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Technical note

# Comparison of pretreatment methods for total lipids extraction from mixed microalgae

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ABSTRACT

Cell disruption can increase the extraction efficiency of total lipids from microalgae for further conversion to biodiesel. Four different pretreatment methods were tested on mixed cultures of microalgae harvested in a stabilization pond system treating sewage: ultrasonication (US), microwaving (MW), autoclave (AC) and electroflotation by alternating current (EFAC). The best results in terms of total lipid yield were: MW (33.7 ± 5.3%), followed by EFAC (24.8 ± 7.1%), AC (15.4 ± 2.3%), and US (13.3 ± 3.0%). However, when both efficiency and costs are considered, EFAC gave the best result and can be an excellent option for simultaneous microalgae harvesting and cell disruption.



## 1. Introduction

The depletion of oil reserves, the resulting 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 [1–3]. Some of the promising alternative energy sources include biohydrogen, biodiesel, bioethanol and biomethane produced from various raw materials, including microalgae biomass [4–6]. Production of biodiesel from microalgae biomass presents some advantages: it can be produced year round (depending on climate and solar radiation); can grow at very high rates; can utilize of a wide variety of water sources (fresh, brackish, seawater and wastewater); and can be produced on marginal land, hence not competing for arable land used to produce food and the production of valuable co-products [6–9].

The lipid content of microalgal cells can vary from 2 to 77% depending on species and environmental/growth conditions [4,10,11]. Lipids extracted from microalgae may be converted into biodiesel with low energy consumption [9] by transesterification, the most common method [12]. Biodiesel can be used in conventional diesel engines without modification and can be mixed with petroleum diesel in any proportion, making it the preferred final product from microalgae [9,13].

Cultivation of microalgae in closed and controlled systems (photobioreactors) usually presents high costs and may not be economically feasible [14,15]. Microalgae cultivation in open systems such as waste stabilization ponds can be achieved at very low costs by using $CO_2$, water and nutrients readily available in sewerage [15]. Waste stabilization ponds can be a cheap option for microalgal biomass and biodiesel production [9,15]. However, their applicability depends on the local climate and land availability. In Brazil, a country with a tropical climate and vast land availability at a low cost, these systems are widely used [16]. For example, in the state of Ceará, located in the Northeast of Brazil, near the equator line, there are approximately 85 waste stabilization pond systems, which correspond to more than 80% of the sewage treatment systems in operation in the state. However, biomass separation and reuse are not widely used in pond systems and microalgae are often discharged directly into water bodies, representing potential hazards to the environment and to human health [17]. In order to effectively couple pollution control with biodiesel production from microalgae it is necessary to separate the biomass from the treated

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sewage and subsequently carry out biomass drying, cell wall disruption, lipid extraction and transesterification [18]. Biomass recovery has often been achieved by coagulation/flocculation, filtration, dissolved air flotation and centrifugation, and all these processes often present very high costs [2,19].

Oil and other intracellular products can be difficult to extract from wet biomass. Dewatering of microalgae is commonly performed to increase the shelf life of biomass feedstock in order to produce biofuels and to enhance the range of possible solvents. Some drying methods used include spray drying, drum drying, freeze-drying and sun drying [7].

Cell disruption can be used to enhance the release of lipids from algae and improving the access of the extracting solvent to fatty acids [4]. Ultrasonication, microwave, bead mill and autoclave pretreatments are the commonly used methods to promote vegetable cell disruption and have been tested in pure cultures of microalgae cells [20–23]. There are no reports in the literature of using electrolytic processes to promote microalgae cell disruption.

This work aims to compare microwave, ultrasonication and autoclave methods on the disruption of microalgae cells harvested from waste stabilization ponds as well as to propose an electroflotation by alternating current as a methodology which combine harvesting and cell disruption steps on this biomass.

## 2. Materials and methods

### 2.1. Microalgae harvesting and identification

Microalgae biomass was harvested from a stabilization pond system treating sewage composed of a mechanically aerated facultative pond, followed by a secondary facultative pond and two maturation ponds in series. The ponds were located in the city of Fortaleza, state of Ceará, Brazil. Samples were collected in the last maturation pond, near the outlet, using a plankton nylon net with a 20 μm opening. Although some microalgae have smaller sizes than the openings used, the filtered volume enables the collection of many species at random, allowing the study of phytoplankton diversity.

Samples were placed in a sterile glass container and fixed with *Transeau* solution (6 parts of water, 3 parts of 95% ethanol and 1 part of formaldehyde) in a 1:1 ratio (effluent:*Transeau*).

Identification of the dominant genus was carried out in quintuplicate with a trinocular optical microscope (L-1000T, Bioval, Brazil) and taxonomic identification guides [24,25].

The *Transeau* solution was used only to preserve the morphology of microalgal specimens and these samples were used exclusively in the microalgae identification assays.

### 2.2. Pretreatment methods

Biomass was harvested from the maturation pond and concentrated to 4 $g \cdot L^{-1}$ using a 20-μm nylon plankton net. Drying was carried out by lyophilization (Liotop, L202, Brazil).

To perform the pretreatments in the modified Bligh and Dyer method, experiments were divided into two blocks, according to Fig. 1. According to Halim et al. (2012) [4], the microalgae cell disruption process step may take place before or after biomass drying, as some methods require a certain amount of water in the biomass to be successful, while others are more efficient with dry biomass. For the group of dried samples, lyophilization was

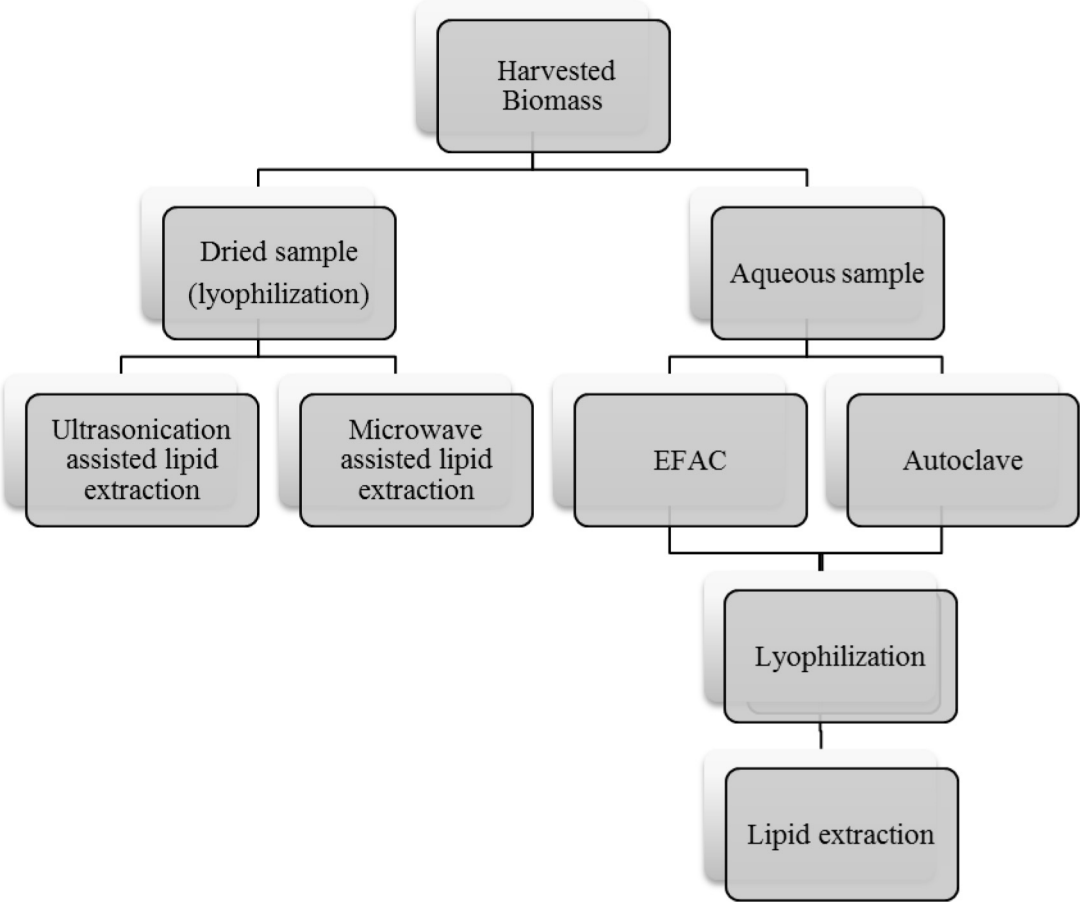


**Fig. 1.** Experimental flow diagram.

performed before cell disruption by ultrasonication and microwave. For the wet biomass group, samples were lyophilized after cell disruption by EFAC and autoclave.

For the control group, cell disruption was not performed. Lipids were extracted by the modified method of Bligh and Dyer (1959) [26]. All samples were analyzed in triplicate, with lipid extraction starting from 500 mg of dried biomass.

### 2.3. Pretreatment of dried biomass: ultrasonication and microwaving

Ultrasonic assisted extraction was carried out using a 80 W ultrasonic processor (Ultra cleaner, 1600A, Unique, Australia) at 40 KHz of frequency. Biomass (500 mg) was re-suspended with 2.5 mL of methanol, 1.25 mL of chloroform and 1 mL of deionized water and then brought to the ultrasonic bath for 40 min. Then, 1.25 mL of chloroform and 1.25 mL of 1.5% sodium sulfate were added and the solution was kept an additional 20 min in the ultrasonic bath.

Microwave assisted extraction was carried out by a 400 W microwave oven (*Mars 5*, *CEM* Corporation, USA). Each 500 mg biomass was diluted with 2.5 mL of methanol, 2.5 mL of chloroform, 1.25 mL of 1.5% sodium sulfate and 1.0 mL of deionized water and then brought to the microwave oven to be heated for 3 cycles. Each cycle used a 70 s radiation ramp temperature stage to reach 100 °C and 45 s at the hold stage. The amount of biomass used during the microwave trial was 1.5 g.

After each pretreatment, samples were centrifuged at 1.000 rpm (Excelsa II 206 BL, Famen, Brazil) for 2 min and the pellet was vacuum filtered. The filtrate was taken to an oven at 100 °C until constant weight.

### 2.4. Pretreatment of wet biomass: electroflotation by alternating current (EFAC) and autoclave

The electrolytic process applied (by alternating current) in this study is based on the principle of superposition of waves, causing a resonance phenomenon [27]. In the frequency range used, a resonance is obtained with the natural frequency of the water molecule (1643.5 $cm^{-1}$, 2127.5 $cm^{-1}$, 3404.0 $cm^{-1}$) which breaks the atoms connections. The fragments $H^+$ and $O^{-2}$ are very reactive and promote the formation of oxidant species ($O_3$, $H_2O_2$ and −OH) which may effectively act on cell disruption.

An electrolytic reactor was developed for the EFAC experiments. The cathode and anode were made of five 316L-stainless steel bars, resistant oxidative action, measuring 15 × 5 cm, thickness 0.2 mm, and spaced at 5 mm.

In electrolysis using alternating current (AC), an electrode is alternately positive (anode) and negative (cathode). Although the motion of an ion in solution is always uncertain, the presence of an electric field inserts a guided movement component and the ions migrate through the solution.

A direct current/alternating current (DC/AC) converter (HY 125 Hobby, Hayama, Brazil) was used applying a voltage of 12 V and a maximum current of 5 A to generate a frequency band from 0 to 1.5 KHz, corresponding to a range of 0–4.000 $cm^{-1}$ wave numbers. Experiments were conducted in batch mode. The amount of biomass used during the EFAC trial in a working volume of 2.9 L was 3.6 g.

The autoclave process was carried out at 100 °C for 10 min in a mini-reactor (BR-300, Berghof, Germany) for 200 mL sample volume. After maintaining samples at the set temperature for 10 min, the reactor was opened; the Teflon vessel was removed and cooled to ambient temperature.

Lyophilization was performed after each pretreatment in this block. Dried biomass (500 mg) was diluted with 2.5 mL of methanol, 1.25 mL of chloroform and 1 mL of deionized water and then homogenized in a shaker for 20 min, after which 1.25 mL of chloroform and 1.25 mL of 1.5% sodium sulfate was added and the solution was held to an additional 2 min of homogenization.

The homogenized sample was centrifuged at 1.000 rpm for 2 min (Excelsa II 206 BL, Famen, Brazil). After that, the chloroform–methanol phase which contains the extracted lipids was separated from the microalgae powder by filtration using a funnel with mild suction followed by the evaporation of the solvent in an oven at 100 °C until constant weight was achieved. The mass of the lipid obtained from each sample was determined gravimetrically.

### 2.5. Data analysis

The efficiency of each pretreatment method was determined based on the lipid yield which was calculated by the ratio of total lipid extracted and the initial weight of microalgae. Additionally, comparison of methods was performed, taking into account the efficiencies, applicability and energy consumption.

In order to verify any significant differences amongst the parameters, the software Statgraphics® Centurion XV (StatPoint, USA) was used. As the number of samples was small, it was not possible to verify their normality. For this reason, the more conservative non-parametric tests of Kruskal–Wallis ANOVA and Mann–Whitney were applied. The evaluation was made based on *p*-values, using a 95% confidence interval.

Descriptive statistics was performed using the softwares Statgraphics® Centurion XV (StatPoint, USA) and Microcal Origin 8.6 (Originlab, USA).

## 3. Results and discussion

### 3.1. Microalgae identification in the stabilization pond effluent

In analyzed samples, 12 genera of algae were identified in the pond effluent and divided into four classes (Table 1). Chlorophyceae and Cyanophyceae showed the highest number of taxa, the first group with five and the second with four, followed by Euglenophyceae with two genera and Bacillariophyceae with one.

The genera found in this study are common in eutrophic environments and have been reported in some studies [16,28–31]. These authors also reported the classes Cyanophyceae and Chlorophyceae as the dominant group, since they are well adapted to polluted waters.

### 3.2. Comparison of pretreatments for lipid yield from microalgal biomass

Statistical analyses showed no significant differences between the pretreatments applied, except for the microwave method

**Table 1**
Main genera found in microalgae harvested in the stabilization pond.

| Class | Genus |
|---|---|
| Chlorophyceae | *Micractinium*<br>*Eudorina*<br>*Chlorella*<br>*Pandorina*<br>*Carteria* |
| Cyanophyceae | *Aphanocapsa*<br>*Microcystis*<br>*Oscillatoria*<br>*Planktothrix* |
| Euglenophyceae | *Hyalophacus*<br>*Phacus* |
| Bacillariophyceae | *Cyclotella* |

which was significantly different to ultrasonication ($p = 0.004$) and autoclave ($p = 0.005$). In general, microwaving and EFAC presented the highest lipid yields, with percentages of 33.7 ± 5.3% and 24.8 ± 7.1%, respectively ($p = 0.15$). These methods were followed by autoclave (15.4 ± 2.3%) and ultrasonication (13.3 ± 3.0%). In the absence of pretreatment, total lipid extraction reached 4.8% efficiency, showing significant differences with all methods evaluated (Fig. 2).

Previous studies have shown better lipid yields by using microwave as compared to other pretreatment methods. Koberg et al. (2011) [5] compared the efficiency of microwaving with ultrasonication in lipid extraction from *Nannochloropsis* using an ultrasonicator with a frequency of 20 KHz for 5 min and a microwave oven operating at 2.45 GHz for 5 min at 70% power (cycle mode of 21 s on and 9 s off). The authors attributed these results to temperature increases that occurred under microwave radiation. Lee et al. (2010) [22] compared five methods (autoclave, bead-beating, microwave, ultrasonication and osmotic shock), and reported that microwaving, at 100 °C and 2.45 GHz for 5 min, was the most efficient method in extracting lipids from all species of microalgae studied (*Botryococcus* sp., *Chlorella vulgaris* and *Scenedesmus* sp.).

However, other authors have reported contrasting results. Prabakaran and Ravindran (2011) [23] described ultrasonication (using a sonicator at a resonance of 50 Hz for 15 min) as the most efficient among five cell disruption methods tested, including microwaving, for extracting lipids from *Chlorella*. Lee et al. (2010) [22] tested autoclaving and microwaving on three different microalgae species and showed that autoclaving outperformed microwaving for lipid extraction from *Chlorella vulgaris*, whereas microwaving outperformed autoclaving when *Botryococcus* sp. and *Scenedesmus* sp. species were used. The results reported by Lee et al. (2010) [22] suggest that lipid extraction efficiency from microalgae depends on the microalgae species and pretreatment methods involved.

The vast majority of open systems used for microalgae production generate biomass with diverse microalgae communities, especially the ones treating wastewater [32]. For such samples it is very difficult to determine the contribution of each species to the total lipid yield and this highlights the need for more investigations on appropriate extraction methods from complex samples. Wahlen et al. (2011) [8] achieved a lipid content of 14.4%, using ultrasonication for 30 s from microalgae collected from a wastewater

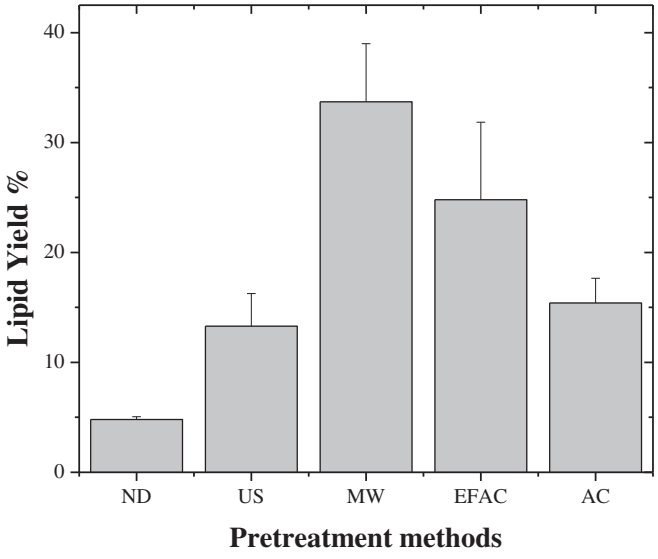


**Fig. 2.** Lipid extraction efficiencies for different pretreatment methods. ND – no disruption; US – ultrasonication; MW – microwave; EFAC – electroflotation by alternating current; AC – autoclave.

**Table 2**
Summary of recent studies comparing pretreatment methods for lipid extraction from different microalgal cultures.

| Microalgal culture | Pretreatments | Lipid yield (%) | Reference |
|---|---|---|---|
| *Nannochloropsis* | Microwave | 32.8 | Koberg et al. (2011) Ref. [5] |
| | Ultrasonication | 18.9 | |
| (Pure cultures) *Botryococcus* sp., *Chlorella vulgaris*, *Scenedesmus* sp. | Autoclave | 5.4–11.9 | Lee et al. (2010) Ref. [22] |
| | Microwave | 10–28.6 | |
| | Ultrasonication | 6.1–8.8 | |
| *Chlorella* sp. | Microwave | 38 | Prabakaran and Ravindran (2011) Ref. [23] |
| | Autoclave | 24 | |
| | Ultrasonication | 40 | |
| Mixed culture from a raceway pond | Ultrasonication | 14.4 | Wahlen et al. (2011) Ref. [8] |
| Mixed culture from stabilization pond | Microwave | 33.7 | This study |
| | Autoclave | 15.4 | |
| | Ultrasonication | 13.3 | |
| | EFAC | 24.8 | |

treatment plant. This value is close to the result obtained in the present study for ultrasonic-assisted extraction (13.3%), although the applied protocols have been significantly different.

Table 2 lists the major results for lipid yield achieved from different microalgal cultures by the above-mentioned authors in comparison with the results achieved in the present study. The current paper describes the first attempt at using EFAC to harvest and disrupt microalgae cells and therefore no direct comparison with previous studies can be made.

## 4. Conclusions

All cell pretreatment methods analyzed were applicable to microalgal biomass originating from waste stabilization ponds, showing lipid extraction efficiencies significantly higher than the control. Microwaving and EFAC achieved the highest lipid extraction efficiencies and showed no statistically difference between each other.

The authors are currently working on further tests that will guide the scaling-up of EFAC for biodiesel production.

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