Comparison of Different Methods of Lipid Extraction from Microalgae *Chlorella pyrenoidosa*.

Ramandeep Kaur [1], Anupama Mahajan[2], Anjana Bhatia[3]
[1, 3] Asst. Professor in Botany Dept. at Hans Raj Mahila Mahavidyalaya, Jalandhar, Punjab .
[1]Research Scholar at.I.K. Gujral Punjab Technical University, Jalandhar.
[2] Asst. Professor in Biotech. Dept. at S.U.S. College of Eng. and Tech., Tangori, Mohali, Punjab.

Abstract

In biofuel production technology, the lipid extraction and purification from microalgal cells is a significant bottleneck. As it demands high energy and high operation cost. Due to lack of standard lipid extraction method from microalgae various extraction methods have been used for different algal species by researchers. Therefore, this study compared different extraction methods and their efficiency for maximum lipid extraction in case of microalgae *Chlorella pyrenoidosa*. These methods included classical Folch method, modified Bligh & Dyer method and Soxhlet extraction. The results highlighted the importance of not only solvent used but ratio of polar and non-polar solvent also. Modified Bligh and Dyer method proves to be better than other methods as it extracted 27.3% of lipid from dry biomass of *C. pyrenoidosa*. While Soxhlet extraction with hexane only extracted only 10.8% of lipids due to lack of polar solvent. Hence study emphasized the further advanced research in algal biofuel field for successful implementation of this technology at commercial production scale.

Keywords: Bligh and dyer, Biofuel, *Chlorella pyrenoidosa*, Lipid extraction, Soxhlet extraction.

I. INTRODUCTION

In present scenario alarming energy crisis and environmental protection are major global issues. In this direction there is need of serious attempts to search for renewable and environmentally safe energy resources (Asif et al., 2007). In past few years, biofuel has drawn attention of many researchers as it is sustainable source of energy. The emergence of 1st and 2nd generation biofuels produced from food crops and lignocellulose respectively have smoothed the problem to small extent but cannot meet the present demand of fuels (Chisti et al.,2007). Microalgae, the 3rd generation biofuels, have been used as the most promising and non-food feedstock for biofuel production (Cooney et al., 2009). They have advantages of faster growth rate, high lipid content and no competition for arable land (Chisti 2008). A no. of microalgal species have been screened which have lipid content ranging 1-70% of dry weight and which can be enhanced to 85% under control stress conditions (Hu et al., 2008). In stress conditions, the production of PUFA (poly unsaturated fatty acids) rich TAG (triacyl glyceride) increases which help microalgae to overcome adverse conditions. These fatty acids are extracted for trans-esterification to produce 'Oilgae' or biofuel from algae (Singh et al., 2010, Cheirslip et al., 2011). Instead of having a number of advantages 'Oilgae' has not commercially replaced the petroleum based fuel (Greenwell et al., 2010, Guschina et al., 2006). Lipid content of microalgae can vary depending on the culture conditions such as pH, temperature, growth media as well as strain of algal used. So for large scale algal cultivation a number of parameters are optimized. Simultaneously reliable and cost effective methods of oil extraction from algal biomass are also taken into consideration in order to utilize microalgae for Oilgae production (Mercer et al., 2011). The solvent used for efficient lipid extraction plays a significant role in biofuel production. Traditionally lipids are extracted using non polar organic solvent such as chloroform, petroleum ether and hexane. 1st successful attempt of lipid extraction was made by Folch by using chloroform and methanol as solvent method (Folch et al., 1957). Using the same track Bligh and Dyer used same solvent but in different ratio for lipid extraction from fish tissue (Bligh et al., 1959). Later on this method become popular for microalgal lipid extraction also. But both of the above methods have major bottle neck of using highly toxic chemicals i.e. chloroform and methanol. There was an urgent need of alternative solvent which should be less harmful to man and environment. Hexane is such non polar solvent which is cheap, easily evaporated and high selective for TAG and neutral lipid (Medina et al., 1998). Hexane also eliminates the non-lipid contaminants present in algal biomass during the lipid extraction. Besides choosing right solvent system the choice of equipment and highly précised extraction methodology is required to use microalgae as biofuel feed stock (Halim et al., 2011). In present study the proficiency of four different solvent systems for lipid extraction from microalgae *Chlorella pyrenoidosa* and their effect on lipid yield was evaluated.

II. MATERIAL AND METHODS:

2.1 Sample collection and isolation of microalgae:

Sample of sewage water was collected from Pholriwal, Waste Water Treatment Plant, Jalandhar, Punjab. To isolate microalgae from the sewage water serial dilution method was used. Each dilution was poured on solidified fogg's media in pteri dishes by streaking (Fogg et al., 1949). The streaked pteri dishes were incubated in photobioreactor under temperature of 25^0C and 16:8 light – dark conditions. After 10days when algal cultures have been raised in pteri dishes, the compound microscope was used to observe different sp. of microalgae. *Chlorella pyrenoidosa* was identified and was used for raising pure culture.

2.2 Raising pure culture and harvesting of *Chlorella pyrenoidosa*:

Chlorella pyrenoidosa isolated by dilution method was cultured in 500ml. culture flasks using Fogg's media with 0.1g/L of urea as nitrogen source to induce lipid accumulation (Kaur et al., 2017). Culture flasks were placed in culture room under standard culture conditions of 25^0C and 16:8 light – dark conditions. The culture flasks were shaken manually 4-5 times in a day to prevent sticking of algal cells with glass walls. After 24 days of culture the harvesting was done using flocculation method. For this chemical flocculent i.e. 250mg of alum/L of solution was used. After adding the alum the harvesting container was left undisturbed for 24 hours. After 24hours the flocculated algal biomass was collected and dried in oven at 80^0C for 2hours to get constant weight (W1).

Fig: 1Pure culture of *Chlorella pyrenoidosa*.

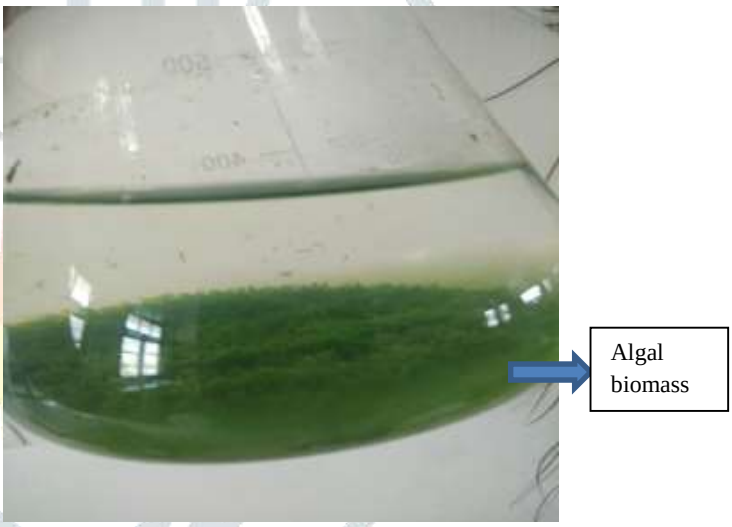

Fig: 2 Chemical flocculation by using alum

2.3 Different methods of lipid extraction from *Chlorella pyrenoidosa*:

2.3.1 Modified Bligh and Dyer method

Bligh and dyer method with minor modification was used for oil extraction from oven dried algal biomass (Bligh et al., 1959, Hajra et al., 1974). In a separating funnel for every 1g of dried algal powder 5ml of chloroform and methanol (1:2 ratio) extraction solution was used. The funnel was vortxed for 2minutes and left undisturbed for 24hours. Then 1ml. of chloroform and 2ml of 1M NaCl was added to prevent binding of denatured lipids to acidic lipids (Hajra, 1974). The funnel was shaken vigoursly for 5minutes, until two layers are visible. The lower layer with lipids is transferred to pre weighed vial (W1). The solvent was evaporated using rotatory evaporator. The lipid content was measured gravimetrically (W2).

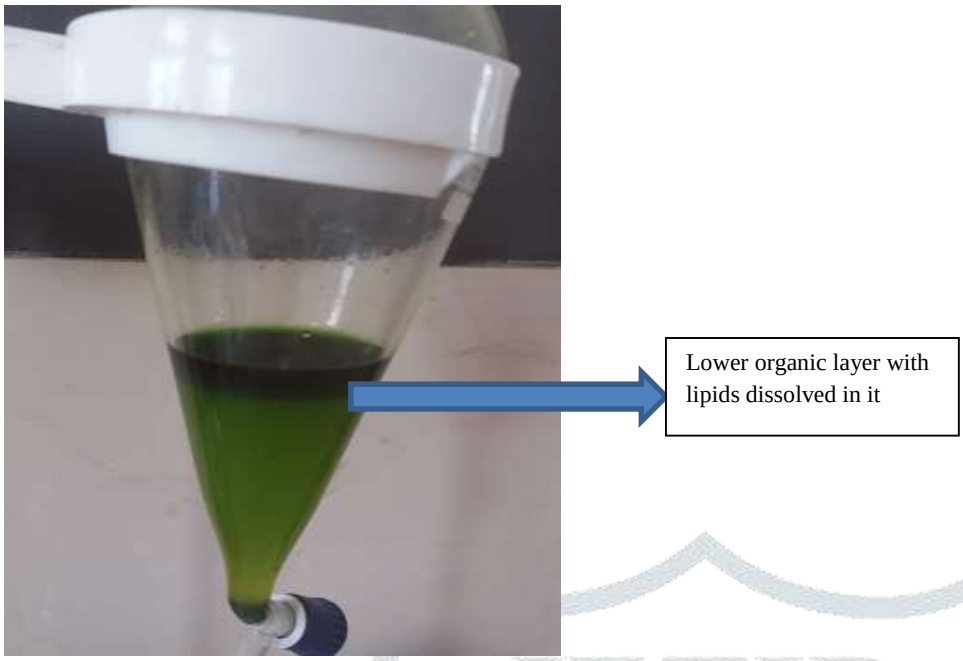

Fig: 3 Bligh and Dyer method with chloroform and methanol in 1:2 ratio.

2.3.2 Folch method

This method was originally purposed and used by Folch in 1957. In this method for each 1g of dried algal powder 5ml of chloroform and methanol solution in 2:1 ratio is used. The rest of method is same as that of Bligh and Dyer method except use of water instead of 1N NaCl.

2.3.3 Soxhlet method (using hexane only)

In Soxhlet extraction, dry algal powder was placed inside the thimble. Hexane was used as extraction solvent. 40ml of hexane was poured in distillation flask. Now solvent is heated for 6hours at 80^0C. After 6hours once the distillation flask cooled down to room temperature, the solvent (hexane) was evaporated by using rotatory evaporator. The residue (microalgal oil) was weighed.

2.3.4 Soxhlet method (using hexane and ethanol)

This method is followed the same steps as described above. Only difference is solvent used for extraction i.e. hexane and ethanol in 3:1 ratio. After the extraction, solvent was evaporated by using rotatory evaporator. The residue (microalgal oil) was weighed (W_2) and lipid content was calculated gravimetrically.

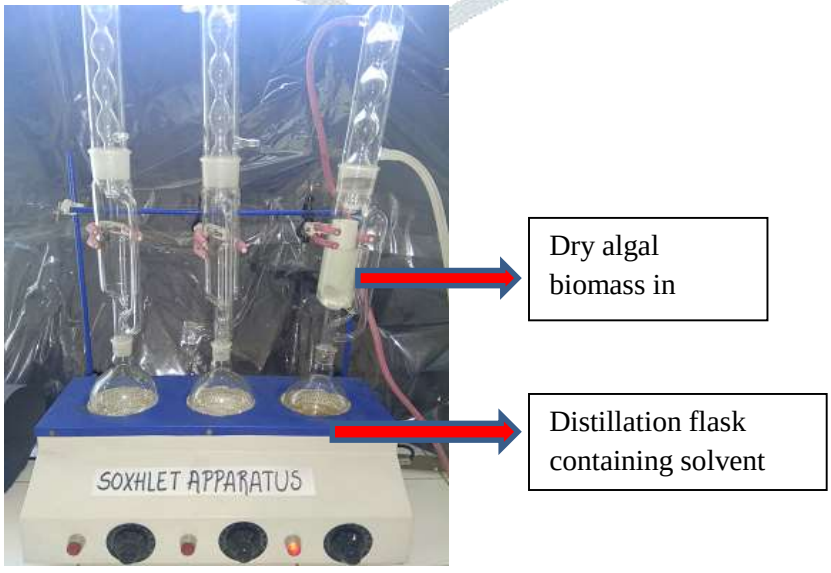

Fig: 4 Soxhlet extraction of lipids from dry algal biomass

2.4 Lipid content analysis

The lipid content was calculated gravimetrically by subtracting weight of residue after lipid extraction (W_2) from dry weight of algal biomass before lipid extraction (W_1). It was expressed as % dry cell weight.

% Lipid content= $(W_1) - (W_2) / (W_1) \times 100$ [Eqn.1].

2.5 Statistical analysis of data

All experiments were carried out in triplicate and results were expressed as the mean ± standard deviation.

III. RESULTS AND DISCUSSIONS

In case of lipid extraction from microalgae, the effectiveness of extraction method depends on both solvent used as well as microalgal species. Previous studies have emphasized the importance of selection of an optimal lipid extraction method which should be suitable for different microalgae with different size, shape, cell wall structure as well as lipid characteristics (Lee, 2010, Ryckebosch et al., 2012, Guschina et al., 2006). Lee et al has used different extraction method and observed significant difference in lipid content from *Chlorella vulgaris, Scenedesmus sp. and Botryococcus sp.* (Lee et al., 2010). In present study different solvent systems were investigated for lipid extraction from microalgae *Chlorella pyrenoidosa*. From the result given in Table: 1 we observed that in modified Bligh and Dyer method lipid recovery was maximum i.e. 27.3% by dry cell weight. This method extracted 3.1%, 5.7% and 16.5% more lipid content in comparison to Folch method, single solvent Soxhlet and two solvents Soxhlet extraction method respectively. Soxhlet method using single solvent hexane resulted in lowest lipid extraction content i.e. 10.8% only.). Soxhlet extraction with a single solvent that is i.e. hexane gave minimum extraction yield of oil because this method involves diffusion process and it does not involve any shear stress to algal biomass.

Table: 1 Lipid recovery (% DCW) in case of *Chlorella pyrenoidosa* in different methods.

	Modified Bligh & Dyer method	Folch method	Soxhlet method (hexane:ethanol)	Soxhlet (with hexane only)
Lipid productivity(% DCW)	27.3± 0.20	25.4±0.15	21.6±0.15	10.8±0.32

This can be explained by fact that some of the neutral lipids are present in cytoplasm as lipid globules and some as polar- lipid complexes. Further these complexes are attached with cell membrane proteins by H-bonding. During lipid extraction the Vander wall forces between non polar solvent and neutral lipids are not sufficient to break H-bonds between protein-lipid complexes. So these lipids are not extracted by using organic non polar solvent only (Ryckbash et al., 2011, Kates 1986). While in case Soxhlet extraction using two solvents i.e. hexane and ethanol in 3:1 ratio gives better result in comparison to hexane only. This is due to fact that by using ethanol with non-polar solvent not only the neutral lipids present in the cells but the membrane-associated complexes, polar lipids (phospholipids and glycolipids) are also extracted (Mercer et al., 2011, Shen et al., 2009). It has been also observed in case of *Tetraselmis* using solvent mixture of hexane and ethanol into 3:1 ratio, has enhanced the liquid recovery by the 50% than the normal hexane extraction method (Rodolfi et al., 2012). Hexane, the non-polar solvent with high stability, no residual, low boiling point and low corrosiveness has been used extensively for the oil extraction throughout the world. Our results agree with results of (Ramluckan et al., 2013, Fajardo et al., 2007).

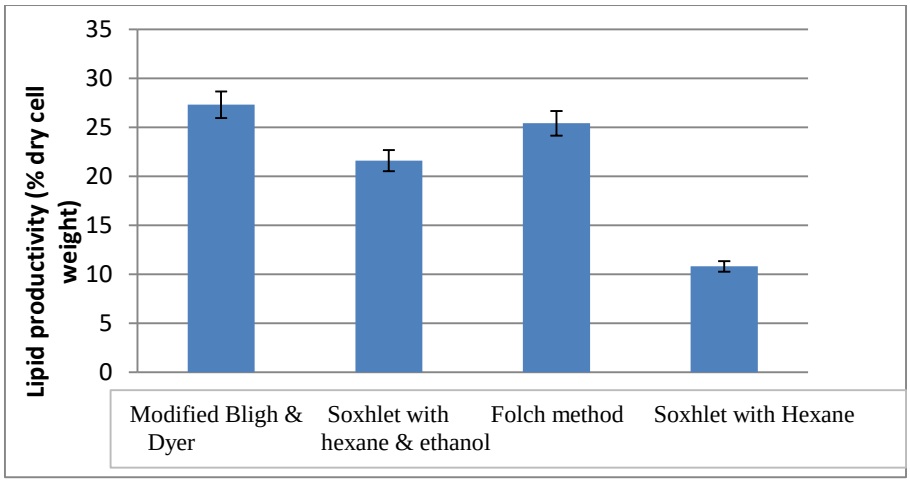

Fig.5 Comparison of lipid recovery using different methods in case of *Chlorella pyrenoidosa*

Fig.5 illustrates the comparative account of different solvent extraction methods used for lipid recovery in case of *Chlorella pyrenoidosa*. The lipid extraction in Bligh and Dyer method is 5.7% more comparison to Soxhlet extraction using hexane (3): ethanol (1). This could be attributed to fact that in Soxhlet extraction high temperature can causes thermo-degradation of long chain poly-unsaturated fatty acids (LC-PUFA) e.g ω-3 fatty acids (Schlechtriem et al., 2003). It can also causes in situ transesterification of fatty acids in presence of alcohol (ethanol) (Mercer et al., 2011, Shen et al., 2009, Schlechtriem et al., 2003). In this respect, Bligh and Dyer method using methanol and chloroform causes less artefacts, so the results obtained with this method in our study are considerably more well-grounded and reproducible (Guckert et al., 1988).

Folch method gives 25.4% of lipid recovery using chloroform and methanol in 2:1 ratio. It has minor difference of lipid recovery i.e. 3.1% only in comparison to highly productive Bligh and dyer method in our study. This is due to fact that the solvents shows good extraction efficiency when are used in fixed ratio but as ratio changes the extraction efficiency deteriorate. This may be due to fact that extraction efficiency of solvents not only depends on vander wall forces between algal biomass and molecules of solvents but also on preferences on different types of polarities. So change in solvents ratio will affect viscosity, which directly affects solubility of target molecules (Burke et al., 1984). So chloroform and methanol in ratio of 1:2 has maximum oil extraction efficiency in comparison to other methods used to compare in case of *Chlorella pyrenoidosa*. Our result agrees with result of Ramluckan et al., 2013 and Zonouzi et al., 2016.

CONCLUSION

Successful implementation of production of biofuel from algae relies not only on microalgal growth but on efficient and maximum lipid extraction recovery from dry biomass also. The present results suggest that modified Bligh and Dyer method with chloroform: methanol (1:2) extracted maximum lipids i.e. 27.3% from dry biomass of *Chlorella pyrenoidosa*. While Soxhlet extraction using hexane only resulted in minimum lipid extraction due to lack of polar solvent. So use of polar and non -polar solvent in appropriate ratio can enhance lipid recovery.

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