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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.

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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, polysaturated 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 polysaturated 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), Magnesium sulphate heptahydrate (25 mg/L), Sodium carbonate (20 mg/L), Sodium silicate (25 mg/L), Ferric chloride (8 mg/L). These media, without vitamins, were autoclaved at 120°C for 20 min. 0.1 mg/L Thiamine, 0.5 μg/L Biotin and 0.5 μg/L Cyanocobalamine 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, Intency 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 (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).

**Determinination 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.

<table>
<thead>
<tr>
<th>Culturing techniques</th>
<th>Diatom biomass dry weight basis&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Crude lipid obtained by sonication method</th>
<th>Crude lipid obtained by soxhlation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shake flask</td>
<td>3.210</td>
<td>0.320</td>
<td>0.251</td>
</tr>
<tr>
<td>Polythene bag</td>
<td>5.124</td>
<td>0.350</td>
<td>0.342</td>
</tr>
<tr>
<td>Photobioreactor</td>
<td>5.945</td>
<td>0.364</td>
<td>0.362</td>
</tr>
</tbody>
</table>

<sup>A</sup> (g/ 100 ml media), <sup>*</sup>(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
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.
Two tables are mentioned in the text:

**Table 2. Physical and chemical analysis of crude oil.**

<table>
<thead>
<tr>
<th>S/N</th>
<th>Analytical parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Density (g/cm³)</td>
<td>0.91</td>
</tr>
<tr>
<td>2</td>
<td>Viscosity (at 31°C), Centistokes</td>
<td>38.42</td>
</tr>
<tr>
<td>3</td>
<td>Iodine Value</td>
<td>89.35</td>
</tr>
<tr>
<td>4</td>
<td>Saponification value</td>
<td>168.44</td>
</tr>
<tr>
<td>5</td>
<td>Acid Value</td>
<td>35.64</td>
</tr>
</tbody>
</table>

**Table 3. Fatty acids composition of crude oil.**

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

biodiesel and can be a major source 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...
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

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

**Table 4.** Triacylglycerol composition.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Triacylglycerol</th>
<th>Relative composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OOL</td>
<td>19.82</td>
</tr>
<tr>
<td>2</td>
<td>OLL</td>
<td>15.62</td>
</tr>
<tr>
<td>3</td>
<td>MPP+OOO</td>
<td>12.25</td>
</tr>
<tr>
<td>4</td>
<td>POL</td>
<td>11.25</td>
</tr>
<tr>
<td>5</td>
<td>POO</td>
<td>8.72</td>
</tr>
<tr>
<td>6</td>
<td>SOO</td>
<td>2.48</td>
</tr>
<tr>
<td>7</td>
<td>PLP+MOP</td>
<td>1.05</td>
</tr>
</tbody>
</table>
number and stability (Kumar et al., 2003). The oil extracted from *N. cryptoecephala* showed the presence of palmitic, oleic, palmitoleic and linoleic acids as main fatty acids detected which can be compared to Jetropha 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. cryptoecephala*, 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.

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