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Research Article

Effect of Various Pretreatment for Extracting Intracellular Lipid from *Nannochloropsis oculata* under Nitrogen Replete and Depleted Conditions

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Microalga is one of the most compelling microbial biomasses for biodiesel production. Various pretreatment processes, namely, enzyme treatment, lysis by acid, ultrasonicator, microwaves, autoclave, and 40% NaCl, for nitrogen replete and depleted algal cultures of *Nannochloropsis oculata* had been carried out to check the most feasible and effective technique to disrupt cells for procuring lipids, for which concentrations were determined. Fatty acid composition, essential functional groups, and cell disruption were analyzed by GC-MS, FT-IR Spectroscopy, and Nile Red fluorescent microscopy, respectively. The present investigation showed that lipid yield was higher in nitrogen depleted cells than that in normally nourished cells. GC-MS revealed the presence of major fatty acids—palmitic, oleic, stearic, arachidic, lauric, and linoleic acids. Highest efficiency was found when cells were pretreated using acid for 3 h. The lipid content was calculated as 33.18% and 54.26% for nitrogen rich cells and nitrogen starved cells, respectively. This work thus aided in identifying the most eligible pretreatment process to avail lipids from cells, to convert them to eco-friendly and nonpolluting biodiesel.

1. Introduction

Increasing population and uncontrolled urbanization have created serious problems of energy requirement. Due to a sudden hike in energy consumption, it is anticipated that there would be deterioration in oil reserves by 2050. Continuous use of fossil fuels resulted in effect on environment by increasing greenhouse gas emission leads to climatic changes [1]. Therefore, there is a current demand to find out the alternative eco-friendly fuel against petrodiesel. Biodiesel has been considered as a major alternative for fossil fuel, as it is a biodegradable, renewable and nontoxic fuel [2]. Fatty acid methyl esters originating from vegetable oils and animal fats are known as biodiesel. It does not contribute net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than the petrodiesel [3]. Plants and algae are good candidates, as alternative energy sources, as they obtain their energy from the sunlight and build up their biomass by removing carbon dioxide from atmosphere through photosynthesis [4]. Recently, there is much interest in lipid production from microalgae because they have multiple advantages over traditional energy crops [5]. Microalgae have a high photosynthetic efficiency, rapid growth rate, shorter doubling time, and higher biomass production rate and utilize very less land than conventional crops [6, 7].

Biodiesel from algae is widely considered as one of the most efficient methods because algae do not compete with food crops [8]. For example, using corn as a feedstock for making ethanol creates a negative competition between human and animal consumption or fuel production [7].

Though algae contain high amount of lipid content, biodiesel production process is not being commercially operated elsewhere; only some companies are involved in commercialization. Since extraction of algal lipids is costly, it is one of the key challenges for the commercial success of algae biofuel [9]. Biodiesel production from microalgae

consists of the following steps including species selection, cultivation, harvest, and cell disruption. Cell disruption is particularly an important step as cell walls are generally thick and consist of multiple layers [6, 9]. Extracting oil from microalgae for biofuel production is one of the principal steps of microalgae-based biodiesel production [10]. Since cell wall and membrane present in algae are formidable barriers to permeation by extraction solvents, cells have to be disrupted prior to extraction [11], which enhances oil recovery. Methods of cell wall disruption and extracting solvents decide the efficiency of oil extraction from microalgae [11].

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 purpose of this study was to compare and evaluate a range of different physical and chemical treatments on the disruption of cells of marine *Nannochloropsis oculata* for lipid recovery. Finding the most appropriate method of cell disruption for *Nannochloropsis oculata* would maximize the lipid concentration and improve the quality of the extracted lipids. Measurements of lipid concentrations were obtained to indicate cell disruption efficiency as a correlating variable [6].

From the literature survey, we have found only few researchers worked on enzymatic cell disruptions, acid hydrolysis, and osmotic shock using NaCl. In this work we had compared different pretreatment process for optimistic extortion of oil from nitrogen availed culture and nitrogen starved culture with the commonly available methods such as ultrasonication, autoclaving, and microwave method. The specific objective of this study was to investigate several pretreatment for loosening the cell wall of microalgae, for intracellular oil extraction and visualizing their cell wall after pretreatment using light microscopy.

2. Materials and Methods

2.1. Culture Condition. *Nannochloropsis oculata* was obtained from CMFRI, Tuticorin, Tamilnadu, India, and cultivated in 25 L photobioreactor using sterile Walne medium under 5000 lux illuminated with white fluorescent bulb for 12 : 12 hr light and dark condition for 15 days. One reactor was filled with nitrogen rich Walne's medium and another medium was supplied with nitrogen for the first 4 days after which the nutrients were added to medium containing nitrogen and medium without nitrogen for scaling up to 25 L.

2.2. Harvesting of Cells. When the culture reached stationary phase, the biomass was harvested by centrifugation at 8500 rpm for 10 min to get thick algal paste. Then the microalgal paste was rinsed with distilled water to remove residual salts and then dried in hot air oven at 60°C for 8 h.

2.3. Pretreatment of Algal Cells for Oil Extraction

2.3.1. Acid Lysis of Microalgae. A quantity of 3 g of dried microalgal biomass was added to sterile sea water, the pH was reduced to 2.0 with HCl, and the solution was shaken for 1 h, 2 h, and 3 h using orbital shaker at 180 rpm.

2.3.2. Enzymatic Treatment. The microalgal suspension was disrupted with cellulase (Hi Media, Ltd, Mumbai, India). A quantity of 2 g of dried microalgal biomass was taken in 250 mL Erlenmeyer flask containing cellulase enzyme solution prepared with 0.1 M sodium citrate buffer and the enzymatic hydrolysis was conducted at 37°C for 1 h, 2 h, and 3 h. The concentration of cellulase enzyme was 5 mg L^{-1}. The pH was adjusted to 5.5 with diluted HCl before disruption. Then cellulase was inactivated by heating at 100°C for 10 min.

2.3.3. Thermal Treatment. The heat treatment was performed for 2 g of biomass using autoclave. In this experiment, the autoclave was maintained at 121°C, 15 lbs pressure for 10 min, 20 min, and 30 min.

2.3.4. Microwave Treatment. This experiment was conducted in the microwave oven (Model-National NN-S557WF) for 5 min, 10 min, and 15 min at 100°C, 900 W, and 2455 MHz.

2.3.5. Pretreatment with 40% NaCl Solution. The algal dried biomass was treated with 40% NaCl solution in an Erlenmeyer flask and kept at 180 rpm in an orbital shaker for 24, 48, and 72 hrs.

2.3.6. Ultrasonic Treatment. The pretreatment process for microalgal cell wall destruction was also performed with Ultrasonicator (VIBRCEL VX400, Sonic Limited, USA) at 24 kHz at a temperature of 50°C for 5 min. The algal biomass was mixed with 15 mL of sterile distilled water and sonicated at 70 aptitude for 5 min, 10 min, and 15 min. To avoid overheating of samples, they were kept in an ice bath during the ultrasonic process. All the experiments were carried out for both microalgal biomass harvested from nitrogen rich and depleted media. After pretreatment, the biomass slurry was subjected to drying to remove excess moisture in hot air oven.

2.4. SEM Analysis. The different pretreated microalgal cells were subjected to morphological analysis to examine cell wall damage. Small amount of sample was taken from the suspension, dried, and observed with Scanning Electron Microscope (SEM).

2.5. Oil Extraction. Cell slurries from acid treatment, enzymatic treatment, ultrasonication, autoclaving, plasmolysis with 40% NaCl, and microwave treatment were subjected to oil extraction by Bligh and Dyer [12] with slight modification. In brief, the biomass suspension was mixed with chloroform: methanol (1 : 2) ratio, vortexed for few minutes, and incubated on ice for 10 minutes. Chloroform was then added, followed by addition of 1 M HCl, and was vortexed again for few minutes. Finally the whole suspension was centrifuged at maximum speed for 2 minutes. Bottom layer containing lipid was transferred into fresh previously weighed beaker. Chloroform was added to reextract the lipid from the aqueous

sample. The solvent system was evaporated using rotary evaporator at 30°C. Finally, the lipids from all the disruption methods were analyzed for fatty acid composition analysis using GC-MS.

2.6. Calculation of Oil Yield. The liquid phase was transferred to preweighed flasks. Thereafter, the flasks were then placed in a hot air oven for complete evaporation of the solvent and were weighed again. The total lipid fraction was calculated after obtaining the differences of final and initial flask weights. The lipid concentration was defined as dry weight ratio of extracted lipids to biomass. According to Suganya and Renganathan [8], the oil extraction yield (%w/w) was determined by the following formula:

$$\text{Oil extraction yield (\%)} = \frac{\text{Weight of extracted oil (g)}}{\text{Weight of algal biomass (g)}} \times 100. \quad (1)$$

The extracted oil from untreated algal biomass (from nitrogen rich medium and nitrogen depletion medium) was considered as control for comparing oil pretreated by different extraction techniques.

2.7. FTIR Analysis. A quantity of 50 mg of dried biomass was taken, mixed with 150 mg of KBR powder, and ground well to fine mixture. The mixture was pressed to a disc using a hydraulic press. The disc was subjected to FTIR spectral measurement in the frequency range of 4000–400 cm^{-1}. The algal powder was characterized using a Fourier Transfer Infrared Spectrophotometer (Bruker Optics, GmBH, Germany).

2.8. Intracellular Lipid Identification by Nile Red Staining. It is a specific stain to identify intracellular lipids present in biological samples. A stock solution of Nile Red stain (9-diethylamino-5H-benzo (α) phenoxa-phenoxazine-5-one) was prepared according to Mohamady et al. [13]. A quantity of 2.5 mg of Nile Red was dissolved in brown bottle containing 100 mL of acetone and this was stored at dark. Each 0.5 mL of microalgae culture broth (both nitrogen rich and nitrogen depletion) was centrifuged at 1500 rpm for 10 minutes and the pellets were washed with sterile distilled water (equal volume) for several times. The cell pellets were then mixed with 0.5 mL of Nile Red solution incubated for 10 min at room temperature. After washing with distilled water, the stained cells were observed under fluorescence microscopy.

2.9. Chlorophyll Content Analysis. The chlorophyll *a* content (mg/L) was estimated according to Su et al. [14]. Two milliliters of culture broth was taken in centrifuge tube, ultrasonic for 10 min in ice bath with two milliliters of 90% methanol overnight. Then the homogenate was centrifuged at 3000 rpm for 5 min. The supernatant was separated and absorbance was read at 665 and the amount of chlorophyll was calculated using the following formula:

$$\text{Chlorophyll } a \left(\text{mg L}^{-1}\right) = 13.43 \times \text{OD}_{665}. \quad (2)$$

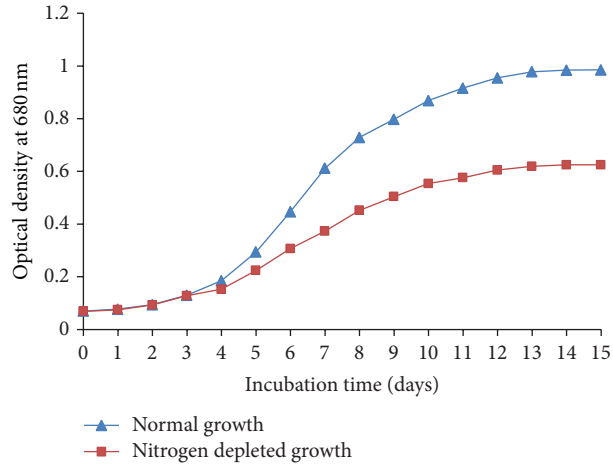


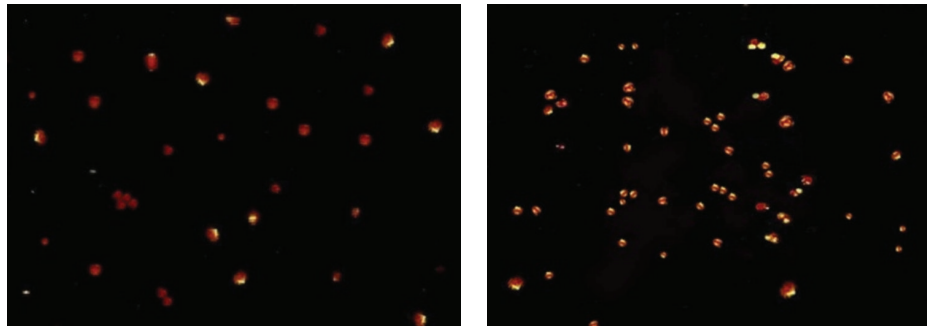
Figure 1: Growth curves of *N. oculata* grown on Walne's medium with nitrogen replete (N^+) and nitrogen depleted (N^-) conditions.

2.10. Total Carbohydrate and Protein Estimation. The total carbohydrate content was determined with DNS method using glucose as reference and the total protein content was estimated according to Lowry's method [15] using bovine serum albumin as standard.

2.11. Gas Chromatography and Mass Spectroscopic Determination of Fatty Acid Components. Fatty acid compositions of oil extracted from both nitrogen rich and nitrogen depleted cultures were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS-QP 2010, Shimadzu) equipped with VF-5 MS capillary column (30 mm length, 0.25 mm diameter, and 0.25 μm film thickness). The column temperature of each run was started at 70°C for 3 min, then raised to 300°C, and maintained at 300°C for 9 min. GC conditions were as follows: column oven temperature: 70°C, injector temperature: 240°C, injection mode split, split ratio: 10, flow control mode-linear velocity, column flow: 1.51 mL/min, carrier gas-helium (99.9995% purity), and injection volume: 1 μL. MS conditions were as follows: ion source temperature: 200°C, interface temperature: 240°C, scan range: 40–1000 m/z, solvent cut time: 5 min, MS start time: 5 min, end time: 35 min, and ionization-EI (−70 eV) and scan speed: 2000.

3. Results and Discussion

3.1. Growth Aspects of N. oculata under Normal and Nitrogen Depleted Conditions. The effect of nitrogen on microalgal growth is shown in Figure 1. The algal growth was increased under nitrogen replete condition, whereas some loss in growth was observed during nitrogen limited or starvation condition [16]. A previous study by Alsull and Wan Omar [17] also resulted in a decreasing yield of biomass when algae were grown without nitrogen. Microalgal growth slows down in lack of nitrogen, hence no synthesis of new membrane compounds takes place. Nitrogen is an essential nutrient for algal growth; hence it was added to the nitrogen depleted cells initially. At the 5th day, the medium was supplied without



(a) (b)

Figure 2: Nile Red stained cells of *Nannochloropsis oculata* under fluorescent microscope. (a) Normal cells. (b) Nitrogen starved cells.

Table 1: Chlorophyll, protein content, and carbohydrate content of *N. oculata* under nitrogen replete and nitrogen depleted condition.

Parameter	N^+	N^-
Chlorophyll content (μg/mL)	9.4	7.38
Protein content (μg/mL)	1.95	0.98
Total carbohydrate (μg/mL)	4.89	2.79

N^+ cells grown in normal medium, N^- cells grown in nitrogen depleted medium.

nitrogen to microalgal culture. The nitrogen-rich cells were dark green pigmented, whereas nitrogen depleted culture grew with same color till lag phase and turned yellow green at log and stationary phases. This indicated that nitrogen was essential in synthesis of chlorophyll for photosynthesis which also enhanced growth of microalgae. Similar result was also found in Beal et al. [18] which showed that nitrogen starved algal paste was yellow green in color and healthy sample was dark green.

3.2. Chlorophyll, Protein, and Total Carbohydrate Analysis. Chlorophyll and protein contents drastically decreased to half of their actual concentration (Table 1). A similar result was obtained by Alsul and Wan Omar et al. [17] for *Tetraselmis* sp. and *Nannochloropsis* sp. The chlorophyll *a* content was decreased, while the total lipid content increased under nitrogen limitation or starvation condition. In case of total carbohydrate, the content decreased moderately than the protein and chlorophyll. In general, during nitrogen limitation conditions the normal carbohydrate and protein metabolic pathways of cells are reverted to lipid synthesis [19], which leads to high amount of intracellular lipid accumulation in microalgae during nitrogen starvation.

3.3. Nile Red Staining for Lipid Identification. Intracellular lipid droplets of *N. oculata* nitrogen rich cells and nitrogen starved cells were observed by Nile Red staining under fluorescent microscope with excitation at 450–490-nm and emission at 515-nm. Neutral lipid or triglycerides appeared as yellow dots, whereas polar lipid and chlorophyll were stained in red colour. Figure 2 shows that nitrogen starved cells contained more lipid droplets with increased cell size than the normal cells. Ahlgren and Hyenstrand [20] and Hoffman et al. [21] reported that under nitrogen-deficient conditions, algal cells often accumulate a surplus of carbon metabolites as neutral lipids more than polar lipids. These neutral lipids are located as lipid bodies in the cytoplasm of microalgal cells (Figure 2). It was also reported that microalgae respond to the nitrogen starvation condition by degrading nitrogen containing macromolecules and accumulating carbon reserve compounds for the maintenance of cells, such as polysaccharides and fats. Current findings support previous research by Elumalai et al. [22] and Pick and Rachutin-Zalogin [23] mentioned that the Nile Red staining technique was a useful tool for rapid determination of lipids in microalgae.

3.4. FTIR Analysis. Analysis of the microalgal biomass was performed by FTIR-Spectrophotometer. Figure 3 shows the FT-IR spectra of dried biomass sample of normally nourished (Figure 3(a)) and nitrogen depleted cells (Figure 3(b)) of *N. oculata*. Table 2 reveals the various functional groups present in the samples. Bands were attributed to –CH stretch, protein band, N–H and C=O stretches of peptide bond, $-CH_2$ stretch of lipids, C=O stretch of ester, and C–O–P, C–C, and C–O stretches of polysaccharides. Both spectra were found to be almost matching with slight disparity in peaks. In Figure 3(b), less intensified absorption bands were observed in the region of 1800–800 cm^{-1}, a region specific for carbohydrate and protein, showing a decrease in their content. This could be a notification of carbohydrate metabolism reverting to that of lipids when cells starved from nitrogen. Also, sharp absorption bands in the region of 3100–2800 cm^{-1} were found in spectrum of nitrogen depleted cells, showing increased lipid content and intensity of the particular band was lesser in nitrogen replete cells. From these spectrograms it was inferred that components present were mostly cis isomers as the bands were between 700 and 3500 cm^{-1} (Phukan [24], Elumalai et al. [22], and Rukminasari [25]).

3.5. Pretreatment of N. oculata for Oil Extraction. The amount of total oil extracted from *N. oculata* was considered as an indication of the efficiency of different cell disruption methods used. A significant difference was found between

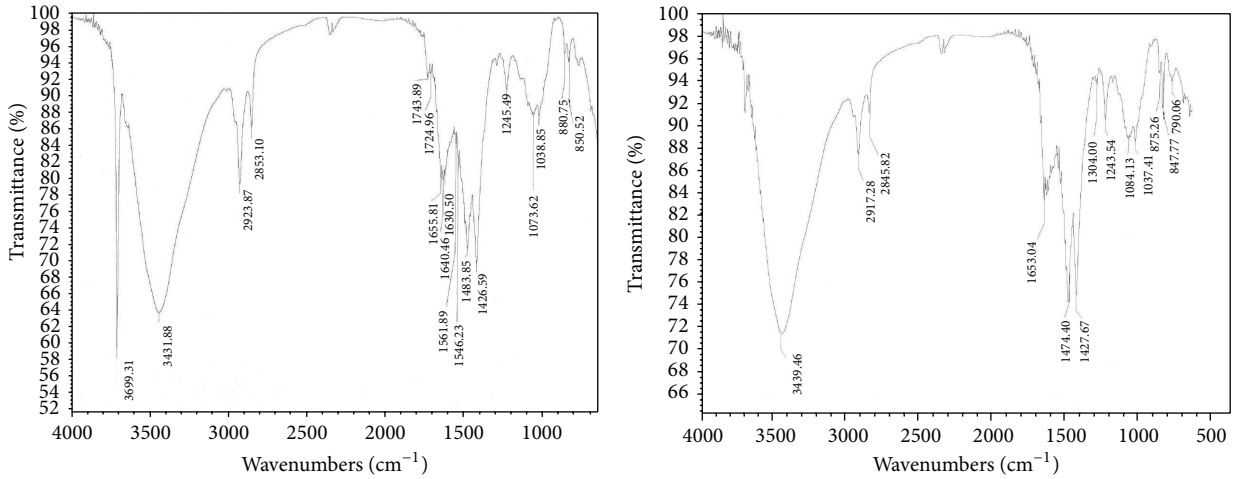


Figure 3: The FTIR spectrum of *N. oculata* grown under nitrogen depleted (a) and replete conditions (b).

Table 2: Representing the functional groups of nitrogen replete and depleted cells of *N. oculata*.

Wave numbers (cm^{-1})	Functional groups
3000–2700	C–H stretching vibrations of –CH_3, >CH_2, CH, CHO
3600–3300	Oleophilic C–H stretching vibration, indicating unsaturation
1653.04	C=O of carboxylic acid and derivatives
1743.89 (Figure 3(a))	C=O of ester group
1800–1500	Characteristic for proteins
1600–1500	Amide II bands, due to N–H stretching vibration
1200–900	C–O, C–C, C–O–C, C–O–P stretching vibrations of polysaccharides CH_3, CH_2 rocking mode of vibrations
3100–2800	Presence of lipid
2922.77 (Figure 3(a)), 2924.50 (Figure 3(b))	CH_2 asymmetric stretching in lipid
2851.32 (Figure 3(a)), 2853.23 (Figure 3(b))	CH_2 symmetric stretching in lipid

the studied techniques and this study proved that all pretreatment methods were able to disrupt *N. oculata* to release intracellular lipids.

Enzymatic pretreatment was carried out using cellulase enzyme (5 mg L^{-1}) for 8 h, 10 h, and 12 h interval. A maximum efficiency by enzymatic pretreatment was found to be at 12 h as 32.74% from nitrogen rich cultures and 51.68% under nitrogen starvation condition. The microalgal cell wall is made up of polysaccharide mainly comprising cellulose that can be hydrolyzed by the enzyme, cellulase [26]. From the data it was clear that the oil extraction was found to be increased with increasing pretreatment time with cellulase. From the literature, the benefit of using enzyme for hydrolyzing microalgal cell wall was evaluated by Zemke-White et al. [27]. Presently, enzymatic cell wall degradation is not widely practiced in industry because cell lysing enzymes have traditionally been cost prohibitive. Though cost intensive, enzyme degradation is important because the rigid cell wall of algal cell is resistant to mechanical methods that will require excess energy usage and multiple passes through disruption equipment. The cost factor can be overcome by immobilization of enzyme. Moreover, an alga does not have lignin in its cell wall, which is a major advantage to perform enzymatic hydrolysis of its polysaccharide components [4]. Fu et al. [26] had successfully hydrolysed *Chlorella* sp. using immobilized cellulase with electrospun polyacrylonitrile (PAN) producing reducing sugar. Therefore use of enzyme to lyse algal cell wall might be advantageous as compared to other methods.

Effect of acid treatment was studied at different time periods —1 h, 2 h, and 3 h. During acid lysis treatment of *N. oculata*, at pH 2.0, there was a high effect on oil released at an incubation of 2 h. The marine microalgae have been used as feed for herbivorous fishes worldwide. The researchers believed that many marine herbivorous fishes must possess acidic gastric condition in their stomach to gain access to the intracellular nutrients from algae for their diet [28]. The present study showed that the most efficient cell disruption occurred after 2 h of incubation giving 33.18% for nitrogen availed culture and 54.26% for nitrogen depleted

condition. A similar result was reported by Zemke-White et al. [27], in which they had analysed 4 macroalgae, namely, *Enteromorpha intestinalis, Ulva rigida, Porphyra* sp., and *Polysiphonia strictissima* and found that at pH 2.0 for 60 min the cell wall pore size of all four algal species increased up to at least 13.5 nm more than the normal cell wall size (8.8 nm). In the present study, after 2 h of treatment, the amount of oil yield was found to be constant. Hence we concluded that the algal cell wall lysed when treated for 2 h at a low pH 2, the porosity of cell wall increased, and intracellular lipid got released. Also this finding was supported by Harun and Danquah [28]. They had reported that acid pretreatment process was most suitable for hydrolyzing the cell wall of *Chlorococcum humicola* to release and convert the polysaccharides, entrapped in microalgal cell wall, into simple sugars for ethanol production.

Ultrasonication is a simple physical method for disrupting *N. oculata*. The cells grown in normal nutrition condition were exposed to various time periods, namely, 5, 10, and 15 min and a maximum of 30.12% oil was extracted at 15 min pretreatment. Similarly nitrogen depleted cells, when ultrasonicated at the above mentioned time intervals, showed higher extraction efficiency of 45.77% at a pretreatment time of 15 min. Higher the processing time, higher would be the oil yield. In contrast to the present study, Lee et al. [10] observed that ultrasonication resulted in least efficiency for *Botryococcus* sp. Suganya and Renganathan [8] reported that, through ultrasonication, higher extraction efficiency was achieved 2.25 times higher than that of direct extraction of oil from *Ulva lactuca*. Ultrasonication is one of the major pretreatments of algal cells for extracting oil; hence higher physical stress due to vibration on prolonged exposure, to extensive release of oil because of cell disruption, enhances the yield of oil for biodiesel production.

Autoclaving of microalgal cells was carried out for extraction of oil at various time intervals 10, 20, and 30 min at 121°C. Higher yield of oil was obtained from nitrogen depleted cells than the normally cultivated microalgal cells. At high thermal treatment, at 30 min, maximum amount of oil (28.81%) was obtained for normal cultures. Likewise for the nitrogen depleted cells the oil content was found to be at its maximum (43.90%) at 30 min. Autoclaving was found to be one of the most efficient methods yielding 7.88% of oil from *Ulva lactuca* by disrupting the membranes of the cells [8]. Lee et al. [10] also reported that extraction of oil from *Chlorella vulgaris* was at its maximum of 7.9% on autoclaving. Therefore, the higher the time of autoclaving at a high temperature, the higher the oil yield.

The effect of 40% NaCl was studied by subjecting the normally nourished and nitrogen starved cells at different time intervals. This higher osmotic shock resulted in cell lysis due to susceptibility of cell membrane of microorganisms. For normal cells 24 h the highest oil content was obtained for 48 h as 26.49%. Similarly, under nitrogen depleted cells the maximum yield oil was 40.56% at 48 h. For determination of optimistic time for oil extraction with NaCl, the cells were treated above 48 h till 72 h, but the amount of oil remained constant. Hence, 48 h was found to be an optimum extraction time for 40% NaCl treatment. However, the osmotic shock is a simple procedure for oil extraction but it did not show much disruption effect on microalgal cells and required longer time duration. The current result was supported by the work of Lee et al. [10] for treating microalgal species, *C. vulgaris* and *Scenedesmus* sp. for 48 h.

Dejoye et al. [29] reported that extraction of oil from microalgae with microwave pretreated microalgae systematically presented higher yields. But in contrast, in this study, microwave pretreated method did not significantly affect cell disruption. Microwave assisted treatment was performed at 5, 10, and 15 min for oil extraction by cell distortion. The maximum oil content using this method was found to be 26.51% at 5 min for normal cells and for nitrogen depleted cells it was 41.28% at 5 min. A decline in oil content was experienced as the time of exposure prolonged. A similar result was found in a previous study that microalgal lipid extraction efficiency was not effective and the oil extracted from microalgae by microwave method became volatile during disruption and extraction process [6] (Table 3).

Higher lipid concentration represented the disruption efficiency in this study and nitrogen depletion enhanced oil content on marine microalgae *N. oculata*, which was compared with the control. Among several methods for pretreating *N. oculata* for oil extraction, the enzymatic method gave maximum efficiency for normal culture and nitrogen depleted culture and also revealed that this method was the best for microalgal cell wall lysis. Acid pretreatment gave second maximum yield. Poor efficiency was shown by ultrasonication, autoclaving, microwave oven, and 40% NaCl pretreatment. Similar results were also found in Zheng et al. [6], who had studied various pretreatment on fresh water microalgae, *C. vulgaris,* showing higher effects of enzymes like lysozyme and cellulose on oil extraction by cell disruption.

In addition, from an extensive literature survey, it was noted that nowadays enzymatic extraction has been widely used in extraction of bioactive compounds from plant based materials. It is more attractive, with advantages like shorter extracting time, less pollution, higher extraction yield, and less decomposition of target compounds [30]. Moreover, enzymatic hydrolysis is specific, gentle and has specific effect on cell wall of algae especially on hemicelluloses and saccharides and accelerates the migration of bioactive compounds. Compared with conventional methods, it utilizes less energy and gave higher extraction yield [30]. Zheng et al. [6] stated that the enzymatic process could be worked at low temperature and could prevent the oxidation of oil, thus improving the biodiesel quality.

3.6. SEM Analysis of Algal Cells. For direct evidence on different pretreatment methods on cell wall damage, microalgal cells can be observed by microscopic study (Figure 4). The undamaged cells of *N. oculata* (Figure 4(a)) showed intact structures and had no indication of cell lysis. In addition, acid treatment 1 M HCl totally disrupted the morphology of microalgal structure appearing completely broken cells under Scanning Electron Microscope (Figure 4(b)). Next to the acid

Table 3: Lipid content at various pretreatment processes of *N. oculata* under nitrogen rich and nitrogen starved conditions.

Various pretreatment	Pretreatment time (min/h)	Normal growth	Nitrogen depleted growth
Control		26.43	40.52
Acid	1 h	31.23	48.38
	2 h	33.18	54.26
	3 h	33.18	54.26
Enzyme	8 h	29.38	45.28
	10 h	30.62	48.52
	12 h	32.74	51.68
Ultrasonication	5 min	28.14	44.16
	10 min	29.32	45.24
	15 min	30.12	45.77
Autoclave	10 min	27.68	42.39
	20 min	28.06	43.38
	30 min	28.81	43.90
Microwave oven	5 min	26.51	41.28
	10 min	22.88	35.36
	15 min	16.79	23.21
40% NaCl	24 h	26.45	40.54
	48 h	26.49	40.56
	72 h	26.49	40.56

Table 4: Fatty acid composition of *N. oculata* FAME.

Lipid number	Common name	Systematic name	Molecular structure	Fatty acid (N^+) %	Fatty acid (N^-) %
C14:0	Myristic acid	Tetradecanoic acid	$C_{12}H_{24}O_2$	9.86	8.94
C16:0	Palmitic acid	Hexadecanoic acid	$C_{16}H_{32}O_2$	19.39	13.83
C18:0	Stearic acid	Octadecanoic acid	$C_{18}H_{36}O_2$	10.76	9.79
C18:1	Oleic acid	9-Octadecenoic acid	$C_{18}H_{34}O_2$	35.21	44.68
C18:2	Linoleic acid	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	8.15	6.92
C20:0	Arachidic acid	Eicosanoic acid	$C_{18}H_{30}O_2$	16.62	15.84

N^+: presence of nitrogen, N^-: absence of nitrogen.

lysis the effective cell disruption happened with enzymatic (cellulase) and ultrasonic disruption (Figures 4(c) and 4(d)).

3.7. Fatty Acid Composition Analysis by GC-MS. The major fatty acid composition of the extracted oil from the different cell disruption methods was determined using GC-MS system (Table 4).

Our experiments found out that various pretreatment methods had beneficial effects on the cell disruption of marine microalga *N. oculata* to extract oilwithout changing fatty acid composition. From the retention time obtained by GC-MS, peak values were analysed and observed as lauric acid (C12:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and arachidic acid (C20:0), which were commonly found in *N. oculata* oil (Table 2). However, under nitrogen starvation condition the lipid content not only doubled but also gradually changed the fatty acid composition of microalgae [3, 31]. In *N. oculata*, the oleic acid content increased from 35.21% to 44.68%. This result was in better agreement with previous study conducted by Zhila et al. [32].

Unsaturated fatty acids have been reported as reasonable balance of fuel properties [6]. The chain length of fatty acids in *N. oculata* was observed between C12 and C20. In a previous report, it was stated that the fatty acids with maximum of C16 and C18 series were recognized as the most common components of biodiesel [33]. Therefore, fatty acids from *N. oculata* were more applicable for producing high quality of biofuel, since it contained high content of C16 (palmitic acid) and C18 (oleic acid).

4. Conclusion

This paper demonstrated the utility of various pretreatment protocols to extract lipids by cell disruption. Amongst all the procedures, acid hydrolysis proved to be the appropriate method. Additionally, it was found that cells when under stressed condition, that is, nitrogen depleted state, produced

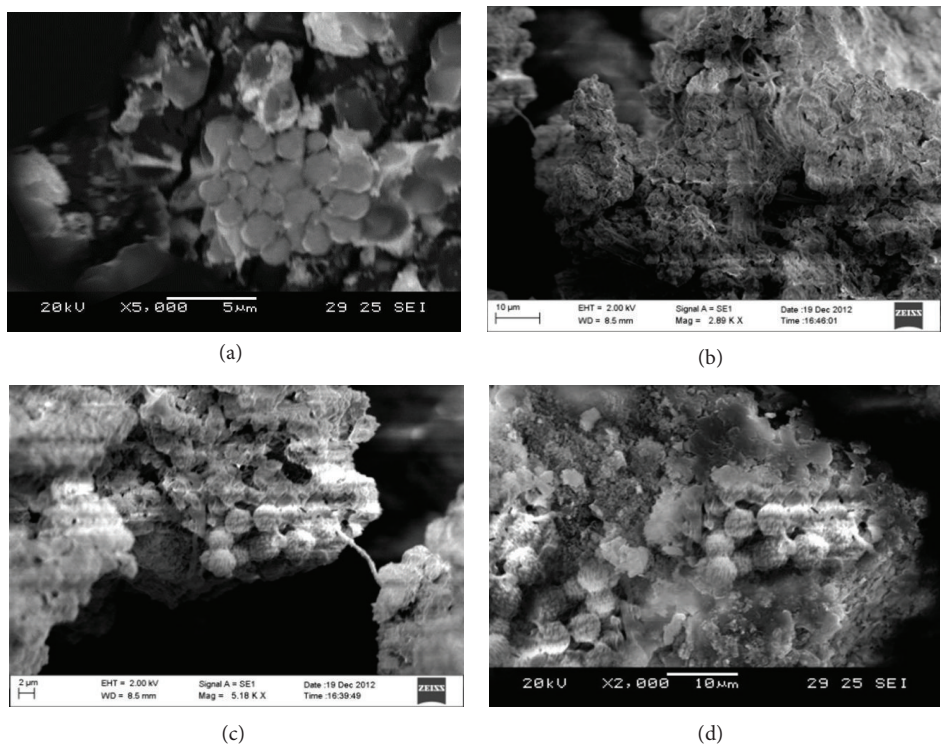


Figure 4: Scanning electron microscopic images of morphological analysis of various pretreatment processes. (a) Control, (b) acid pretreatment, (c) enzyme pretreatment, and (d) ultrasonication.

lipids at a higher rate due to the reversion of carbohydrate, protein metabolism to that of lipid, which was clearly depicted by FT-IR spectral analysis. This technique could be used for the large scale production and utilize lipids to generate biodiesel.

Conflict of Interests

The authors claim no conflict of interests.

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