J. Bangladesh Acad. Sci., Vol. 40, No. 2, 147-153, 2016

TRIACYLGLYCEROL PROFILE OF A MICROALGA *CHLOROCOCCUM* SP. AS A POTENTIAL BIOFUEL FEEDSTOCK

CHOWDHURY MD. MONIRUL HASAN, SHEIKH AFTABUDDIN[*1], MISKAT SHARIF AND MALA KHAN[2]

Department of Biochemistry & Molecular Biology, University of Chittagong, Chittagong-4331, Bangladesh

ABSTRACT

Chlorococcum sp. has been studied as its possibility for biofuel feedstock. This alga was grown in Zarrouk media. After harvesting, lipid extraction was done by pure n-hexane and mixture of chloroform: methanol (2 : 1)) and hexane: ethanol (3 : 1). Highest oil recovery (21.20%) derived from chloroform: methanol (2 : 1) solvent system from dry biomass whereas the lowest (2.83%) came from n-hexane: ethanol solvent system from wet biomass. GC/MS used for the analysis of extracted lipid revealed that, palmitic acid methyl ester ($C_{17}H_{34}O_2$), 9-octadecenoic acid methyl ester ($C_{19}H_{36}O_2$) were major contents of this biofuel. The acquired fatty acid profile indicates that *Chlorococcum* sp. could be used as promising biofuel feedstock in near future.

Key words: Potential biofuel, Microalgae, Feedstock, *Chlorococcum*

INTRODUCTION

Energy sector is most important for development of a nation because of increase in industrialization and population explosion and demand of this energy is increasing constantly. Fossil fuels (petroleum, coal and natural gas), hydro and nuclear are major sources of energy (Kulkarni and Dala 2006). Fuels provide around 70% of the total global energy requirements, particularly in transportation, manufacturing and domestic heating (Pandit *et al*. 2006). Electricity only accounts at present for 30% of global energy consumption (Pandit *et al*. 2006). The sources of fossil are limited and will be finished in near future (Dermibas 2005). Those fossil fuels have difficulties in their extraction, processing, leading to an increase of its cost. Moreover, those fossil fuels are responsible for global worming as their burning release CO_2 (Sharif *et al.* 2008). The need for renewable sources of portable, liquid fuel is receiving great attention, and much of this attention has been focused on biomass-derived liquid fuels or biofuels.

[*]Corresponding author: <aftabims@cu.ac.bd>.
[1]Institute of Marine Sciences and Fisheries, University of Chittagong, Chittagong-4331, Bangladesh.
[2]Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Qudrat-i-Khuda Road Dhanmondi, Dhaka-1205, Bangladesh.

Biofuels are sometimes referred to as first, second or third-generation biofuels. First-generation biofuels are made from sugar, starch, vegetable oil, or animal fats. Second-generation biofuels are produced from non-food crops, such as cellulosic biofuels and waste biomass (stalks of wheat and corn, and wood) using conventional technology. Third-generation biofuels are produced from extracting oil of algae-sometimes referred to as "oilgae". Biofuel is nontoxic and has low CO_2 emission profile as compared to petroleum diesel (Ramos *et al.* 2009) which can reduce emissions of total hydrocarbons, particulates and carbon monoxide up to 55, 53% and 48%, respectively (Haas *et al*. 2001).

Microalgae are a diverse group of single-celled organisms that have the potential to offer a variety of solutions for our liquid transportation fuel requirements through a number of avenues (Hannon *et al*. 2010). Algal species grow in a wide range of aquatic environments, from fresh water through saturated saline water. Algae efficiently use CO_2, and are responsible for more than 40% of the global carbon fixation, with the majority of this productivity coming from marine microalgae (Mata *et al*. 2010). They can produce biomass very rapidly, with some species doubling in as few as 6 hour, and many exhibiting two doublings per day (Huesemann *et al*. 2009). That algae are one of the best sources of biodiesel was reported by Shay (1993). These offer forty times higher oil production than terrestrial oil seed crops such as soy, canola under controlled conditions (Sheehan *et al.* 1998). Production of fuel from microalgae is renewable and environment friendly.

Chlorococcum sp. belonging to the family Chlorococcaceae, is unicellular green microalga and grows in both aquatic and terrestrial habitats. Cells are spherical or slightly oblong in shape and variable in size. This group of algae is non-motile, but some taxa produce motile cells (planospores) in the reproductive state can be cultivated under photoautotrophic and mixotrophic conditions. The present study reports the lipid extraction from the *Chlorococcum* sp. and quantitative analysis of fatty acid by Gas chromatography-mass spectrometry (GC-MS). This fatty acid profile is very important for the evaluation of algal biomass as biofuel feedstock.

MATERIAL AND METHODS

All chemicals used were analytical grade and obtained from Sigma-Aldrich and Merck, Germany. Pure strain of *Chlorococcum* sp. was collected from the Botany Department, Rajshahi University in Rajshahi, Bangladesh. This experiment was carried out in the Department of Biochemistry and Molecular Biology and Institute of Marine Sciences and Fisheries, University of Chittagong, Chittagong, Bangladesh.

The initial stock cultures of *Chlorococcum* sp. was maintained in Zarrouk media (Zarrouk 1966) and incubated for 10 days at 25°C under 14 : 10 light: dark (L : D) photoperiod in culture room. Ten ml of stock culture was transferred to three 100 ml conical flasks containing zarrouk media. The primary culture suspension was covered with aluminum foil to avoid any contamination. Within 7 - 10 days, the flask culture showed their maximum growth with dark green color. Then, culture was transferred to 1 liter of Erlenmeyer flask. For the mass culture, transparent 200 liter FRP container was used. For detailed procedures Astri *et al.* (2013) and Aftab and Zafar (2006) were followed. All the test tubes with media were autoclaved for sterilization. Inoculation process at the flask level carried out in Laminar flow cabinet.

Whatman filter paper was used to remove water from the biomass and wet algal biomass from the filter paper was collected with a spatula and transferred in Petri dishes. Then the biomass was dried at 80°C for 2 hours and kept in the desiccator for overnight in order to confirm that no liquid was present in the sample. Instantly, the dry mass was ground and weighed. In case of wet extraction process, the drying step was avoided.

Soxlet apparatus was used to investigate the effects of three different solvents, i.e. n-hexane, the mixture of chloroform and methanol at 2 : 1 v/v ratio and n-hexane and ethanol at 3 : 1 v/v ratio of dry biomass of *Chlorococcum* sp. For this, 5 g of dry weight and 10 g of wet fresh algae paste were taken into the extraction chamber separately. The chamber was enclosed both ends with cotton balls to withstand any solid algae discharge. One hundred and eighty ml of one of the three different solvents were added to the bulb and heated at 80°C. Progressively the solvent started to vaporize and after condensation, algae and lipids were stored into the extraction chamber. The solvent-lipid mixture then reached a critical stature within the chamber and the siphoning process was initialized i.e. the mixture was drawn back to the bulb. By constantly boiling and condensing, the system recalculates the solvent. A mixture of solvent and oil was left in the bulb and the algal biomass was left in the chamber at the end of this extraction process. Detail process was followed according to Kumer *et al.* (2011) and Sharmin *et al.* (2016).

For evaporation process, the solvent-oil mixture was placed into a pre-weighed flask, exposed to a vacuum, and then heated at 60°C by using a vacuum pump Rotary Evaporator (RE 200, Bibby Sterling, UK). Final weight of the flask was taken and compared with the first weight after evaporation. Finally, dichloromethane was used to recover oil and collected into a small vial. The percentage of oil extraction was determined by the following formula:

$$\% \text{ of total oil recovered} = \frac{\text{Weight of crude lipid extracted}}{\text{Weight of dry algae biomass}} \times 100$$

Fatty acid methyl ester (FAME) composition was determined using gas chromatography-mass spectrometry (GC-MS). The fatty oils were esterified with methanol prior to GC-MS analysis to make the fatty oils more volatile and to avoid the acidic attack to the stationary phase/column. 1 µl sample was injected in spilt less mode. The inlet temperature was set at 260°C and oven temperature was programmed as 70°C (0 min); 10, 150°C (5 min); 12, 200°C (15 min); 12, 220°C (5 min). Total run time was 38.833 minute and column flow rate 0.6 ml/min helium gas. The aux (GC to MS interface) temperature was set to 280°C. The MS was set in scan mode; the ionization mode was EI (electron ionization) type. The mass range was set in the range of 50 - 550 m/hz. Vieler *et al.* (2007), Jones *et al.* (2012) and Sharmin *et al.* (2016) were followed for necessary information.

RESULTS AND DISCUSSION

For the determination of extraction of lipid content of algae Vieler *et al.* (2007) used chloroform and methanol as a solvent, while Miao and Wu (2006) used hexane as a solvent in some others.

Result presented in Table 1 shows that, every solvent system gives maximum lipid recovery for dry sample and low lipid recovery for wet sample. For dry sample highest lipid recovery, (21.2 %) comes from chloroform and methanol solvent system and lowest (14.7%) from hexane and ethanol solvent system.

For wet sample, highest lipid recovery (5.53%) obtained from chloroform and methanol solvent system and lowest (2.8%) from hexane and ethanol solvent system. This is because chloroform and methanol is a mixture of polar (methanol) and non-polar (chloroform) solvents, thus both neutral and polar lipids could then be extracted. On the other hand, such non-polar solvent as hexane could preferably dissolve only non-polar lipids in the microalgae. The polarity of ethanol is higher than methanol. Ethanols react with water content of sample. For this reason hexane and ethanol solvent system have lowest lipid recovery.

Fatty acid profile is very important for the evaluation of extracted lipid as biofuel feedstock. The most common fatty acids of microalgae are palmitic-(hexadecanoic-C16:0), stearic-(octadecanoic-C18:0), oleic (octadecenoic-C18:1), linoleic-(octadeca-dienoic-C18:2) and linolenic-(octadecatrienoic-C18:3) acids. Most algae have only small amounts of eicosapentaenoic acid (EPA) (C20:5) and docosahexaenoic acid (DHA) (C22:6), however, in some species of particular genera these PUFAs can accumulate inappreciable quantities depending on cultivation conditions (Li *et al.* 2014).

The results obtained in this work (Table 2) show that FAME obtained from the lipids of *Chlorococcum* sp. mainly composed of saturated esters, among which palmitic

(hexadecanoic-C16:0) is the most significant with a relative percentage of 14.71. Other fatty acids namely octadecanoic acid (8.78%), tetradecanoic acid (1.5%) were significant for biofuel (Schenk *et al.* 2008). Additionally, a few fatty acid namely 9-octadecenoic acid, octadecanoic acid, methoxylate ester of methyl oleate 4, 5-dihydro-4-oxoindeno [1,2-b] indole-3-carbaldehyde-ester are also important for biodiesel production (Zhang *et al.* 1998, Knothe 2008, Gouveia and Oliveira 2009).

Table 1. Percentage of total oil recovered by the three different solvents.

Solvent	Physical condition	Sample wt. (g)	% of oil content
n-hexane	Dry	5.00 ± 0.00	19.133 ± 3.557
	Wet	10.00 ± 0.00	4.800 ± 1.300
$CHCl_3$: MeOH (2:1)	Dry	5.00 ± 0.00	21.200 ± 1.200
	Wet	10.00 ± 0.00	5.533 ± 1.350
n-hexane: EtOH (3:1)	Dry	5.00 ± 0.00	14.733 ± 2.013
	Wet	10.00 ± 0.00	2.833 ± 1.040

Table 2. Fatty acid methyl ester analysis of *Chlorococcum* sp.

S. No.	Retention time	Area%	Compound name
1	6.960	10.71	Benzaldehyde
2	10.638	6.46	1,2-Benzenedicarboxylic acid
3	11.887	1.60	Phenol, 2,4-bis(1,1-dimethylethyl)- (CAS)
4	12.025	1.56	Phenol, 2,6-bis(1,1-dimethylethyl))-4-methyl-ester
5	16.572	1.50	Tetradecanoic acid (myristic acid), methyl ester
6	19.736	14.71	Hexadecanoic acid (palmitic acid), methyl ester
7	22.161	2.73	Phthalic acid, methyl tridecyl ester
8	22.830	1.68	2,4-dimethyl-3-(3'-methylbuta-1',2'-dienyl)aniline
9	24.000	2.18	9-Octadecenoic acid (oleic acid), methyl ester
10	24.845	8.78	Octadecanoic acid, methyl ester
11	30.589	7.77	Methoxylate ester of methyl oleate 4,5-dihydro-4-oxoindeno[1,2-b]indole-3-carbaldehyde

CONCLUSION

The result provides an effective approach of lipid extraction from *Chlorococcum* sp. which may be potential source for biodiesel production. The targeted fatty acids in biodiesel for quality were C-16:0 and C-18:1, which were major contents of produced biodiesel. Further research should be carried out with microalgae and be compared with the ratio of biodiesel production after chemical analysis.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Dr. Sabrina Naz, Department of Botany, University of Rajshahi, and Dr. Md. Atiar Rahman, Department of Biochemistry and Molecular Biology, University of Chittagong for their kind support in intensification of the research for providing the microalgal strain.

REFERENCES

Aftab Uddin, S. and M. Zafar. 2006. Live feed production in different nutritive condition as diet for *Penaeus monodon* in shrimp hatchery, Bangladesh. *Int. J. Agri.Biol*. **8**(4): 493-495.

Astri, R., K. Edwan, D. I. Astuti and D. Kania. 2013. Growth response and chlorophyll content of *Scenedesmus oliquus* cultivated in different artificial media. *Asian J. Environ. Biol*. **1**(1): 1-9.

Dermibas, A. 2005. Biodiesel production from vegetable oils via catalytic and non-catalytic supercritical methanol transesterification methods. *Prog. Energy Combust. Sci*. **31**: 466-487.

Gouveia, L. and A. Oliveira. 2009. Microalgae as a raw material for biofuels production. *J. Ind. Microbiol. Biotechnol*. **36**(2): 269-74.

Haas, M. J., K. M. Scott, T. L. Alleman and R. L. McCormick. 2001. Engine performance of biodiesel fuel prepared from soybean soap stock: A high quality renewable fuel produced from waste feedstock. *Energy and Fuels* **15**: 1207-1212.

Hannon, M., J. Gimpel, M. Tran, B. Rasala and S. Mayfield. 2010. Biofuels from algae: Challenges and potential. *Biofuels* **1**(5): 763-784.

Huesemann, M. H., T. S. Hausmann, R. Bartha, M. Aksoy, J. C. Weissman and J. R. Benemann. 2009. Biomass productivities in wild type and pigment mutant of *Cyclotella* sp. (diatom), *Appl. Biochem. Biotechnol*. **157**(3): 507-526.

Jones, J., S. Manning, M. Montoya, K. Keller and M. Poenie. 2012. Extraction of algal lipids and their analysis by HPLC and mass spectrometry. *J. Amer. Oil Chemists Soc*. **89** (8): 1371-1381.

Knothe, G. 2008. "Designer" biodiesel: Optimizing fatty ester composition to improve fuel properties. *Energy Fuels* **22**: 1358-64.

Kulkarni, M. G. and A. K. Dala. 2006. Waste cooking oil - An economical source for biodiesel: A review. *Ind. Eng. Chem. Res*. **45**: 2901-2913.

Kumar, P., M. R. Suseela and K. Toppo. 2011. Physico-chemical characterization of algal oil: a potential biofuel. *Asian J. Exp. Biol. Sci*. **2**(3): 493-497.

Li, H.Y., Y. Lu, J. W. Zheng, W. D. Yang, J. S. Liu. 2014. Biochemical and genetic engineering of diatoms for polyunsaturated fatty acid biosynthesis. *Mar. Drugs.* **12**: 153-166.

Mata, T. M., A. A. Martins and N. S. Caetano. 2010. Microalgae for biodiesel production and other applications: A review. *Renew. Sustainable Energy Rev*. **14**: 217-232.

Miao, X. and Q. Wu. 2006. Biodiesel production from heterotrophic microalgal oil. *Bioresour. Technol*. **97**: 841-846.

Pandit, A., H.J.M. de Groot and A. Holzwarth. 2006. *Harnessing solar energy for the production of clean fuels* (European Science Foundation, Strasbourg, France, 2006)

Ramos, M. J., C. M. Fernandez, A. Casas, L. Rodriguez and A. Perez. 2009. Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresour. Technol*. **100**: 261-268.

Schenk, P. M., S. R. Thomas-Hall, E. Stephens, U. C. Marx, J. H. Mussgnug, C. Posten, O. Kruse and B. Hankamer. 2008. Second generation biofuels: High-efficiency microalgae for biodiesel production. *Bioenergy Res*. **1**: 20-43.

Sharmin, T., C. M. M. Hasan, S. Aftabuddin, M. A. Rahman and M. Khan. 2016. Growth, fatty acid, and lipid composition of marine microalgae *Skeletonema costatum* available in Bangladesh coast: Consideration as biodiesel feedstock. *J. Mar. Biol*. **2016**: 1-8.

Sharif Hossain, A.B.M., A. Salleh, A. N. Boyce, P. Chowdhury and M. Naqiuddin. 2008. Biodiesel fuel production from algae as renewable energy. *Am. J. Biochem. Biotechnol* **4**(3): 250-254.

Shay, E. G. 1993. Diesel fuel from vegetable oils: Status and opportunities. *Biomass Bioenergy* **4**: 227-242.

Sheehan, J., T. Dunahay, J. Benemann and P. Roessler. 1998. A look back at the U.S. Department of Energy's Aquatic Species Program-Biodiesel from Algae. National Renewable Energy Laboratory (NREL) Report NREL/TP.580-24190.Golden.CO.

Vieler, A., C. Wilhelm, R. Goss, R. Suß and J. Schiller. 2007. The lipid composition of the unicellular green alga *Chlamydomonas reinhardtii* and the diatom *Cyclotella meneghiniana* investigated by MALDITOF MS and TLC. *Chem. Phys. Lipids*. **150**:143-155.

Zarrouk, C. 1966. Contribution à l'étude d'une cyanophycée. Influence de divers facteurs physiques et chimiques sur la croissance et photosynthèse de *Spirulina maxima* Geitler. Ph.D. Thesis. University of Paris. France.

Zhang, X., C. Peterson, D. Reece, R. Haws and G. Möller. 1998. Biodegradability of biodiesel in the aquatic environment. *Amer. Soc. Agrl. Engineers* **41**(5): 1423-1430.

(*Received revised manuscript on 5 October, 2016*)