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Biodiesel production from *Halamphora coffeaeformis* microalga oil by supercritical ethanol transesterification

Pablo E. Hegel[a,b,*], Lucas A. Martín[c], Cecilia A. Popovich[c,d], Cecilia Damiani[c], Patricia I. Leonardi[c]

[a] Departamento de Ingeniería Química, Universidad Nacional del Sur (UNS), Argentina
[b] Planta Piloto de Ingeniería Química - PLAPIQUI (UNS-CONICET), Bahía Blanca, Argentina
[c] Centro de Recursos Naturales Renovables de la Zona Semiárida (CERZOS), CONICET- UNS, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Bahía Blanca, Argentina
[d] Centro de Emprendedorismo y Desarrollo Territorial Sostenible (CEDETS-CIC-UPSO), Bahía Blanca, Argentina

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ABSTRACT

The marine benthic diatom *Halamphora coffeaeformis* is a potential feedstock for biodiesel production. This species shows high growth rates, important triacylglycerol (TAG) contents and grows in seawater making large-scale cultivation advantageous. Moreover, sustainable biofuel production in future biorefineries requires the implementation of technologies that employ renewable solvents. Thus, the goal of this work was to evaluate ethanol usage as extraction solvent and reaction medium for biodiesel production from *H. coffeaeformis*. In a first step a bio-oil extraction was carried out comparing ethanol and n-hexane to investigate the performance of ethanol with respect to a conventional solvent. Then, a free-catalyst supercritical ethanol transesterification of the bio-oils was carried out to obtain biodiesel. Higher lipid extraction yields were obtained using ethanol respect to n-hexane (26 wt.% vs 21.1 wt.%). The transesterification of crude lipids extracted with ethanol as solvent at 305 °C and 40 min. produced up to 15.9 wt.% of biodiesel respect to dried biomass processed. Comparable biodiesel yields were obtained using non-renewable organic solvents and a conventional catalytic technology. Thus, ethanol extraction and subsequent supercritical transesterification of *H. coffeaeformis* oil proved to be technically feasible and environmental friendly technology for the production of biodiesel.

1. Introduction

Microalgae are receiving an increasing attention worldwide during the last years because they are promising for the sustainable reduction in the consumption of fossil fuels. Different biofuels can be obtained from microalgae such as biodiesel, bioethanol, biogas, bio-hydrogen, as well as valuable co-products with applications in human nutrition, animal feed, pollution control, and bio-fertilizers [1]. Regarding biodiesel production, microalga oil yields in terms of land used for its cultivation exceed the yield of the best oilseed crops because they can be cultivated during all year [2]. Martín et al. [3,4] evaluated the cultivation of the marine benthic diatom *Halamphora coffeaeformis* for biodiesel production. They determined the quality, quantity and productivity of lipids and triacylglycerols (TAG) at different operating conditions and concluded that *H. coffeaeformis* is an interesting biomass to produce biodiesel at large scale [3]. This species can be cultivated in marine media with low contamination risks, it accumulates up to 29 wt.% of TAG, and possesses a high tendency to decant which reduces the harvesting costs [4]. In addition, the cell wall or frustule of *H. coffeaeformis* represents up to 25% of the harvested biomass, being a potential source of silica materials for industrial applications [5,6]. Diatomite, a fossil source of frustules, presents several commercial applications, including uses in filtration, insulation, absorption, building materials, mineral fillers, and as a fine abrasive [7]. Moreover, frustules obtained from diatom cultures show advantages over diatomite in terms of both sustainability and quality, which make them suitable for bio-sensing, medical, bio-engineering and nano-technological applications [8,9].

The most expensive costs associated to biodiesel production from microalgae according to the literature [1,2] are related to dewatering and drying processes, as well as to oil extraction and subsequent transesterification to obtain biodiesel [1]. Conventional technologies for biodiesel production that use catalysts for the transesterification, such as NaOH or H_2SO_4, cannot process raw materials with high

* Corresponding author.
E-mail address: phegel@plapiqui.edu.ar (P.E. Hegel).

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contents of water or free fatty acids [10]. This represents an important problem in microalga processing due to the high associated costs of drying pretreatments [2,11]. Besides, these impurities make purification steps of the reaction products after transesterification quite complex due to the presence of the catalyst, also facing unavoidable environmental problems [12]. Therefore, different authors are developing cost-effective technologies to process biomass [13]. An interesting method proposed in the literature to overcome these problems of transesterification is the free-catalyst direct supercritical alcohol transesterification of microalgae. This technique allows the direct processing of wet microalgae avoiding both exhaust drying and oil extraction steps, which may reduce considerably biodiesel production costs [14–18].

An alternative to maximize profits in biorefineries is considering an oil extraction step, and also fractionation units for different co-products [19,20]. The extraction can be carried out by mechanical methods in an expeller or press machine, where microalgae can be processed dry or wet, normally being followed by a chemical solvent extraction to maximize yields [21]. Solvents used in a biorefinery context should be cost-effective for oil extraction of microalgae or microorganisms at industrial scale, and both the environment and human health should also be considered [21–24]. Thus, solvents like chloroform and methanol employed in Bligh and Dyer's method for the extraction of microorganism oils in laboratory studies [21,25], should be avoided at industrial scale due to environmental concerns [20,22–24]. Moreover, petroleum solvents like hexane or diethyl ether currently used at the industry for extraction of vegetable oils from crops should be excluded in future biorefineries because of increasing worldwide restrictions regarding the use of toxic non-renewable solvents [20,22–24]. Supercritical CO_2 extraction of microalga oils is a promising technology because CO_2 is a cheap non-flammable innocuous solvent that can be used in biorefineries to extract microalga oils or to fractionate valuable co-products [20]. The main disadvantages of this technology are the high initial capital cost and the need for exhaustive drying pretreatment to process feedstock [21,24,26].

Ethanol is a renewable solvent that has also been proposed for the extraction of oils from vegetable crops and microorganisms [22,23,27]. This solvent can be used even with wet microalgae due to the natural affinity between water and ethanol that results beneficial for the extraction [23]. Moreover, ethanol can be employed as a reactant later in the transesterification of the microalga oil to produce the biodiesel, being unnecessary a complete removal of the solvent after the extraction, which can reduce the processing costs. Thus, the main goal of this work is to study the biodiesel production from *H. coffeaeformis* by a two-step process based on a solvent lipid extraction step and subsequent supercritical ethanol transesterification of the extracted lipids. *Halamphora coffeaeformis* crude lipids were extracted using ethanol and n-hexane as solvents media in order to compare solvent power and selectivity of ethanol with respect to a conventional hydrocarbon solvent. Then, supercritical ethanol transesterifications of the microalgal crude lipids extracted with both solvents were carried out in a batch reactor at different operating conditions to evaluate the biodiesel yields.

2. Material and methods

2.1. Materials

Ethanol (99.6 wt.%) purchased from Ciccarelli SA was used in extractions and transesterification reactions. N-hexane (99.9%) from Dorwill was used in Soxhlet extractions, separations and for the preparation of GC standard solutions and samples. Methyl heptadecanoate (99.99 wt.%) and tetradecane (99.99 wt.%) purchased from Sigma-Aldrich were used as analytical standards.

2.2. Halamphora coffeaeformis biomass

H. coffeaeformis (C. Agardh) Levkov was isolated from Bahía Blanca Estuary (38° 45′ S, 62° 22′ W). This strain is maintained in stock cultures at the Laboratorio de Estudios Básicos y Biotecnológicos en Algas (LEBBA), CERZOS – CONICET, Bahía Blanca, Argentina. Cultures of *H. coffeaeformis* were carried out in a hybrid two-stage culture according to methods described in previous work [4]. Briefly, the species was cultured in a two-stage culture integrated by a photobioreactor and an indoor raceway pond in order to obtain biomass rich in TAG. The experiment in the raceway pond was performed with 100 L of culture at a depth of 0.3 m. Seawater was supplemented with $NaNO_3$ (N), K_2HPO_4 (P), Na_2SiO_3 (Si), and trace metals according to f/2 medium [4]. On day 32, the biomass was harvested by autoflocculation of the suspended cells. After about 2 h, the cell-free supernatant was removed by siphoning and flocculated cells were collected by scraping. The harvested pellet was washed with distilled water, centrifuged (10 min at 3600 *g*) and dried in a convection oven at 60 °C during 6 h. Final water content in the biomass of 18 wt.% was determined by a gravimetric analysis (Sartorius moisture analyzer MA 35). *H. coffeaeformis* triglyceride content was determined following methods reported in previous works [4], the biomass processed in the experiments presented 22.0 (± 0.6) wt.% of triglycerides on a dry basis.

2.3. Experimental procedure

2.3.1. Lipid extraction of Halamphora coffeaeformis

Lipid extractions were carried out in a Soxhlet apparatus assembled with a 100 mL round bottom flask and an Allihn (straight type) condenser. Since Soxhlet extractor is well known as a reference for the assessment of solid-liquid extraction, a general description of the technique can be found in previous studies [27]. In this work the biomass sample (2 g ± 0.01 g; 18 wt.% humidity) was set into an envelope filter paper (pore size 1.6 microns) and placed in a 25 mL cellulose thimble-holder. Later, the main extraction chamber of the Soxhlet apparatus was filled with glass beads (1 mm diameter, porosity: 0.4) to reduce the dead volume. Extractions were carried out at increasing operating times in order to evaluate the kinetics along the full extraction process. Heating at the round bottom flask was graduated at a rate of eight refluxes per hour for both solvents (ethanol and n-hexane) in order to have an equivalent number extraction cycles against time. During the operation, after a given time, the extraction was discontinued and the solvent removed from the round bottom flask in a roto-evaporator operated at 50 °C under vacuum until a constant weight of the sample. The crude lipid obtained in the extraction was quantified gravimetrically using a precision mass balance (± 0.1 mg). Finally, the samples were collected in glass vials using ethanol as solvent and stored at 4 °C for the reaction studies.

Scanning electron microscopy (SEM) of biomass residues obtained in extractions were sputter–coated with Au–Pd and examined in a LEO, EVO-40XVP Scanning Electron Microscope (CC – CONICET, Bahía Blanca).

2.3.2. Supercritical ethanol transesterification

Supercritical ethanol reactions were carried out in a high pressure stainless steel batch reactor of 7.6 mL capacity. A general description of the equipment and auxiliary instruments were described in a previous work [17]. The reactor basically consists in a high pressure stainless steel tube with proper connections for temperature and pressure sensors. A high temperature tin bath pre-heated at the reaction temperature is employed to control the reaction temperature using an electric heating cartridge of 400 W and a Novus 480D controller. Crude lipids extracted were mixed with ethanol in a ratio of 8.5 g ethanol/g crude lipids and placed inside the high pressure tube reactor. The total mass of solution loaded to the reactor was 3.04 g to assess a single phase condition in the reactor at pressures greater than 150 bar (± 3 bar) and

temperatures between 270 °C and 305 °C [28]. A gentle N_2 stream was used to remove the air inside the reactor. The desired reaction temperatures, 270 °C and 305 °C (± 1 °C), were reached in ≈5 min and ≈8 min, respectively. After getting the desired temperature, the reaction time was varied between ≈20 min and ≈40 min to investigate the effect of this variable on lipid conversion to fatty acid esters. Crude lipid extracts obtained at different degrees of extraction (≈12 wt.% / ≈26 wt.%) were processed to evaluate the effect of this variable on the transesterification yields. The reactor was quenched in a water bath to stop the reaction (30 s) and the products were collected at room temperature in a round bottom flask. The excess ethanol was removed in a roto-evaporator at 50 °C under vacuum and the mass of reaction products was determined gravimetrically. N-hexane was used as solvent to collect the sample in a 50 mL flask for quantification of fatty acid ethyl esters (FAEE) by gas chromatography.

In order to compare transesterification yields obtained by supercritical ethanol with a conventional technique, an acid catalyst transesterification of the crude lipids extracted by ethanol was performed according to a previous work [29]. Briefly, crude lipids (0.465 g) and excess ethanol (40 mL) were placed in a round bottom flask of 100 mL using H_2SO_4 (0.1 M in ethanol) as catalyst. An electric heater was used to control the reaction temperature at 80 °C and a condenser on the top of the flask was used to avoid ethanol evaporation. The reaction was performed during 12 h to ensure a complete conversion of the lipids through fatty acid esters. After the reaction, excess ethanol was removed using a roto-evaporator at 50 °C under vacuum and products were washed with water and hexane to eliminate H_2SO_4. Reaction products soluble in hexane were analyzed by GC to evaluate lipids-fatty acid ester conversion.

Reaction yields (Y_1) reported as % dry biomass converted to FAEE (Eq. 1) were estimated from the FAEE content in bio-oils reaction product samples analyzed by GC ($FAEE_{bio\text{-}oil}$ wt.%) and biomass processed. Acid catalyst has proved to be able to convert to total microalgae lipids in biodiesel (triglycerides and polar compounds such us fatty acids, phospholipids and glycolipids) [30,31]. Thus, reaction yields obtained in supercritical ethanol transesterifications were compared with acid catalyst reaction in order to evaluate the efficiency of the supercritical method.

$$Y_1 = \frac{FAEE\ (g)}{dry\ biomass\ (g)} 100 \tag{1}$$

$$Y_2 = \frac{FAEE\ Supercritical\ (g)}{FAEE\ Acid\ catalyst\ (g)} 100 = \frac{Y_1\ supercritical}{Y_1 Acid\ catalyst} 100 \tag{2}$$

2.3.3. Gas chromatography

The fatty acid esters concentration in the non-volatile bio-oils reaction products was determined by gas chromatography in a GC Agilent – 7820A. The GC was assembled with a capillary column (J&W Scientific, model HP-5 ms, 30 m length, 0.25 mm inner diameter, and 0.25 µm film thickness), a FID detector set at 340 °C, and a split/splitless injector temperature set at 280 °C with a split ratio of 20:1. The oven was programmed at 70 °C for 1 min and have a ramp of 15 °C/min to 180 °C, a ramp of 7 °C/min to 230 °C, and a ramp of 10 °C/min to 310 °C, where the temperature was maintained for 10 min. before concluding the analysis. Methyl heptadecanoate was use as internal standard reference for fatty acid esters quantification. A stock solution of n-hexane with a known amount of internal standard was prepared (~10 mg/mL). The bio-oil sample solution was prepared diluting the reaction product sample (previously weighted in an analytical balance) in a known volume of hexane (to obtain ≈20 mg/mL). The sample injected to the chromatograph consisted of 2 µL of a solution prepared with 0.1 ml of the internal standard stock solution, 0.1 ml of bio-oil sample solution, 0.1 ml of silylating agent (MSTFA) and 0.1 ml of hexane. Fatty acid esters content in the samples was evaluated in weight fraction ($FAEE_{bio\text{-}oil}$ wt.% = g FAEE/g bio-oil %). GC analysis of fatty acid esters in the bio-oil exhibit a deviation of ca. 1.5 wt.% in their concentration.

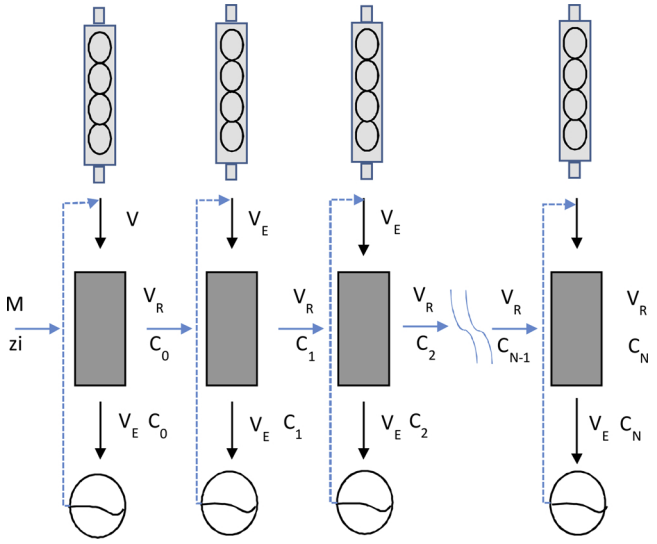

Fig. 1. Schematic diagram of the co-current extraction process that represents the extraction kinetics model. V_E: solvent volume refluxed, V_R: solvent volume retained, $C_{i=0\ to\ N}$: crude lipid concentration in the solvent, M: mass of processed microalga, Zi: mass fraction of crude lipids in the studied species.

3. Mathematical modeling

3.1. Lipid extraction kinetics

Extractions were modeled according to a co-current process, as shown schematically in Fig. 1, to correlate the observed extraction kinetics with both solvents (ethanol and n-hexane). Basically, the model considers negligible the external mass transfer resistance, and assumes the diffusion of crude lipids from the intact microalga cells as the limiting step. The single sphere model (Eq. 3) as reported by Esquivel et al. [32] was used to calculate the fraction of crude lipids extracted from the intact cells in each cycle considering an extraction time of 7.5 min/cycle. The mass balance also considers up to 15% of the solvent is retained in the Soxhlet extraction chamber after each cycle (Eq. 5), as observed experimentally during extractions. The concentration of crude lipids in the solvent refluxed after each Soxhlet cycle (C_i) is given by Eq. (4), which is derived from a mass balance of the extraction process (Fig. 1). The mass of extracted crude lipids (m_{ext}) after a given number of cycles is estimated from Eq. (7), as a function of crude lipid concentration in the solvent at each cycle and the partial volume of solvent (V_E) refluxed to the round bottom balloon during the Soxhlet extraction. Analytical Eqs. (3–8) were programmed in Microsoft Excel, and the effective diffusivity parameter (D_e) was fitted by minimizing absolute errors between the experimental and the calculated mass of extracted crude lipids.

$$y = 1 - \frac{6}{\pi} \sum_{j=1}^{\infty} \frac{1}{j^2} \exp\left(-\frac{D_e j^2 \pi^2 t}{R^2}\right) \tag{3}$$

$$C_i = C_0 \sum_{i=0}^{N} \frac{(1-y)^{N-1}}{(1+B)^i} \tag{4}$$

$$C_0 = \frac{M\ Zi\ y}{V} \tag{5}$$

$$B = \frac{V_E}{V_R} \tag{6}$$

$$m_{ext} = V_E \sum_{i=0}^{N} C_i \tag{7}$$

$$Y_{ext}^{(N)} = \frac{m_{ext}}{M} \tag{8}$$

Where y is the fraction of crude lipids extracted by diffusion from the intact cells during each cycle, D_e is the effective diffusion parameter (fitting parameter), j is number of particles, t is the extraction time, R is the radius of sphere particle, C_0 is the initial crude lipid concentration, M is the mass of dried microalga, Z_i is the mass fraction of crude lipids in the biomass, N is the number of cycles, and Y_{ext}^{N} is the extraction yield for a given number of cycles.

3.2. Supercritical ethanol transesterification

To compare experimental results obtained in this work with previous studies reported in the literature and to evaluate the effect of temperature and reaction time, the experimental data obtained in the supercritical ethanol transesterification from *H. coffeaeformis* oil were correlated according to a first- order reaction kinetics (Eq. 9). This simplified model has been used by several authors and it can be considered valid for high molar ratios of ethanol to TAG in the reaction system [28,33–38]. The model basically assumes the reaction proceeds as if it were first order with respect to the concentration of unreacted or non-esterified glycerides (triglycerides, diglycerides and monoglycerides) phospholipids, and free-fatty acids (uEE). Thus, the rate constant (k) was estimated as a function of the reaction time (t), the initial concentration of crude lipids that can be transesterified (estimated from acid catalyst transesterification), and FAEE content in the reaction products analyzed by gas chromatography. Finally, the apparent activation energy (Ea) was estimated from the rate constant values at different temperatures using Arrhenius equation (Eq. 10).

$$-\frac{\ln(uEE)}{\ln(uEE_0)} = k\,t \tag{9}$$

$$k = A\,e^{\left(\frac{Ea}{RT}\right)} \tag{10}$$

4. Results and discussion

4.1. Lipid extraction from H. coffeaeformis with ethanol and n-hexane

Fig. 2 shows the results about the lipid extraction from *H. coffeaeformis* with both solvents, and the extraction kinetics modeling. In general, ethanol was more efficient than n-hexane to perform the extraction. Thus, a higher extraction yield was obtained for a given number of Soxhlet cycles with ethanol in comparison with n-hexane. As it can be seen, a yield of 16 wt.% of crude lipids/dry biomass was obtained using ethanol after 20 extraction cycles (120 min), while a much lower extraction yield (7.3 wt.%) was observed in n-hexane tests. Practically, a complete extraction (24.5 wt.%) of crude lipids from *H. coffeaeformis* was achieved in ethanol experiments after 80 extraction cycles, and up to 26.4 wt.% yield was obtained after 100 cycles. N-hexane solvent extractions at 80 extraction cycles showed a lower yield (18 wt.%) and even after 124 cycles up to 21.1 wt.% extraction yield was obtained. It is worth to mention no-disruption methods were performed on the biomass before the extraction tests (Fig. 3A). Thus, intact cells were extracted during the experiments (Fig. 3B) in both solvents. On the other hand, the solvents used did not affect the presence of extracellular exopolysaccharides (EPSs) covering the cell walls (Fig. 3B, arrowheads). The absence of pretreatments in *H. coffeaeformis* may explain the higher time required to extract total lipids in comparison with previous microalga studies reported in the literature [21,24].

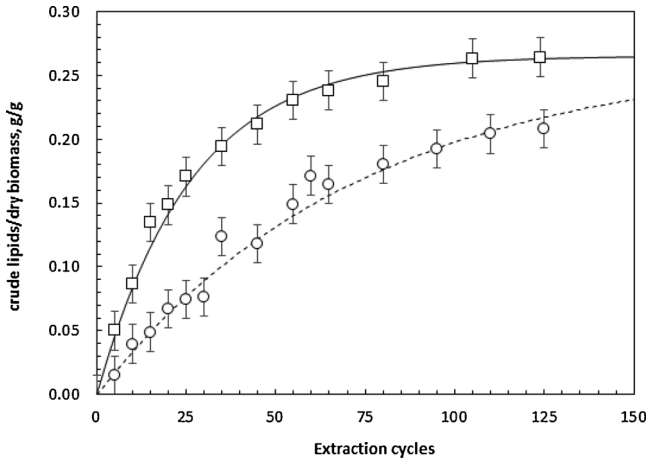

Fig. 2. Lipid extraction kinetics of *H. coffeaeformis* using ethanol (□) and n-hexane (○).

The mathematical modeling of the extraction kinetics by coupling the single sphere model with a co-current configuration shows in general a good agreement with the experimental results. As expected, a lower effective diffusion parameter was obtained for hexane (D_e: 1×10^{-16} m^2/s) in comparison with ethanol (D_e: 7×10^{-16} m^2/s). This higher effective diffusion observed for ethanol can be related with the higher solvent affinity to extract lipids bounded to proteins in the cytoplasm [24]. Polar solvents, like ethanol or isopropanol, are able to disrupt the lipid-protein associations favoring the extraction of neutral and polar lipids [22]. In general, the low values of effective diffusivity show that the system exhibits an important mass transfer resistance for the oil extraction in comparison with results obtained for conventional vegetable crops. For example, Esquivel et al. [30] in the supercritical CO_2 extraction of oils from olive husks reported effective diffusivities between 10^{-13} and 10^{-12} m^2/s. A volumetric solvent to biomass ratio of nearly 10 mL solvent/ g microalga was used during the Soxhlet cycles. This variable may have influence on the extraction efficiency if the solvent gets eventually saturated with lipids during the extraction cycles. In fact, most authors report crude lipid extractions using higher solvent volumes [21,22,24]. However, experimental and modeling results pointed out that lipid concentrations were lower than 0.1 wt.% (g lipids/g solvent), which are far lower than the solubility of triglycerides in ethanol informed in the literature [26].

Regarding lipid extraction from microalgae with Soxhlet technique, different results have been reported in the literature. Cheng et al. [39] obtained a low efficiency in *Pavlova* sp. extractions performed with hexane. They indicated extraction yields between 13.5 wt.% and 18.5 wt.% of crude lipids with respect to the dry biomass after 15 h and 100 h of extraction time, respectively. At the same time, more polar solvent mixtures, like ethyl acetate/methanol mixtures, showed a greater solvent power, increasing the yields up to 44.7 wt.% after only 3 h extraction time. Liau et al. [40] obtained up to 5.8 wt.% lipids/dry biomass in *Nannochloropsis oculata* using hexane as solvent after 16 h of extraction time. However, the extraction performed with ethanol showed yields of up to 44 wt.% during a similar extraction time. Converti et al. [41] studied the lipid production in *N. oculata* cultures. They obtained crude lipid yields of 7 wt.% using petroleum ether by 4 h extraction, whereas 8 wt.% was obtained with the classic Folch method by 1.5 h extraction. In the present work, results after 15 h extraction time using ethanol and hexane as solvents show extraction yields of up to 26.4 wt.% and 21.1 wt.% crude lipids/dry biomass, respectively. High extraction yields have also been reported using non-polar solvents, like hexane and $SCCO_2$. Tal et al. [42] obtained an extraction yield of crude lipids from *Schizochytrium limacinum* of 45 wt.% in hexane Soxhlet experiments after 8 h of extraction, whereas supercritical CO_2 + ethanol (1:1) showed a yield of ≈34 wt.% after 2 h extraction time. Patil et al. [43] studied the CO_2 + azeotropic co-solvent (hexane-ethanol) extraction of *Nannochloropsis salina* dry biomass subjected to microwave pretreatment to disrupt the microalga cells. They reported a maximum

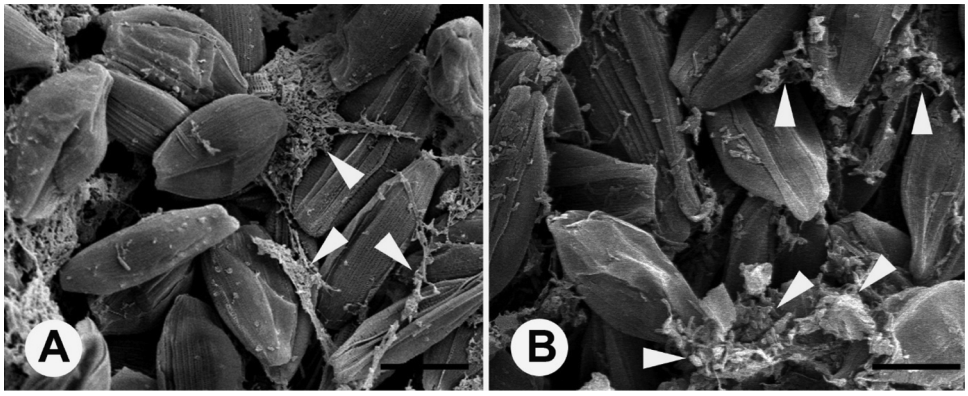

Fig. 3. Scanning electron microscopy images of frustules before the extraction test (A) and residues after lipid extraction with ethanol (B). The arrowheads indicate exopolysaccharides. Scale bars: A, B = 10 µm.

yield of 31.37 wt.% of lipid working at 340 bar and 80 °C, using ethanol + hexane as co-solvent (12 to 1 solvent/microalga) during 80 min of extraction time. In *H. coffeaeformis*, lipids (polar and neutral lipids) seem to be accessible to the solvent during the extraction due to the permeable wall cells of the microalga [44], which can explain the higher yields obtained with n-hexane in this study in comparison with results coming from other microalga species [39–41].

4.2. Supercritical ethanol transesterification of H. coffeaeformis crude lipids

Table 1 shows a summary of the studied reaction conditions and results obtained in the supercritical ethanol transesterification of *H. coffeaeformis* crude lipids extracted with ethanol and n-hexane. As it can be seen, the concentration of fatty acid ethyl esters in the bio-oil ($FAEE_{bio\text{-}oil}$ wt.%) determined in GC analysis changes notably with the operating conditions. Moreover, results varying remarkably for a given operating condition according to the type of processed crude lipids (ethanol or n-hexane extracted). The FAEE concentrations in transesterified bio-oils previously extracted with ethanol show amounts between 20.1 and 56.1 wt.% FAEE. Meanwhile, supercritical transesterification of bio-oils extracted with n-hexane show higher FAEE concentrations with values between 35.0 wt.% and 71.7 wt.% FAEE. In general, the highest concentrations of FAEE were obtained at the longest reaction time, and this effect was more important in the transesterification of bio-oils extracted with ethanol. Thus, FAEE concentration in the reaction products processed at 270 °C from ethanol-extracted bio-oils increased more than two-fold from 20 to 40 min (20.1 wt.% FAEE to 42.3 wt.% FAEE). On the other hand, the effect of temperature was more important in the processing of crude lipids extracted with n-hexane, where FAEE concentration in the reaction products after 40 min. of reaction time increased from 49.1 wt.% at 270 °C to 71.7 wt.% at 305 °C.

The processing of microalga lipids obtained from the partial extraction of *H. coffeaeformis* (12 wt.%) also produced interesting results (Table 1). Transesterification of these lipids was carried out at 305 °C by 40 min. A lower FAEE content was mainly determined in the reaction products in comparison with results obtained from lipids processed after a complete extraction (26 wt.% ethanol or 21% n-hexane). This reduction in the FAEE concentration was more relevant in lipids extracted by ethanol, where only 17.8 wt.% FAEE content was determined in the bio-oil after the supercritical ethanolysis. Ethanol extracts exhibit a darkest green color in comparison with lipids obtained by hexane which can be pointing out a greater concentration of pigments in crude lipids extracts. Previous works [30,31] has showed the presence of polar lipids and pigments reduce reaction yields explaining the lower FAEE content found in supercritical bio-oil products from lipids extracted by ethanol.

Table 2 shows the main fatty acids profile of the biodiesel determined in GC analysis of different crude lipids extracted either by n-hexane or ethanol transesterified by supercritical ethanol at 305 °C and 40 min. In fact, biofuels produced from the ethanol transesterifications of crude lipids under different operating conditions, or from acid catalyzed transesterification, exhibit similar fatty acid profiles. The biofuel fatty acid profile determined in this study is in agreement with results reported in previous works [3,4]. GC analysis shows that the main fatty acid esters were the saturated palmitic acid ethyl esters (C16:0 = 20.1/23.5%), the monounsaturated palmitoleic acid ethyl ester (C16:1 = 27.8/32.2%) and the polyunsaturated eicosapentaenoic fatty acid ethyl esters (C20:5 = 17.6/21.3%). Biofuel properties such as viscosity, heating value, cloud/pour point, and cetane number are highly correlated to biodiesel fatty acid ester profile. Thus, the biodiesel from *H. coffeaeformis* shows a high cetane number (> 54) and heating

Table 1
Summary of supercritical ethanol transesterification for the biodiesel production from *H. coffeaeformis* lipids extracted by ethanol or n-hexane. Concentration of fatty acid ethyl esters determined in bio-oil reaction products by GC analysis (FAEE, wt.%).

Temperature	time	Extraction solvent	Extraction yield	$FAEE_{bio\text{-}oil}$
°C	min.	–	(Y_{ext} g/g %)	wt.%
270	20	Ethanol	*26.4* ± 2.1	20.1 ± 1.2
305	20			37.8 ± 1.5
270	40			42.3 ± 1.3
305	40			56.1 ± 2.1
305	40		12.2 ± 1.3*	17.8 ± 1.7*
270	20	n-Hexane	*21.1* ± 1.9	35.0 ± 1.3
305	20			59.5 ± 1.1
270	40			49.1 ± 1.6
305	40			71.7 ± 1.5
305	40		12.0 ± 1.3*	44.7 ± 1.7*

* Transesterification of crude lipids obtained in partial extractions of *H. coffeaeformis*: ethanol 14 extraction cycles and n-hexane 40 extraction cycles.

Table 2
Main fatty acid profile of the biofuel produced from *H. coffeaeformis* oil transesterifications of hexane and ethanol crude lipids extracts.

Fatty acid profile	Ethanol extract FAEE A/A, %	Hexane extract FAEE A/A, %
C14:0	9.1	12.6
C16:0	20.1	23.5
C16:1	27.8	32.2
C18:0	2.5	1.9
C18:1/C18:2/C18:3	11.5	6.5
C20:2	3.4	1.5
C20:4	2.0	0.5
C20:5	23.6	21.3

Table 3
FAEE content from acid catalyzed transesterification determined by GC analysis ($FAEE_{bio-oil}$ wt.%) and FAEE yield respect to biomass processed (Y_1).

	Hexane	Ethanol
Extraction yield (wt.%)	21.1 ± 1.9	26.1 ± 2.1
$FAEE_{bio-oil}$ wt.% (g FAEE/ g bio-oil)	86.5 ± 1.5	85.3 ± 1.5
Y_1 (g FAEE/ g biomass %)	18.3 ± 0.5	22.5 ± 0.5

values (40 Mj/kg) [3], with a relatively low cloud point (-4.6 °C) [4].

Table 3 shows maximum FAEE yield obtained in the acid catalyzed transesterification of crude lipids extracted by ethanol and n-hexane. FAEE concentrations of 86.5 wt.% and 85.3 wt.% were analyzed in the reaction products obtained in the acid catalyst ethanol transesterification of the crude lipids extracted by n-hexane and ethanol, respectively. These higher concentrations of FAEE can mainly be related to the water washing liquid-liquid fractionation step that could isolate the acid catalyst non-transesterified compounds. It is worth mentioning that FAEE concentrations reported for the supercritical ethanol transesterifications are based on total products obtained in the reactions without any fractionation or concentration step (only subjected to ethanol evaporation after reaction). Besides, it may be certainly related to a higher conversion of lipids through FAEE due to the presence of the catalyst (H_2SO_4) and the longer reaction time (6 h) employed in this technique. FAEE yields obtained in the acid catalyst transesterification of crude lipids extracted by ethanol are pointing out a complete conversion of triglycerides in the biomass (22 wt.%). On the other hand, catalyst transesterification of crude lipids extracted by hexane shows a conversion of nearly 80% of triglycerides present in the biomass (Fig. 4).

Fig. 4 shows supercritical transesterification results in terms of FAEE yield obtained in reaction products (Y_1, FAEE to biomass processed in the extraction). A lower yield was mainly obtained in the supercritical transesterification of crude lipids extracted with ethanol at a given operating condition, the exception being at 270 °C and 40 min., where 11.4 wt.% of the biomass was converted to FAEE. Better yields were obtained at 305 °C and 40 min., where as much as 16.4 wt.% of the processed biomass was converted to FAEE in the transesterification of crude lipids extracted by n-hexane. Equivalent yields can be obtained in the reactions operating at 305 °C and 20 min., or at 270 °C and 40 min. (Fig. 4).

The higher conversion of crude lipids obtained with n-hexane as solvent can be related to a greater concentration of triglycerides and a minor presence of pigments, polysaccharides, proteins and other non-lipids materials in the bio-oil [30]. Results reported in extraction tests show ethanol has a greater efficiency for bio-oil extraction (Fig. 2), which can be attributed to the hydrogen bonding of the solvent that associates with microalga membranes enabling the extraction of more bio-oil [21,22,24]. However, non-lipid materials co-extracted with crude lipids may also interfere with the biofuel production during the transesterification due to non-desirable side reactions [30,31].

According to previous works [45], acid catalyst transesterification has found to be useful for the conversion to biodiesel of total microalga lipids (polar, non-polar glycerides and free fatty acids). Thus, FAEE yields (Y_1) obtained in supercritical transesterification can be compared with acid catalyst transesterification (Y_2, Eq. 2) in order to estimate the efficiency of the supercritical process. The supercritical ethanol transesterification of crude lipids extracted by n-hexane at 305 °C and 40 min. shows a proximate yield with respect to acid catalyst reaction (89.9% of FAEE / FAEE A.C.). Supercritical reaction of crude lipids extracted by ethanol shows a lower efficiency to convert crude lipids. Accordingly, a maximum FAEE yield (Y_2) of 70.6% was obtained in the supercritical process at 305 °C and 40 min. in comparison to the catalytic reaction. In terms of total yield this lower efficiency in the lipids conversion is compensated by the higher amount of crude lipids produced in ethanol extraction relative to n-hexane. Hence, the FAEE yield Y_1 obtained in supercritical ethanol transesterification of crude lipids extracted by ethanol and hexane are very proximate (16.4 wt.% vs. 15.9 wt.%, Fig. 4).

Tobar and Núñez [46] recently reported similar FAEE production yields in the supercritical ethanol transesterification of *Spirulina platensis* oil that was extracted by cold press. FAEE yields between 64 wt.% and 68 wt.% of FAEE/oil were indicated operating the reactor at 300 °C and 200 bar, during 30 min. of residence time and using CO_2 as co-solvent between 0.0003 and 0.001 g CO_2/g ethanol. Liu et al. [36] studied the continuous supercritical methanol transesterification of *Chlorella protothecoides* oil at temperatures and pressures between 300 °C and 400 °C and 150 bar to 300 bar, respectively. The authors found that pressure can only play a relevant role in the transesterification between 150 bar and 200 bar. It was also reported up to 24 wt.% of triglycerides conversion to fatty acid methyl esters working at 300 °C and 10 min. of reaction time. High biodiesel yields (80 to 95 wt.%) were obtained operating the reactor between 350 and 400 °C and low residence reaction times (4 min.). However, a high degradation of fatty acid esters has been reported at these high temperatures in the transesterification of vegetable oils [47,48]. Patil et al. [49] studied the production of biodiesel from microalga oil using supercritical methyl acetate (40 to 1 acetate/oil) obtaining a fatty acid methyl ester yield of 72 wt.% at 310 °C, 100 bar, and 60 min. of reaction time. Fushimi and Umeda [50] studied the supercritical methanol transesterification of *Fistulifera solaris* JPCC DA0580 oil obtained by hydrothermal liquefaction at 300 °C. Their results show FAME yields of ≈80 wt.% with

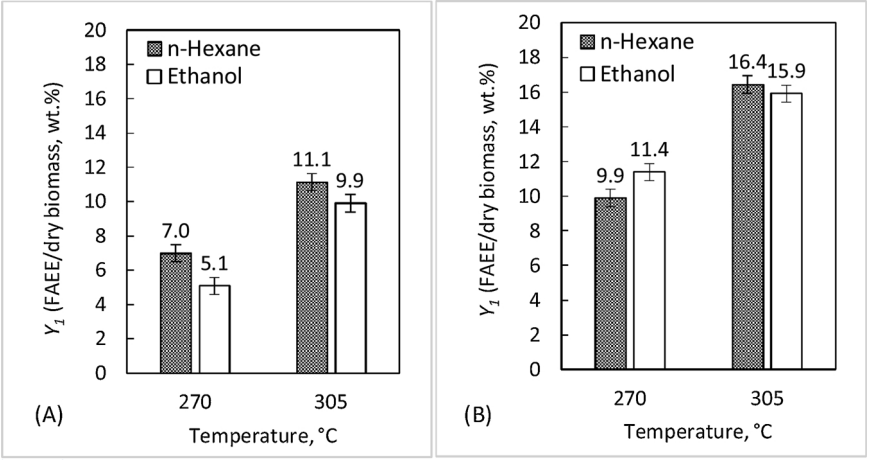

Fig. 4. Biodiesel production yields based on dry biomass processed at A) 20 min. and B) 40 min. of reaction time.

Table 4
First-order rate constants estimated from experimental data.

	Temperature range, °C	
	270	305
Ethanol crude lipids	0.023	0.034
Hexane crude lipids	0.020	0.038

respect to the neutral lipid content (16 wt.% FAME/biomass) in transesterification studies at 320 °C and 130 bar after 30 min. of reaction time. This high reaction yield can be attributed to a high content of free fatty acids produced during the hydrothermal oil extraction. Nan et al. [51] reported up to 87.8 wt.% of FAEE yield working at 340 °C and 170 bar, with 33 to 1 ethanol to oil molar ratio.

Similar FAEE yields have also been reported in the supercritical transesterification of different edible and non-edible vegetable oils [52]. Gonzales et al. [53] indicated 72.7 wt.% FAEE yield in the supercritical ethanol transesterification of soybean fried oil at 300 °C and 200 bar. The yield increased at these operating conditions to nearly 82 wt.% FAEE due to the addition of up to 10 wt.% water to the system. Vieitez et al. [47] found 53 wt.% FAEE yield in the supercritical ethanol transesterification of soybean oil at 300 °C and 200 bar.

Table 4 shows kinetic rate constants estimated from experimental data obtained in this work for the supercritical ethanol transesterification *H. coffeaeformis* lipids extracted with ethanol and n-hexane. A one-step first-order irreversible kinetic model was assumed considering the concentration of ethanol is in large excess in the system, being negligible the amount of ethanol consumed during the transesterification. Similar rate constants were obtained at different reaction temperatures, 270 °C and 305 °C, for the transesterification of lipids extracted with n-hexane and ethanol. However, transesterification of n-hexane extracted lipids shows at greater effect of temperature. Thus, apparent activation energies estimated from the fitting of rate constants by Arrhenius equation shows a higher value for the conversion of hexane crude lipids to biodiesel (47.3 Kj/mol) respect to results obtained for ethanol crude lipids transesterification (29.2 Kj/mol). Results reported by different authors in the literature [28,37,38] for the supercritical ethanol transesterification of vegetable oils from crops shows higher activation energy values (68 to 79 Kj/mol). Liu et al. [36] studied the supercritical methanol transesterification kinetics of *C. protothecoides* oil according to different kinetic models. The authors estimated an activation energy value of 90 Kj/mol using the single – step irreversible model, indicating a greater effect of temperature in comparison with the results obtained in this work. The difference in apparent activation energy values obtained for *H. coffeaeformis* lipids extracted by n-hexane or ethanol can certainly be related to the total lipid composition and fatty acid profile observed in biofuels [30]. A higher amount of complex polar lipids and non-lipid compounds in the bio-oils can influence reaction results as shown in previous works [30,31]. The free-catalyst transesterification of microalga oils can reduce costs with respect to the traditional process since it is a robust process that avoids the use of catalyst and makes it possible to process raw materials with high concentration of free fatty acids and water contents [35,47,51]. As shown in this work, crude lipids + ethanol solution obtained in the lipid extraction of *H. coffeaeformis* may be processed to obtain the biodiesel after ethanol partial evaporation.

In the case of extraction/reaction experiments carried out in this study, a simple analysis shows after extraction it would be necessary to evaporate ≈ 76% of the ethanol used in this step to process later a concentrated miscela in the reactor. In fact, there is a considerable difference between solvent to crude lipid ratio in the miscela (36 g /g) at the extractor outlet and the feeding of supercritical reactor (8.5 g ethanol/g crude lipids). The energy required for the solvent evaporation step in the ethanol process (23 kJ/g crude lipid) is still high in comparison with n-hexane (10 kJ/g crude lipids) due to the much higher vaporization enthalpy of ethanol (838.3 J/g) respect to n-hexane (334.9 J/g). Thus, results are pointing out extraction step should be optimized to reduce the amount of alcohol used in the process. A heat integration analysis as proposed in a previous work [54] can be carried out to analyzed different flow schemes alternatives in order to minimize operating costs. The global process avoids the exhaustive drying of biomass, decreases extraction time, and reduces reaction time in comparison with acid catalyst method. The most important advantage is that it is environmentally safe.

5. Conclusions

Crude lipids from *H. coffeaeformis* diatom were extracted using ethanol and n-hexane as solvents. The extraction of partially dried microalga with ethanol produced higher extraction yields in shorter extraction times in comparison with n-hexane. A maximum yield of 26.4 wt.% of crude lipids with respect to the dry biomass was obtained using ethanol as extraction solvent after 15 h, whereas n-hexane produced a yield of 21.1 wt.%. Extraction kinetics modeled using the single sphere model indicate a high mass transfer resistance in the system. The non-catalytic supercritical transesterification at 305 °C and 40 min. of *H. coffeaeformis* crude lipids extracted whether with ethanol or hexane shows in general a good conversion to biodiesel. The supercritical technology promotes the conversion of nearly 16 wt.% of dry microalga biomass to biodiesel. The extraction of partially dried biomass using ethanol in a first step can be a feasible sustainable process to obtain biolipids from microalga, while the remaining frustules and EPSs may be used under a biorefinery model. Thus, using ethanol as extraction solvent and supercritical reaction medium can be an interesting alternative to obtain biodiesel from *H. coffeaeformis*. It is a green technology that can produce comparable biodiesel yields with respect to conventional non-renewable organic solvents.

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