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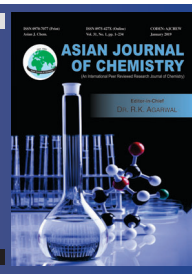

Ultrasonication Assisted Bligh and Dyer Method for Extraction of Lipids from Green Algae

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We have modified the original Bligh and Dyer method by introducing ultrasonication for enhancing the lipid extraction from green algae and compared with Soxhlet extractor. The Bligh and Dyer method coupled with ultrasonication increased the lipid extraction from green algae, *Oedogonium* sp. by up to 3.95 %, *Botyrococcus braunii* KUTZING NIES 2199 up to 8.5 %, *Cladophora* sp. up to 4.5 %. Therefore, ultrasonication assisted Bligh and Dyer method might be an important development for the removal of lipids from green algal biomass.

Keywords: Lipid extraction, Bligh and Dyer method, Green algae, *Botyrococcus braunii*, *Oedogonium* sp., *Cladophora* sp.

INTRODUCTION

The rapid increase in the demand for fuel energy has encouraged the scientific community worldwide to search for more reliable energy sources other than the conventional fuels. The biofuels hold a promising future to sustain the equilibrium between demand and supply of the energy. The competition of the first generation biofuels with the livestock feed gave rise to second generation of biofuels, wherein there was no need for compromising on the side of food resources [1-4]. The yield and efficiency of the second generation biofuels was required to increase to meet the demand. This gave rise to the third generation of biofuels, where both the problems were attempted to solve. The primary stock of the third generation is the microalgae. The growth of algae is not subjected to specificity of place or time of the year. This unique property is an added advantage for algae based fuels for continuous production throughout the years. Although algae provide us with drastically high efficiency of yields and no binding on time and place of growth [5-7], competitive commercialization of algae biofuels is yet to be realized. Fuels derived from algae cost much more than its conventional counterparts. The main hurdle in its commercial viability is biological challenges [8-10]. Harnessing energy from algae implies benefitting from the biological properties of algae. The research to overcome problems involves amplifying the algal properties, which are harnessed in the form of fuels. Biomass is considered as one of the best renewable source of energy which not only helps as an alternative source but also contributes to removal of carbon dioxide from atmosphere. Biodiesel produced from biomass is one such alternative fuel, which is obtained by the transesterification of triglycerides present in biomass. It is non-toxic and biodegradable and does not pose any health hazards. Lipid extraction using suitable and efficient solvents is the first primary step for increasing production of biodiesel from biomass. Among various biomass sources, microalgae usually have higher photosynthetic efficiency. Elumalai *et al*. [11] reported that algae is considered as a superior source for biodiesel production when compared with plant sources. All algae primarily comprise of carbohydrates, proteins, fats and nucleic acid. While the percentages differs based on the type of algae. Dried biomass of some type of algae comprises up to 40 % of fatty acids [12]. These fatty acids contain triglycerides which can thereby use for conversion into biodiesel.

Oedogonium is a filamentous green algae which can be identified by a distinctive rings at the apical ends of certain cells. It lives in quiet, fresh water and can be used to extract biodiesel. Zhang *et al*. [13] reported that *Oedogonium* sp. has about 45.38 % of lipids by dry weight. Other green algae's like

Botyrococcus brauni KUTZING NIES 2199 and *Cladophora* sp. has more than 30-40 % of lipids [14] and 40 % [15] of lipids by dry weight, respectively.

Extraction techniques like the Soxhlet method and Bligh and Dyer method are commonly used for lipid extractions from biomass. The Soxhlet method for lipid extraction is done using hexane, petroleum ether or ethyl acetate. The disadvantages of Soxhlet method include its long duration and wastage of large amount of solvents. Disposal of solvents is not only expensive but also causes additional threat to the environment.

The Bligh and Dyer method is considered as one of the best method for polar lipid extraction. It has wide applications in environmental engineering for analyzing lipid content of the samples [9-7]. The method mainly consists of extraction of fats using polar solvent mixtures (chloroform, methanol and water (1:2:0.8)). After extraction, the chloroform layer was removed and evaporated for collection of residual fats. Since the introduction of this method, many scientists have used it in various fields for extraction of polar ad neutral lipids [11,12]. Ranjan *et al*. [16] has compared Bligh and Dryer method, Soxhlet method and ultrasonicaation assisted Bligh and Dryer method for removal of lipids from Scenedesmus sp. Upto 8 g of lipids can be extracted from 100 g of dry biomass of *Scenedesmus* sp. [17]. *Botyrococcus brauni* can give 30 g of lipids per 100 g of dry biomass [18].

In present study, the original Bligh and Dyer method has been modified by introducing three changes including the change in the composition of solvents, increasing the mechanical homogenization time and introducing ultrasonication for lipid extraction for *Botyrococcus brauni*, *Oedogonium* sp. and *Cladophora* sp. The study also involves comparing the results with conventional Soxhlet method and original Bligh and Dyer method [10-12].

EXPERIMENTAL

Hill and Machlis media for algal growth (*viz.* $CuSO_4 \cdot 5H_2O$, $Na_2B_4O_7 \cdot 10H_2O$, $CoCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$ KNO_3, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, $N(CH_2COOH)_3$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4 and vitamin B12), chloroform and methanol (Merck, India). Distilled water was used throughout the experiments (Millipore Sigma India).

Maintenance of green algae cultures: Bulk biomass of *Oedogonium* Sp. and *Cladophora* Sp. were obtained directly from the pond (Latitude: 13º4′ 27.21″ N, Longitude: 77º34′ 47.7156″ E) present in University of Agricultural Sciences, GKVK Campus, Bengaluru, India. The bulk biomass of *Oedogonium* sp. and *Cladophora* sp. were dried and kept for storage at -4 ºC for further experimentation.

Botyrococcus braunii KUTZING NIES 2199 was procured from National Institute for Environmental Studies, Microbial Culture Collection, Tsukuba, Japan. *Botyrococcus braunii* KUTZING NIES 2199 was maintained in 250 mL Erlenmeyer flasks. Modified CHU 13 media (Table-1) medium (150 mL) was added to Erlenmeyer flasks and plugged with cotton and incubated at 25 ºC. Bulk biomass for lipid extraction was produced using autoclavable polypropylene bags. The light intensity was maintained at 1200 lux and pH at 7.5 for the modified CHU 13 media inside polypropylene bags. The concentration of *Botyrococcus braunii* KUTZING NIES 2199 in terms of number density was found using UV-visible spectrophotometer at 686 nm. *Botyrococcus braunii* KUTZING NIES 2199 was harvested after 28 to 30 days from the polypropylene bags once it reaches maximum absorbance.

Harvesting of biomass: Biomass was harvested by centrifugation and drying. The medium containing algal biomass was taken in polyethylene centrifuge tubes and centrifuged for 10 min at 6000 rpm (Remi Centrifuse, India). After centrifugation, the resultant supernatant was discarded. The biomass present at the bottom of centrifugal tubes was kept at 45 ºC for 4 to 5 days in a hot air oven. The dried biomass of microalgae was powdered using mortar and pestle before using it for lipid extraction [16].

Lipid extraction procedure: The dried biomass of three green algae (*Botyrococcus braunii* KUTZING NIES 2199, *Oedogonium* sp. and *Cladophora* sp.) was treated by three methods for extraction of lipids as described below:

Bligh and Dyer method: The Bligh and Dyer method was proposed in 1959 for extraction of lipids from fish muscle. Algae biomass of 2.5 g was homogenized in a blender for 2 min with 20 mL of chloroform and 40 mL of methanol. To this mixture again 20 mL of chloroform was added and continued blending for 30 s. To this mixture, 20 mL of distilled water was added and again blended for 30 s. The final solution was filtered using Whatman no. 1 filter paper. The solution was kept in a separating funnel for 20 min. The residual biomass along with filter paper was blended with 20 mL chloroform. The mixture is filtered and rinsed with 10 mL chloroform. Both chloroform solutions were added and evaporated for extraction of lipids. The final weight of lipid was measured.

Ultrasonication assisted Bligh and Dyer method: Algal biomass weighing 2.5 g on dry basis was taken for ultrasonication assisted Bligh and Dyer method. Methanol (40 mL) and chloroform (40 mL) was added to algal biomass and blended for 10 min. To this mixture, additional 20 mL chloroform and 20 mL water was added and homogenized for 10 min and ultrasonicated for another 10 min. The Erlenmeyer flask was covered with a cap to stop chloroform from evaporating and cooled in

TABLE-1
MODIFIED CHU 13 MEDIA (NIES, JAPAN)

Compounds	Grams	Compounds	Grams
Potassium nitrate (KNO_3)	240	Manganese(II) chloride tetrahydrate ($MnCl_2$ $4H_2O$)	2.172
Dipotassium phosphate (K_2HPO_4)	48	Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	0.264
Calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$)	64.2	Copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$)	0.096
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	120	0.072 N H_2SO_4 (sulfuric acid)	1 drop
Ferric citrate ($C_6H_5FeO_7$)	12	Cobalt(II) chloride ($CoCl_2$)	0.012
Citric acid ($C_6H_8O_7$)	60	Boric acid (H_3BO_3)	3.432

TABLE-2
EXTRACTION OF LIPIDS FROM THREE GREEN ALGAE USING VARIOUS METHODS

Method	Green algae (grams lipid per 2 g of algae biomass)		
	Botyrococcus braunii KUTZING NIES 2199	*Oedogonium sp.*	*Cladophora sp.*
Bligh and Dryer method	0.218	0.072	0.315
Ultrasonication Assisted Bligh and Dyer method	0.322	0.083	0.482
Soxhlet method	0.168	0.056	0.218

water bath with ice [19]. The final solution was filtered using Whatman no. 1 filter paper. The residues were treated similar to the original Bligh and Dyer method [20].

Soxhlet method: The soxhlet flasks were dried in oven at 100 ºC for 1 h and cooled for 30 min inside desiccators. The algal biomass weighing 2.5 g was taken in Soxhlet extraction tube. Approximately 100 mL of chloroform was added to Soxhlet flask and the sample was extracted for 24 h. The condensation rate was about 2 to 3 drops per second. The chloroform was distilled and the flask was dried in a hot air oven for 2 h at 105 ºC. The flask was again cooled and weighted for final lipid concentration.

RESULTS AND DISCUSSION

Extraction of lipids from green algae (*Botyrococcus braunii* KUTZING NIES 2199, *Oedogonium* sp. and *Cladophora* sp.): The three methods (Bligh and dryer method, ultrasonication assisted Bligh and Dryer method and Soxhlet method) were used for lipid extraction using three green algae (*Botyrococcus braunii* KUTZING NIES 2199, *Oedogonium* sp. and *Cladophora* sp.). It is quite evident that considerable improvement in the lipid extraction was observed in case of ultrasonication assisted mediated Bligh and dryer method when compared with original Bligh and Dryer method and Soxhlet method. It is also observed that more lipids can be easily extracted from *Botyrococcus braunii* KUTZING NIES 2199 and *Cladophora* sp. when compared with *Oedogonium* sp. Morphologically, *Oedogonium* sp. and the alga cells are hard to break when compared with *Botyrococcus Braunii* and *Cladophora* sp. About 47 % increase in the lipid content was observed for ultrasonication assisted Bligh and Dryer method in case of *Botyrococcus braunii* KUTZING NIES 2199 and 15 % increase was observed for *Oedogonium* sp. and 53 % increase was observed for *Cladophora* sp. when compared with original Bligh and Dryer method. Soxhlet method produced poor results (Table-2). Thus, ultrasonication assisted Bligh and Dryer method proved to be best when compared with original Bligh and Dryer method and Soxhlet method.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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