Maximizing Lipid Extraction from *Chlorella pyrenoidosa*: A Study on the Effectiveness of Cell Disruption Techniques

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Abstract
Microalgae are one of the most eco-friendly and promising raw material sources for the production of biofuel. Proteins, carbohydrates, lipids and nucleic acid are the major components of algal biomass depending upon the species. The present study aimed to determine the lipid extraction and transesterification from Chlorella pyrenoidosa microalgae. Different methods and solvents are used for the disruption of the cell-wall and extraction of lipid content. The methodology involved ultrasonication, microwave digestion, Soxhlet extraction and autoclave while using different solvents as methanol, chloroform, hexane, dichloromethane and heptane. We compared various cell disruption methods to improve lipid extraction yields and the highest lipid extraction yields were obtained using Soxhlet method with hexane.

The obtained lipid converted into fatty acid methyl esters (FAMEs) through transesterification process. The identification of fatty acid was compared by retention time and individual pattern of use from caprylic acid (C8:0) to lignoceric acid (C24:0) in form of methyl ester. The Soxhlet method using methanol and chloroform and FAME analysis showed a highest percentage of palmitic acid (C16:0) and lowest percentage of myristic acid (C14:1).

Keywords: *Chlorella pyrenoidosa*, Cell distribution, Lipid extraction, FAME, Transesterification.

Introduction

Microalgae are unicellular organisms known for extremely tough cell wall, very difficult to erade to obtain lipid. These algae can accumulate 20-50% lipid as part of cytoplasm[18]. Number of mechanisms are needed to break the cell wall as high-pressure homogenization, ultrasonication and microwave digestion besides the application of organic solvents. It has identified various uses of lipids as food additives, animal feed, plant fertilizers and biofuels. In total, algae specially microalgae hold numerous utilities that are considered eco-friendly and are economically viable source that can be maintained in any climate condition[4].

The microalgae *Chlorella*, single-cell green algae, is seen spherically shaped with 2-10 microns in diameter in size having no flagellum. It bears chloroplasts with green photosynthetic pigments: *Chlorella vulgaris*, *Chlorella pyrenoidosa* and *Chlorella ellipsoidea*, *Chlorella emersonii* and *Chlorella minutissima*. *Chlorella* is classified as prominent species according to the shape of cell, characteristics of chlorophyll and other variables[7]. The present study carries comparative approaches of lipid extraction from *Chlorella pyrenoidosa.*

Four extraction methods were applied viz. Soxhlet extraction, microwave digestion, autoclave and ultrasonication using different solvents mixture- chloroform, methanol, heptane, dichloromethane and hexane. One approach is to convert algal triglycerides to biodiesel where the lipids were extracted using organic solvents as hexanes, chloroform, methanol, dichloromethane and heptane[2]. These solvents were then removed through distillation and the triglycerides were reacted with acid or base and an alcohol to make the fatty acid methyl esters[3,21]. Gas chromatography was used to quantify individual total fatty acid present in the obtained lipid extract. This is normally achieved by converting it to FAME by the process known as transesterification[10].

Efficiency of lipid is dependent on the polarity of the solvent[8,9]. The extraction followed by mixtures of polar and non-polar solvents extracts higher amount of lipids from microalgae. In this research, combinations of chloroform and methanol, chloroform: heptane (2:1), chloroform: hexane (1:1) and chloroform: hexane (2:1) were applied[19].

The Bligh and Dyer method was applied using chloroform and methanol for lipid extraction[17].The use of chloroform and ethanol with 1:1 ratio yielded maximum lipid. The combination of dichloromethane and ethanol increased the lipid extraction efficiency by 25% from *Chlorella sp.*[16] Different genus of species have tendency to produce different amounts of lipids, for instance *Chlorella vulgaris* 14-22%, *Chlorella ellipsoidea* 4.49% and *Chlorella pyrenoidosa* 2- 11.9%[11].

Material and Methods

Techniques for rupturing microalgal cells to release the lipid bodies that have been accumulated there, are the basis of mechanical methods for lipid extraction. Solvent-free extraction is considered to be a potential method for the commercial production of primary extracted lipids, despite the fact that these techniques are not yet fully established. Mechanical techniques offer a viable and dependable

alternative because they are unaffected by the type of microalgae. Additionally, using no-solvent considerably reduces the risk of contamination. However, occasionally the heat used to speed up the process may damage the extracted lipids. The extraction of lipids from microalgae is basically a mass transfer operation which depends on the nature of solute and solvent, selectivity of solvent and the level of convection in medium. Cracking of cell walls by mechanical methods would increase the accessibility of lipids for solvents[1].

Microalgae cultivation and harvest: *Chlorella pyrenoidosa* was procured from NCIM, Pune and cultured in BG-11 medium at a temperature of 26°C with light-dark cycles for 20 days. The harvested biomass of microalgae cells was determined by measuring the mass of the wet microalgae, followed by drying in an oven set to 60ºC. Various methods including autoclaving, microwave digestion, ultrasonication and Soxhlet extraction were examined to disrupt the cells. Different polar and non-polar solvents were used in these methods.

High frequency sound waves and Microwave digestion method: To extract lipids from the biomass, two different methods were employed: high frequency sound wave-assisted extraction (sonication) and microwave-assisted digestion. In the sonication method, 0.5 g of dried biomass was mixed with 30 ml of various solvent mixtures such as chloroform: methanol (2:1), hexane, dichloroform and others and subjected to 20 cycles of 5-second sonication at room temperature using a probe sonicator. The resulting mixture was centrifuged and the solvent was evaporated in a microwave oven at 60ºC for 8 to 9 hours. The lipid content was then determined gravimetrically.

In the microwave-assisted digestion method, 0.5 g of dried biomass was dissolved in 6 ml of water and loaded into teflon vessels, which were sealed and subjected to microwave digestion at 175ºC to 50ºC and 40 bar pressure for 26 minutes. After cooling, the digested sample was mixed with 30 ml of various solvent mixtures such as chloroform: methanol (2:1), hexane, dichloroform and others and centrifuged at 4000 rpm for 15 minutes. The resulting mixture was separated into organic and aqueous phases and the organic phase was collected and evaporated in a microwave oven at 60ºC for 6 to 8 hours. The lipid content was then determined gravimetrically.

Overall, while both methods used the same amount of biomass and solvents, they differ in the way the solvents were mixed with the biomass. The sonication method used high frequency sound waves to enhance the extraction efficiency, while the microwave-assisted digestion method relied on high pressure and temperature to break down the biomass. Furthermore, the sonication method evaporated the solvent mixture as a whole while the microwave-assisted digestion method separated the organic phase and evaporated it separately. By using these complementary methods, we were able to obtain a comprehensive and accurate measurement of the lipid content in the biomass.

Soxhlet method: Soxhlet set up has been designed for continuous extraction of lipid in the presence of organic solvents. Soxhlet lipid extraction used 1 g of dry algae biomass collected in cellulose thimble. Later, different solvent ratios as chloroform: methanol (2:1), hexane, dichloromethanol, chloroform: heptane (2:1), chloroform: hexane (1:1), chloroform: hexane (2:1) were added in solvent vessels. The extraction method was performed continuously at varying temperatures ranging from 120ºC to 140 ºC for 6 hours. Then the whole solvent was evaporated in microwave oven for 6-8 hrs at 60ºC. The lipid was measured gravimetrically[13].

Lipid extraction using Soxhlet for transesterification process: 10 g of dry algae biomass was taken in a cellulose thimble. The extraction was performed with 150 mL of solvent mixture ratio chloroform: methanol (1:1). The extraction method was performed continuously at varying temperatures ranging from at 120ºC to 140 ºC for 6 hours. Then the whole solvent was evaporated in microwave oven for 6-8 hrs at 60ºC. The lipid was measured gravimetrically[11].

Analytical method: The FAME qualitative composition of biodiesel was determined using gas chromatography and GC-FID as follows: injector: SPL, 205ºC, linear velocity mode, column flow rate 0.75 mL/min, injection volume 1.5 µL; column:cp-sil88 (60.0 mm length × 0.25 mm inner diameter × 0.25µm film thickness), oven temperature of 240ºC, with total analysis time of 43 min. Fatty acid methyl ester contents was detected by comparison of their peak areas with those of standards[11].

Results and Discussion

The comparative analysis of five different solvents was applied with four different methods for lipid extraction of NCIM 2738 *Chlorella pyrenoidosa* microalgae. Different methods were examined and compared with regard to lipid content obtained through each process. Lipid extraction percentages from dry biomass using various solvent and different methods from *Chlorella pyrenoidosa* microalgae vividly highlighted the extraction using Soxhlet method to carry the highest amount of lipid content with the application of hexane as solvent.

The laboratory findings infer that different cell disruption methods bring varying efficiency and effects on lipid yield; different solvents were also compared to obtain effects of lipid yield. The main parameters considered in the choosing of a solvent for the extraction of lipids from microalgae are polarity or extractability, lipid solubility, water miscibility (ability for two-phase systems) and low toxicity[1]. Lee et al[8] found that the lipid recoveries from *Chlorella vulgaris* were maximum with autoclave pre-treatment method.

Table 1
Comparison of different method for lipid extraction

Solvents	Ultrasonication method	Microwave method	Soxhlet method
Chloroform: methanol (2:1)	2.30	7.51	9.01
Hexane	4.29	3.48	10.97
Dichloromethane	3.01	2.66	7.54
Chloroform: heptane (2:1)	5.12	5.80	9.40
Chloroform: hexane (1:1)	5.64	3.40	4.36
Chloroform: hexane (2:1)	4.82	2.70	6.74

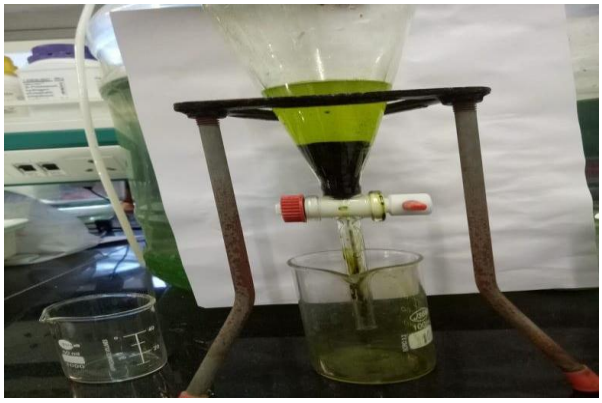

Figure 1: Separation of organic and aqueous phase

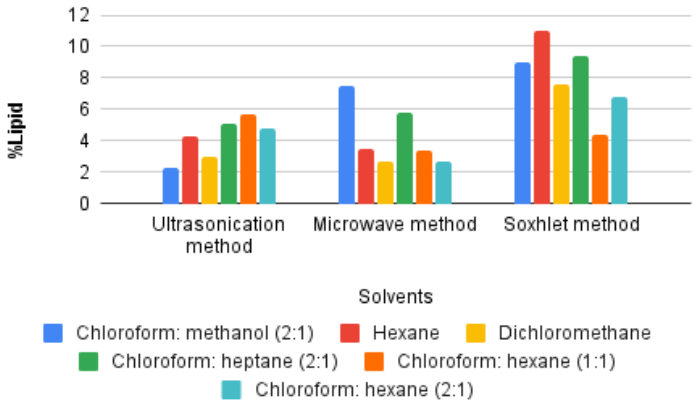

Figure 2: Comparison of different method with different solvent

Table 2
Profile of fatty acid methyl ester lipid of *C.Pyrenoidosa*

Peak	Ret. time	Name	Area	Area%
1	26.781	Myristic	12764	0.53
2	29.612	Palmitic	592511	24.63
3	30.26	Palmatolic	189841	7.89
4	31.987	Stearic	39588	1.65
5	32.45	Oleic	557847	23.19
6	33.25	Linoleic	397669	16.53
7	34.196	Linolenic	432586	17.98
10	34.813	Arachidic	17947	0.75
Total			2405287	100

De Souza Silva et al[5] noted that autoclave and microwave pre-treatment of microalgal biomass result in lipid yields of 15.40% and 33.7% respectively[5]. *Chlorella sorokiniana* resulted in the low lipid yields compared to other laboratory techniques such as bead beating and sonication[23]. The energy that causes temperature and pressure changes on the cell wall that result in cell wall, ruptures the cell wall to come out of lipid content[6].

According to fig. 2, the best yield of extracted lipid is shown in microwave digestion method using chloroform: heptane (2:1) The highest lipid content is 17.64 % and the lowest lipid content is 2.7 % with chloroform: hexane (2:1) in 26 minutes of treatment. Cell disruption techniques for lipid extraction from *Botryococcus sp*., *Chlorella vulgaris* and *Scenedesmus sp*. reported increased lipid yields while increasing sonication time as a result of enhanced cell disruption efficiency[8,20]. An increasing temperature from 30ºC to 40ºC recovered slightly higher lipid after 5-10 min[15].

The Soxhlet method in *Scenedesmus sp*. 11.3 % in methanol and chloroform followed Wiyarno et al method[20]. *Chlorella pyrenoidosa* was obtained with ethanol 19.01 % and chloroform 16.20 %[11]. Extraction with methanol was effective for ultrasonication method obtaining 17.59 % lipid. Soxhlet extraction was best performed with ethanol 19.01 % and chloroform 16.20 %[12]. Depending on the kind of lipids to be extracted, polar and non-polar solvents can be used during the extraction process. Hexane, benzene, toluene, diethyl ether and chloroform are the non-polar solvents that are used most frequently while methanol, acetone, ethyl acetate and ethanol are examples of polar solvents.

The most popular organic solvent to extract lipid from algal biomass is a mixture of chloroform and methanol (1 to 2 v/v) which yields a high yield with a short extraction time. The performance of five different solvents (chloroform/ methanol, hexane/ isopropanol, dichloromethane/methanol, dichloromethane/ethanol and acetone/dichloromethane) reported a higher lipid yield of 28.6% for the chloroform/methanol (2/1, v/v)[15].

Transesterification of lipids from *C.pyrenoidosa*: The fatty acid compositions as well as extraction performances of the described solvent mixtures are shown in table 2 and it is evident that the fatty acid compositions of lipid extracts were similar regardless of extraction method. The resulted FAMEs were analyzed in gas chromatography. The main fatty acids in the lipid extracts were methyl esters of myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3) and arachidic acid (C20).

The identification of fatty acid was performed and compared by retention time and individual pattern from caprylic acid (C8:0) to lignoceric acid (C24:0) in form of methyl ester. The Soxhlet method using methanol and chloroform showed a highest percentage of palmitic acid (C16:0) and oleic acid (C18:1) and lowest percentage of myristic acid (C14:1) and arachidic acid (C20) for FAME of *C. pyrenoidosa* (NCIM 2758).

The fatty acid methyl ester highest amount was 24.63% of palmitic acid. Marcelo et al[11] observed 24.9% in palmitic acid (C16:0). In this study, the lowest amount observed in myristic acid (C14:0) was 0.53%. Lowest amount of myristic acid ranging from 0.45 to 1.55 % is present into all the samples. According to Petkov and Garcia[14], the percentage of 14:0 in fresh water microalgae does not exceed 1%, linoleic acid (C18:3) was detected as 17.98 % and linoleic acid (C18:3) was detected as 20.4 %.

Conclusion

In conclusion, this study aimed to determine the most effective method for extracting lipids from *Chlorella pyrenoidosa* microalgae which can be used for biofuel production. Various cell disruption methods were compared and the Soxhlet method using hexane was found to be the most effective for lipid extraction. Fatty acid methyl esters (FAMEs) were obtained through transesterification and the composition of fatty acids was identified by retention time and individual pattern.

Palmitic acid (C16:0) was found to be the most abundant fatty acid in the extracted lipids. These findings provide valuable information for the development of efficient and sustainable biofuel production from microalgae.

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