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Effect of various pretreatment methods prior to extraction of omega 3 fatty acids from *Nannochloropsis gaditana*

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Abstract
Microalgae constitute a source of bioactive compounds offering a variety of nutraceutical and pharmaceutical applications. Amongst them, the omega-3 long-chain polyunsaturated fatty acids, such as eicosapentaenoic (EPA, 20:5 *n*-3), and docosahexaenoic (DHA, 22:6 *n*-3) acids are known for their beneficial effects on human health. There is currently a large demand for microalgae in the nutraceutical and pharmaceutical industry due to their health-promoting effects. High scale economically feasible microalgae based oil depends on optimizing the entire production process. To make it more economically attractive, a feasible cell disruption method should be established to ensure a low operating cost, high product recovery, and high quality of the recovered lipids. The aim of this study was to compare different pretreatment methods that enhance the lipid yield from the extraction process. In the present study, it was found that ultrasonic assisted extraction produces the highest lipid yield of 12 g followed by enzymatic treatment with 8.8 g per 100 g of dry wt. EPA yield also found to be high in ultrasonic disruption showing 32.5 mg/g, while, the lowest yield quantified in control and acid treatment with 18.2 mg and 18.9 mg/g respectively. Through further investigations, it was justified that pretreatment methods have the potential to reduce energy cost required for the conventional functional lipid extraction.

Keywords: EPA, *Nannochloropsis gaditana*, PUFA, pretreatment, ultrasonication

1. Introduction
Environmental concerns and alarming energy crises are the major issues in the 21st century. The depletion of oil reserves, the resulting an increase in fossil fuel prices and the international awareness of the environmental impact of greenhouse gas emissions have contributed to worldwide interest in developing sustainable alternative energy sources to meet current and future demands. The cultivation of microalgae can make an important contribution to the transition to a more sustainable society. Many algae produces important compounds like pigments, proteins, vitamins, antimicrobial colorants, and bio oils [1]. Marine algae are rich and varied source of bioactive natural products, so it has been studied as potential biocidal and pharmaceutical agents. Microalgae are the primary producers of EPA and/or DHA in the marine environment and are thus the most promising alternative source for omega-3 LC-PUFA. Khozin-Goldberg *et al.* and Ryckebosch *et al.* [2, 3] studied a total lipid extracts of several microalgae, such as *Isochrysis* (DHA), *Nannochloropsis* and *Phaeodactylum* (EPA) and *Pavlova* and *Thalassiosira* (EPA and DHA) are sufficiently rich in omega-3 LC-PUFA to serve as a potential alternative for fish oil. Furthermore, microalgae oils also contain phytosterols and carotenoids, components that may provide added value to the microalgae omega-3 LC-PUFA oils because of their nutritional importance and, in the case of carotenoids, also their protective effect against omega-3 LCPUFA oxidation. *Nannochloropsis* is well appreciated in aquaculture due to its nutritional value and the ability to produce valuable chemical compounds, such as pigments and polyunsaturated fatty acids (EPA) [4]. However, lipid extraction methods for microalgae cells are not well established, and there is currently no standard extraction method for the determination of the fatty acid content of microalgae [5]. This has caused a few problems in microlagal biofuel research due to the bias derived from different extraction methods. A lot of research is being carried out for developing microalgal biodiesel technology by performing bioprospecting of high-lipid-

Containing strains as well as by inducing higher lipid production by various physiological and genetic strain improvement methods. Ryckebosch *et al.* also showed, specifically for microalgae, that a mixture of chloroform and methanol yields the highest extraction efficiency [6]. This solvent mixture, however, has the disadvantage that chloroform and methanol are toxic solvents. Therefore, large scale and workable lipid extraction using this method is prohibited due to environmental and health risks and as such other solvents have been evaluated [7]. Therefore, lipid extraction is an extremely important process for the production of microalgal biodiesel. There are also other methods such as algal biorefinery for the production of multiple algal products and thermochemical technology for the production of biocrude. As fuels are a commodity product, extraction of lipids from algae is technically and economically viable even in integrated concepts. When produced in huge quantities, extraction of lipid for biodiesel production from strains containing even around 10% lipid content will be feasible [8]. The thermochemical method is the most commonly used pretreatment for lipid extraction of microalgal biomass. Nevertheless, research using pretreatment methods that require less energy such as mechanical and biological should be stimulated [9]. The the present study was to evaluate effects of algal pretreatment methods prior to omega3 fatty acid extraction methods and to exploit effective and simple protocol for extracting polyunsaturated fatty acids.

2. Materials and Methods

The fresh culture of *Nannochloropsis gaditana* was collected from the Pondicherry coastal area and further mass cultivated using double strength F2 algal media [10] in artificial sea water and grown at 25 °C under fluorescent tubes with 2000 lux at 16 h light and 8 h dark cycle. At the stationary phase algal biomass were collected by centrifugation at 8500 rpm for 10 min and then air dried. The dried alga was subjected to lipid extraction methods.

2.1 Pretreatment of algal cells prior to FAME extraction

Pretreatment of algal biomass was found to be an important step to facilitate easier and faster lipid recovery methods for fatty acid extraction. Pretreatment methods had beneficial effects on the cell disruption of marine microalga to extract oil without changing the fatty acid composition [11].

2.2 Acid hydrolysis

A quantity of 1 g of dry alga was subjected to acid hydrolysis by adding sterile water, reducing pH to 2.0 using hydrochloric acid and then kept at orbital shaker for 15 min [12].

2.3 Enzymatic treatment

Microalgal suspension was added to cellulase enzyme prepared in sodium acetate buffer. Then the mixture was shaken and kept at 37 °C for 15 min [13, 14].

2.4 Thermal treatment

Algal suspension was autoclaved for 15 min at 121 °C with 15 lbs pressure [15].

2.5 Microwave treatment

The experiment was conducted in microwave oven for 15 min (Model: SAMSUNG MW73AD) 800 W and operating frequency of 2450 MHz [16].

2.6 Ultrasonic treatment

Algal suspension was sonicated at 42 kHz, 170 W, temperature of 60 °C. To avoid overheating the samples were kept in an ice bath during the process [17].

2.7 Lipid extraction

Algal lipids were extracted by the procedures similar to the Folch method [18] Dichloromethane/methanol (2:1,v/v) containing 0.1% butylated hydroxytoluene (as antioxidant) was added and mixed vigorously for 1 min then left at 4 °C overnight. Add 1 mL of 0.9% NaCl was added and mixed again. The organic phase containing lipids was collected. The residual extract was extracted with 2 mL dichloromethane. Organic phase extracts were pooled and dried under nitrogen and subjected to saponification by mixing with sodium hydroxide and water at 100 °C for 30 min. The mixture is cooled and poured into crushed ice and 40 mL of water added. The mixture was shaken with 4 mL ethyl ether to wash of the unsaponified materials [19]. Further, the suspension was subjected to methylation by adding methanolic hydrochloride solution. Fatty acid methyl esters were then collected by mixing with the extraction solvent containing hexane and ter butyl methyl ether. Finally the solvent phase was evaporated and stored for chromatography analysis.

2.8 Thin Layer chromatography

Fatty acid methyl ester mixtures extracted from different pretreatment methods were charged on a thin layer of silica gel and were developed by ascending technique using three solvent systems, Petroleum ether: ether (60:40) (v/v), Hexane: ether (80:20) (v/v), Toluene : Ethyl acetate (90:10) (v/v) [20-23]. After developing, the plates dry at room temperature and placed in iodine chamber. The fatty acid methyl ester gave dark brown colored spot with iodine vapour. The coloured spots are marked and the R_f values of the spots are calculated. Chromatography with standard methyl ester is carried out and R_f values are compared.

2.9 Fatty acid estimation by gas chromatography

Gas chromatography analyses were performed with HP 5890 Gas Chromatograph equipped with a split/splitless capillary (1:40) injector and a flame ionization detector. Analytical separation was achieved on an Agilent capillary column (25 m × 0.32 mm i.d.) with 0.2 µm film thickness. Nitrogen was used as carrier gas (with a constant flow rate of 1 mL/min). Temperature setting was controlled at injector 250 ºC, and detector 300 ºC. The oven temperature was held at 190 ºC [24-26]. Quantification of omega 3 fatty acid content in the unknown samples were calculated using EPA methyl ester (Eicosapentaenoic acid), DHA methyl ester (Docosahexaenoic acid) as external standards.

3. Results and discussion

3.1 Gravimetric quantification of lipids

The filtrate collected from lipid extraction was transferred into a funnel and sufficient water and hexane were added to induce biphasic layers. After settling, the mixture partitioned into two phases, a top dark-green hexane layer containing most of the extracted lipid, and the bottom layer contained all the co-extracted non-lipid contaminants. The hexane phase was transferred to a pre-weighted flask and heated to dryness in the oven to enable gravimetric

quantification of the extract. The lipid was dissolved in hexane and sealed for storage [27].

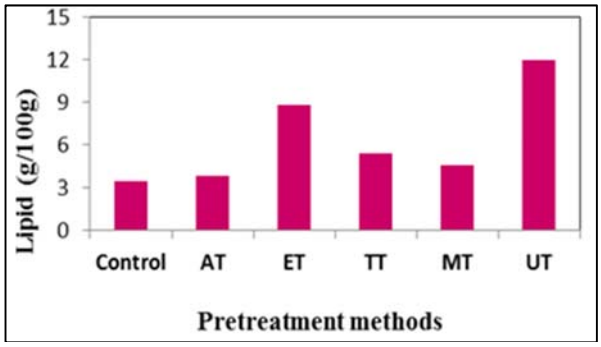

Fig 1: Fatty acid determination by thin layer chromatography**.**

(AT-Acid treatment, ET-Enzymatic treatment, TT-Thermal treatment, MT-Microwave treatment, UT-Ultrasonic treatment).

3.2 R_f value determination by thin layer chromatography

Thin layer chromatography of the isolated Eicosapentaenoic Acid (EPA) was carried

Out in three different solvent systems and R_f values were compared with the standard values.

Table 1: R_f values of different solvent systems by thin layer chromatography

S. No	Solvent system	R_f value
1	EPA Standard	0.62
2	Petroleum ether : Ether (60:40)	0.54
3	Hexane : Ether (80:20)	0.58
4	Toluene : Ethyl acetate (90:10)	0.61

3.3 GC estimation of omega 3 fatty acids

Table 2 shows the EPA content extracted from different pretreatment methods which were measured from the chromatogram obtained using gas chromatography. EPA belongs to a group of fatty acids that are part of the phospholipids, which serve as structural components in the cell wall. Under nutritional limitations, such as nitrogen, cells are unable to resynthesize them or keep the concentration of these components constant. However, with adequate nutrition, cells are capable of synthesizing high amounts of energy rich PUFAs, such as EPA.

Table 2: Eicosapentaenoic acid (EPA) quantification from different pretreatment methods

S. No	Pretreatment methods	EPA content (% dry wt)
1	Control	1.82
2	Acid hydrolysis	1.89
3	Enzymatic treatment	2.8
4	Thermal treatment	2.36
5	Microwave treatment	1.97
6	Ultrasonic treatment	3.25

Nannochloropsis gaditana was isolated from the coastal areas of Pondicherry and cultivated in F2 Guillard medium for twenty days in light and dark period under fluorescent tubes with 2000lux. Biomass was harvested by centrifugation and algal paste subjected to different pretreatment methods prior to fatty acid extraction.

The amount of EPA extracted with the different pretreatment methods is summarized in Table.2. Solvent extraction was presently suitable and efficient method for lipid extraction. The highest EPA recovery of 3.25% was obtained with ultrasonic treatment, while EPA recovery with all other treatment ranged from 1.82 to 2.8%. Elena Cequier *et al.* [28] reported that dichloromethane-methanol could readily replace the commonly employed chloroform-methanol, thus avoiding the major health, security, and regulatory problems associated with the use of chloroform. The results from gravimetric lipid estimation shown that oil content were high from ultrasonic disruption followed by enzymatic degradation. The low lipid content observed from untreated and acid hydrolysis samples. However, microwave treatment has the disadvantage of maintenance cost, especially in commercial production [29]. Gary Witman *et al.* [30] reported the significance of TLC technology for quantitative lipid detection and found it was possible to detect as little as 25 ng of phospholipids, 25 ng of cholesterol, and 50 ng of neutral lipids and fatty acids. Solvent system toluene and ethyl acetate were found to be effective with R_f value 0.61 equal to the standard.

One of the major obstacles for producing bioactive compounds from microalgae is extracting intracellular lipids, which requires penetration of solvents into the cell wall and membrane. Janda *et al.* [31] reported lipid yield extraction increased from 0.246 to 0.311 g per dry g of *C. vulgaris* when the cells were pretreated with ultrasonication, which is equivalent to a 26.4% increase. Similarly, Anna Patrícya Florentino de Souza Silva *et al.* [32] achieved a 13.3% increase in lipid yield by ultrasonic assisted extraction. Mubarak *et al.* 2016 [33], shown high lipid yield from aquatic feed *Salvinia molesta* and confirmed that ultrasonic pretreatment would be the most effective treatment prior to extraction for biodiesel. Wiltshire *et al*. [57] reported a 90% extraction efficiency of fatty acids and pigments from the species *Scenedesmus obliquus* using ultrasound extraction. Ranjan *et al*. [58] reported that ultrasound assisted microalgae lipid extraction demonstrated more distorted clusters of biomass on micrographs, in comparison to cells with solvent penetration. Cravotto *et al*. [59] noted that ultrasound assisted lipid extraction from *Crypthecodinium cohnii* resulted in an increase in lipid yield of 21.1% from hexane solvent extraction. The results obtained from this study testified that highest EPA (20:5) yield achieved using ultrasonication followed by enzymatic digestion.

4. Conclusion

In the present study, cell disruption methods were found to be efficient for algal oil extraction. All pretreatment methods in the study shown high lipid extraction efficiency than the untreated control. Dichloromethane would be the better lipid extraction solvent substituting chloroform which reduces health risks during downstream process. Ultrasonication was found to be the efficient pretreatment method for lipid extraction. Further studies would be suggested applying dual pretreatment techniques such as ultrasonic assisted enzymatic digestion may result in 100% lipid recovery and economical energy consumption.

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7. References

1. Hans Wlkers. Microalgae, the green gold of the future, Large scale sustainable cultivation of microalgae for the production of bulk commodities, www.groenegrondstoffen.nl
2. Khozin-Goldberg I, Iskandarov U, Cohen Z. LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Appl Microbiol Biotechnol. 2011; 91:905-915.
3. Ryckebosch E, Muylaert K, Foubert I. Optimization of an analytical procedure for extraction of lipids from microalgae. J Am Oil Chem Soc. 2012; 89:189-198.
4. Rocha JM. Growth aspects of the marine microalga *Nannochloropsis gaditana*, Biomol Eng. 2003; 20(46):23742.
5. Yan Li. A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. Microbial Cell Factories. 2014; 13:14.
6. Ryckebosch E, Bruneel C, Termote-Verhalle R, Muylaert K, Foubert I. Nutritional evaluation of omega-3 LC-PUFA rich microalgae oils as an alternative for fish oil. Food Chem. Under review, 2013.
7. Molina Grima E, Ibanez Gonzalez MJ, Gimenez GA. Solvent extraction for microalgae lipids. In: Borowitzka MA, Moheimani NR (eds) Algae for biofuels and energy. Springer, Dordrecht, 187-206.
8. Ranjith Kumar. Lipid extraction methods from microalgae: a comprehensive review, Frontiers in energy research, 2015, 2.
9. Viviane T de. Influence of lipid extraction methods as pretreatment of microalgal biomass for biogas production, Renewable and Sustainable Energy Reviews. 2016; 59:160-165.
10. Ksenia Gulyaeva, Development of protocols for the extraction, separation and characterization of lipids, Norwegian University of Science and Technology Department of Biotechnology, 2015.
11. Duraiarasan Surendhiran, Mani Vija. Effect of various pretreatment for extracting lipid from *Nannochloropsis oculata* under nitrogen repleted and depleted conditions, Bioelectrochemical Laboratory, Department of Chemical Engineering, Faculty of Engineering and Technology, Annamalai University.
12. Laurens LML. Acid-catalyzed algal biomass pretreatment for integrated lipid and carbohydrate-based biofuels production, Green Chem. 2015; 17:1145-1158.
13. Dongqin Wang. Combined enzymatic and mechanical cell disruption and lipid extraction of green alga *Neochloris oleoabundans,* Int. J Mol. Sci. 2015; 16:7707-7722.
14. Kyle Sander Ganti S. Murthy, Enzymatic degradation of microalgal cell walls, An ASABE meeting presentation paper number: 1035636, 2009.
15. Michael D, Marsolek. Thermal pretreatment of algal biomass and lipid-extracted algal byproduct for enhanced biogas generation, Energy Challenges & Mechanics, 2014.
16. Azadmard-Damirchi. Microwave pretreatment of seeds to extract high quality vegetable oil, International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering. 2011; 5:9.
17. Ji-Yeon Park. Sonication-assisted homogenization system for improved lipid extraction from Chlorella Vulgaris, J.-Y. Park *et al*. / Renewable Energy 2015; 79:3-8.
18. Jordi Folch. A simple method for the isolation and purification of total lipids from animal tissues. J Biol. Chem. 1957; 226:497-509.
19. Chitra Som RS. Bioactivity Profile of Polyunsaturated Fatty acid extracts from *Sardinella longiceps* and *Sardinella fimbriata* - A Comparative Study Thesis submitted to the Cochin University of Science and technology, 2010.
20. Barma KS, Goswami BC. Identification and estimation of fatty acids in fresh water fish *Anabus testudineous,* Annals of Biological Research, 2013; 4(11):18-24
21. Shilpa Deshpande. Microbial Conversion of Plant Based Polyunsaturated Fatty Acid (PUFA) To Long Chain PUFA and Its Identification by Gas Chromatography, J Biotechnol Biomaterial, 2013.
22. Avanti Polar lipids Inc, Solvent systems -lipid migration, www.avantilipids.com.
23. Kajal Chakraborty. Preparation of eicosapentaenoic acid concentrates from sardine oil by *Bacillus circulans* lipase, Food Chemistry 2010; 120:433-442.
24. Príncipe de Vergara, Determination of trans unsaturated fatty acids by capillary column gas chromatography, International olive oil council, COI/T.20/Doc.no.17/Rev. 12001.
25. Jing X Kang, Jingdong Wang. A simplified method for analysis of polyunsaturated fatty acids, *BMC Biochemistry* 2005; 6:5 doi:10.1186/1471-2091-6-5.
26. Jagannathan N. Production of Omega-3 Fatty Acids in *Cymbella* sp, Adv. Biores. 2010, 1.
27. Dr Claude Leray. Estimation of total lipids, Fats and Oils Cyberlipid center, 2016.
28. Elena Cequier-S Anchez. Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty Acids from samples of different natures, J. Agric. Food Chem., 2008; 56:12.
29. Ramanathan Ranjith Kumar. Lipid extraction methods from microalgae: a comprehensive review, Front. Energy Res., 2015.
30. Dr. Gary Witman, Dr. Mark Moskovitz. Thin Layer Chromatography in the purification of Lipids, Dynamic Adsorbents, 2016.
31. Janda Danielle *L*, MS. Evaluation of lipid extraction methods from microalgae *Chlorella vulgaris*, San Diego State University, 2015; 1584548.
32. Anna Patrícya, Florentino, de Souza Silva. Comparison of pretreatment methods for total lipids extraction from mixed microalgae, Renewable Energy 2014; 63:762-766
33. Mubarak M, Ultrasonication: An effective pre-treatment method for extracting lipid from *Salvinia molesta* for biodiesel production, Resource-Efficient Technologies 2016; 2:126-132.
34. Wiltshire KH, Boersma M, Moller A, Buhtz H. Extraction of pigments and fatty acids from the green alga Scenedesmus obliquus (Chlorophyceae). Aquatic Ecology. 2000; 34:119-126.

35. Ranjan A, Patil C, Moholkar SV. Mechanistic assessment of microalgal lipid extraction. Industrial Engg Chem Res. 2010; 49:2979-2985.
36. Cravotto G, Boffa L, Mantegna S, Perego P. Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. Ultrasonics Sonochem. 2008; 15:898-902.