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Molecular approach for identification of algal isolate using 18S rRNA Phylogenetic analysis and determining its oil content

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Abstract

Increasing greenhouse gases, ozone depletion, and continued decline of fossil fuel reserves have necessitated researchers to search for a low input, high output, non-palatable fuel crop which should not compete with food crops for agricultural land and water. The only organisms that come close enough to possess all the above characteristic features are microalgae. The unicellular green alga *Chlorella vulgaris* was isolated from local water bodies and maintained under controlled culture conditions. Algal biomass obtained by cultivating within bioreactor system was subjected to n-hexane soxhlet extraction which revealed 22.2% oil productivity. Molecular identification of the algal isolate was carried out through 18S rRNA sequencing employing ITS-1, ITS-4 universal primers, comparative analysis of amplification product revealed an overlapping region of 621 bp corresponding to 18S rRNA (Accession no. KX363808). Multiple sequence alignment of the amplified sequence using Nt-NCBI-BLAST revealed 92-94% sequence similarity to *Chlorella* Sp. USTB-01 The fatty acid composition was analysed using Perkin Elmer 500GC-MS which confirmed the presence of several FAME, thus evidencing its potential as an alternate fuel crop.

Keywords: *Chlorella*, Microalga, NCBI-BLAST, Phylogenetic analysis, 18S rRNA genes.

Introduction

Fossil fuels have been the lifeline of all industrial civilization driving their progress and development yet their excessive use have delocalized large carbon reserves into the atmosphere (Schlagermann *et al*., 2012; Althor *et al*., 2016). More than 80% of the world energy demand is being subsidized by fossil fuels constantly adding greenhouse gases within the ecosystem (Chisti, 2007; Patil *et al*., 2008; Schlagermann *et al*., 2012; Hansen *et al*., 2016). This has led to the development of a new challenge in the form of greenhouse effect, ozone depletion and environmental pollution paving way towards natural calamities (Ahmed *et al*., 2011; Althor *et al*., 2016). The increased emission of carbon dioxide, carbon monoxide, has greatly elevated Earth global temperature leading to melting of polar ice caps and increase in sea level which pose a serious threat to low lying areas, being drowned under water (Schlagermann *et al*, 2012; Hansen *et al*., 2016). The dependence on fossil fuel and their ever increasing demand has greatly intensified research on alternate renewable energy sources especially biofuels (Patil *et al*., 2008; Ahmed *et al*., 2011; Prommuak *et al*., 2012; Mubarak *et al*., 2015).

In the quest to achieve success for alternate fuels attempts has been made from time to time (Patil *et al*., 2008; Chen *et al*., 2009; Hare *et al*., 2009; Verma *et al*., 2016). Introduction of biomass derived bio-fuels could not only solve the problem of greenhouse effect but also lead to economic and environmental self-sustenance. Oil plants (Mustard, Coconut, etc) have been used for biofuel productivity since ages but have resulted in a conflict between food and fuel. Moreover, use of economically important crops as bio-fuel source had led to price hike, hampered livestock maintenance and influenced people living under poverty (Patil *et al*., 2009; Mubarak *et al*., 2015). Further, studies were taken up to explore for a high output, non-palatable fuel crop which could grow in aquatic environment without competing for agricultural land (Chisti, 2007; Hare *et al*., 2009). Independent scientific studies across the globe have synonymously recognized microalgal strains (*Chlorella vulgaris*, *Chlorella minutissima, Botryococcus braunii*) as the most prominent game changer in the field of biomass derived renewable energy sources (Banerjee *et al*., 2002; Chen *et al*., 2009; Radakovits *et al*., 2010; Ahmed *et al*., 2011; Pazos and Izquierdo, 2011; Ahmed *et al*., 2012; Prabakaran and Ravindran, 2013; Gupta *et al*., 2015).

Algae constitute a diverse group of photoautotrophic eukaryotic organisms ranging from unicellular to multi-cellular thalli with the ability to convert CO_2 into bio-fuel, food and feed stock (Banerjee *et al*., 2002; Singh *et al*., 2005; Hu *et al*., 2008). Algal species could be categorized into macro or microalgae growing usually in aquatic

environments. Macro algae are multicellular algae inhabiting generally marine water grouped under Brown Seaweed (Phaeophyceae), Red Seaweeds (Rhodophyceae) and Green Seaweeds (Chlorophyceae) (Wang *et al*., 2008; Schulze *et al*., 2014). The most widely studied algae belongs to Chlorophyceae as these are photoautotrophic and could effectively fix large amount of carbon dioxide into stored lipids (Chisti, 2007; Hu *et al*., 2008; Chen *et al*., 2009). Large scale cultivation of microalgae as a source of oil for biodiesel production can effectively lead to a net decrease in carbon emission solving twin problem of global warming and environmental pollution (Chisti, 2007; Wang *et al*., 2008; Sharma *et al*., 2012). More than 40,000 species of microalgae are estimated to occur out of which hardly 40-50% had been investigated thus offering tremendous scope for the discovery of an ideal algal strain (Hu *et al*., 2008; Radakovits *et al*., 2010; Gupta *et al*., 2015). The present study was conducted to isolate a potential algal strain as a source of oil, determining its cultural characteristics, phylogenetic position and lipid productivity.

Materials and methods:

1. Collection of Samples

Samples were collected from local water bodies' ponds, river banks and local reservoirs of Mathura-Vrindavan region. Water samples were collected both from the surface and from a depth of one foot. Samples were then transferred to the laboratory aseptically in sample bottles and stored at 4° C till further investigation.

From the samples collected, 10 µl of inoculum was taken serially diluted and sub cultured over algal growth media. Algal isolate were obtained in the form of pure culture through streak plate method, intermittent UV light exposure and use of broad spectrum antibiotics. BG-11 media, BB media, CHU media and *Spirulina* based media were used as selectable media for the isolation of algal strains (Prabakaran and Ravindran, 2013). As advocated by the review literature only a unicellular, endemic and fastidiously growing microalgal strain was selected for further studies (Radakovits *et al*., 2010; Sharma *et al*., 2012).

2. *Cultural Characterization of Isolated Microalgae*

Once pure culture of microalga was obtained, culture characteristics such as optimum growth conditions (Optimum temperature, light intensity, pH), growth curve (under optimum condition), pattern of Chlorophyll depletion were determined over different growth media. The isolated algal specie was grown in both broth and solid cultures above BG-11, BB media, CHU media and Spirulina based media. Comparative growth analysis was performed by incubating selected algal strain at varying temperature range (25^{o}C-28^{o}C) under 24 hrs light illumination of (1900 Lux and 2700 Lux) for 20 days under conditions of neutral pH. The purity of the culture was investigated by repeated microscopic examination under Mitzer bright field microscope at 100 X magnification; Gram's staining at regular intervals.

3. Growth Kinetics of the selected Algal species

The selected microalga isolate was cultured under optimized growth condition within broth cultures of 150 ml each in three different 250 ml emerson conical flasks (in triplet). Once the purity of culture maintenance was established primary inocula was prepared by growing it for seven to eight days until 0.6 OD was recorded at 750 nm (Ahmed *et al*., 2012). Primary inoculum was used in scaling up reaction to obtain microalga biomass by cultivating it within the bioreactor system under optimized conditions (Fig. 1). Growth analysis was recorded through spectrophotometric method which involved sampling on every next day to determine UV absorption at 750 nm up to nineteen days over a Lab India, Spectrophotometer.

Figure. 1. Algal growth within the bioreactor under optimum cultural condition

4. Algae harvesting and lipid extraction

The selected algal specie was harvested by decantation first as it was benthic dweller, separated media was reused after addition of trace metal components. Further reduction of media components was carried out through slow and steady percolation. Algal cells were harvested from the bioreactor as the culture reached its optimum OD on the 17th day of incubation. Following percolation, centrifugation was carried out at 5000 rpm for 5 minutes. 10 ml of the algal media was up taken in each centrifuge tube, after centrifugation supernatant was discarded, and the wet wt. of harvested algae determined. Harvested alga was dried within hot air oven at 60 ºC to determine its dry wt.

Solvent extraction system was used for lipid extraction. The solute was extracted through leaching which involved treating a finely powered algal biomass with a liquid to dissolve and remove the lipids contained within the algal cells. Solvent extraction method was employed using n-hexane as a solvent which distilled over and effectively separated algal oil from algal biomass (Ahmed *et al*., 2013; Dianursanti *et al*., 2015; Mubarak *et al*., 2015). After extraction n-hexane was separated and recovered from algal oil via distillation and condensation so as to make the extraction economical.

5. Molecular Identification of the selected Algal specie

The selected microalga was identified by the amplification of conserved 18S rRNA encoding sequence using ITS-1 and ITS-4 universal primers (Vorobyev *et al*., 2009). Primers used during PCR amplification reaction are depicted in Table 1 and their binding sites stated in Fig. 2.

Table 1. Primers used during PCR amplification of consensus 18S rRNA encoding sequence.

S. No.	Primers	Type	Primer Sequence
1.	ITS-1	Forward	5'-TCCGTAGGTGAACCTGCGG-3'
2.	ITS-4	Reverse	5'-TCCTCCGCCTTATTGATATCC-3'

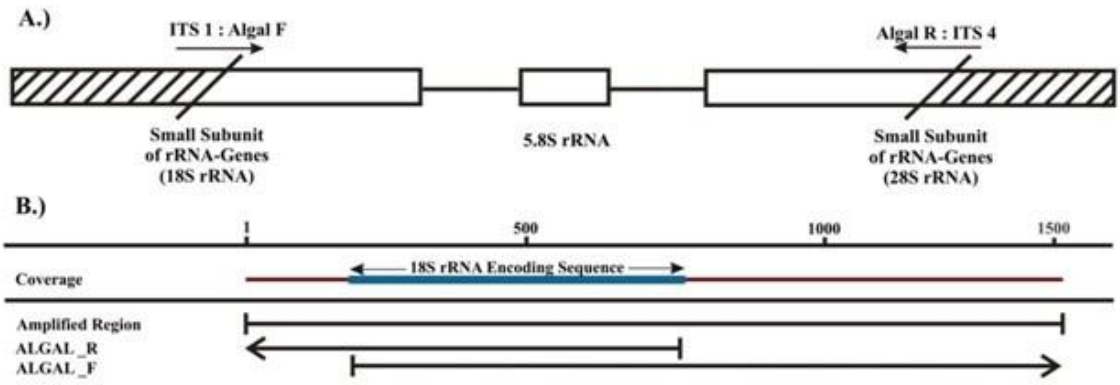

Figure 2. (A) Figure depicting the specific binding sites for universal primers (ITS-1 & ITS-4). (B) Amplified 18S rRNAencoding sequence using Algal_R&Algal_F primers.

Algal genomic DNA was isolated using Insta Gene TM Matrix Genomic DNA isolation kit (Cat. No. 732-6030). The isolated DNA was subjected to PCR amplification. A positive control in the form of genomic DNA and negative control in the form of PCR primer alone were included in parallel reactions. PCR conditions included, initial denaturation of 2 min at 94 °C for 60 sec, annealing step at 55 °C, 60 °C and extension step at 72 °C.

Removal of unincorporated nucleotides and primers from the PCR product was achieved using Montage PCR clean-up kit (Millipore), and analysed through 0.8% Agarose gel electrophoresis. The PCR products were subsequently sequenced by Yaazh Genomics using ABI PRISM Big Dye TM Terminator Cycle Sequencing Kits with Ampli Taq DNA Polymerase (Applied Biosystems). A 621 bp long DNA sequence, an overlap region of the amplified PCR product obtained through PCR reaction was used to determine the phylogenetic position of the unknown algae through Phylogene software, using Muscle for multiple alignment, G Blocks for alignment curation, while Phylogenetic tree was constructed using Phy ML (Dereeper *et al*., 2008; Dereeper *et al*., 2010).

6. Biochemical Lipid Analysis through GC

Fatty acid composition was analysed using a Perkin Elmer 500 GC-MS equipped with flame ionizer detector using Elite-5 MS column (30mm X 0.25mm X 0.25mm film thickness) (Eltgroth *et al*, 2005; Pazos and Izquierdo, 2011). Nitrogen was used as the carrier gas at a flow rate of 1 ml/min. Conditions maintained in the GC was 250 °C for injector port, 280 °C for detector and 100 °C for capillary column. There were thirty four different standards used including both fatty acids and fatty acid methyl esters (FAME). The GC was performed at ARBRO Pharmaceuticals Ltd. (Analytical division), New Delhi, India. The composition of transesterified lipid was determined by comparing peak retention time with that of standard FAME.
The fatty acids were quantified based on the Equation:

$$\boldsymbol{Fattyacid}(\%) = \frac{\boldsymbol{Ai}}{\sum \boldsymbol{A}} \quad \text{..... [} \qquad \text{Eq.1]}$$

Where *Ai* is the area of the peak corresponding to the component *i*, while $\sum A$is the sum of the areas of all the peaks.

As the formation of triglyceride molecule was accomplished by the combination of three fatty acids and a molecule of glycerol along with the liberation of three water molecules, so the average molecular weight of the microalgae oil was determined using the formula:

$$\boldsymbol{Av.MolWt.ofTriglyceride} = (Av.molwt.ofFatty\ Acid\ X\ Mol.wtofGlycerol) - 3(Mol.wtof H_2O)$$

Results:

1. Determining Cultural characteristics and Optimum growth conditions

Colony characteristic of the isolated alga strain on the petri plate were found to be punctiform, pulvinate elevation with entire margins. Purified algal cultivar was sub cultured over BG-11, BB Media, CHU, Spirulina based media which displayed optimum growth over BB-media in terms of no. of colonies appeared, intensity of growth. The

optimum light intensity for the culture was determined to be 2700 Lux at 28 °C under conditions of neutral pH keeping other conditions constant (Fig. 3). Thus BB-media was selected for all further experiments.

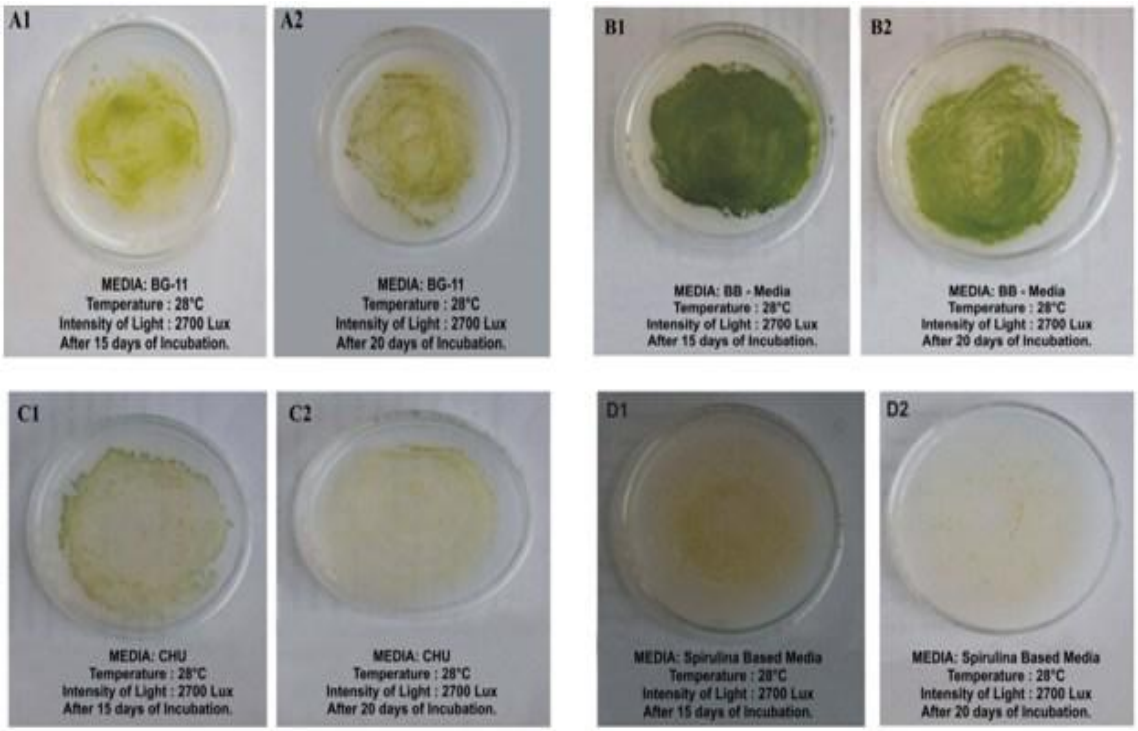

Figure 3. (A-D) Comparative analysis of growth pattern of *Chlorella* Spp. over BG-11 media (A1-A2), BB media (B1-B2), CHU based media (C1-C2) & *Spirulina* based media (D1-D2) under optimum conditions of temperature and neutral pH.

2. Morphological Examination and growth pattern of Chlorella sp. *strain*

Microscopic examination of the isolated Algal strain revealed it to be colonial, unicellular with an average cell size of 2.965 µm. The size of a daughter cell was recorded as 1.329 µm while that of a mature cell being 4.58 µm (Fig. 4. B). Average cell size, colony characteristics and morphological characteristics made it resemble to *Chlorella* Sp.

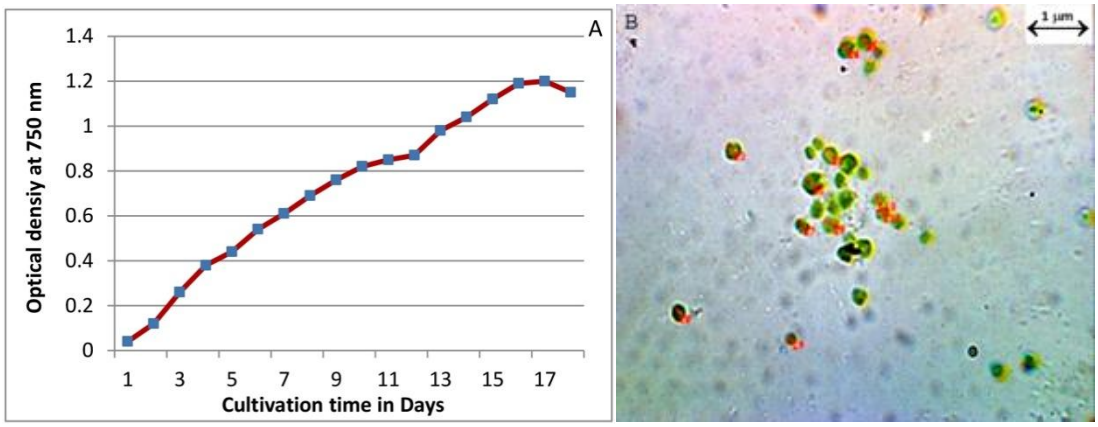

Figure 4. (A) Growth curve analysis of the isolated *Chlorella* Spp. at 750 nm under optimum cultural conditions. (B).Microscopic examination of isolated species depicting higher cell count, lower dry biomass and cell size.

The growth pattern of *Chlorella* sp was determined by measuring UV absorption at 750 nm. It revealed that the growth of *Chlorella* sp was found to be optimum on the seventeenth day of incubation, beyond which a decline in growth curve occurred due to chlorophyll depletion (Fig. 4. A). The pattern of chlorophyll depletion was determined by

incubating petri plate with *Chlorella* Sp cultures till 25 days, while sub culturing was simultaneously performed to determine the viability of the cultures. *Chlorella* sp. displayed a centrifugal pattern of Chlorophyll depletion i.e. the depletion of chlorophyll starts from the centre towards the periphery (Fig. 5. A). Further beyond 20th day of incubation the viability of the *Chlorella* strain greatly decreases and became completely dead upon the 25th day of incubation (Fig. 5. B).

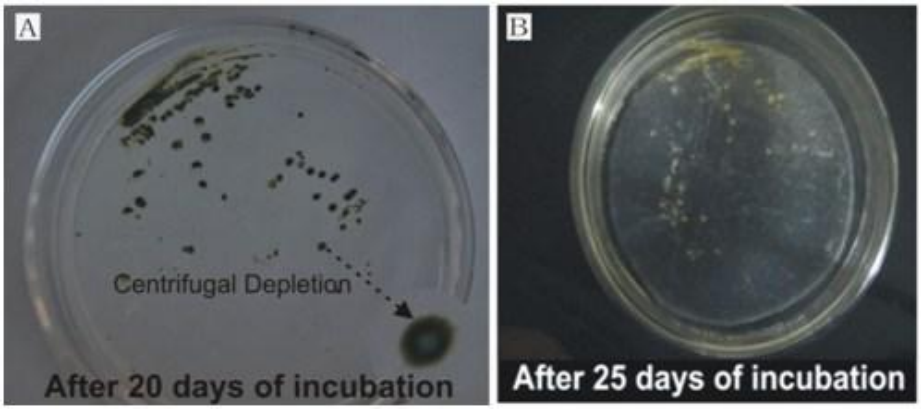

Figure 5. (A) Centrifugal pattern of chlorophyll depletion in isolated *Chlorella* spp. (B) Loss in viability of *Chlorella* after 25 days of incubation.

3. Biomass and Oil productivity of Chlorella Sp.

The algal cells were harvested; wet and dry weights were determined to be 21.5 mg/ml and 14.75 mg/ml respectively. Algal oil was extracted via n-hexane mediated soxhlet extraction method (Fig. 6) and the percent oil productivity of dried algal biomass by wt. was found to be 22.2% (approx) (Table 2.) After algal oil extraction n-hexane was recovered back to a maximum of 41.8% through distillation at 68 ºC.

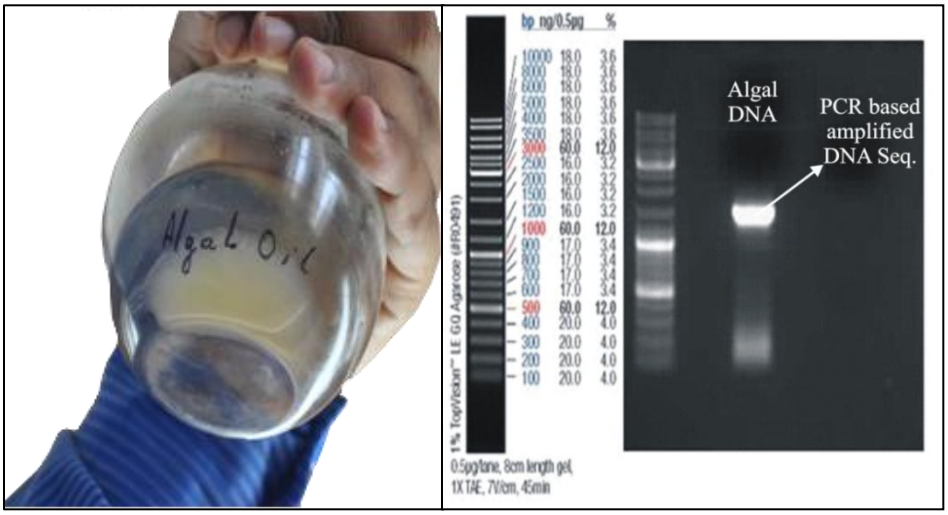

Figure 6. (A) Isolated Algal oil through soxhlet extraction using n-hexane as a solvent. **(B)** Electrophoresis bands of the amplified 18S rRNA encoding genomic DNA seq. of the *Chlorella* Spp.

Table 2. Algal Oil productivity of *Chlorella* spp. per mg of dried biomass.

S. No.	Wet Wt. of harvested alga (mg/ml)	Dry Wt. of harvested algal (mg/ml)	Algal Oil productivity (wt/wt of dry biomass)	Oil Productivity percentage by wt. of dried biomass
1.	28 mg/ml	20 mg/ml	4.473	22.3 %
2.	15 mg/ml	10 mg/ml	2.04	20.4 %
3.	20 mg/ml	13 mg/ml	3.3	25.4 %
4.	23 mg/ml	16 mg/ml	3.296	20.6 %
Average Yield	**21.5 mg/ml** S.D.: + 6.5 /- 1.5	**14.75 mg/ml** S.D.: + 5.25/ - 4.75	**3.27**	**22.175%**
Average Oil Productivity per mg of the dried algal Culture				**22.2 %**

4. Molecular Identification of the Algal strain

The isolated algal strain was genetically characterized using 18S rRNA encoding conserved DNA sequences of the nuclear genome. PCR products obtained were subsequently sequences to obtain two different DNA sequences, a 1359 bp sequence from *Algal_R_ITS1* primer and 874 bp sequences from *Algal_L_ITS4* primer. Comparative analysis of both the sequences revealed an overlapping region of 621 bp corresponding to highly conserved region within the algal genome.

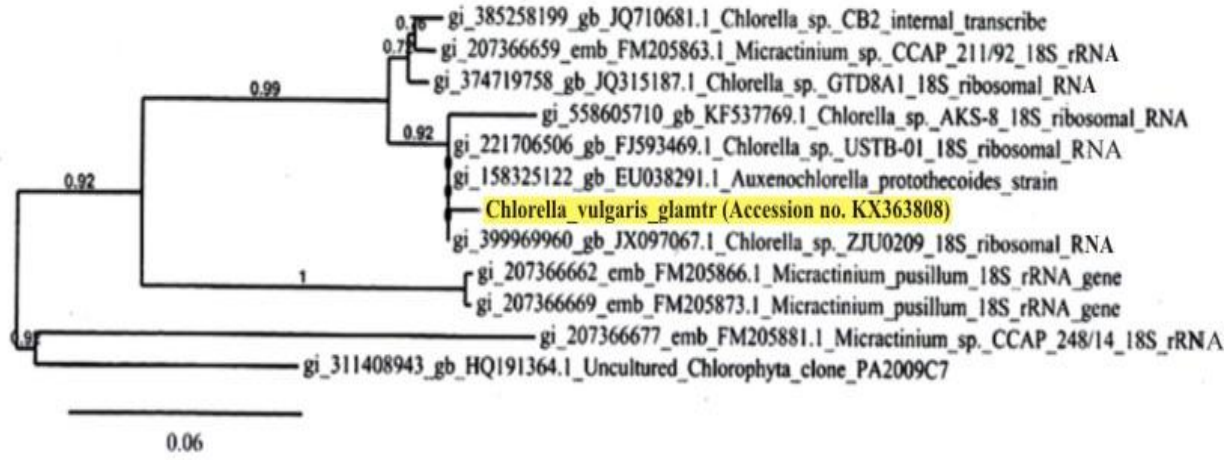

Figure 7. Phylogenetic analysis of the isolated *Chlorella* spp. depicting close resemblance to *Chlorella* sp. USTB-01; *Auxenochlorella protothecoides* and *Chlorella* sp. ZJU0209.

Phylogenetic analysis of the conserved 621 bp sequence using Phylogene software revealed maximum identity to *Chlorella sp. USTB-01* and isolated alga was named as *Chlorella vulgaris* glamtr strain (Fig. 7). 18S rRNA encoding genomic sequence was submitted to NCBI and assigned an accession no. KX363808.

5. Fatty Acid Profile of Algal Oil

Fatty acid profile of microalgal oil after transesterification was obtained through GC analysis (Fig. 8). Table 3 depicts the result obtained which reports that the most abundant FAME was Arachidonic methyl esters such as Methyl Arachidonate (10.57%), Heptadecanoic acid (9.73%) a well established biodiesel component (Table 3). The average molecular wt. of FAME was found to be 154.91 Da. Using the formula (Eq. 1) as advocated by Prommuak *et al*. (2012) the average molecular weight of microalgal oil was estimated to be 583.62 g/mole (Fig. 8).

Table 3. Major components of the Fatty Acid Methyl Esters (FAME-analysis) within Gas Chromatogram.

Fatty acid	Retention time	Molecular wt. (g/mol)[a]	Distribution in Sample (%)[b]	$(axb)/100$
Methyl Butyrate	3.99	102.13	2.5	2.55
Pentadecanoic Acid	23.93-24.36	242.39	7.19	17.42
Heptadecanoic Acid	28.05-28.23	270.45	9.73	26.3
Cis-9-Oleic Acid Met	29.77	296.49	2.06	6.1
Methyl Arachidate	31.88	326.5	3.15	10.28
Gamma linolinic acid	32.12	278.4	2.14	5.95
Methyl heneicosanoat	33.83	340.5	4.12	14.02
Methyl Erucate	35.57	352.59	3.88	13.68
Methyl tricosanoate	36.74	368.64	3.05	11.24
Cis-13, 16 docosadien	37.36	336.3	4.47	15.03
Methyl Arachidonate	39.07	318.5	10.57	33.66
Methyl Nervonate	39.31	380.6	3.35	12.75
Average molecular weight of fatty acids (FA)				**154.91**

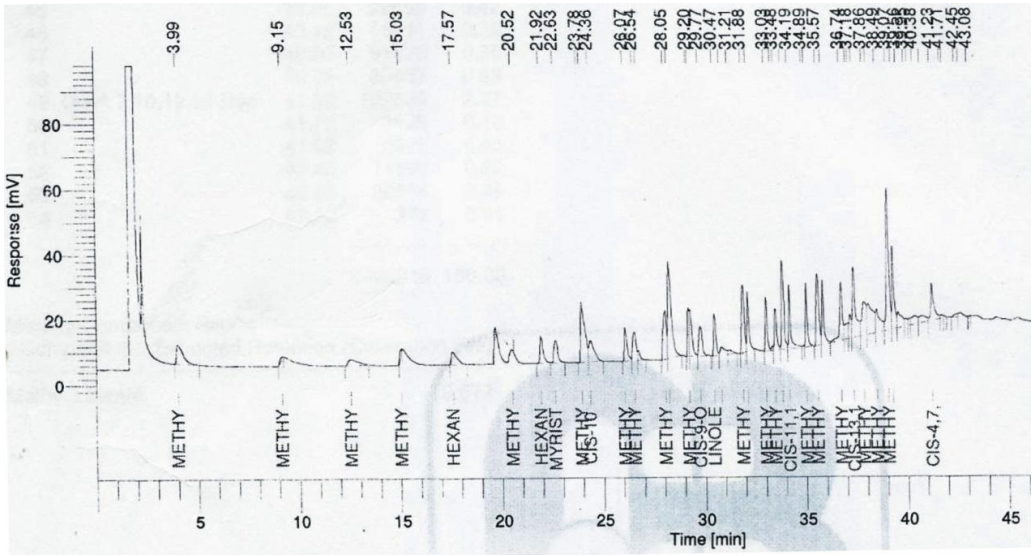

Figure 8. Gas Chromatogram of the transesterified algal oil isolated from *Chlorella* Spp.

Discussion:

The successful implementation of biofuel program greatly depends upon the bio energy feedstock being used hence an attempt was made to isolate endemic microalgae with high oil content and to determine its phylogenetic position. Algal growth was found to be directly proportional to the light intensity provided up to a certain limit (2900 Lux) beyond which the negative effect of solarisation predominated generating harmful free radicals and inhibiting growth. Isolated alga displayed higher cell count and lower dry cell mass indicating the ability of the species to reach high cellular density for biomass productivity. Results highlighted the importance of growth conditions such as light intensity, growth media composition which can drastically reduce generation time thus facilitating biomass productivity.

n-hexane solvent extraction system was employed for oil extraction to abridge the procedure towards industrial application as conventional Bligh & Dyer method being complex and time consuming (Bligh and Dyer, 1959). GC analysis of the oil predicts the presence of diversified categories of lipids particularly long chain polyunsaturated fatty acid (PUFA) in abundance. PUFA includes the derivative of arachidonic and linolenic acid, a clearly important biodiesel precursor indicating that algal oil could be used as a biodiesel source (Prabakaran and Ravindran, 2013; Pazos and Izquierdo, 2011; Ahmed *et al*., 2012).

Presence of essential fatty acids such as Arachidonic acid, Linolenic acid and their derivatives highlights the potential of microalgae to act as an important source of dietary lipids in nutritional and pharmaceutical industry (Shahidi and Wanasundara, 1998; Horrocks and Yeo, 1999). The presence of Eicosapentaenic acid (EPA, 20:5 ω3), docasahexaenoic acid (DHA, 22:6 ω3) and arachidonic acid (AA, 20:4 ω6) derivatives indicates importance of algal oil in treatment of arthrosclerosis, cancer, rheumatoid arthritis, Alzheimer's, etc (Simopoulos, 1999; Walker *et al*., 2005; Amtul *et al*., 2011). The most prominent biochemical reactions involved in Algal metabolism is photosynthesis, converting atmospheric CO_2 into fixed carbon sources (sugars) which are utilized via Kreb Cycle & electron transport chain (ETC) to biosynthesize ATP molecules. ATP triggers protein biosynthesis leading to cell division and growth. The increase in lipid productivity with regard to nutrient deprivation indicates a switch over in cellular metabolism diverting biochemical flux of Acetyl CoA from cellular respiration (Kreb Cycle, ETC) in direction of fatty acid biosynthesis. Fatty acid biosynthesis restores photosynthetic machinery by regenerating $NADP^+$ and AMP which would work as a substrate for light reaction to form $NADPH+H^+$; ATP, ultimately powering dark reactions of photosynthetic pathway (Hu *et al*., 2008; Radakoviys *et al*., 2010; Sharma *et al*., 2012). Thus large scale algal cultivation can not only prevent Earth warming but also provide a scope for sustainable development, meeting the ever increasing demand of energy. The works opens new challenges for future researchers to determine the interrelationship between triacylglycerol production and cell cycle on molecular level, genes encoding enzymes for lipid productivity and their regulatory sequences.

Acknowledgement:

We are thankful Prof. DS Chauhan, our beloved Vice-Vhancellor for his tremendous support in initiating algal bio-fuel research work at GLA University, Mathura. We would like to pay our regards to Arbor Pharmaceutical Ltd., New Delhi to carry out the GC analysis. We are highly grateful to the University Grants Commission (Grant # No. F.2-13/2011 SA-I), Government of India, New Delhi for providing financial support to carry out the above work.

References:

Ahmed, A.L., Yasin, N.H.M., Derek, C.J.C. and Lim, J.K. 2011. Microalgae as a suitable energy source for biodiesel production: A review. *Renewable and Sus. Energy Rev*. **15**: 584-593.

Ahmed, F., Khan, A.U. and Yasar, A. 2013. Transesterification of oil extracted from different species of algae for biodiesel production. *Afr. J. of Env. Sci. and tech*. **7**: 358-364.

Ahmed, I., Fatima, Z., Yazdani, S.S. and Kumar, S. 2012. DNA barcode and lipid analysis of new marine algae potential for biofuel. *Algal Res.* **2**: 10-15.

Amtul, Z., Uhrig, M., Rozmahel, R.F. and Beyreuther, K. 2011. Structural insight into the differential effects of Omega-3 and Omega-6 fatty acids on the production of A*β* peptides and amyloid plaques. *J. Biol. Chem.* **286**: 6100-6107.

Althor, G., Watson, J.E.M. and Fuller, R.A. 2016. Global mismatch between greenhouse gas emissions and the burden of climate change. *Sci. Rep.* **6**: 1-6.

Banerjee, A., Sharma, R., Chisti, Y. and Banerjee, U.C. 2002. *Botryococcus braunii*: A renewable source of hydrocarbons and other chemicals. *Crit. Rev. Biotechnol.* **22**: 245-279.

Bligh, E.G. and Dyer, W.J. 1959. A rapid method for total lipid extraction and purification. *Canad. J. Biochem. & Physio.* **37**: 911-917.

Chen, W., Zhang, C., Song, L., Sommerfeld, M. and Hu, Q. 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *J. Microbiol. Methods.* **77**: 41-47.

Chisti, Y. 2007. Biodiesel from Microalgae, *Biotech. Adv.* **25**: 294-306.

Dereeper, A., Audic, S., Claveroie, J.M. and Blanc, G. 2010. BLAST-Explorer help you building datasets for phylogenetic analysis. *BMC Evol. Bio.* **10**: 1-6.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Letort, V., Lescot, M., Claverie, J.M., and Gascuel, O. 2008. Phylogene fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acid Res.* **36**: W465-W469.

Dianursanti, Religia, P and Wijanarko, A. 2015. Utilization of n-hexane as co-solvent to increase biodiesel yield on direct transesterification reaction from marine microalgae. *Procedia Env. Sci.* **23**: 412-420.

Eltgroth, M.L., Watwood, R.L. and Wolfe, G.V. 2005. Production and cellular localization of neutral long-chain lipids in the haptophyte algae *Isochrysis galbana* and *Emiliania huxleyi*. *J. Phychol.* **41**: 1000-1009.

Gupta, P.L., Lee, S.M. and Choi, H.J. 2015. A mini review: Photobioreactors for large scale algal cultivation. *World J. Microbio Biotech.* **31**: 1409-1417.

Hansen, J., Sato, M., Hearty, P., Ruedy, R., Kelley, M., Delmotte, V.M., Russell, G., Tselioudis, G., Cao, J., Rignot, E., Velicogna, I., Tormay, B., Donovan, B., Kandiano, E., Schuckmann, K.V., Kharecha, P., Legrande, A.N., Bauer, M and Lo, K.W. 2016. Ice melt, sea level rise and superstroms: evidence from paleoclimate data, climate modelling, and modern observations that 2 ºC global warming could be dangerous. *Atmos. Chem. Phys.* **16**: 3761-3812.

Hare, M.O., Plevin, R.J., Martin, J.I., Jones, A.D., Kendall, A., and Hopson, E. 2009. Proper accounting for time increases crop-based biofuels greenhouse gas deficit versus petroleum. *Env. Res. Letters.* **4**: 1-7.

Horrocks, L.A. and Yeo, Y.K. 1999. Health benefit of docosahexaenic acid (DHA). *Pharmcol. Res.* **40**: 211-225.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. and Darzins, A. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J.* **54**: 621-639.

Mubarak, M., Shaija, A. and Suchithra, T.V. 2015. A review on the extraction of lipids from microalgae for biodiesel production. *Algal res*. **7**: 117-123.

Patil, P.D., Gude, V.G. and Deng, S. 2009. Biodiesel production from *Jatropha Curcas*, Waste Cooking, and *Camelina sativa* oils. *Ind. Eng. Chem. res*. **48**: 10850-10856.

Patil, V., Tran, K.Q. and Giserod, H.R. 2008. Towards sustainable production of biofuels from microalgae. *Int. J. Mol. Sci.* **9**: 1188-1195.

Pazos, J.V.P. and Izquierdo, P.F. 2011. Synthesis of neutral lipids in *Chlorella* sp. under different light and carbonate conditions. *Ciencia, Tecno. & Futuro.* **4**: 47-57.

Prabakaran, P. and Ravindran, D. 2013. Selection of microalgae for accumulation of lipid production under different growth conditions. *Carib. J. Sci. Tech*. **1**: 131-137.

Prommuak, C., Pavasant, P., Quitain, A.T., Goto, M. and Shotiprul, A. 2012. Microalgal Lipid Extraction and Evaluation of Single-Step Biodiesel Production. *Engineer. J.* **16**: 157-166.

Radakovits, R., Jinkerson, R.E., Darzin, A. and Posewitz, M.C. 2010. Genetic Engineering of Algae for enhanced biofuel production. *Eukaryotic Cell* **9**: 486-501.

Schlagermann, P., Gottlicher, G., Dillschneider, R., Sastre, R.R. and Posten, C. 2012. Composition of Algal Oil and its potential as Biofuel. *J. of Combustion*. **285185**: 1-15.

Schulze, P.S.C., Barriera, L.A., Pereira, H.G.C., Perales, J.A. and Varela, J.C.S. 2014. Light emitting diode applied to microalgae production. *Trends Biotech.* **32**: 422-430.

Shahidi, F. and Wanasundara, U.N. 1998. Omega- 3 Fatty acid concentrates: Nutritional aspects and production technologies. *Trends Food Sci. Tech*. **9**: 230-240.

Sharma, K.K., Schuhmann, H. and Schenk, P.M. 2012. High lipid induction in microalgae for biodiesel production. *Energies*. **5**: 1532-1553.

Simopoulos, A.P. 1999. Essential fatty acids in health and Chronic disease. *Am. J. Clin. Nutr.* **70**: 560S-569S.

Singh, S., Kate, B.N. and Banerjee, U.C. 2005. Bioactive compounds from cyanobacteria and microalgae: An overview. *Crit. Rev. Biotech*. **25**: 73-95.

Verma, D., Raj, J., Pal, A. and Jain, M. 2016. A critical review on production of biodiesel from various feedstocks. *J. Sci. and Innov. Res.* **5**: 51-58.

Vorobyev, K., Andronov, E., Rautian, M., Skoblo, I., Migunova, A. and Kvitko, K. 2009. An atypical *Chlorella* symbiont from *Paramecium bursaria*. *Protistology* **6**: 39-44.

Walker, T.L., Purton, S., Becker, D.K. and Collet, C. 2005. Microalgae as bioreactor. Plant Cell Rep. *Microalgae as bioreactor*. **24**: 629-641.

Wang, B., Li, Y., Wu, N. and Lan, C.Q. 2008. CO_2 bio-mitigation using microalgae, *App. Microbiol. Biotech.* **79**: 707-718.