Effect of harvesting processes on the lipid yield and fatty acid profile of the marine microalga *Nannochloropsis oculata*

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A B S T R A C T

The effect of different methods of biomass concentration and salt removal on the lipid yield and fatty acid profile of the marine microalga *Nannochloropsis oculata* was evaluated. The microalgae were subjected to nine treatments: (1) centrifugation, (2) centrifugation + washing with H_2O, (3) centrifugation + washing with ammonium formate, (4) flocculation with NaOH, (5) flocculation with NaOH + washing with H_2O, (6) flocculation with NaOH + washing with ammonium formate, (7) flocculation with NaOH and neutralization with HCl, (8) flocculation with NaOH, neutralization with HCl + washing with H_2O, and (9) flocculation with NaOH, neutralization with HCl + washing with ammonium formate. Flocculation with the addition of NaOH was effective at concentrating biomass (>90%) but interfered with lipid extraction because it exhibited a lower lipid yield ($4.40 \pm 0.1\%$) compared with centrifugation ($45.4 \pm 0.8\%$). Moreover, important polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA C20:5) and eicosatetraenoic acid (ETA C20:4), disappeared when NaOH was used. Centrifugation plus washing the biomass with ammonium formate was more effective than other treatments, leading to higher lipid yields, as well as larger amounts of the polyunsaturated fatty acids EPA and ETA. However, it is a very expensive harvesting method. Thus, the main finding of the present study is that microalgae concentration processes can affect lipid and fatty acid extraction in massive microalgae production; therefore, it is necessary to carefully choose the harvest process to be used in large-scale microalgae production.

1. Introduction

Microalgae have recently received much attention because they have been recognized as an alternative source for renewable energy production. These organisms can be used for biodiesel production due to their high lipid productivity (Chisti, 2007). However, there are some barriers in different phases of the massive production process. One of the "bottlenecks" is the lack of an effective method for harvesting and concentrating the generated biomass because the cells are small and normally diluted in large volumes of water (Uduman et al., 2010).

Currently, microalgae harvesting involves mechanical, chemical, biological and electrical methods or a combination of two or more of these processes (Barros et al., 2015). The well-established mechanical processes of dewatering, such as filtration and centrifugation, are energetically expensive and require high operational costs (Uduman et al., 2010). Chemical processes (coagulation/flocculation) are the main option to concentrate large volumes of biomass at a relatively low cost (Barros et al., 2015). Adding metals, bases and polymers to the culture medium, causes cells to coagulate and form flocs that are easily separated through sedimentation or flotation (Uduman et al., 2010).

Coagulation results from the addition of electrolytes or the adjustment of the pH, which destabilizes the negative charges on the microalgae cell walls or membranes and reduces electrostatic repulsion. Flocculation may occur in three ways: by electrostatic patch, when a charged polymer binds to an oppositely charged particle; by bridging, when polymers create a bridge by linking the surface of two particles; and by sweeping, when particles get trapped in a mineral precipitation (Vandamme et al., 2013). Ideally, chemical coagulation/flocculation should be uncontaminated, non-toxic and highly efficient and should allow the reuse of the culture medium with low environmental impact (Grima et al., 2003).

The latest trend in microalgae harvesting is autoflocculation by pH increase (Brady et al., 2014). This inexpensive method consumes a small amount of energy, and is non-toxic to microalgal cells. Thus, the biomass can be further used as food in aquaculture

Table 1
Lipid yield (mean% ± SD) obtained after harvesting *Nannochloropsis oculata* biomass using different treatments.

	Treatments	Lipid
1	Centrifugation	21.4 ± 1.2^c
2	Centrifugation and washing with H_2O	30.8 ± 2.5^b
3	Centrifugation and washing with formate	45.4 ± 0.8^a
4	Flocculation with NaOH	5.3 ± 0.7^e
5	Flocculation with NaOH and washingd with H_2O	3.5 ± 0.1^e
6	Flocculation with addition of NaOH and washed with formate	4.4 ± 0.1^e
7	Flocculation with of NaOH and neutralized with HCl	11.1 ± 1.2^d
8	Flocculation with addition of NaOH, neutralized with HCl and washing with H_2O	10.9 ± 1.4^d
9	Flocculation addition of NaOH, neutralized with HCl, and washing with formate	21.9 ± 0.0^c

Different letters indicate significant differences among processes ($p < 0.05$).

or as feedstock in food and pharmaceutical industries. The method also avoids the use of flocculants (Vandamme et al., 2013). In marine cultures, when the pH is raised above 10, algae autoflocculate due to electrostatic interactions between anionic algae and divalent cations, with the formation of calcium or magnesium precipitates (Vandamme et al., 2013, 2015; Brady et al., 2014). This process can occur naturally, associated with an increase in pH due to photosynthetic carbon dioxide consumption (Uduman et al., 2010; Sales and Abreu, 2015), or by adjusting the pH in the culture medium with bases such as sodium hydroxide (NaOH) (Knuckey et al., 2006). Despite the high efficiency of NaOH for flocculating microalgae biomass (Vandamme et al., 2012, 2015; Wu et al., 2012, 2015; Besson and Guiraud 2013; Pirwitz et al., 2015), no information is available about the possible effects of this compound on marine microalgae biochemical composition, particularly on the total lipid concentration and the fatty acid profile, major co-products of the microalgae biomass.

Another constraint in the production of biodiesel from marine microalgae is the presence of salts in the biomass. The salt content overestimates the total biomass weight, affecting the lipid yield and fatty acid calculations. Moreover, the presence of salts may negatively affect further microalgae biomass processing for the production of bioproducts. Thus, washing the salts in the biomass is recommended (Zhu and Lee, 1997).

The objective of this study was to evaluate if different methods of concentration and inorganic salt removal could affect the lipid yield and fatty acid profile of the marine microalga *Nannochloropsis oculata* biomass.

2. Materials and methods

2.1. Inoculum

The microalgae *Nannochloropsis oculata* (Eustigmatophyceae) used in this study was obtained from the microalgae collection at the Phytoplankton and Aquatic Microorganisms Laboratory of the Federal University of Rio Grande (Catalogue name NANN OCUL-1). The microalgae was grown in open 2000 L raceways until the cell abundance was approximately 3000×10^4 cells mL^{-1}. The cells were cultivated at salinity 34 ± 1, pH 8.5 ± 0.4, temperature $19.4 \pm 4.6\,^{\circ}$C, and with a mean sunlight of $210.9 \pm 49.7\,\mu$mol m^{-2} s^{-1}. The medium used was natural seawater enriched with ammonium sulfate, urea, calcium superphosphate, iron chloride and a vitamin mix (cyanocobalamin, biotin and thiamine), adapted from Yamashita and Magalhães (1984).

2.2. Experimental design

The experiment was performed at the Aquaculture Marine Station at the Institute of Oceanography of the Federal University of Rio Grande (FURG) using a completely randomized design. Nine treatments were performed in triplicates, using following processes to harvest the algae biomass and to wash the inorganic salts: (1) centrifugation – 6 L of biomass concentrated through centrifugation; (2) centrifugation + H_2O – 6 L of biomass concentrated through centrifugation and then washed with distilled water to eliminate salts; (3) centrifugation + formate – 6 L of biomass concentrated through centrifugation and then washed with a 0.5 M solution of NH_4HCO_2 (ammonium formate) to eliminate salts; (4) flocculation + NaOH – 3 L of culture flocculated after a pH increase with the addition of a 6 M NaOH solution; (5) flocculation + NaOH + H_2O – 3 L of culture flocculated after a pH increase with the addition of a 6 M NaOH solution, and elimination of salts by washing the concentrated biomass with distilled water; (6) flocculation + NaOH + formate – 3 L of culture flocculated after a pH increase with the addition of a 6 M NaOH solution, and elimination of salts by washing the biomass with a 0.5 M NH_4HCO_2 solution; (7) flocculation + NaOH + HCl – 3 L of culture flocculated after a pH increase with the addition of a 6 M NaOH solution and further neutralization of the sample by the addition of a 6 M HCl solution; (8) flocculation + NaOH + HCl + H_2O – 3 L of culture flocculated after a pH increase with the addition of a 6 N NaOH solution, further neutralization of the sample by the addition of a 6 N HCl solution, and elimination of salts with distilled water; and (9) flocculation + NaOH + HCl + formate – 3 L of culture flocculated after a pH increase with the addition of a 6 N NaOH solution, further neutralization of the sample by the addition of a 6 N HCl solution, and elimination of salts by the addition of a 0.5 M NH_4HCO_2 solution.

2.3. Biomass concentration

The biomass was concentrated from 36 L of a *Nannochloropsis oculata* massive culture. This volume was distributed among the different treatments in sub-samples. In treatments 1, 2 and 3, the biomass was concentrated in a Fanem® Excelsa 4 (model 280R) centrifuge at 2276 g for 25 min. In treatments 4, 5 and 6, the biomass was concentrated by increasing the pH: a few milliliters of a 6 M NaOH solution were added to each sample with constant stirring until the pH = 10. The cultures were stirred until a white precipitate appeared. Then, the stirring was stopped, and the cultures were maintained at rest for approximately 1 h until the biomass settled. Treatments 7, 8 and 9, followed the same process as treatments 4 to 6; however, after the biomass settled, the remaining solution (supernatant) was discarded, and a 6 M HCl solution was added until the pH returned to 8.

The concentrated biomass was placed on a cellulose filter for washing to eliminate inorganic salts. In treatments 2, 5 and 8, the biomass was washed with distilled water; in treatments 3, 6 and 9, the biomass was washed with a 0.5 M NH_4HCO_2 solution (Zhu and Lee, 1997). In treatments 1, 4 and 7, the biomass was not washed.

Table 2
Total fatty acid composition (mean% ± SD of total lipids) obtained after harvesting *Nannochloropsis oculata* biomass, using different treatments: (1) Centrifugation; (2) Centrifugation washed with H_2O; (3) Centrifugation washed with formate; (4) Flocculation with addition of NaOH; (5) Flocculation with addition of NaOH and washed with H_2O; (6) Flocculation with addition of NaOH and washed with formate; (7) Flocculation with addition of NaOH and neutralized with HCl; (8) Flocculation with addition of NaOH, neutralized with HCl and washed with H_2O; (9) Flocculation with addition of NaOH, neutralized with HCl, and washed with formate.

Treatments	C14:0	C15:0+C17:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:4	C20:5
1	7.3 ± 0.1[a]	1.1 ± 0.1[a]	32.7 ± 0.2[a]	24.7 ± 0.2[a]	0.9 ± 0.0[a]	14.1 ± 0.0[a]	4.1 ± 0.0[a]	2.5 ± 0.0[a]	12.1 ± 0.3[a]
2	6.8 ± 0.5[b]	1.2 ± 0.1[a]	22.7 ± 2.9[b]	21.5 ± 0.3[b]	0.6 ± 0.3[b]	13.4 ± 0.5[a]	4.9 ± 0.0[b]	4.9 ± 1.0[b]	24.2 ± 3.3[b]
3	7.9 ± 0.1[c]	0.9 ± 0.1[b]	28.8 ± 0.0[c]	22.2 ± 0.0[c]	1.1 ± 0.0[a,c]	14.4 ± 0.0[a]	4.8 ± 0.0[b]	2.8 ± 0.0[c]	17.2 ± 0.1[c]
4	15.9 ± 0.1[d]	0.0[c]	60.8 ± 0.1[d]	6.3 ± 0.2[d]	2.6 ± 0.3[d]	12.0 ± 0.7[a]	2.5 ± 0.0[c]	0.0[d]	0.0[d]
5	8.1 ± 0.1[c]	0.0[c]	49.1 ± 0.5[e]	17.3 ± 0.6[e]	2.7 ± 0.2[d]	18.8 ± 1.1[b]	4.1 ± 0.2[a]	0.0[d]	0.0[d]
6	10.5 ± 0.0[e]	0.8 ± 0.1[b]	58.9 ± 0.2[f]	14.4 ± 0.0[f]	1.9 ± 0.3[e]	11.2 ± 0.4[c]	2.4 ± 0.0[c]	0.0[d]	0.0[d]
7	6.4 ± 0.2[f]	0.4 ± 0.1[d]	26.5 ± 0.1[g]	22.1 ± 0.2[c]	1.3 ± 0.0[c]	15.7 ± 0.2[d]	4.9 ± 0.1[b]	2.0 ± 0.0[e]	20.5 ± 0.1[e]
8	4.5 ± 0.1[g]	0.4 ± 0.0[d]	22.2 ± 0.2[h]	21.3 ± 0.2[b]	0.8 ± 0.0[a,b]	14.7 ± 0.0[a]	5.2 ± 0.0[d]	3.2 ± 0.0[f]	27.6 ± 0.3[f]
9	7.6 ± 0.1[a]	0.5 ± 0.0[d]	22.8 ± 0.2[b]	21.2 ± 0.1[b]	1.0 ± 0.1[a]	13.3 ± 0.1[a]	4.6 ± 0.2[b]	4.1 ± 0.0[g]	25.0 ± 0.4[g]

Different letters in the same column indicate significant differences between processes ($p < 0.05$).
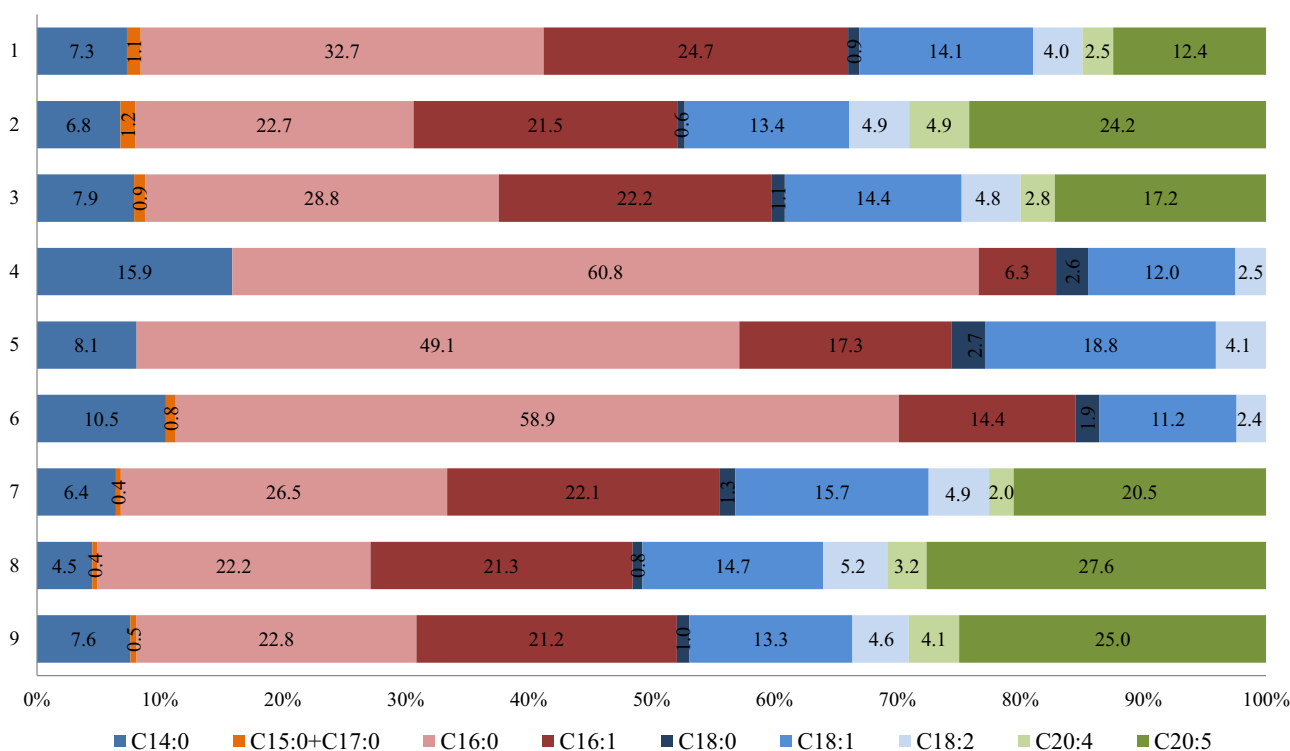

Fig. 1. Fatty acids profile of *Nannochloropsis oculata* for each treatment (% of total fatty acids). (1) Centrifugation; (2) Centrifugation washed with H_2O; (3) Centrifugation washed with formate; (4) Flocculation with addition of NaOH; (5) Flocculation with addition of NaOH and washed with H_2O; (6) Flocculation with addition of NaOH and washed with formate; (7) Flocculation with addition of NaOH and neutralized with HCl; (8) Flocculation with addition of NaOH, neutralized with HCl and washed with H_2O; (9) Flocculation with addition of NaOH, neutralized with HCl, and washed with formate.
*The results relate to the average of three repetitions.

2.4. Lipid extraction and quantification

Lipids were extracted from the dry biomass (dried at 60 °C for 24 h) using a method adapted from the literature (Bligh and Dyer 1959; Zhu et al., 2002; Montes D'Oca et al., 2011). Dry biomass (0.5 g) was crushed and placed into test tubes (in three replicates) with 1.5 mL of a chloroform-methanol (2:1) mixture. The solution was ultrasonicated for 20 min. Next, the mixture was centrifuged for 2 min at 1851g. The lipid extraction process was repeated three times for each tube. The liquid phase was transferred to previously weighed flasks. Afterwards, the solvent was evaporated under vacuum in a rotary evaporator, and the flasks were then reweighed. The total lipid fraction was determined based on the flask weight differences, and the lipid content was calculated based on the percentage of the initial dry weight.

2.5. Fatty acid profiles

The lipid samples (in triplicates) were esterified using a method described in the literature (Metcalfe and Schmitz, 1961). First, the sample was placed in a tube with 2 mL of a BF_3 MeOH solution. The mixture was heated at 70 °C for 20 min. To recover the derivatized esters, the mixture was extracted into a separatory funnel with 15 mL of hexane and 20 mL of distilled water. The separatory funnel was shaken vigorously and then left undisturbed until the organic and aqueous phases separated. The aqueous phase was discarded. The organic phase containing the fatty esters was filtered, and the solvent was evaporated.

The fatty acid profile was characterized using gas chromatography mass spectrometry (GC–MS)-based detection. Analyses were performed using a GCMS-QP2010 Plus chromatographic system equipped with a split/splitless injector coupled with a mass detec-

tor. The detector operating temperatures were 280 °C for the interface and 230 °C for the source. Detection was performed using a full scan from *m/z* 30 to *m/z* 500 with the scan time of 0.20 s. The ionization mode electron impact was at 70 eV. The operating conditions of the chromatograph were as follows: injector, 250 °C; column, 80 °C (initial temperature, 0 min), followed by a gradient of 10 °C/min to 180 °C and then 7 °C/min to the final temperature of 330 °C; gas flow, 1.3 mL/min; pressure, 88.5 kPa; average linear velocity, 42 cm/s; and 1 mL injection volume with the split ratio 1:100. A Crossbond 5% dimethyl polysiloxane diphenyl 95% column (30 m × 0.25 mm × 0.25 μm Restek) was used. The fatty acid methyl esters were identified by comparison with known standards and were quantified using the standardized areas method.

2.6. Statistical analysis

Data normality and homoscedasticity were evaluated using Kolmogorov-Smirnov and Cochran's tests, respectively. Differences among treatments were determined by a one-way ANOVA ($\alpha = 0.05$), followed by Tukey's post-hoc multiple comparison test for the lipid content, and Duncan's post-hoc multiple comparison test ($\alpha = 0.05\%$) for the determination of differences in fatty acids in each treatment (Zar, 1996).

3. Results

Treatments in which the biomass was harvested by centrifugation (1, 2, 3) showed a higher lipid yield (Table 1). The biomass concentrated through centrifugation followed by washing with ammonium formate achieved the highest lipid yield ($45.42 \pm 0.8\%$).

Flocculation with NaOH was effective at concentrating biomass, with more than 90% efficiency. However, treatments that were not neutralized (4, 5, 6) resulted in significantly lower lipid contents (3.46–5.29%) than the other treatments (Table 1). The treatments that were flocculated with NaOH and neutralized by the addition of HCl (7, 8, 9), regardless of the washing method, showed higher lipid yields than the treatments where the NaOH was not neutralized (Table 1). In general, treatments in which the biomass was washed with ammonium formate showed higher lipid yields compared with treatments using distilled water, or no washing (Table 1).

The most abundant fatty acids in all of the *N. oculata* profiles were C16:0, C16:1, C18:1 and C20:5. The samples that were centrifuged or flocculated with NaOH and neutralized by the addition of HCl, showed higher percentages of long-chain polyunsaturated fatty acids (Table 2). Important polyunsaturated fatty acids, such as EPA (eicosapentaenoic acid, C20:5) and ETA (eicosatetraenoic acid, C20:4), were not found in treatments flocculated with NaOH (4, 5, 6); however, the highest values of C20:5 occurred in treatments where cells were flocculated with NaOH followed by neutralization with HCl (7, 8, 9) (Fig. 1). The percentage of saturated fatty acids, such as C14:0, C16:0 and C18:0, was significantly higher in treatments using only NaOH (Table 2).

4. Discussion

The results of our study demonstrate that NaOH highly interferes with the lipid extraction and the fatty acid profile of the biomass of *N. oculata*. The measurements taken from samples treated with NaOH (regardless of the washing method) showed a lower lipid yield. However, this was not the case in treatments where NaOH was neutralized by a strong acid (HCl), leading to high lipid yields. We hypothesize that NaOH interacts with the biomass, binding to the cell membrane/wall and forming a "layer" that prevents organic solvent activity and membrane lipid extraction, perhaps due to the saponification of these lipids. Thus, the

HCl probably neutralizes the saponification products. However, the action of the HCl is only partial because the neutralized treatments did not yield the same results as those observed in the centrifugation treatments. Therefore, although NaOH is an efficient and low-cost flocculant (Wu et al., 2012; Besson and Guiraud 2013; Pirwitz et al., 2015), this compound can reduce the lipid yield and affect the fatty acid profile of the marine microalgae biomass.

The bound NaOH affected the fatty acid composition, leading to larger amounts of saturated fatty acids in samples where NaOH was added and not neutralized with HCl. Samples flocculated with NaOH did not present the polyunsaturated C20:4 and C20:5 fatty acids, which are membrane-forming elements in *N. oculata* (Sukenik et al., 1993) and which have high market value mainly due to their use in the food and pharmaceutical industries (Chauton et al., 2015). As observed in other studies (Borges et al., 2011; Alves Sobrinho et al., 2015), the most abundant fatty acids in all of the *N. oculata* profiles were C16:0, C16:1, C18:1 and C20:5. However, the proportion of C16:0, which is a reserve lipid (triglyceride) in this species (Sukenik et al., 1993), was higher in treatments using only NaOH.

In a previous study, Borges et al. (2011) evaluated the effect of cationic and anionic flocculants in the lipid and fatty acid profile of *N. oculata*. In that study, NaOH was used only to adjust the pH before the addition of the polyacrylamides. The lipid content (approximately 4%) was similar to that obtained from treatments using NaOH in the present study. Perhaps the use of NaOH, prior to the addition of flocculants, may have contributed to these low values.

In the present study, higher lipid yields where obtained from samples washed with ammonium formate. It is likely that washing the concentrated biomass with ammonium formate, which is an isotonic solution, removed inorganic salts without altering the osmotic pressure, contrary to distilled water, which could rupture the cells and thus decrease the lipid levels. Moreover, the presence of salts overestimates the biomass weight, affecting the lipid yield calculation. Thus, washing the salts with ammonium formate allows the correct determination of the microalgae biomass and, consequently, of the lipid yield, as reported in the literature (Zhu and Lee, 1997) and confirmed herein.

In conclusion, our results suggest that in the flocculation process, NaOH interferes with the lipid extraction and the fatty acid profile of the microalgae biomass. Although it is an effective method for harvesting microalgae, the use of NaOH should be carefully considered. Moreover, it was observed that the lipid content could be underestimated by the presence of salts in the dried biomass because the salt content overestimates the total concentrated biomass.

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