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Biofuel from Bryophyta as an Alternative Fuel for Future

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ABSTRACT

Today's growing demand for energy has emphasized the need for the search for renewable resources. This demand can be met with by using alternative resources such as biofuel, rather than just depending on non-renewable resources. Therefore, the present study has been undertaken to extract lipid from a species of Bryophyta, i.e. *Marchantia polymorpha*. 0.044 g of lipid was extracted from 8 g of the bryophyte. Bligh and Dyer method was used for the extraction of lipid. It is a multi-step process in which methanol, chloroform, and NaCl play an important role in the extraction of bioproduct in the form of oily bodies. This small step taken towards energy utilization and conservation will open the new vista to mitigate the problem of energy crisis.

INTRODUCTION

Most of the fuel resources that we use are confined. Since the Earth's population is increasing, the demand for fuel is also increasing exponentially. And as a result of the consumption of such carbon-based fuels, pollution is choking the earth atmosphere and is creating a worldwide catastrophe. Since petroleum-based fuels are limited in quantity and also cause global warming due to the accumulation of carbon dioxide, a great emphasis is put on the search for renewable resources.

Therefore, more research impetus is laid on an alternative source such as biofuels, to compensate this ever-increasing demand for petroleum products. Biofuels are renewable in nature, therefore, can be used frequently and easily substituted. There are three categories of biofuels. The first generation, the second generation, and the third generation (Dragone et al. 2010). First generation biofuels contain edible feedstocks, for example soya beans, wheat, corn, rapeseed, oil crops, maize, sugarcane and sugar beet. While, the second-generation biofuels are derived from wastes as well as from lignocellulosic feedstocks, for example, switch-grass and Jatropha. One of the major disadvantages of both the first and second generation is that the cultivation of the food or non-food crops as biofuel feedstock might compete for limited arable farmland, which should be utilized to cultivate crops as food feedstocks. Therefore, if biofuel is derived from food or non-food, we cannot consider them as sustainable. Biofuel production of food crops grown in farmland will affect prices, while the cultivation of non-food energy crops will result in competition with food crops for farmland. On the other hand, liverworts as third generation biofuel feedstock have some distinguishable features, such as high photosynthetic efficiency, rapid growth, high lipid content, high carbon dioxide, mitigation efficiency, non-competition with food crops for farmland and less water demand than croplands. Being photosynthetic organisms, liverworts are able to capture solar energy and use water and atmospheric carbon dioxide to accumulate biomass in forms of organic ingredients such as lipids (Alam et al. 2012).

Sustainable biofuel resources: The most suitable and alternative form of fuel, biofuel, is produced from algal biomass. Algae have an ability to produce oil which is around 100 times more per acre as compared to any other plant (Mubarak et al. 2015) (Table 1).

Many useful products can be produced from algae which can be helpful in reducing the cost of biofuels. On the other side, there are many industrial and medicinal applications of algae (Elegbede et al. 2016, Gendy et al. 2013, Alam et al. 2012). Microalgae produce many different kinds of lipids, complex oil, and different hydrocarbons, which are solely dependent on species (Table 2). Oil content ranges from 20-50 percent for many algal species. The condition of culturing helps in creating the variations in fatty acid as well as lipid content. By optimizing the growth determining factor, the lipid concentration may get improved up to 80 percent (Hu et al. 2008). The oil content of the biomass and the algal growth rate are the main key factors which determine

Table 1: Total oil yield from different sources (Gouveia et al. 2009).

Source	Oil Yield (L/ha)
Corn	172
Soybean	446
Canola	1190
Jatropha	1892
Coconut	2689
Palm	5950
Microalgae (70% oil by weight)	136900
Microalgae (30% oil by weight)	58700

Table 2: Different lipid content of various microalgae (Gouveia et al. 2009).

S.No.	Species	Lipids (% dry weight)
1.	*Scenedesmus obliquus*	11-22/35-55
2.	*Scenedesmus dimorphus*	6-7/16-40
3.	*Chlorella vulgaris*	14-40/56
4.	*Chlorella emersonii*	63
5.	*Chlorella protothecoides*	23/55
6.	*Chlorella sorokiana*	22
7.	*Chlorella minutissima*	57
8.	*Dunaliella bioculata*	8
9.	*Dunaliella salina*	14-20
10.	*Neochloris oleoabundans*	35-65
11.	*Spirulina maxima*	4-9

the oil productivity per day. In comparison to other crops, the amount of oil produced by algal biomass is remarkably higher, specifically 100000 L/ha. On the other hand, the oil content in soybean is 446 L/ha, in sunflower 952 L/ha, in rapeseed 1200 L/ha, in castor 1413 L/ha, in coconut 2689 L/ha, and in the palm around 5950 L/ha (Kleinová et al. 2012).

The upcoming future generation will become a regular user of biofuel solely produced from algae. Talking about renewable biodiesel, microalgae will be the only capable source of biofuel that will completely eradicate the shortage of transport fuel. With the help of thermochemical as well as biochemical methods, microalgae could be easily converted into biomethane, biohydrogen, biomethanol and bio-oil. The only true source of biodiesel is microalgae (Li et al. 2014).

For the production of biodiesel as well as high value fatty acids, microalgae play an important role as they have the capability to assemble lipids in the form of triglycerides.

Botrycoccus braunii, *Chlorella vulgaris*, and *Scenedesmus* sp. are the three microalgae containing high biomass as well as high lipid productivity that are cultivated in the presence of 10 percent carbon dioxide and flue gas. Under 10 percent carbon dioxide concentration, for *Scenedesmus* sp. the biomass productivity is 217.50 mg/L/d, and lipid productivity is 20.65 mg/L/d (9 percent of biomass). Similarly, for *Botryococcus braunii*, the biomass and lipid productivity was 26.55 mg/L/d and 5.51 mg/L/d (21 percent of biomass). For *Scenedesmus* sp. and *Botryococcus braunii*, the lipid productivity was increased up to 1.9 fold (39.44 mg/L/d) and 3.7 fold (20.65 mg/L/d) respectively when observed under the influence of flue gas (Yoo et al. 2010). Under optimum conditions, *Chlamydomonas* sp. KO-7267 and PK-7195, *Chlorella* sp. KS-7300 and *Desmodesmus* sp. BK-7291 are considered as propitious strains as they have the ability to form lipid in the range lying between 14.7- 45.7 percent dry weight (Santosa et al. 2010).

Bryophytes are the plants that are better evolved than halophytes, yet bryophytes lack true roots, stem and leaves. They may possess root-like, stem-like or leaf-like structures. Bryophytes are thallus like and prostate or erect and attach to the substratum by unicellular or multicellular rhizoids. Bryophytes live on land and they need water for sexual reproduction, this makes them amphibians of the plant kingdom. Bryophytes, consisting of liverworts, mosses, and hornworts represent the earliest diverging group of land plants.

The most widely described liverwort is *Marchantia*. More specifically, *Marchantia polymorpha* can be used for the production of biofuel which contains lipid in the form of oily bodies. *Marchantia polymorpha* is a common, easily cultivated, dioecious liverwort species, and is found in western Himalayas, Assam and other north eastern belts of India. More specifically, *Marchantia polymorpha* can be utilized for the production of biofuel which contains lipids in the form of oily bodies and is emerging as an experimental model organism. Because of its peculiar characteristics and concert, it can be widely used in for the study of molecular genetics, such as the introduction of reporter constructs overexpression, gene silencing and targeted gene modification are accessible (Ishizaki et al. 2015).

One of the most prominent contents of *Marchantia polymorpha* is the lipid. Lipids are biologically originated substances that are soluble in a non-polar solvent (Demirbas et al. 2008). It includes a group of naturally occurring substances such as fats, steroids and vitamins. There are carbohydrate, protein, and phosphate-containing lipids which are known as glycolipids, proteolipids and phospholipids respectively or also known as modified lipids. Lipids perform many functions like signalling, storing energy and are the main component of the cell membrane (Fahy et al. 2009, Subramaniam et al. 2011). Lipids have a wide range of applications in different sectors like food industries, cosmet-

ics, and nanotechnology (Mashaghi et al. 2013).

Significance of the extraction technique: Due to lack of a proper and effective method for the extraction of lipid, researchers have been trying different ways for the extraction which eventually create bias in reproducibility. The primitive Bligh and Dyer method and other chemical based lipid extraction such as saponification and sonication were used for the analysis of fatty acid as well as extraction from *Tetraselmis* sp. M8 (Li et al. 2014). To figure out the importance of solvent polarity, Soxhlet method was used for the oil extraction from algae. There was a difference observed in the yield of extract as well as in the composition of fatty acids. The most favourable method for the extraction of long-chain unsaturated fatty acids from microalgae was supercritical extraction technique (Li et al. 2014).

Other than the obligatory cell disruption technique for oil extraction (Li et al. 2014, Burja et al. 2007), polarity of the organic solvent, as well as the solvent mixture is also the primary requirement for the coherent extraction of lipids from microalgae. Generally, a substantial amount of lipid could be extracted by using a homogenate of polar and nonpolar solvent (Ryckebosch et al. 2012). In case of Bligh and Dyer method, chloroform as a nonpolar solvent and methanol as a polar solvent are mixed with water to form a homogenate which is helpful for extraction of lipids from a broad range of biological samples (Ryckebosch et al. 2012). More emphasis is laid on the use of dichloromethane which is the form of nontoxic solvent, which helps in reducing the issues related to the biosafety (Cequier-Saìnchez et al. 2008). Saponification is one of the alternative solvent methods for the revival of lipids from different microalgae (Burja et al. 2007). Other than saponification a more beneficial technology for the extraction of lipid has been pronounced as supercritical fluid technology. Basically, this technology is meant for pharmaceutical bioproducts. The advantage of supercritical technology over primitive solvent methods is that it is nontoxic, thermally stable, bioproducts are easily separable with high diffusion rate (Couto et al. 2010, Wang et al. 2007).

Whilst the Soxhlet method is extremely helpful in lipid extraction from different biological species, yet it is a highly time-taking process (Cravotto et al. 2008, Lam et al. 2012, Mercer et al. 2011). Moreover, it can thermally degrade LC-PUFAs (Li et al. 2014). Still, we can improve the efficiency of yield in Soxhlet method by using solvent mixtures.

Soxhlet method and liquid-liquid extraction methods are compared for determining the lipid content in food samples. The time taken by the Soxhlet method for extraction process of lipid was studied by using different solvent mixtures. There are certain limitations with Soxhlet method as only solid samples can be detected for the presence of lipid (Manirakiza et al. 2001). Lipid can be extracted from microalgae by the means of the mechanical and the chemical methods. The chemical methods include Soxhlet extraction, accelerated solvent extraction and supercritical fluid extraction. While mechanical methods are ultrasonic assisted extraction, oil expeller, and microwave-assisted extraction. Pre-treatment of mechanical as well as chemical methods could increase the yield of lipids from certain microalgae (Mubarak et al. 2015).

Bligh and Dyer extraction and Soxhlet extraction both are the chemical methods for extraction of lipid in which the former uses chloroform/methanol compounds while the later use acetone/hexane compounds. Lipid, as well as polychlorinated biphenyls (PCBs), are extracted using these two methods from fish, more accurately muscle tissue in 4 different species of fish; herring (*Clupea harengus*), salmon (*Salmo salar*), cod (*Gadus morhua*) and Northern pike (*Esox lucius*). In the Bligh and Dyer method, the lipid is extracted in a far better amount as compared to PCBs which is opposite to that of the Soxhlet method (Ewald et al. 1998).

Extraction and purification of lipid can be done by a simple and rapid method from biological materials. This method was developed when lipid decomposition was studied in a frozen fish. This method is really efficient as it takes less time and highly reproducible. Chloroform and methanol are used to amalgamate wet tissue to form a miscible system. Two layers are formed on diluting the homogenate mixture with chloroform and water. The lower chloroform layer contains all the lipid, and the upper methanol layer contains all the non-lipids. The chloroform layer is isolated for pure lipid extraction. This method is easily adopted for different tissues (Bligh et al. 1959).

In the present study, we intend to analyse the extraction and quantification of lipid (oily body) from Bryophyta (*Marchantia polymorpha*) through Bligh and Dyer method.

MATERIALS AND METHODS

Materials: *Marchantia polymorpha* used for this experiment has been garnered from Jain Scientific Industries, Agra. And the collected samples are authenticated by Dr. Ashok Kumar, Botany Department, Chaudhary Charan Singh University (CCS University), Meerut, Uttar Pradesh, India (Fig. 1). All chemicals used for experimentation were from HiMedia, India.

The chemical process is more effective on a laboratory scale mainly for the analysis and extraction from the freeze-dried sample of Bryophyta which is usually being performed with chloroform or methanol or both. This process was first postulated by Bligh and Dyer in 1959 (Kitagawa et al. 1986).

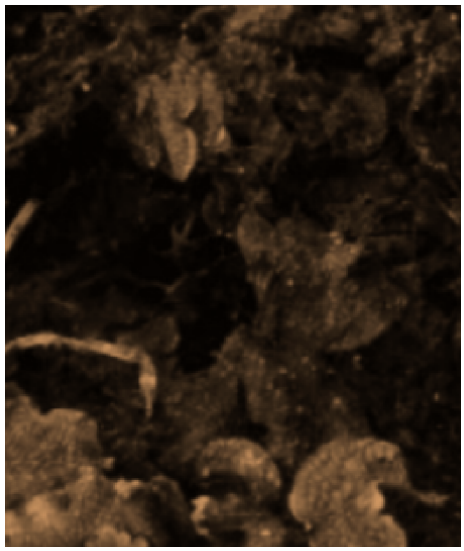

Fig. 1: Sample of *Marchantia.*

Fig. 2: Filtering of sample.

Methods: Eight grams of wet sample was weighed and then transferred to a mortar pastel for squashing and grinding. Ten mL of chloroform and 20 mL of methanol was mixed to prepare a homogenized solution. The sample was ground by continuous addition of the homogenized mixture to convert it into a slurry. Another 10 mL of chloroform and 20 mL of methanol was added and the sample was ground for 2 minutes. Finally, another 20 mL of chloroform was added into the mixture. A 17.4 mL saturated sodium chloride solution was prepared by adding 6 g of sodium chloride with 11.4 mL of distilled water. Saturated sodium chloride solution was added into the slurry and thoroughly mixed for another 5 minutes. Using methanol, a pre-wetted filter paper was used and the slurry was gauzed through the glass funnel using the filter paper (Fig. 2). The liquid phase of the slurry was then collected into a sidearm flask. From the sidearm flask, the solution was transferred into a graduated cylinder and was left undisturbed until it gets completely separated into 2 phases (Fig. 3). The separated phase contains methanol as an upper layer and chloroform as the lower layer. The amount of chloroform layer was noted. The methanol layer was discarded using a micropipette. Some amount of chloroform was compromised to make sure that methanol was completely removed. Aluminium boat was prepared and weighed using analytical balance (Fig. 4a). The remaining chloroform was transferred into the aluminium boat. Aluminium boat was kept over a hot iron plate at 80-degree Celcius so that all the amount of chloroform could get vaporized. After vaporization of chloroform, only the desired lipid in the form of an oily body was left over the aluminium boat. The initial weight of the aluminium boat was then compared with the weight of the aluminium boat containing the oily bodies.

Fig. 3: The different layers after separation.

During the crushing of the sample, methanol and chloroform were thoroughly added to extract the desired lipid (oiling bodies) from the sample. Chloroform and methanol results in breaking the cell wall and membrane, thus releasing oily bodies from the sample. The sample is then kept undisturbed until it gets separated into two phases.

Once the 2 phases get completely separated, the two layers can be differentiated by their colour. The upper metha-

Fig. 4: a. Aluminum boat, b. Oil droplets over aluminum boat

nol layer is whitish fuzzy and blurry in appearance, whilst the lower chloroform layer is green in appearance.

On heating the aluminium boat, all the chloroform gets evaporated, so in the end, greenish patches will appear, unevenly distributed on the surface of the boats (Fig. 4b).

RESULTS AND DISCUSSION

The results of this experiment can be studied by comparing the initial and final weight of the aluminium boats. On heating the aluminium boats on a hot iron plate, the remnant was lipid in the form of an oily body.

Added the difference of the weight found in the boat. On totalling, the amount came out to be 0.044 g. So, 0.044 g of an oily body was obtained from 8 g of *Marchantia polymorpha* (Table 3).

However, 0.044 g was not the exact value as some amount of oil droplets were lost during the filtration of slurry, which was done using filter paper as well as lost in the methanol fraction which was removed by pipetting.

Nations are quite concerned over the use of renewable energy and have put emphasis on transport and other sectors. Biofuels can be used as a source of renewable energy. Raw material such as biomass, which is considered as second-generation biofuel, compete with the feedstock of higher vertebrates such as human and animals. Bryophytes, generally fall into the category of the third generation. Biofuels do not compete with the feedstock and can be utilized wisely (Alam et al. 2015).

Biofuel extraction is not new to this world, and the majority of species have been used to produce biofuel on the laboratory scale. But to find a prominent source for the production of biofuel has always been a topic of concern for the fellow researchers. The heterogeneity in the value of the lipid content was observed when various methods are used for the extraction of lipids from different species. Three species that are prominently used were *Botryococcus* sp., *Chlorella vulgaris* and *Scenedesmus* sp. When lipid is extracted by using an autoclave, the content ranged between 5.4g/L and 11.9 g/L; when lipid is extracted using bead beating, then the range came out to be 7.9-8.1 g/L; similarly by using the microwave, sonication, and 10 percent NaCl, the range stand out from 10.0-28.6 g/L, 6.1-8.8 g/L, and 6.8-10.9 g/L respectively (Lee et al. 2010). Oil content of some microalgae is given in Table 4 (Gouveia et al. 2009).

For the last century, gasoline and diesel both were dominant over all kinds of other fuels. These fuels are created with the help of fractional distillation while biofuels are created by reacting the alcohol with lipids. The difference between traditional fuel and biofuel is basically dependent on their non-renewable and renewable sources.

Biofuel is a domestic fuel and can definitely contribute to a more stable and reliable supply of energy. The biofuel

Table 3: Weight of aluminum boats with oily bodies and without oily bodies on it.

Aluminum boat	Initial Weight (grams)	Final Weight (grams)	Difference (grams)
1.	0.667	0.680	0.013
2.	0.359	0.369	0.010
3.	0.714	0.735	0.021

Table 4: Biomass oil content of different microalgae (Gouveia et al. 2009).

S.No	Species	Oil Content (%) (AFDW)
1.	*Spirulina maxima*	4.1
2.	*Chlorella vulgaris*	5.1
3.	*Scenedesmus obliquus*	17.7
4.	*Dunaliella tertiolecta*	16.7
5.	*Nannochloropsis* sp.	28.7
6.	*Neochloris oleabundans*	29.0

production process has evolved dramatically to control the original problem with viscosity. At the present day, biofuel is an increasingly non-toxic, biodegradable fossil fuel alternative that can be produced from a variety of renewable sources (Ali et al. 2013).

Insufficient quantities or the unreasonable price of petroleum fuels is a matter of concern, whereas the renewable energy resource is a promising alternative solution because it is clean and environmentally safe (Demirbas et al. 2008). There are many alternative sources of fuel like for example biogas, biomass and primary alcohol which are all renewable in nature (Ali et al. 2013). Fossil fuel is directly related to air pollution, land and water degradation. In these circumstances, biofuel from renewable sources can be an alternative to reduce our dependency on fossil fuel and assist to maintain the healthy global environment and economic sustainability (Alam et al. 2012).

CONCLUSION

Lipid was extracted in this experiment from *Marchantia polymorpha*, and amount of the oily body was calculated.

The lipid present in this Bryophyte is in minute quantity, but it can be seen as a source of biofuel for the future. At the laboratory level, 8 g of *Marchantia polymorpha* yields 0.044 g of the oily body. This figure may get improved if we perform the same experiment on an industrial level. To yield a litre of an oily body, around 190 kg of the sample is required. This amount is enormous for laboratory level but can be easily handled on an industrial level. The remnant is completely organic and eco-friendly, therefore it can be used for manure production.

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