Nitrogen starvation condition results in more lipid accumulation[23-25]. However, total lipid yield was examined maximum in BG-11 (191.53 ± 6.70 mg/l), followed by BBM (191.29 ± 8.64 mg/l), Fog's medium (162.01±5.13 mg/l) and M_4N (153.95 ± 3.06 mg/l). The selection of culture medium depends on several factors: the target product, the growth rate, and medium cost. Because lipid production is the major aim of algae production in this study, BG-11 and BBM are the ideal culture media. According to some researchers, lipid production can also be increased by nitrogen starvation conditions[25-26] and Bg-11 have more potential to increase lipid production by applying nitrogen starvation condition. Therefore, BG-11 is considered the best culture medium for *Chlorella sp.* based on results from this study.

3.1. Feasibility of cultivating microalgae in polybag and Glass photobioreactor under outdoor conditions

Microalgae cultured outdoors can use natural sunlight to grow, thereby decreasing the overall cost of biodiesel production from algae. Therefore, the feasibility of microalgae culture under outdoor conditions should be tested in 1 litter polybag and 7 litter capacity photobioreactor (figure 9). The culture medium was used BG 11 (selected and optimized in section 3.2). The cells grew well and reached high biomass productivity in the photobioreactor within 14 days (Figure 5 and 6). As shown in figure 5, *Chlorella sp.* achieved 59.81 mg/L/d and 36.70 mg/L/d of biomass productivity with14.67% and 13.48% in 7 litter capacity and one litter polybag photobioreactor respectively. The biomass productivity of *C. zofingiensis* (58.4 mg/L/d) was obtained in outdoor condition using 60 L flat photobioreactor by Feng et al., 2011[27]. Zheng et al. (2012) and Oh et al. (2010) observed that the lipid contents of *Chlorella sorokiniana* and *Chlorella minutissima* were 13.1% and 12.8%, respectively, under autotrophic conditions in large scale photobioreactors[28, 29]. Specific growth, doubling time and lipid yield of *Chlorella sp.* was shown in table 1. *Chlorella sp.* was harvested by centrifuged and dried at 60^{o}C in oven to get dry biomass. The dried biomass was used for further lipid extraction.

3.3 *Comparison of lipid extraction methods*

Figure 7 shows the comparative analysis of different lipid extraction methods in relation to the total lipid content in the dry biomass. Chloroform: methanol (2:1) was the best solvent system for lipid extraction from *Chlorella sp.* (14.67±.99%), whereas minimum lipid content was found in hexane (6.53±0.92%).This value is similar to the total lipid of *Chlorella sp*. reported in the literature, which lies between 5% and 40% under phototrophic culture condition[30]. This can be explained by the chemistry concept of 'like

dissolving like'**.** Furthermore, the efficient extraction of lipid is highly dependent on the polarity of the organic solvent or solvent mixture. Lipids that are largely hydrophobic (e.g., neutral lipids) will favorably interact with the relatively non-polar solvent molecules (e.g.,chloroform,), while membrane-associated polar lipids will require polar solvent molecules (e.g., methanol) to disrupt the hydrogen bonding and electrostatic forces between the lipids and proteins [31].

3.4 *Fatty acid composition and physico-chemical properties of biodiesel*

Figure 8 shows the FA profile of *Chlorella sp.* grown 7 liter vertical glass column photobioreactor. The FA profile of alga was determined by the quantification of FAME content which reveals the abundance of FA with carbon chain length of C16 and C18 similar to *Jatropha*. The physico-chemical properties of biodiesel are highly affected by the Fatty acid profile of the algae. The total amount of saturated, monounsaturated and polyunsaturated FAME were found 49.5%, 8.32% and 37.48% respectively. Fuel properties of microalgae biodiesel was shown in Table 2. Degree of unsaturation is the sum of monounsaturated and polyunsaturated FAME content and influence the oxidation stability of biodiesel. The examined oxidation stability of *Chlorella sp.* biodiesel was 5.73 which is slightly lower EN 14214 standards (minimum 6 hour) but follow ASTM D 6751 (minimum 3 hour).Cold filter plugging point (CFPP) values of biodiesel CFPP is a filterability test for cooled fuels already containing some solids. It can be described by the high amounts of the saturated fatty acid alkyl esters, because the unsaturated fatty acid alkyl esters have lower melting points than the saturated fatty acid alkyl esters[32]. Biodiesel obtained from *Chlorella sp.* has CFPP 5.77^{o}C (Table 2). CN is one of the most important indicators for determining combustion behavior of diesel [33]. The CN of a fuel is related to the ignition delay time (the time between injection and ignition). The shorter the ignition delay time, the higher the CN, and *vice versa*. According to the ASTM D6751-02 and EN14214 standard for biodiesel, the minimum CN should be 47.0 and 51.0, respectively, whereas the IV is set to a maximum of 120 g I2/100 g fat. The iodine value and Cetane no. of *Chlorella sp.* based biodiesel was found 95.84 and 51.83 respectively, which meets the fuel standards.

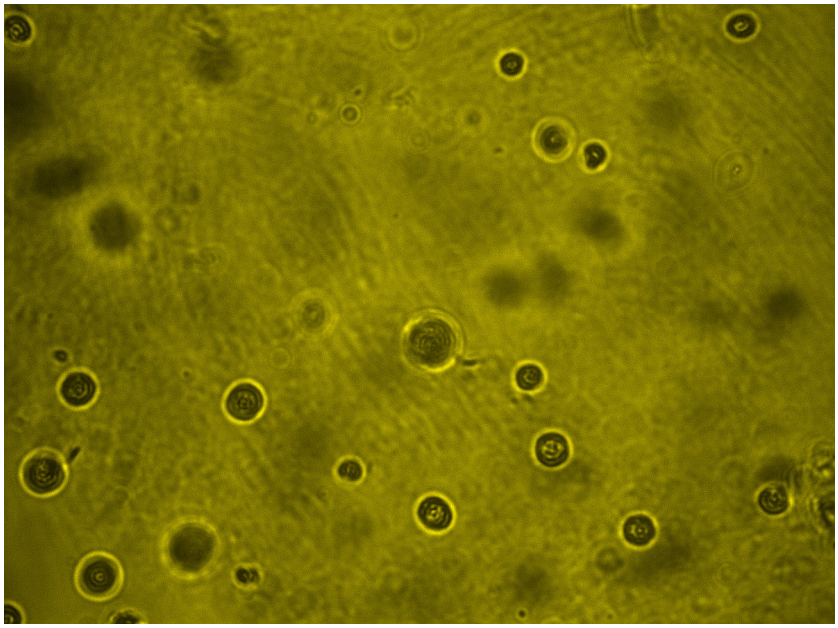

Figure 1
Light microscope picture of the tested microalgal isolates.

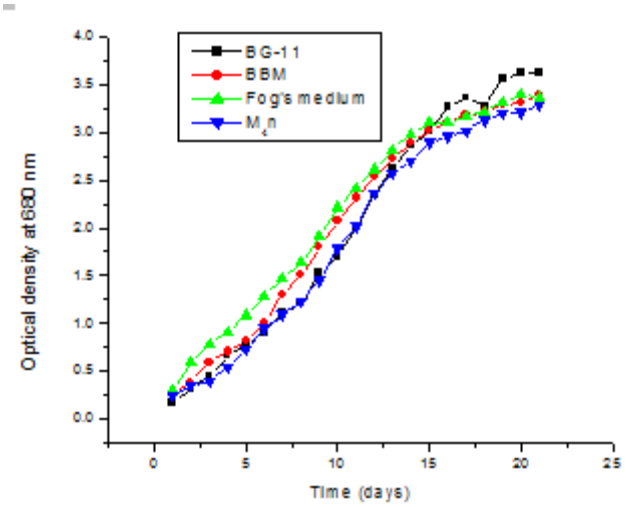

Figure 2
Growth Kinetics of Chlorella sp. at 680 nm absorbance

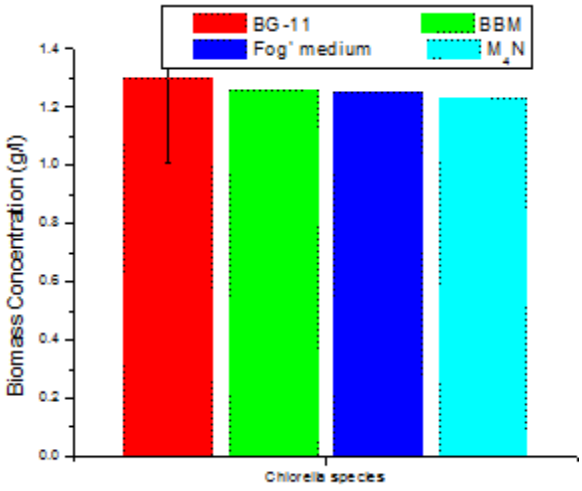

Figure 3
Biomass concentratio of Chlorella sp. in different medium

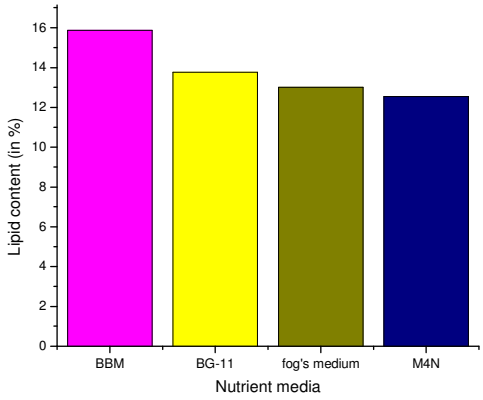

Figure 4
Lipid Content of Chlorella species in different medium

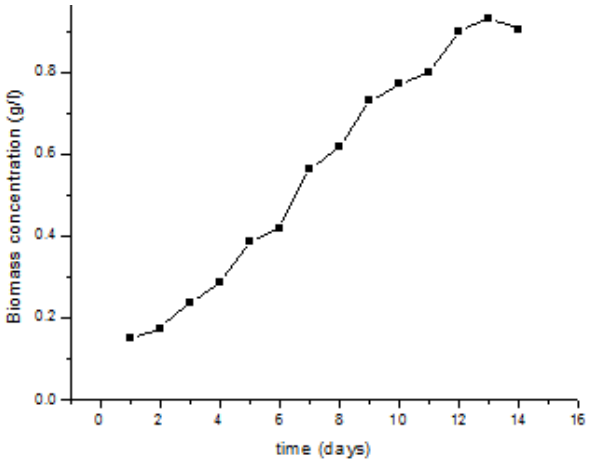

Figure 5
Biomass concentration of Chlorella sp. in one litter polybag photobioreactor inoutdoor condition

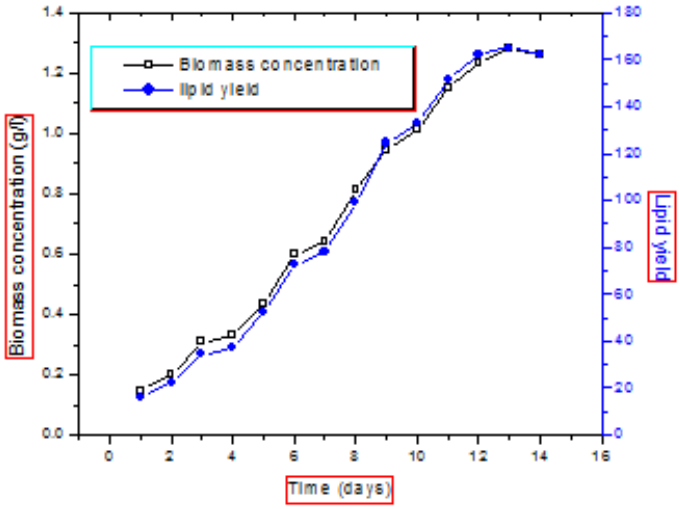

Figure 6
Biomass concentration of Chlorella sp. in 7 litter glass photobioreactor in outdoor condition

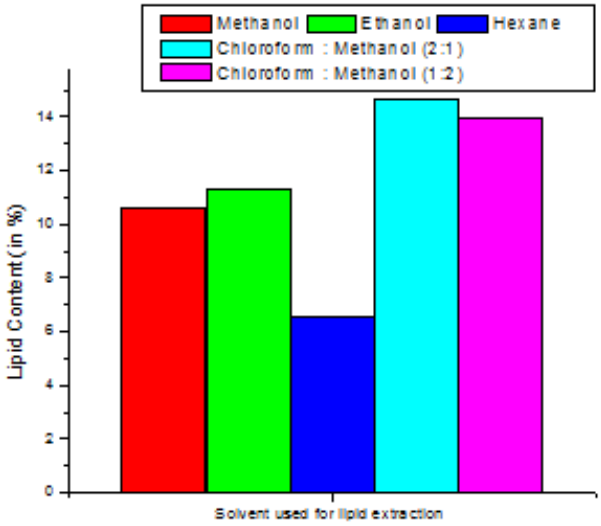

Figure 7
Lipid content of Chlorella sp. using different extraction methods

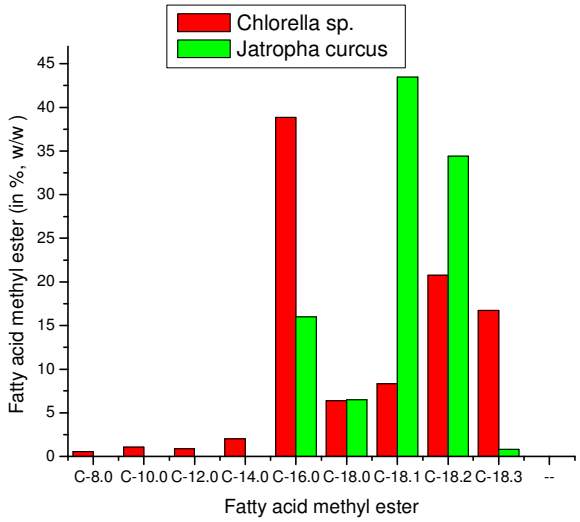

Figure 8
Fatty acid methyl ester composition of Chlorella sp. based biodiesel

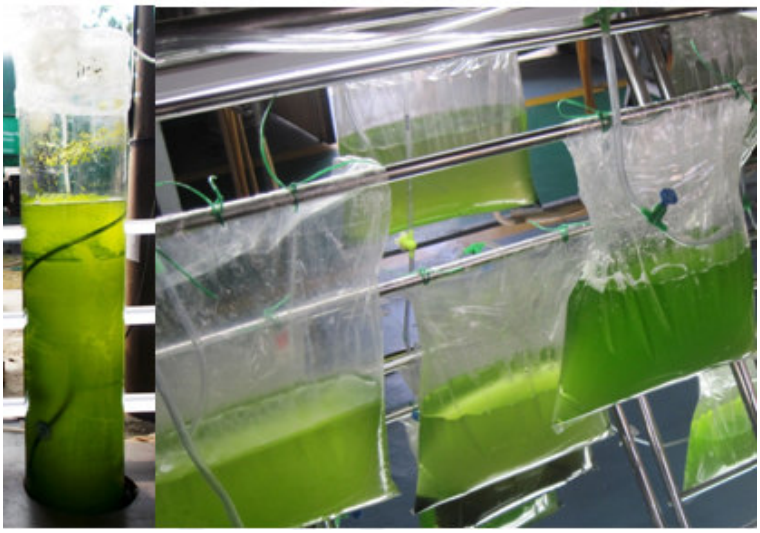

Figure 9
Culture of Chlorella sp. in one litter polybag and and 7 liter glass photobioreactor

Table 1
Growth parameter of Chlorella sp. in one litter polybag and 7 litter glass photobioreactor

Growth parameter	Biomass concentration	Specific Growth rate	Doubling Time	Lipid content	Lipid Yield
One liter polybag photobioreactor	.90	0.146516	4.73	13.48	126.72
7 liter glassphotobioreactor	1.26	0.165149	4.19	14.67	185.23

Table 2
Phisco-chemical properties of biodiesel derived from of Chlorella sp.

Properties	SV	IV	CN	DU	CFPP	Oxidation stability	References
Chlorella sp.	201.42	95.85	49.52	83.28	5.78	5.74	This study
***Chlamydomonas* sp.**	206	26	56.7	27	17.6	20.2	(33)
Chlorella vulgaris	189	50	50	56	8.8	14.3	(33)
Jatropha Curcus	195.09*	100.29*	52.71029*	113.9*	-1.23976*	5.94077*	(34)

SV=Saponification value,IV = iodine value, CN=Cetane number, DU= Degree of unsaturation, CFPP= Cold filter plugging point

*Calculated on the basis of fatty acid composition of jatropha curcus in Sahoo et al (32)

4. CONCLUSION

On the basis of biomass concentration, lipid content, lipid productivity and cost of culture media, it can be observed that BG-11 culture media is the best for biodiesel production among four tested media. Furthermore, Chloroform: MeOH (2:1) was found the best lipid extraction method. Biodiesel obtained from *Chlorella sp.* met the criteria of National Petroleum Agency (ANP255), European biodiesel standardEN14214, ASTM D6751. Therefore, it can be concluded that *Chlorella sp.* is the good candidate for Biodiesel production.

ACKNOWLEDGEMENT

Authors are highly thankful to Chancellor UPES, Dr. S J Chopra and Vice- Chancellor, Dr. Parag Diwan for constant encouragement, necessary laboratory facilities, financial assistance and valuable guidance timely.

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