

Full Length Research Paper

Isolation of diatom *Navicula cryptocephala* and characterization of oil extracted for biodiesel production

Sanjay K. R.*, Nagendra Prasad M. N., Anupama S.#, Yashaswi B. R.# and Deepak B.#

Department of Biotechnology, Sri Jayachamarajendra College of Engineering, Mysore- 570006, India.

Accepted 17 August, 2012

Over the past decade diatoms are screened for high lipid content. Geologists claim that much of crude oil comes from diatoms. In this study diatom *Navicula cryptocephala*, isolated from fresh water source was grown on suitable media for extracting and characterizing the oil for biodiesel production. Three methods namely, shake flask, polythene bag and photobioreactor were adopted for culturing the diatom. The maximum yield of biomass was obtained in photobioreactor (5.95 g/ 100 ml of media). Compound microscope and electron microscopic studies were carried out to identify the diatom. Both soxhlet and sonication methods were used for extraction of oil from the diatom biomass. The yield of oil is higher in sonication method (0.364 g/g of dry biomass). Further characterization of oil using gas chromatography showed the presence of Palmitic acid, Oleic acid, Palmitolic acid and linoleic acid as major fatty acids. HPLC analysis of crude oil showed the presence of OLL (dilinoleoyloleoylglycerol) and OOL (linoleoyldioleoylglycerol) as major triacylglycerols (TAGs).

Key words: Diatom, biodiesel, triacylglycerols, photobioreactor.

INTRODUCTION

Biodiesel is a domestic, renewable fuel derived from natural oils and fats. Biodiesel offers economic, environmental, fuel quality and energy security benefits vs. petroleum-based diesel (Knothe et al., 1997). Natural oils can be converted to biodiesel by a relatively simple refining process called transesterification. In this process vegetable oils or animal fats or microalgal oils are esterified using alcohol (ethanol or methanol) in the presence of a catalyst (sodium or potassium hydroxide) to form fatty esters (ethyl or methyl ester) (Vasudevan and Briggs, 2008).

Over the past few decades, more than thousand species of algae including diatoms have been screened

in search of high lipid content. It was found that, on an average, polyunsaturated fatty acid constitutes approximately 25% of algal mass (Hu et al., 2006). This content may vary noticeably between species, and interestingly, the lipid content increases when cells are subjected to unfavorable culture conditions, such as photo-oxidative stress or nutrient starvation. This is due to the shift in lipid metabolism from membrane lipid synthesis to the storage of neutral lipids (Qiang et al., 2008).

Diatoms were shown under appropriate conditions to produce up to 60% of their cellular mass as triacylglycerols (TAGs) under certain growth conditions. These TAGs can be easily converted into biodiesel through a transesterification reaction (Sheehan et al., 1998). Diatoms produce oil intracellularly as reserve food material during the vegetative period of growth. Besides high lipid and fatty acid content, there is an abundance of eicosapentaenoic acid, a polyunsaturated fatty acid (PUFA) in diatoms (Lebeau and Robert, 2003).

*Corresponding author. E-mail: sanjayrajesh@gmail.com. Tel: +91 821-2548285.

#Equal contribution to the work.

Theoretical calculations based on the photosynthetic ability and growth potential of diatoms has shown that they can yield more than 30,000 liters of oil per hectare per annum. Diatoms can yield 100 to 200 times as much oil as soybean, 10 to 200 times more than oil seeds and 7 to 31 times more than oil palm, which is considered as the next best source of oil. Therefore, sustainable energy could be made from diatoms. This requires development of appropriate cultivation and extraction of oil, using advanced technologies that mimic the natural process while cutting down the time period involved in oil formation (Ramachandra et al., 2009). Despite many years of research on the relationship between diatoms and crude oil, we still know very little about the oil inside diatoms itself. To conduct a proper chemical analysis of the oil inside diatom oil droplets, a method for separating out oil droplets inside diatoms from the shell and cytoplasm must be developed. The mechanisms of crude oil formation by natural phenomena are now partially understood, and technology for crude oil synthesis is in the budding stage (Ramachandra et al., 2009). The manipulation of diatom lipid quantity using available biochemistry and quality could be very significant and help us in effectively using this renewable resource as energy. The present work explains, isolation, culturing and identification of diatom *Navicula cryptocephala* species from fresh water sources, and extraction and characterization of lipids from its biomass for biodiesel production.

MATERIALS AND METHODS

Culturing of diatoms

Water samples were collected for isolation of diatoms from seven different fresh water sources in and around Mysore, Southern India. The collected samples were transferred to Chu medium containing Calcium nitrate (40 mg/L), Dipotassium hydrogen phosphate (10 mg.L⁻¹), Magnesium sulphate heptahydrate (25 mg.L⁻¹), Sodium carbonate (20 mg.L⁻¹), Sodium silicate (25 mg.L⁻¹), Ferric chloride (8 mg.L⁻¹). These media, without vitamins, were autoclaved at 120°C for 20 min. 0.1 mg.L⁻¹ Thiamine, 0.5 µg.L⁻¹ Biotin and 0.5 µg.L⁻¹ Cyanocobalamin were added in the same proportions to both media. Vitamin solutions and the oligo-element solution were filtered (0.2 µm) and frozen before use.

Three methods of culturing were followed for diatoms such as shake flask, polythene bag and photobioreactor culturing. In shake flask method, 1000 ml of Chu medium was prepared and transferred to 500 ml conical flasks (100 ml for each flask). The flasks were sterilized in autoclave at 121°C, 15 lbs pressure for 15 minutes. 10 ml of collected samples were transferred aseptically to the conical flasks. The flasks were kept in incubator shaker provided with illuminator (LT IL, Scigenics Biotec, Intensity 6 Watts) at 20°C and 120 rpm for 10 days. After 10 days diatoms was identified using stereomicroscope and isolated from other algal species using capillary tube method (Blanco et al., 2008; Debenest et al., 2009). The isolated species of *Navicula* were inoculated in to fresh medium for further multiplication and used as inoculum for further studies.

In polythene bag culturing method autoclaveable polythene bags

(22 cm × 35 cm Breadth and Length respectively) were used. 200 ml of medium was transferred to each bags and sterilized at 121°C, 15 lbs pressure for 15 min. The bags were inoculated with the 20 ml of pure culture of *Navicula* aseptically and kept at 20°C below the light source (6 Watts). Aerators with the sterile filters were used for sufficient aeration for the growth of diatoms.

Vertical photobioreactor made up of glass chamber (80 cm × 40 cm × 50 cm) was designed for culturing of diatoms. The reactor was provided with a source of light of (12 W), aerator (with the sterile filters), 2.5 l/min, voltage AC 220/240- 50 Hz., Power-3 W), thermometer, inlet and outlets. 15 L of sterilized Chu medium was transferred to the reactor and 1.5 L of inoculum was added in aseptic condition. The temperature was maintained at 20°C, with sufficient aeration and light and incubated for 15 days.

The diatom biomass was harvested by centrifugation at 10,000 rpm for 10 min (Ronald et al., 2011). The biomass was transferred to pre-weighed clean Petri plate and dried at 50°C to determine the dry weight of the biomass and stored under refrigerated condition.

Identification of diatoms

Diatoms were identified in compound microscope according to the method of Karthick et al. (2010). The sample was mixed with concentrated HCl in 1:1 ratio and kept for 24 h at room temperature. After 24 h, two to three drops of hydrogen peroxide was added and samples were observed under microscope. For electron microscope studies dried samples were sprinkled over double-stick tape pasted on the copper stubs used for mounting specimens for scanning electron microscope, and coated with about 20 nm gold particles in a sputter coater (EMS-550). The sample were observed and photographed with a JEOL 100CX-II electron microscope fitted with a scanning attachment (ASID-4D) at 20 kV.

Extraction of lipids from diatom biomass

The dried biomass of diatom was ground to fine powder in a mortar pestle. The lipid was extracted from dried biomass by sonication (David and Stuart, 1998) and soxhlation methods (Ronald et al., 2011). Sonication was carried out in a probe sonicator (model). An aliquote of sample (2.5 g) was suspended in 5 ml of hexane and subjected to sonication for 5 min at 31 Amp. The diatom biomass (2.5 g) was subjected to soxhlation using hexane as solvent for 16 h.

Chemical and physical analysis of diatom oil

The weight of oil extracted per gram of biomass was measured to determine the lipid content. Results were expressed as the percentage of oil in the dry matter of diatom biomass. Further the oil was characterized for its density (ASTM D 4052-96), kinematic viscosity (ASTM D 445-06), acid value (AOAC Official Method Cd 3a- 63), saponification value (MPOB Official Test Method, 2004) and peroxide value (AOAC Official Method 965.33).

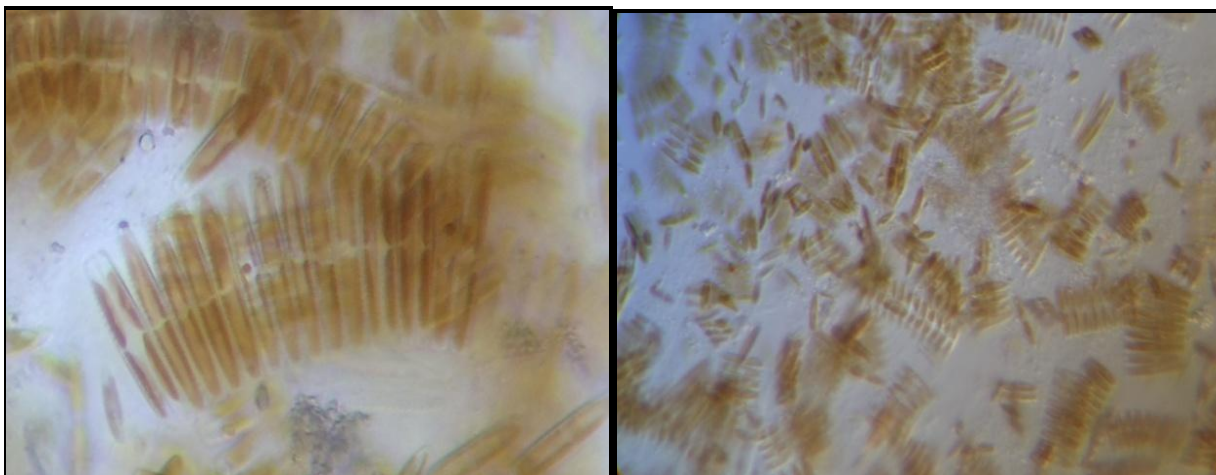
Determination of fatty acid composition

The free fatty acids were characterized using gas chromatography (Shimadzu 2010 plus, Japan) equipped with flame ionizing detector using RTX/1 bonded with Poly (Dimethyl siloxane) stain less steel capillary column (30 mm × 0.25 mm × 0.25 mm). Injector and detector temperatures were set as 220 and 230°C respectively, with

Table 1. Yield of diatom biomass and total lipid content.

Culturing techniques	Diatom biomass dry weight basis ^A	Crude lipid obtained by sonication method	Crude lipid obtained by soxhlation method
Shake flask	3.210	0.320	0.251
Polythene bag	5.124	0.350	0.342
Photobioreactor	5.945	0.364	0.362

^A (g/ 100 ml media), *(g/g of dry biomass).

**Figure 1.** Structure of *N. cryptocephala* under light microscope.

flow rate of 0.8 ml/min. The temperature programmed to 120°C for 5 min and 5°C increase/min up to 280°C.

Detection of TAGs composition

Thin layer chromatography was carried out to detect the TAGs using hexane: Ether: acetic acid (60:35:5) as a solvent system, along with the standard TAGs. Further determination of TAGs was carried out by HPLC (Shimadzu LC) equipped with ELSD 800 detector (altech). The TAGs was separated using commercial C-18 Column from waters (250 × 4.6 mm). The mobile phase was a mixture of acetonitril: dichlormethane (60:40) set at a flow rate of 1.0 ml/min, with pressure 2.3 bar. TAGs peaks were identified based on the retention time of available commercial TAGs (Sigma Aldrich, Germany).

RESULTS

Culturing of diatoms

For culturing of diatoms, initially, three media namely WC, Chu and Polytomella media were used. Out of the three, Chu medium was seen to facilitate the growth of diatoms very well. In the Chu medium, visible growth of diatom biomass was seen in 72 h after inoculation. It was observed that the growth of diatoms in three culturing

techniques (shake flak, polythene bag and photobioreactor culture) was dependent upon factors such as surface area, amount of inoculum added, temperature and degree of aeration. Table 1 shows the yield of diatom biomass by different culturing techniques.

Identification of diatoms

The samples collected from different water sources were subjected to microscopic studies (Figure 1). In all the samples collected the major diatom species identified was *Navicula cryptocephala* (Kingdom-Chromista, Phylum- Ochrophyta, Class -Bacillariophyceae, Family- *Naviculaceae*, Genus- *Navicula*). In this species the cells are either free or united into ribbon-like colonies. Frustules are generally linear in nature; lateral longitudinal ribs may be present in some forms. A characteristic feature of diatom cells is that they are encased within a unique cell wall made of silica (hydrated silicon dioxide) called frustules (Figure 2).

In pennates, 'sex' involves two adult cells lining up alongside each other, dividing and then swapping one daughter cell each. The new pairs of daughter cells fuse, giving two cells that are half of each 'parent'. They then

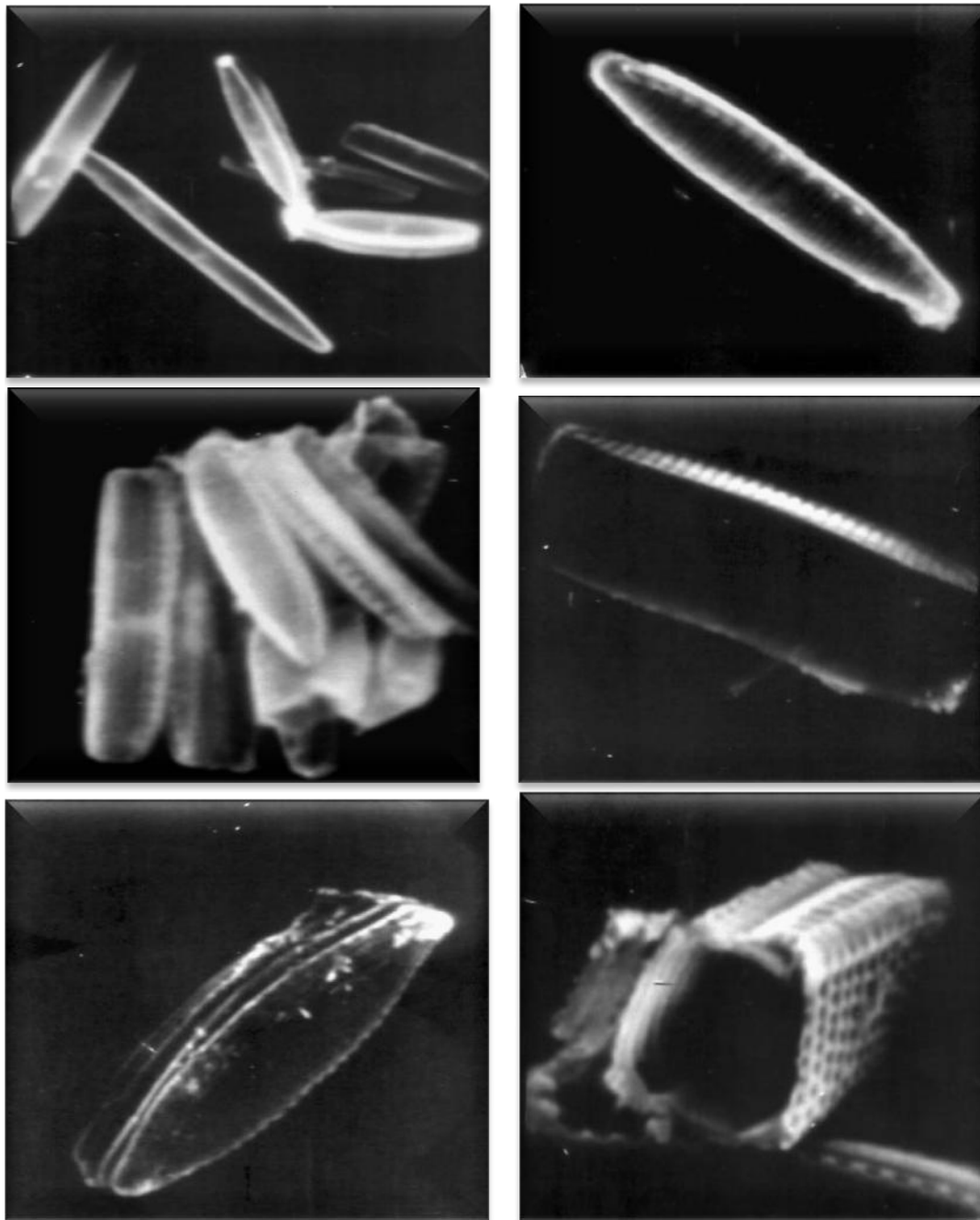


Figure 2. Scanning electron microscopic pictures of *N. cryptocephala*.

produce new, large frustules and glide away (Figure 2).

Lipids from diatoms biomass

The results of different culturing methods of diatoms showed that the yield of biomass is comparatively higher (5.954 g/100 ml of media used) in photobioreactor than

the other two methods. This may be due to effect of availability of surface area and aeration. The lipids from diatom biomass was extracted by sonication and soxhlation method, the amount of crude lipid obtained from sonication and soxhlation method with different culturing method was shown in Table 1. Sonication method was found to be more effective compare to soxhlation method for extraction of lipids from diatom

Table 2. Physical and chemical analysis of crude oil.

S/N	Analytical parameter	Value
1	Density (g/cm ³)	0.91
2	Viscosity (at 31°C), Centistokes	38.42
3	Iodine Value	89.35
4	Saponification value	168.44
5	Acid Value	35.64

Table 3. Fatty acids composition of crude oil.

S/N	Fatty acid	Retention time	Area	Concentration (%)
1	Myristic acid	18.48	63796	3.412
2	Palmitoleic acid	22.23	204084	10.916
3	Palmitic acid	22.73	635475	33.989
4	Linoleic acid	25.84	151128	8.083
5	Oleic acid	26.02	562564	30.089
6	Stearic acid	26.53	111163	5.946

biomass.

Characterization of crude oil obtained from diatom biomass

The results of physical and chemical analysis such as density, kinematic viscosity, acid value, saponification value and peroxide value of crude oil extracted from diatom biomass are shown in Table 2. Gas chromatogram results for free fatty acid detection showed that the major free fatty acids present in the oil extracted from diatom biomass were myristic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid and stearic acid (Table 3). Out of six fatty acids, palmitic and oleic acid were found as major fatty acids (Figure 3). Results of HPLC analysis for estimation of TAGs content in the oil extracted from diatom biomass showed that the presence of OOL (19.82%), followed by OLL (Oleoyl-dilinoleoylglycerol) (15.62%), POL (Palmitoyl-oleoyl-linoleoyl) (11.25%), SOO (Stearoyl-dioleoylglycerol) (2.48%). Monounsaturated that has been detected are MPP+OOO (12.25%), POO (palmitoyl-dioleoyl-glycerol) (8.72%), and PLP+MOP (1.05%) (Table 4).

DISCUSSION

The basic line of reasoning of geologists in attributing crude oil to diatoms is that they comprise the bulk of the ocean phytoplankton, so they must be a major source of the oil (Levorsen and Bery, 1967). The pattern of fatty

acids in recent sediments matches that of diatoms (Volkman and Joans, 1977) and the 24-norcholestane biomarkers are indicators of diatom- formed oil (Holba et al., 1998; Rampson et al., 2007).

In preliminary studies three media were used for culturing diatoms, however the Chu No. 10 medium was selected for its suitability. The comparison of modified freshwater "WC" medium (Guillard and Lorenzen, 1972) with Chu No. 10 medium indicated that freshwater "WC" medium was rapidly colonized by cyanobacteria and other algae (green algae) (Nichols, 1973). Such contamination may be related to the absence of inhibitor elements in the micronutrient solution, such as vanadium for cyanobacteria (Nalewajko et al., 1995). Although the diatom biomass growth rate was higher in freshwater "WC" medium, the contamination of the culture by other microorganisms made it unsuitable for further ecotoxicological experiments. These microorganisms produce EPS that can agglomerate diatoms and other algae and thus disturb diatom cellular observations under the microscope. In contrast, Chu No. 10 allowed sufficient production of diatom biomass (Debenest et al., 2009).

Diatoms have been regarded as useful neutral lipid sources of liquid-fuel precursors. Besides high lipid and fatty acid content, there is an abundance of eicosapentaenoic acid (polyunsaturated fatty acids in diatoms) (Renaud et al., 1994). *Nitzschia laevis* is a potential producer of eicosapentaenoic acid, as shown by extracting the lipid and analyzing it via thin layer chromatography (TLC) and gas chromatography (GC) (Chen et al., 2007). The lipids present are neutral lipids (accounting for, 75%), glycolipids, and phospholipids. Fatty acids that dominate the organisms include

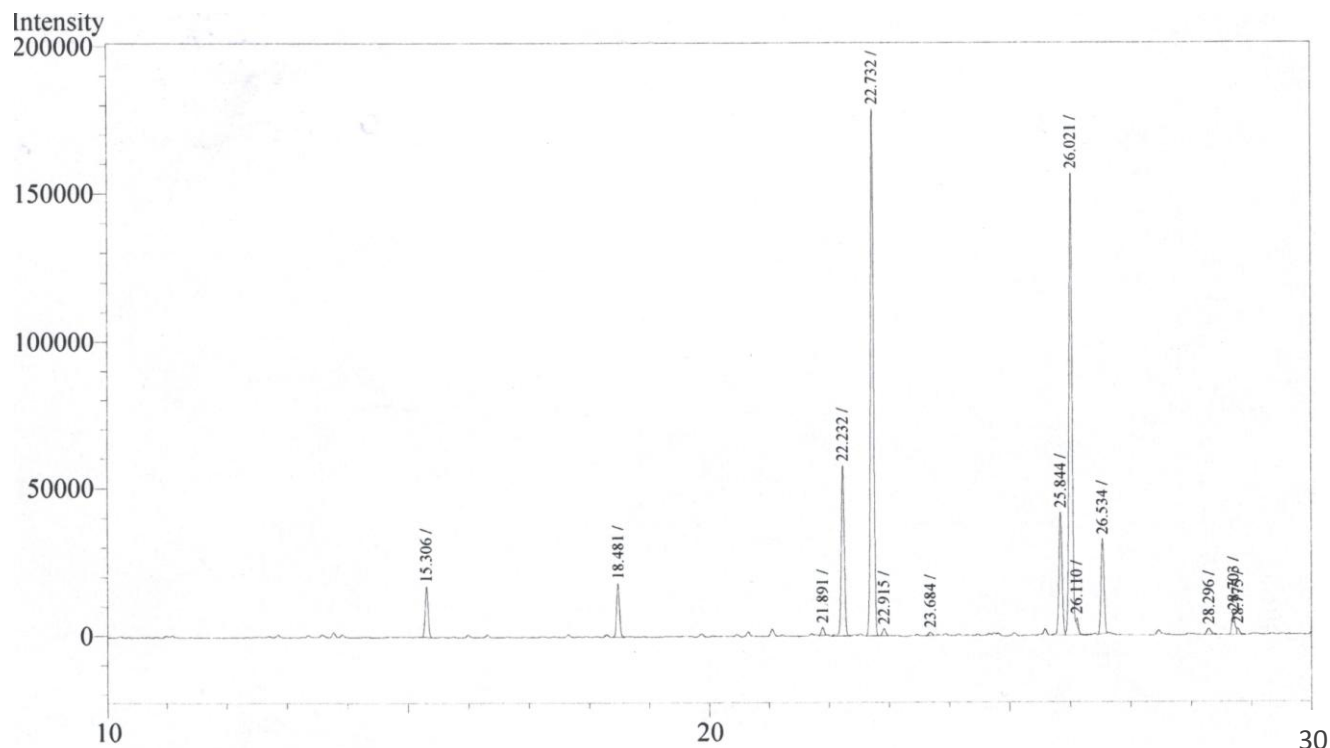


Figure 3. Gas chromatogram of diatom oil showing fatty acid composition.

Table 4. Triacylglycerol composition.

S/N	Triacylglycerol	Relative composition (%)
1	OOL	19.82
2	OLL	15.62
3	MPP+OOO	12.25
4	POL	11.25
5	POO	8.72
6	SOO	2.48
7	PLP+MOP	1.05

tetradecanoic acid, hexadecanoic acid and palmiloteleic acid. Many of the C25 (haslene) and C30 (rhizene) alkenes are biosynthesized by a restricted number of diatom genera, particularly some species of *Haslea*, *Rhizosolenia*, *Pleurosigma*, and *Navicula* (Grossi et al., 2004). In *Haslea ostrearia*, highly branched isoprenoid alkene (haslene) biosynthesis proceeds even under axenic conditions, indicating de novo biosynthesis (Wraige et al., 1999).

Lipid valorization as biodiesel using diatoms was reported with *Hantzschia* DI-6067 and *Chaetoceros muelleri* (McGinnis et al., 1997). The production of fuel (diesel, gasoline) through the transesterification and catalytic cracking of lipids accumulated in algal cells has

been reported, including diatoms (Nagle and Lemke, 1990). The main raw material for diatom-based biodiesel is the enormous range of triglycerides (monoglycerides, diglycerides, and triglycerides), which are indeed compounds of fatty acids and glycerol. In the transesterification process, an alcohol (such as methanol) reacts with the triglyceride oils that are contained in diatom fats, forming fatty acid alkyl esters (biodiesel) and glycerin (Lebeau and Robert, 2003).

The fatty acid composition of oil plays an important role in the performance of biodiesel in diesel engines. Saturation fatty acid methyl esters increase the cloud point, octane number, and improve stability whereas more polyunsaturation reduces the cloud point, cetane

number and stability (Kumar et al., 2003). The oil extracted from *N. cryptocephala* showed the presence of palmitic, oleic, palmitoleic and linoleic acids as main fatty acids detected which can be compared to *Jatropha* oil and rape seed oil used for biodiesel production. The free fatty acid content is one of the important factors for alkali-catalyzed transesterification. This is due to the free fatty acids reacting with the alkaline catalyst to produce soap, which inhibits the reaction and this result in the reduction of biodiesel yield (Meher et al., 2006).

Total lipid content on dry weight basis and total lipids made up of triglycerides were comparable with the other algal and plant sources used for biodiesel production. An additional algal characteristic for biodiesel production is the suitability of lipids for biodiesel in terms of the type and amount produced by an algal species, e.g. chain length, degree of saturation and proportion of total lipid made up by triglycerides. These influence the quality of biodiesel produced. The majority of lipid-producing algal species have a similar lipid profile, generally equivalent to vegetable oil from land plants suitable for biodiesel production (Xu et al., 2006). The proportion of various lipid classes (particularly triglycerides) varies widely with environmental conditions (Rodolfi et al., 2008), making it difficult to compare algal species across experimental conditions (Molina Grima et al., 1994). Lipid productivity can be calculated as the product of biomass productivity (grams dry weight per liter per day) and lipid content (%dw) to give an indicator of oil produced on a basis of both volume and time. High lipid content may, however, improve the efficiency of biomass processing (Rodolfi et al., 2008).

Conclusion

Diatoms would seem to fare, in average dry weight that they can synthesize as lipids, only a little better than green algae (24.5% vs. 17.1%), although their average dry weight is enhanced by a factor of 2 to 3 by nitrogen deprivation. However in spite of all these evidences there are only few reports on extraction and characterization of lipids from diatoms for biodiesel production. This study resulted in isolation of diatom *N. cryptocephala*, further the characterization of oil extracted showed presence of fatty acids and triglycerides content suitable for biodiesel production by transesterification. Further studies on mass culturing of diatom, transesterification process of oil extracted and characteristics of biodiesel produced are in progress.

ACKNOWLEDGEMENTS

The authors acknowledge Department of Science and Technology, Govt. of India for their gracious funding of the project, Principal SJCE, Mysore and Head,

Department of Biotechnology, SJ College of Engineering, Mysore, India for his constant support and guidance.

REFERENCES

- Blanco S, Alvarez I, Cejudo C (2008). A test on different aspect of diatoms processing techniques. *J. Appl. Phycol.* 20:445-450.
- Chen GQ, Jiang Y, Chen F (2007). Fatty acid and lipid class composition of the eicosapentaenoic acid-producing microalga *Nitzschia laevis*. *Food Chem.* 104(4):1580-1585.
- David S, Stuart H (1998). Extraction and Quantification of chlorophyll A from Freshwater Green Algae. *J. Sci. Technol. Charles Sturt Univ.* 32:2220-2223.
- Debenest T, Silvestre J, Coste M, Delmas F, Pinelli E (2009). A New Cell Primo-culture Method for Freshwater Benthic Diatoms communities. *J. Appl. Phycol.* 21:65-73.
- Grossi V, Beker B, Geenevasen JAJ, Schouten S, Raphael D, Fontaine MF, Sinninghe Damste JS (2004). C25 highly branched isoprenoid alkenes from the marine benthic diatom *Pleurosigma strigosum*. *Phytochemistry* 65(22):3049-3055.
- Guillard RRL, Lorenzen CJ (1972). Yellow-green algae with chlorophyllide. *J. Phycol.* 8:10-14.
- Holba AG, Tegelaar EW, Huizinga BJ, Moldowan JM, Singletary MS, McCaffrey MA, Dzou LIP (1998). 24-norcholestanes as age-sensitive molecular fossils. *Geology* 26(9):783-786.
- Hu Q, Zhang CW, Sommerfeld M (2006). Biodiesel from Algae: Lessons Learned Over the Past 60 Years and Future Perspectives. Juneau, Alaska: Annual Meeting of the Phycological Society of America, July 7-12:40-41 (Abstract).
- Karthick B, Taylor JC, Mahesh MK, Ramachandra TV (2010). Protocol for Collection, preservation and Enumeration of Diatoms from Aquatic Habitats for Water Quality Monitoring in India. *Icfa Univ. J. Soil Water Sci.* 3:25-60.
- Knothe G, Dunn RO, Bagby MO (1997). Biodiesel: The use of vegetable oils and their derivatives as alternative diesel fuels. *ACS Symp. Ser.* 666:172.
- Kumar MS, Ramesh A, Nagalingam B (2003). An experimental comparison of methods to use methanol and *Jatropha* oil in a compression ignition engine. *Biomass Bioenergy* 25:309-318.
- Lebeau T, Robert JM (2003). Diatom cultivation and biotechnologically relevant products. Part II: current and putative products. *Appl. Microbiol. Biotechnol.* 60(6):624-632.
- Levorsen AI, Berry (1967). *FAF Geology of Petroleum*. In: Freeman WH and Co, 2nd Edition.: San Francisco, CA, 1967.
- McGinnis KM, Dempster TA, Sommerfeld MR (1997). Characterization of the growth and lipid content of the diatom *Chaetoceros muelleri*. *J. Appl. Phycol.* 9(1):19-24.
- Meher LC, Sagar DV, Naik SN (2006). Optimization of alkali-catalyzed transesterification of *Pongamia pinnata* oil for production of biodiesel. *Bioresour. Technol.* 97:1392-1397.
- Molina Grima E, Robles Medina A, Gimenez Gimenez A, Sanchez Perez JA, Garcia Camacho F, Garcia Sanchez JL (1994). Comparison between extraction of lipids and fatty acids from micro algal biomass. *J. Am. Oil Chem. Soc.* 71:955-959.
- Nagle N, Lemke P (1990). Production of methyl ester fuel from microalgae. *Appl. Biochem. Biotechnol.* 25(1):355-361.
- Nalawajko C, Lee K, Jack TR (1995). Effects of vanadium on freshwater phytoplankton photosynthesis. *Water Air Soil Pollut.* 81(1-2):93-105.
- Nichols HW (1973). Growth media-freshwater. In: Stein JR (ed) *Handbook of phycological methods: culture methods and growth measurements*. Cambridge University Press, New York.
- Qiang H, Milton S, Eric J, Maria G, Matthew P, Michael S, Darzins A (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J.* 54:621-639.
- Ramachandra TV, Mahapatra DM, Karthick B (2009). Milking diatoms for sustainable energy: Biochemistry engineering versus gasoline-secreting diatoms solar panels. *Ind. Eng. Chem. Res.* 48(19):8769-8788.
- Rampsen SW, Schouten S, Abbas B, Panoto FE, Muiyzer G, Campbell

- CN, Fehling JS, Damste JS (2007). On the origin of 24-norcholestanes and their use as age-diagnostic biomarkers. *Geology* 35(5):419-422.
- Renaud SM, Parry DL, Thinh LV (1994). Microalgae for use in tropical aquaculture I: Gross chemical composition and fatty acid composition of twelve species of microalgae from the Northern Territory, Australia. *J. Appl. Phycol.* 6(3):337-345.
- Rodolfi L, Zittelli GC, Bassi N, Padovani G, Biondi N, Bonini G, Tredici MR (2008). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol. Bioeng.* 102(1):100-112.
- Ronald H, Brendan G, Michael K D, Paul AW (2011). Oil extraction from microalgae for biodiesel production. *Bioresour. Technol.* 102:178-185.
- Sheehan J, Dunahay T, Benemann J, Roessler PG (1998). US Department of Energy's Office of Fuels Development, July 1998. A Look Back at the US Department of Energy's Aquatic Species Program – Biodiesel from Algae, Close Out Report TP-580-24190. Golden, CO: National Renewable Energy Laboratory.
- Vasudevan PT, Briggs M (2008). Biodiesel production-current state of the art and challenges. *J. Indian Microbiol. Biotechnol.* 35:421-430. PMID: 18205018.
- Volkman JK, Johns RB (1977). The geochemical significance of positional isomers of unsaturated acids from an intertidal zone sediment. *Nature* 267:5613-693.
- Wraige EJ, Johns L, Belt S T, Masse G, Robert JM, Rowland S (1999). Highly branched C25 isoprenoids in axenic cultures of *Hasleaostrearia*. *Phytochemistry* 51(1):69-73.
- Xu H, Miao X, Wu Q (2006). Biodiesel production from heterotrophic microalgal oil. *Bioresour. Technol.* 97:841-846.