

Comparison of lipid extraction methods for the microalgae *Acutodesmus obliquus*

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Abstract: Microalgae are widely used in the pharmaceutical and energy industries, therefore the conditions for their cultivation and extraction methods play an important role in the profiling and acquisition of lipids. The efficiency of lipid extraction from microalgae has attracted great interest from industry because of the wide variety of lipids and amounts that can be obtained. *Acutodesmus obliquus* (*Scenedesmus obliquus* UTEX 393) was used in this study. It was cultivated in Bold 3N medium modified with 75% nitrogen at 25 °C, pH 6.8, 125 r/min and a photoperiod of 18/6 h and illuminated with white light provided by a Light-Emitting Diode Surface Mount Device extensions (LED SMD) with an intensity of 1200 $\mu\text{E}/(\text{m}^2 \text{ s})$. The cells were stained with the Red Nile (RN) technique to indicate lipid production. Four extraction methods were compared, classical, microwave (MW), Soxhlet, and ultrasound (US), using the same solvent proportions (hexane: chloroform: methanol=1:2:3). All samples were analyzed with Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography coupled to Mass Spectrometry (GC-MS). The results showed: 1) lipid production detected by RN was consistent with microalgal growth; 2) the MW technique was the best extraction method, according to the statistical analysis through Randomized Complete Block (RCB) design and performance of 4.6%; and 3) the presence of saturated and unsaturated acids was indicated by FTIR spectra. GC-MS was able to identify palmitic and linoleic acids as the likely major constituents of the sample.

Keywords: *Acutodesmus obliquus*, lipid extraction, trans-esterification, Red Nile (RN)

DOI: 10.25165/ijabe.20181105.3748

Citation: Hurtado D X, Garzón-Castro C L, Cortés-Romero J, Tello E. Comparison of lipid extraction methods for the microalgae *Acutodesmus obliquus*. Int J Agric & Biol Eng, 2018; 11(5): 211–217.

1 Introduction

Microalgae are unicellular organisms that assimilate CO₂, nitrogen (N) and phosphorus (P) to form their biomass^[1]. They contain significant amounts of micronutrients, such as proteins, carbohydrates and lipids, as well as pigments, polyphenols and minerals. These metabolites can be transformed into products of interest of the energy^[2,3], nutraceutical^[4], cosmetic^[5] and food industries^[6]. Additionally, they are used in diverse applications related to environmental restoration^[7,8].

One of the principal difficulties in harvesting lipids from microalgae is the extraction procedure^[9]. The establishment of an adequate extraction method for different applications is not an easy task because it requires chemical or physical techniques, thermal treatment and the use of special solvents^[10]. A wide variety of extraction methods exist; the selection of one is based on efficiency,

cost-efficiency, ease of application, precision and reproducibility^[11]. Because of this, the scientific community is interested in the development of efficient and economical extraction techniques for lipids, mainly because of their great applicability in biofuel production^[9].

The objective of this study was the establishment of the best method for extracting lipids from the microalgae *A. obliquus* due to its biomass potential for the biodiesel production, as well as, the higher potential to be used as lipid producer.

2 Materials and methods

2.1 Microorganism and crop medium

The green microalgae *A. obliquus* (*Scenedesmus obliquus* UTEX 393) was initially inoculated at 1.00×10^4 cells/mL in a Modified Bold 3N broth medium as suggested by UTEX. A modified Bristol's medium containing, in g/L: NaNO₃, 0.75; K₂HPO₄, 0.075; KH₂PO₄, 0.175; MgSO₄·7H₂O, 0.075; CaCl₂·2H₂O, 0.025; NaCl, 0.025; and the following additives, in mL/L: PI-V metal solution, 6; soil water GR+medium, 40; vitamin B12, 1; biotin vitamin, 1; thiamine vitamin, 1 was also used. Cultures of microalgae were grown with oxygen-free CO₂.

2.2 Experimental procedure

The laboratory at Universidad de La Sabana has a prototype system that was designed and implemented using a PLC S7 1200 Siemens (Republic of Austria). It allows control of temperature, light/dark cycle, and pH monitoring. Figure 1 illustrates the connections between the components of the system: a) PLC, b) Boards, c) sensors and actuators, d) Human-Machine Interface

Received date: 2017-08-25 **Accepted date:** 2018-08-01

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freeze-drying, followed by extraction with a solvent mixture of hexane: chloroform: methanol in a 1:2:3 proportion. In each extraction, 12 mL of solvent was applied to 2.0 g of freeze-dried biomass.

2.6.1 Classical extraction

Two gram of biomass was extracted twice with 12 mL of solvent for 12 h for each extraction. Then the organic phase was filtered and subjected to rotary evaporation under a vacuum and until the moment the trans-esterification procedure was carried out.

2.6.2 Microwave extraction

Microwave extraction (1.4 kW, 2.45 GHz, LG, MH1443XAR, South Korea) was performed with 2.0 g of biomass and 12 mL of solvent using 4 pulses of 15 s each with 90 s of rest time between pulses and the microwave was set to a 50% power level.

2.6.3 Soxhlet extraction

Soxhlet extraction was performed in a 25-mL vessel, using 2.0 g of biomass and 12 mL of solvent for eight hours at 65 °C using a cellulose dash that was 5 cm long by 2 cm wide.

2.6.4 Ultrasound extraction

Ultrasound extraction was performed in an ELMA, Trasonic TI-H 10 (750 W, Germany), using 2.0 g of biomass and 12 mL of solvent and a frequency of 45 kHz for 15 min, the equipment was set to a 100% power level and 20 °C. Each sample was extracted twice, with a rest time of 5 min.

2.7 Lipid identification

The crude extracts obtained were individually trans-esterified using 3.5 mL of BF₃ MeOH (14%), 85 °C, for 50 min in an oil bath. Then, the samples were brought to room temperature and 3 mL of hexane was added; two phases were then separated. The organic phase was concentrated under a vacuum to obtain methyl esters, which were stored in vials for subsequent analysis.

Methyl esters were analyzed by FTIR (Termo Scientific®, Nicolet iS10). The composition of the FAMES was further analyzed with an Agilent 7890B GC (Agilent Technologies Inc., USA) coupled to an Agilent 5977A Mass Selective Detector, an HP-5 MS column (50 m length × 0.25 mm i.d. × 0.25 μm, stationary phase) with a split injection of 10:1 and an injection volume of 1 μL. The FAMES Mix C8-C24 was used as an external standard. The samples were dissolved in hexane. The carrier gas was helium at a flow rate 0.6 mL/min. The column oven temperature program began at 60 °C and was increased to 200 °C at 10 °C/min. After 200 °C, the ramp changed to 5 °C/min until 250 °C was reached. The ramp was changed back to 10 °C/min from 250 °C to 300 °C. The total program time was 30.5 min.

2.8 Statistical analysis

All extractions were realized in quadruplicated to confirm their reproducibility though RCB design, for comparing four treatments in four blocks, with each treatment appearing exactly once in every block. A Statistical Analysis Software (SAS) used to the validation of the normality for Shapiro-Wilk test and analyze the difference between the weigh and percentage of lipids for each treatment.

3 Results and discussion

3.1 Optimization of experimental conditions

The optimal medium to grow *A. obliquus* microalgae was chosen from two different media recommended in UTEX^[14] for the growth of this species. The results showed that best medium was Modified Bold 3N, according to the biomass productivity.

The temperature was maintained at 25 °C because a study by Li

et al.^[15], indicated that the production of biomass in the microalgal culture was significantly higher at 20 °C, 25 °C and 30 °C (313.3 ± 6.7 g; 340.0 ± 13.3 g; 340.0 ± 6.7 g) than at 10 °C (200.0 ± 6.7 g). The pH of the medium was maintained at 6.8, as recommended by the UTEX Culture Collection of Algae^[16]; however, Hodaifa et al.^[17] showed that pH variations change the lipid profile. For example, linoleic acid was higher at pH 8.0, but considerably lower at pH 9.0. It has also been shown that monounsaturated fatty acids were present in a greater amount at pH 6.0, while a greater amount of the saturated acids was observed at pH 7.0. An irradiance of 1200 μE/(m² s) was used because it was the maximum for the white SMD LED.

Another condition considered was the percentage of N in the medium, Shen et al.^[18] concluded that a reduction in N did not cause a notable decrease in the microalgal growth rate. However, a lack of N increased the amount of lipids produced compared to lipid production in N-saturated medium. Therefore, in presented work 500 μL samples of microalgae were taken every 36 h to monitor growth, as indicated by the living cells produced by *A. obliquus* over 15 d (Figure 2). The results showed that the production rate of living cells was higher at 75% N than at 100% N; however, at the end of the experiment, the number of living cells was similar for both N levels. This result is noteworthy because the percentage of N used allows larger quantities of biomass to be produced in less time.

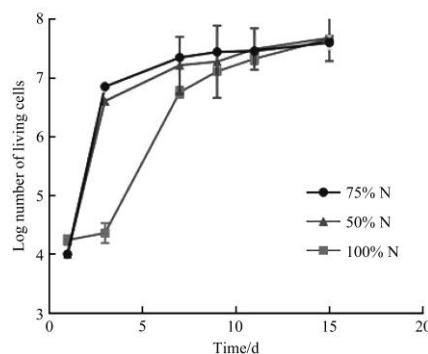


Figure 2 Number of living cells produced during the 15 d experiment with different nitrogen concentrations

3.2 Lipid vacuoles determination

The NR fluorophore (9-diethylamino-5H-benzo[*a*] phenoxazine-5-one) is a hydrophobic and metachromatic coloring agent that allows the detection and quantification of the microalgal neutral lipid content^[17–20]. Lipid accumulation in the microalgae was periodically established by using this technique. The lipid vacuoles had a fluorescent yellow color, while chlorophyll was stained red. The results showed that the cell size and lipid production became saturated after 15 d, so the experiment was terminated at this time (Figure 3).

In Figure 3, the changes in the lipids in the microalgae at different cultivation times are shown. The greatest increase occurred between the 7th and 11th days. Fewer yellow dots were evident on the first day (Figure 3a), while at the 7th day (Figure 3c), the yellow lipid vacuoles were grouped and easily observed. Between the 11th and 15th days (Figures 3e and 3f), the vacuoles showed the greatest lipid production and an intense yellow color and greater cell size was evident. These results indicate that high cell growth does not necessarily imply high lipid production because, as Figure 3 shows, at day 9 with 75% N, the culture had nearly reached stationary phase, but maximum lipid production began on day 11.

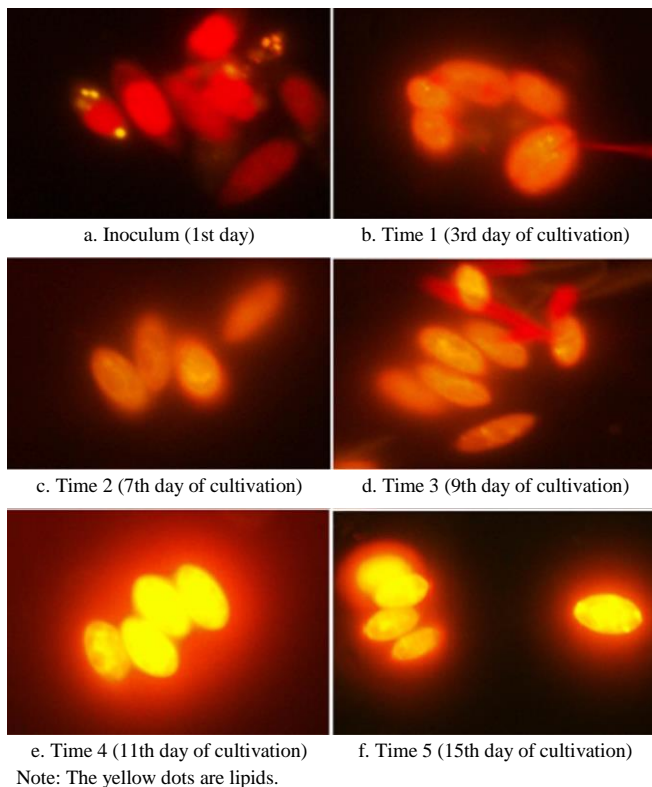


Figure 3 *A. obliquus* cell stained with Nile Red

The protein percentage produced during 15 days of experimentation is shown in Figure 4. As expected, the protein content increased with increased cell growth. Additionally, a marked similarity with lipid vacuole production during the experiment exists, which could be related to the biosynthetic process in *A. obliquus* that produces lipids as a result of catabolism. At the end of the experiment, a decreasing production of protein was observed, which could be associated with the energy requirements of the cells.

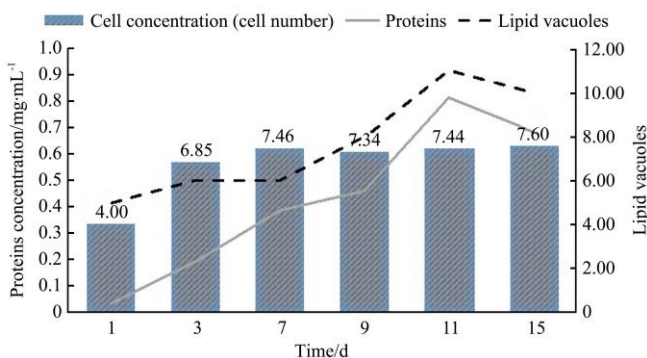


Figure 4 Cell concentration and protein concentration

3.3 Extraction methods comparison

At the end of the experiment, the biomass was decanted and removed from the medium, centrifuged, frozen and lyophilized. Forty grams of dry biomass was obtained from this PBR experiment. The biomass was split into homogeneous quantities of 2 g, which were used in the four extraction methods (classical, MW, Soxhlet and US). Table 1 shows the results for each method.

The crude extract is the product obtained after rotary evaporation, which contains other metabolites that may be extractable with the solvents employed. Table 1 indicates that the classical extraction was the best method, and Soxhlet produced the least amount of crude extract, while the MW and US extractions

produced similar amounts. Although classical extraction produced the greatest amount of crude extract, this result does not imply that it is the best lipid extraction method because in addition to lipids, the crude extract may contain terpenes, proteins, phenolics compounds, among other substances.

Table 1 Biomass and lipids extracted from different types of extractions

Product	Extraction methods			
	Classical	Microwave	Ultrasonic	Soxhlet
Crude extract/mg L ⁻¹	281.81	113.46	112.09	50.51
Average lipid content/mg L ⁻¹	581.73	610.26	605.23	621.55
Lipid productivity/mg (L d) ⁻¹	38.78	40.68	40.35	41.44

Data were evaluated under a RCB design^[23]. The Shapiro-Wilk test for normality has a *p*-value of 0.9767, which reflects the normal nature of the residuals and the analysis of variance showed a significant effect at 0.0495 for the treatments. The best extraction method was determined according to the amount of lipids obtained. The comparison of means between treatments showed that the extractions with wave treatment (MW and US) are better than the classic treatments (Classical and Soxhlet), with a probability of 0.0113, while the others comparisons presented in Table 2 do not have significant differences. As Table 1 and Figure 5 indicate, all of the methods produced approximately the same amount of lipids, but the best method in this study was extraction by MW. Although Soxhlet produced a greater amount, it was not significantly different from MW. However, the Soxhlet method takes eight hours to produce a product, but MW requires 4 pulses of 15 s with 90 s of rest time. This result is in agreement with a report by Balasubramanian et al.^[24] Similarly, classical extraction takes 48 h and requires twice the amount of solvent.

Table 2 Statistical probability for comparisons of treatments

Parameter	<i>t</i> value	Pr > <i>t</i>
Waves vs Classic	-3.17	0.0113
MW vs US	0.17	0.8722
Soxhlet vs Classic	1.24	0.2460
MW vs others	-1.97	0.0807
US vs others	-1.70	0.1240

Note: *t* = Student's *t*-distribution, Pr = Probability.

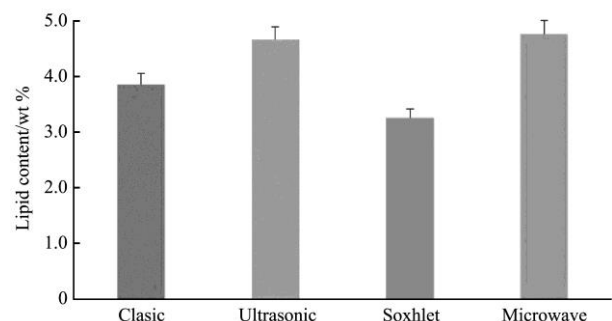
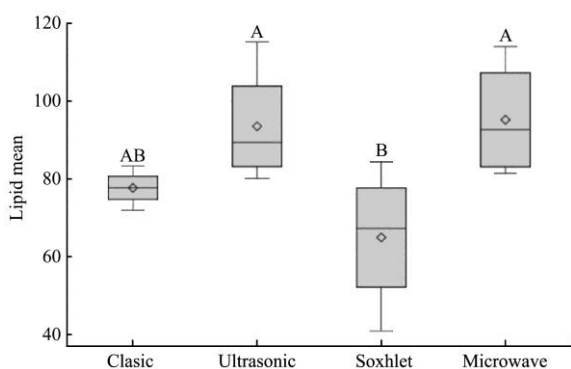


Figure 5 Lipid extraction efficiency according to method

According to Figure 6, the samples treated by classical extraction were not differentiated from the others extraction; therefore, these belong to both groups A and B. This means that the statistics of MW and US treatments are equal, classical is intermediate and Soxhlet is the treatment with lower yield. The US and MW extractions yield a similar amount of lipids (4.6%), but US takes approximately 35 min and MW requires only 60 s of

irradiation. Furthermore, less time (1 min for MW) versus 35 min (US), 8 h (Soxhlet), and 24 h (classical) was required to yield a similar lipid recovery, demonstrating the advantages of MW compared with the other lipid extraction methods. These results are in accord with other results reported in the literature. For example, Balasubramanian et al.^[24] found that extraction by MW drastically reduced the process time by approximately 10 times for a species of *A. obliquus* microalgae compared with Soxhlet extraction and a warm bath. Florentino de Souza Silva et al.^[25] obtained the best results in terms of the lipid yield using MW (33.7% ±5.3%), followed by Electroflocculation with an alternating current (24.8% ±7.1%), autoclave (15.4% ±2.3%), and US (13.3% ±3.0%). Prabakaran and Ravindran^[26] found that US and MW extraction were more efficient for *Chlorella* sp. and *Nostoc* sp.



Note: Means with the same letter are not significantly different.

Figure 6 Box and whiskers diagram for the means of lipid weight of each treatments.

3.4 Lipid characterization

It was frequently reported in literature that the extraction time affects the fatty acid profile; e.g., Balasubramanian et al.^[24] found that the oil obtained from a Soxhlet extraction contained a large

percentage of saturated fatty acids (86%) and a very low percentage of unsaturated fatty acids (14%). They noticed that the oil extracted when microwaves were used as a heat source resulted in a high percentage of ω -3 and ω -6 fatty acids compared to other methods. Kobberg et al.^[27] compared the efficiency of the MW versus the US extraction of lipids from the algae *Nannochloropsis* and found that palmitic (C16:0) and palmitoleic (C16:1) acids were present in the highest proportion using MW and US extraction, indicating that a high concentration of saturated fatty acids was obtained. Prabakaran and Ravindran^[26] used different extraction methods (autoclave, bead beating, WW, US, and treatment with NaCl solution to 10%) in *Chlorella* sp., *Nostoc* sp. and *Tolyphothrix* sp. and found a large amount of oleic acid from *Nostoc* sp. and *Tolyphothrix* sp., while linoleic acid predominated with *Chlorella* sp.

The previous discussion emphasizes the need for a comprehensive investigation of the lipids produced by *A. obliquus*. The lipids obtained via the four methods (classical, MW, Soxhlet, and US) were compared with FTIR spectroscopy and GC-MS. FTIR analyses were used to establish chemical and structural differences between the lipids. The spectra for the four extractions are shown in Figure 7. The broad band between 2900 cm^{-1} and 3600 cm^{-1} represents the stretch of the O—H bond, the band at 1045 cm^{-1} indicates a C—O stretch anti-symmetrically coupled to the C—C stretch, and the band at 649 cm^{-1} is the O—H wag, all of these bands are characteristic of alcohols, carboxylic acids and free fatty acids, which were principally found in the traditional extraction.

The band located at 2920 cm^{-1} was assigned to $-\text{CH}_2$ antisymmetric stretching; this band together with the band located at 1045-1074 cm^{-1} is characteristic of primary alcohols. The band located at 2855 cm^{-1} is the $-\text{CH}_3$ symmetric stretch and bands between 1450 cm^{-1} and 1460 cm^{-1} represent the antisymmetric bend of the CH_3 group. These bands are characteristic for MW, US and Soxhlet extractions.

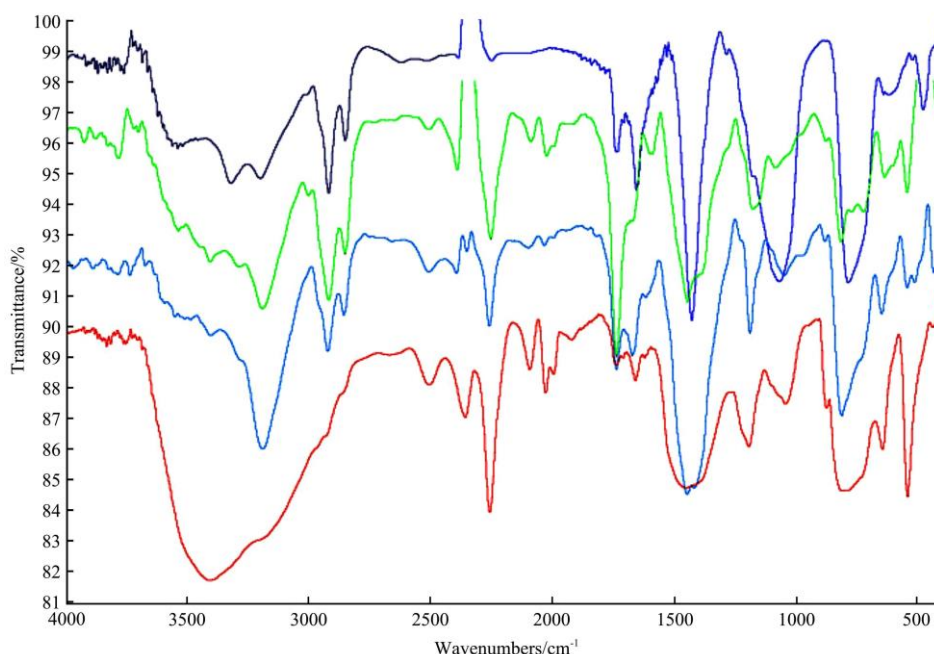


Figure 7 Fourier Transform Infrared spectrum: Ultrasonic (green line), Classical (orange line), Microwave (blue line), Soxhlet (black line)

Only in the US extraction, was a strong band found near 1735-1750 cm^{-1} , which is characteristic of esters, representing the C=O stretch in a saturated ester. Another important band between 3400 cm^{-1} and 3250 cm^{-1} in the US and Soxhlet extraction profile

was the band at 3310 cm^{-1} , which represents the N-H stretch of a secondary amine, and the strong band at 810 cm^{-1} is also due to the N-H wag for a secondary amine. On the other hand, MW extraction shows two bands at 3335 cm^{-1} and 3190 cm^{-1} that,

respectively, represent the antisymmetric N-H stretch and symmetric N-H stretch in a primary amine. Additionally, the band at 1660 cm^{-1} indicates the N-H bending vibration, and the strong band at 782 cm^{-1} is due to the N-H wag of a primary amine. All of these bands are characteristic of lipids and related to the olefinic and nonolefinic fatty acids.

The major fatty acid composition of the tested microalgae was determined using GC analysis. The pattern of the FAMES esters containing a fatty acid methyl carbon chain with a length between 8 and 24 carbon atoms was used.

The analysis showed that the MW and US extraction methods had a similar fatty acid composition. The same result was seen in the FTIR spectra. Palmitic acid was the most abundant fatty acid for these extraction methods, except for Soxhlet, in which it could not be extracted. The best method for extracting unsaturated fatty acids was MW, which extracted more oleic and linoleic acids compared to the other methods. Classical extraction yielded a similar amount of stearic acid as US, but stearic acid was low in the MW and Soxhlet extractions, and in the sample extracted by Soxhlet, only stearic acid was present (Table 3).

In all of the samples, the most representative acids were palmitic acid, which is used in the soap and cosmetics industries, and oleic and linoleic acids (ω -6 and ω -9), which have great importance in human health and are essential in the diet because they are not produced by the body. Saturated stearic acid is, also, of great importance in the cosmetics industry. The above results lead to the conclusion that GC-MS analysis was consistent with the FTIR results, and both techniques proved to be very useful for the analysis of lipids in microalgae.

Table 3 Relative amounts of fatty acids obtained with the different extraction methods

Fatty acid type	Classical extraction	Microwave	Soxhlet	Ultrasound
Palmitic acid (C16:0)	30.62	44.25	-	52.58
Palmitoleic acid (C16:1 ω 7c)	-	0.51	-	-
Linoleic acid (C18:2 ω 6c)	18.89	26.99	-	15.41
Oleic acid (C18:1 ω 9c)	11.92	15.16	-	7.96
γ -Linolenic acid	5.00	6.61	-	4.33
Stearic acid	33.57	5.17	100.00	19.72
10-octadecenoic acid	-	1.30	-	-

4 Conclusions

The optimal medium for the growth of *A. obliquus* was Bold 3N modified medium because this medium allowed a higher biomass production. A greater number of living cells was produced during the exponential phase under 75% nitrogen; however, at the end of the experiment (15 d), the number of cells was similar (75%-100% of N). The above results are in agreement with the lipid content established by the Red Nile method, which showed that the highest rate of lipid production occurred in the exponential phase, but similar to biomass, the lipid content was highest in the stationary phase.

All lipid extraction methods yielded similar amounts of lipids and the statistics of MW and US treatments are equal, however, MW extraction drastically reduced the extraction time compared to the other methods (1 min (MW) versus 35 min (US), 8 h (Soxhlet), and 24 h (classical) without affecting lipid production. The extraction time affected the profile of the fatty acids obtained. The unsaturated acids were extracted better by US and MW than by classical extraction. MW and US produced a similar fatty acid

composition that only varied in the proportion of lipids obtained. Palmitic acid was obtained at the highest proportion with MW and US extraction as opposed to Soxhlet and classical extraction. On the other hand, stearic acid was extracted at high amounts by the classical and Soxhlet methods as opposed to MW and US extraction. Palmitic and stearic acid are very important for cosmetic industry. Oleic and Linoleic acids were produced by three methods, but MW extraction was the best method for the extraction of these lipids, which are very important in human health and diet because humans do not produce them.

Acknowledgments

The authors would like to thank at Universidad de La Sabana by the financing for the development of ING-140-2013 “Aplicación de técnicas de control proporcional integral generalizado al crecimiento de la microalga *Scenedesmus obliquus*” project. Additionally, they thank COLCIENCIAS for the “Doctorado Nacional-617-2” Scholarship given to C. L. Garzón-Castro. The authors would also like to thank at PhD Bioscience Program of the Universidad de La Sabana and at Universidad Nacional de Colombia.

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