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Microbial Oil Derived from Filamentous Cyanobacterium *Trichormus* sp. as Feedstock to Yield Fatty Acid Ethyl Esters by Enzymatic Synthesis

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this study was to optimize the cultivation conditions of *Trichormus* sp. CENA77 and to evaluate the lipid feedstock to generate ethyl esters via enzymatic route using Novozym® 435 as catalyst. Under optimized cultivation conditions (1.5 g L^{-1} Na_2CO_3 and 150 µmol photon $m^{-2}s^{-1}$ light intensity), biomass productivity of 286.5 ± 3.6 mg.L^{-1}.day^{-1}, lipid contents of 14.5 ± 2.8% and lipid productivity of 41.5 ± 0.4 mg.$L^{-1}day^{-1}$ were achieved. Enzymatic transesterification was performed in a microwave reactor using a 1:12 molar ratio (oil/ethanol) at 50°C assessing iso-octane as solvent for 12 h. The viscosity values for the microbial oil (51.9 $mm^2.s^{-1}$) sharply decreased to 10.7 mm^2s^{-1}, upon the progress of transesterification reaction. The maximum fatty acid ethyl esters yield (80%) achieved was due to the presence of non-lipid compounds, which may have affected

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the biocatalyst activity. Evidence of the Novozym® 435 deactivation was demonstrated by the assistance of Fourier transform infrared spectroscopy and scanning electron microscopy analyses.

Keywords: Biodiesel; fatty acid ethyl esters (FAEE); cyanobacterium, Trichormus sp; microwave irradiation; lipase.

1. INTRODUCTION

Cyanobacteria are oxygenic photoautotrophic microorganisms which are widely distributed bacterial phyla. The systematic of Cyanobacteria stated to come into bacteriological focus upon confirmation of these organizations' prokaryotic origin, based on ultrastructure, and biochemical and molecular studies [1].

The genera *Trichormus* are classified based on the morphology of the filament, vegetative cells, heterocytes and akinetes that belong to the order of Nostocales, Nostocaceae family [2] and subsection IV.I of the bacteriological classification [3]. *Trichormus* was separated from the genus *Anabaena* and included into the subfamily Nostocoidae [4].

One major advantage of cyanobacteria over microalgae is that they produce lipids which are accumulated in the thylakoid membranes due to high levels of photosynthesis and rapid growth rate [5], while microalgae produces them as a reserve material under stress conditions and slow growth rate [6]. Thus, cyanobacteria are of great interest in the bioenergy scenario, since they produce lipids at high-speed growth.

Depending on the strain and culture conditions, they can synthesize up to 45% of lipids [7,8]. In a previous screening assay, three cyanobacterial isolates (*Synechococcus* sp. PCC7942, *Microcystis aeruginosa* NPCD-1 and *Trichormus* sp. CENA77) that do not produce toxins showed suitable properties to be used as feedstock for biodiesel synthesis [5]. However, practical investigation of the growth of filamentous oleaginous cyanobacterium *Trichormus* sp. CENA77 was required to exploit the properties fully as a biodiesel feedstock.

In order to establish a process to yield biodiesel from microbial feedstock, several parameters should be carefully evaluated. In addition to obtaining high biomass productivity, there are technological challenges related to harvesting and oil extraction from microbial biomass that should be taken into account, e.g. for some strains 20% of the total biodiesel production cost is associate to the harvesting step [9]. Thus, the use of the filamentous *Trichormus* sp. strain becomes even more attractive, since it can be easily recovered by filtration after the culture, which reduces the great energy demand that is usually required to recover biomass by centrifugation [10,11].

Furthermore, microbial oils normally contain high free fatty acid levels, which is another critical problem for transesterification via traditional alkaline methods [12]. Such limitation can be easily overcome by using lipases that can catalyze both the esterification of free fatty acids and the transesterification of triacylglycerols with no soap formation. Therefore, enzymatic catalysis is typically used to yield biodiesel from microbial oils, as previously reported [13,14,15]. Although the enzymatic technique offers a number of environmental and economic advantages over the chemical route, its reaction rate is slower, hence decreasing biodiesel productivity. Microwave irradiation can overcome such limitations by providing substrates with higher enzyme selectivity, enabling high yields in shorter reaction times and, consequently, greater volumetric productivity of the target product [16].

In this context, the present work performed a systematic study aimed to establish the culture conditions of *Trichormus* sp. CENA77 to obtain high cell productivity and lipid content. To this end, a 2^2 full factorial design was carried out. The lipid feedstock obtained by optimized culture was further evaluated for biodiesel synthesis using Novozym® 435 as catalyst, owing to its proven catalytic properties to synthesize biodiesel (fatty acid ethyl esters -FAEE) from different microbial and vegetable oils [13,15,17,18,19]. In order to enhance the reaction rate, the transesterification was conducted under microwave irradiation.

2. MATERIALS AND METHODS

2.1 Materials

A filamentous cyanobaterium strain, *Trichormus* sp., with heterocytes from the culture collection

of the Laboratory of Molecular Ecology of Cyanobacteria (CENA/USP, São Paulo, Brazil), isolated from a contaminated environment containing propanil was used in this work [5]. Novozym® 435 was supplied by Sigma-Aldrich Chemical Co (Milwaukee, WI, USA) to be used as biocatalyst. The reaction control was carried with refined macaw palm oil, provided by the Association of Small Farmers D'Antas (Minas Gerais, Brazil), with the following fatty acid composition (% w/v): 0.1% lauric, 0.1% mirystic, 17.6% palmitic, 2.0% stearic, 58.6% oleic, 16.1% linoleic acid and 5.5% of others, with an average molecular mass of 886 $g.mol^{-1}$. Other characteristics of macaw palm oil are: acid index of 16.1 mg KOH g^{-1}, high free fatty acid (FFA) content of 9.2%, iodine value of 28 g I_2 100 g^{-1} of oil and saponification value of 223 mg KOH g^{-1} of oil. Anhydrous ethanol (minimum 99.8%) and isooctane were supplied by Cromoline (SP, Brazil), chloroform and methanol were supplied by Synth (SP, Brazil) and oleic acid was supplied by Reagen (RJ, Brazil).

2.2 Cyanobacterium Culture Conditions

The influence of the variables (light intensity and Na_2CO_3 concentration) on the lipids content (%) was analyzed according to a 2^2 full factorial design. The strain was inoculated at 10% (v/v) in flasks containing 8 L of BG-11 culture media [5] supplemented with Na_2CO_3 concentrations (0.5-1.5 $g.L^{-1}$). Cultures were sparged with sterile air during 10 days at 24±1ºC using different light intensities (50-150 photon m^{-2} s^{-1}). The cells were harvested by filtration with a sieve (mesh sizes ranged from 250 to 300 µm), which were subsequently lyophilized. The Design expert (version 6.0 - Stat-Ease Corporation, USA) and Statistica (version 8.0 - StatSoft Inc., USA) softwares were used for regression and graphical analyses of the obtained data. Lipid content (%) was considered as response variable.

2.3 Lipid Extraction

Lipids were extracted from dry biomass cells (lyophilized and grounded) through modified Folch's methodology [20] by using a mixture of chloroform, methanol and water as extracting solvent, in the following respective ratio: 1:2:0.8 (v/v/v). The extractions were performed by ultrasound with 200 W ultrasonic processors (Model-UP200S/ UP400S, Hielscher-Ultrasound Technology). The obtained extract was dried in a rotatory evaporator to remove the solvent, and the microbial oil was dried at 60℃ until constant weight was reached. The oil yield was calculated according to equation 1. Biomass productivity and lipid productivity was calculated by the ratio between biomass content (mg L^{-1}) and lipid content (mg L^{-1}) per day of culture. Thus, $mg.L^{-1}.day^{-1}$ is the unit being used for biomass and lipid productivities.

$$\% \, Lipid = \frac{Lipid \; mass}{Mass \; of \; the \; dry \; biomass} * 100 \qquad (1)$$

2.4 Measuring the Effect of Biomass Moisture on Microbial Oil Yield

The effect of moisture contents (10, 20, 35 and 45%) on the biomass harvested from the culture of *Trichormus* sp. supplemented with 1.5 g L^{-1} of Na_2CO_3 under 150 $\mu mol.m^{-2}.s^{-1}$ light intensity has been studied. Biomass samples were dried in order to attain moisture at the required level (10-45%) and the lipid extraction was carried out by following the aforementioned protocol.

2.5 Microbial Oil Characterization

The microbial oil fatty acid composition was determined by gas chromatography (GC) according to the AOCS Ce 1-62 method [21] using capillary gas chromatography (CGC Agilent 6850 Series GC System): Agilent capillary column (50% cyanopropyl methylpolysiloxane), dimensions: 60 m, Ø int: 0.25 mm, film: 0.25 µm. The oven temperature was 110℃/5 min, 110-215℃ (5℃ min^{-1}), and 215℃/24 min; detector temperature: 280℃; injector temperature: 250℃; carrier gas: helium; split ratio: 1:50; injection volume: 1.0 µL. The qualitative composition was determined by comparing the peak retention times with the respective standards for fatty acids. The AOCS's method (Cd 3d-63) was used for the determination of total free fatty acids (FFA), which was expressed in terms of free oleic acid (%).

2.6 Fatty Acid Esters Synthesis

Reactions were performed in a microwave reactor (Model Discover/University-Wave, Cem Corporation) consisting of a cylindrical internal chamber of 75 mm in diameter and 100 mm in height, containing mixtures of lipid feedstocks (microbial oil or macaw palm oil) and anhydrous ethanol at fixed oil:alcohol molar ratio (1:12) and iso-octane as solvent [13]. Novozym® 435 was inserted at proportions of 20% (w/w) according to the total mass of added reactants, and the

reactions were carried out for at least 12 h. A magnetic stirrer was used to qualitatively mix the reaction medium. The maximum power of operation was about 100 W. The temperature was controlled through blowing compressed air into the chamber. This cooling air was assigned to operate at different pressure levels, either on a continuous or intermittent flow mode. The reaction temperature was monitored by an infrared sensor located at the lower part of the chamber. The ChemDriver software was used to record data for each run, including the variation in microwave flux and temperature evolution. Fig. 1 shows the schematic diagram of the experimental procedure used in this work, starting from the cultivation of *Trichormus* sp up to the formation of ethyl esters by enzymatic transesterification under microwave irradiations.

2.7 Fatty Acid Ethyl Ester Analysis (FAEE)

Aliquot samples were taken at various time intervals for quantifying the ethyl esters formed by FID gas chromatography through a Varian CG 3800 model (Varian, Inc. Corporate Headquarters, Palo Alto, CA, USA) equipped with a flame-ionization detector and 5% a DEGS CHR-WHP 80/100 mesh 6 ft 2.0 mm ID in OD in a stainless steel packed column (Restek, Frankel Commerce of Analytic Instruments Ltd., SP, Brazil). Nitrogen was used as the carrier gas at a flow rate of 25 mL min^{-1}. Temperature programming was performed. The column temperature was maintained at 90℃ for 3 min, heated to 120℃ at 25℃ min^{-1} and kept constant for 10 min. Afterwards, the temperature was programmed at 25℃ min^{-1} to 170℃ and kept constant for 15 min. The temperatures of the injector and detector were set at 250℃. Data collection and analyses were performed by the software Galaxie Chromatography Data System, version 1.9. Hexanol was used as internal standard. The reaction yield was calculated by taking into account the mass of the ester content obtained by the GC analysis and the total theoretical ester mass was based on the reaction molar ratio [22].

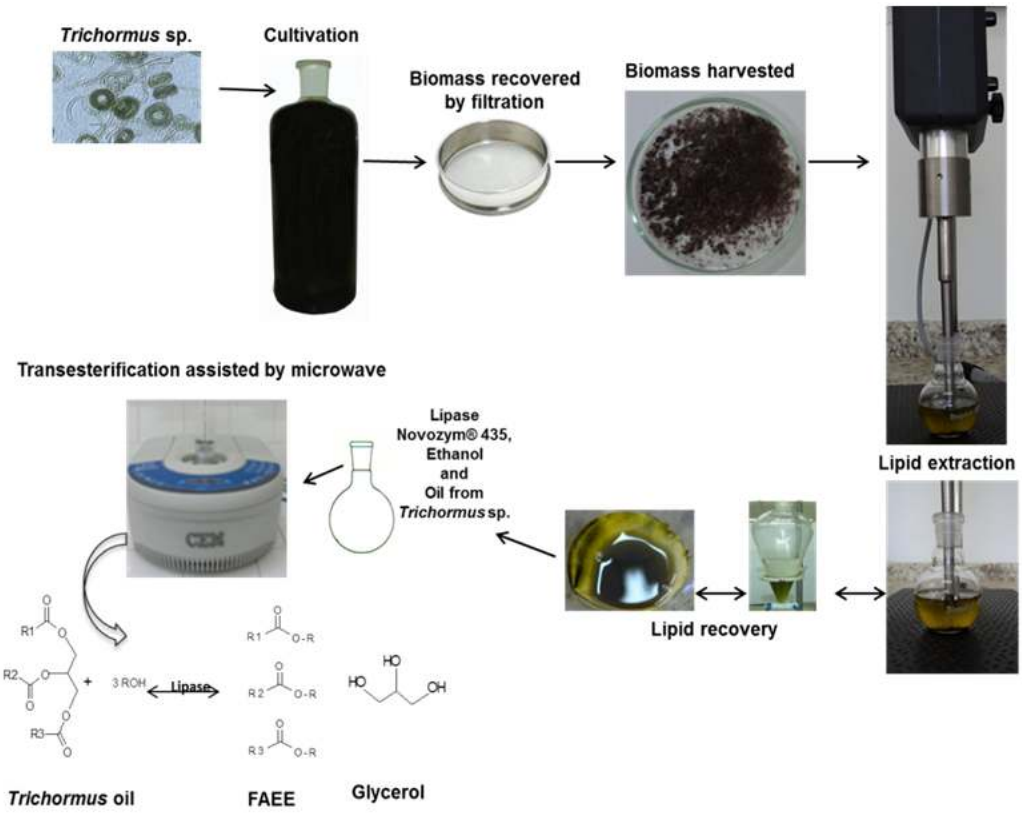


Fig. 1. Experimental scheme for producing ethyl fatty esters from microbial oil by enzymatic route under microwave irradiations

2.8 Downstream Procedure

After the transesterification reaction, the biocatalyst was recovered by centrifugation and the liquid phase was purified by traditional aqueous washing procedure according to the methodology described by Da Rós et al. [5]. The viscosity values of the purified ethyl esters were determined by a Brookfield viscosimeter (Brookfield Viscometers Ltd, England) through a CP 42 cone. Residual monoacylglycerols and diacylglycerols were quantified by HPLC in an Agilent 1200 Series liquid chromatograph (Agilent Technologies, USA) equipped with ELSD (Evaporative Light Scattering Detector) and a Gemini C_{18} (5 μm × 150 × 4.6 mm, 110 Å) column at 40℃, according to the methodology described by Carvalho et al. [15].

2.9 Biocatalyst Analysis

The catalytic activities of the biocatalysts (original and recovered) were assessed through the esterification reaction of oleic acid with ethanol by following the methodology described by Pinto et al. [23]. One unit of enzymatic activity (1 IU or 1 U) corresponds to the amount of enzyme necessary to form 1 μmol of ethyl oleate per minute under the presently selected conditions (40℃). IR spectra were obtained from a FT-IR Spectrum GX (Perkin Elmer, SP, Brazil) with a spectral resolution of 4 cm^{-1} [5] and the biocatalyst morphology was obtained by high-resolution scanning electron microscopy (LEO Model 1450 VP, ZEISS).

2.10 Bioprospecting of Biodiesel Properties Based on Fatty Acids Profile

Biodiesel properties based on the fatty acids profile from microbial oils obtained under the conditions established in the present work (culture medium enriched with Na_2CO_3 and high luminous intensity-150 $\mu mol.m^{-2}.s^{-1}$) and under standard culture conditions [5] were estimated through the Biodiesel Analyzer© ver. 1.1 [24,25].

3. RESULTS AND DISCUSSION

3.1 Lipid Content from *Trichormus* sp.

Lipids are part of the essential macromolecules from cyanobacteria metabolism. The diversity of growth conditions and habitats has significantly influenced lipid content production by each strain. Part of fixed CO_2 is stored in various lipid forms, including triacylglycerols (TAGs), free fatty acids, sterols and wax esters − with up to 60% of dry cell material in some strains [26].

In a previous work, which had used standard culture conditions, biomass and lipid productivities for *Trichormus* sp. CENA77 were 30.8 and 7.3 mg L^{-1} day^{-1}, respectively [5]. In order to increase lipid productivity from the *Trichormus* sp. biomass, a 2^2 full factorial design was proposed by taking light intensity (X_1) and Na_2CO_3 content (X_2) in the growth medium as independent variables. The lipid content (%) reached after 10 days of culture was taken as the experimental design response variable. The total weight of the lipids was quantified by the percentage of lipid in g 100 g^{-1} biomass. The experimental design together with the results are shown in Table 1.

Culture conditions have significantly affected lipid content, and the highest value (14.5 ± 2.8%) was achieved when both variables were set at their highest levels (run 4), which indicates a strong influence of both variables on lipid content. Under these conditions, a lipid productivity of 41.5 $mg.L^{-1}.day^{-1}$ could be reached.

The statistical analysis confirms the effect suggested by the direct data analysis. In agreement with Student's t test (Table 2), it was verified that lipid content was significant on both studied variables at 90% confidence level, while the interaction between them was not statistically significant (p=0.6369) at the same confidence level.

From these data, it was possible to generate a square graph (Fig. 2) which shows the effect of the variables within the studied range (-1 to +1) on the analyzed response variable: lipid content (%). It demonstrates that, in order to obtain high lipid content from *Trichormus* sp., it is necessary to increase both the light intensity and the Na_2CO_3 content in the culture medium, thus indicating that these variables have a positive effect on the response variable. These results suggested that both variables are essential to increase biomass productivity and lipid content in the culture of *Trichormus* sp.

This is illustrated in Table 3, which shows comparative parameters of cultures carried out under standard and optimized conditions.

Table 1. Lipid contents obtained from *Trichormus* sp. cultivated under different conditions

Run	Light intensity (µmol.m^{-2}.s^{-1})	Carbon – source Na_2CO_3 (g L^{-1})	Lipid content (%)
1	50	0.5	3.1 ± 1.05
2	150	0.5	6.8 ± 0.94
3	50	1.5	11.8 ± 1.60
4	150	1.5	14.5 ± 2.80
5	100	1.0	8.6 ± 1.40
6	100	1.0	9.3 ± 0.98
7	100	1.0	7.5 ± 1.50

Table 2. Estimated effects, standard errors and Student's t test for lipid content from *Trichormus* sp. using the 2^2 full factorial design

Variables	Effects	Standard errors	t	*p*< 0.10
Mean	8.80	± 0.34	25.66	0.0015*
Light intensity (x_1)	3.20	± 0.90	9.04	0.0120*
Na_2CO_3 (x_2)	8.20	± 0.90	3.52	0.0718*
x_1x_2	-0.50	± 0.90	-0.55	0.6369

**significant at 90% confidence level*

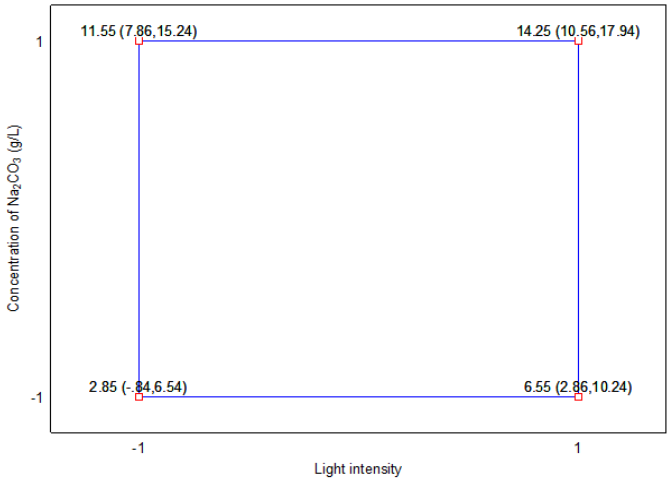


Fig. 2. Square graphic for the obtained lipid content (%) showing the effect of the following variables: light intensity and Na_2CO_3 concentration in a *Trichormus* sp. culture medium

Table 3. Comparative performance of the filamentous cyanobacterium *Trichormus* sp. cultivated in BG 11 medium under different conditions

Parameter	Medium cultivation	
	Optimized medium (this study)	Standard BG 11 Da Rós et al.[5]
Light intensity (µmol m^{-2}s^{-1})	150	100
Carbon source (Na_2CO_3)	1.5 g L^{-1}	Absence
Biomass (X)	2.86 g L^{-1}	0.62 g L^{-1}
Lipid concentration (P)	415 mg L^{-1}	146 mg L^{-1}
Volumetric lipid production rate (QP)	41.5 mg L^{-1} day^{-1}	7.3 mg L^{-1} day^{-1}
Volumetric biomass production rate (Q_X)	286.5 mg L^{-1} day^{-1}	30.8 mg L^{-1} day^{-1}
Specific rate of lipid production (qP)	0.015 mg lipid mg biomass^{-1} day^{-1}	0.011 mg lipid mg biomass^{-1} day^{-1}

As can be observed, the lipid concentration (P) increased approximately 3 times by setting the light intensity to 150 µmol $m^{-2}s^{-1}$ and supplementing the culture medium with Na_2CO_3. Moreover, the volumetric lipid production rate (QP) and volumetric biomass production rate (Q_X) were approximately 6 and 9 times greater for *Trichormus* sp. cultivated under optimum conditions established in this work.

3.2 Effect of Biomass Moisture Content on Oil Yield

Water plays a significant role in microbial oil yields from biomass extractions by creating a water layer on the cell wall [27]. In an ultrasonic process, acoustic cavitation occurs when a sufficiently strong ultrasonic forcing propagates in a liquid. When these cavitation bubbles collapse, a large amount of energy is released. This is reflected in the rupture of cells [28]. Moreover, the sonication process increases the system temperature, which facilitates the penetration of the solvent into the cell hydration shell. Thus, it becomes possible to extract larger amounts of fatty acids, mostly unsaturated membrane lipids, from a highly hydrated biomass.

To assess the influence of biomass moisture content on microbial oil yields, the biomass recovered from run 4, in which the culture medium was enriched with 1.5 g L^{-1} of Na_2CO_3 and kept under light intensity of 150 µmol.m^{-2}.s^{-1}, was submitted to lipid extraction by Folch's method. Four levels of moisture content (10, 20, 35 and 45%) were investigated, whose results are shown in Fig. 3.

By comparing the total microbial oil extracted from the examined hydrated samples, it was possible to observe that the biomass containing 45% of water led to optimal microbial oil extraction (21.6 ± 1.1%). This confirms that a cavitation phenomenon produced by ultrasonic waves easily disrupts highly hydrated cells by Folch's method, thus allowing better microbial oil extraction. A slightly lower yield (19.4 ± 0.9%) was achieved when the biomass exhibited 35% of moisture content. On the other hand, for biomass containing lower moisture levels (10 and 20%), extraction yields dropped to values (≈ 15%) close to those attained with dried biomass, which suggests a strong influence of biomass moisture when subjected to extraction techniques (Fig. 3). Such results are in agreement with those reported in literature [15], which can be considered an advantage since drying the biomass prior to lipid extraction saves energy.

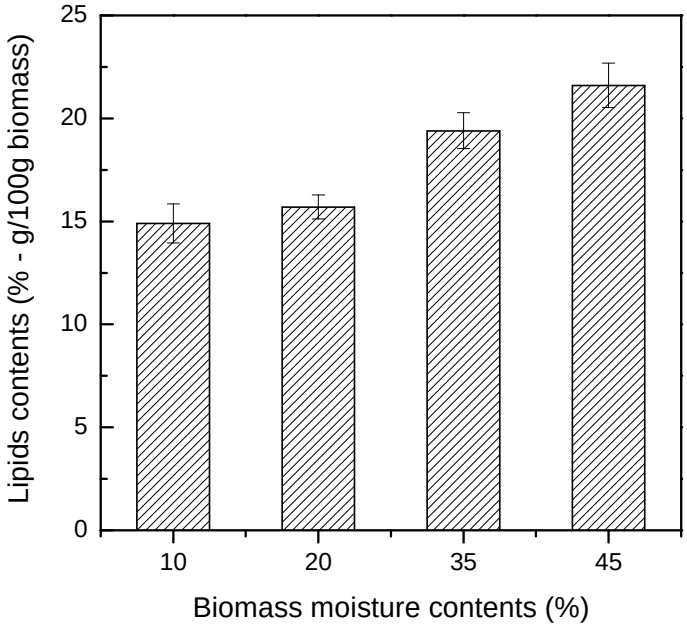


Fig. 3. Influence of *Trichormus* sp. biomass containing different moisture contents (10, 20, 35 and 45%) on the lipid yield

3.3 Microbial Oil Characterization

The lipid material resulting from the extraction of biomass containing 45% of moisture was further characterized with regard to its fatty acid profile, with the main fatty acids composition being displayed in Fig. 4. The microbial oil derived from *Trichormus* sp. showed elevated proportions of unsaturated fatty acids in its composition (81.5%). Those obtained at higher proportions were the oleic (C18:1), linoleic (C18:2) and palmitic (C16:0) acids. Values lower than 4% were attained for the following fatty acids: stearic (C18:0), linolenic (C18: 3), myristic (C14:0) and lauric (C12:0) acids. Other fatty acids obtained at smaller proportions were: the caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), pentadecanoic acid (C15:0), arachidic acid (C20:0), behenic acid (C22:0), among others.

In a previous work under different culture conditions [5], the fatty acids profile from the *Trichormus* sp. lipids showed similarity with respect to the formation of the main fatty acids, but at different proportions. Under the conditions used in the present work (culture medium enriched with Na_2CO_3 and high light intensity-150 $\mu mol.m^{-2}.s^{-1}$), a sharp increase in the unsaturated fatty acids content (especially linoleic and oleic acids) and a reduction of the saturated fatty acids (palmitic and lauric acids) were observed.

Estimated properties of the biodiesel based on the fatty acids profile from *Trichormus* sp. lipids obtained previous the optimization [5] and under the conditions established in the present work are displayed in Table 4.

Biodiesel quality is largely determined by the ratio of saturated to unsaturated fatty acids present in the feedstock. Saturated fatty acids are resistant to degradation and, therefore, increase biodiesel stability, while unsaturated FAs enhance cold flow characteristics. Saturated fatty acids, on the other hand, increase biodiesel resistance to oxidation under hot weather conditions [24].

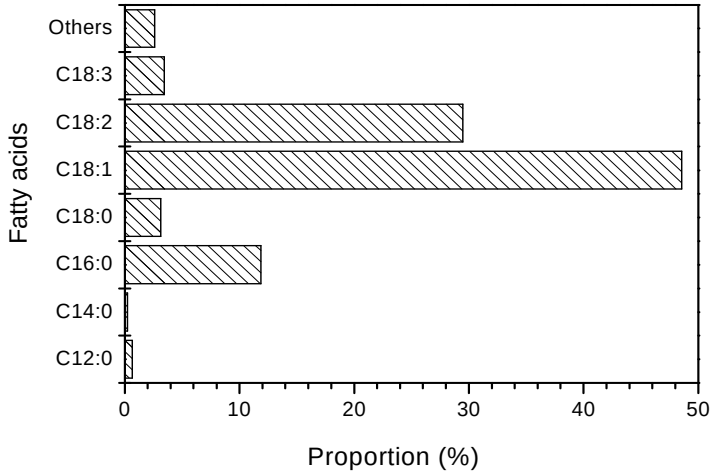


Fig. 4. Fatty acid profile of the microbial oil derived from the *Trichormus* sp. biomass

Table 4. Estimated biodiesel properties from *Trichormus* sp. lipids cultured under different conditions

Biodiesel property	*Trichormus* sp. (this study)	*Trichormus* sp. Da Rós et al. [5]
Degree of Unsaturation (DU)	112.0	67.0
Saponification Value (SV)	189.0	194.4
Iodine Value (IV)	103.9	65.2
Cetane Number (CN)	51.8	59.7
Long-Chain Saturated Factor (LCSF)	2.6	3.9
Cold Filter Plugging Point (CFPP) (℃)	-8.3	-4.2
Cloud point (CP) (℃)	0.8	7.6
Oxidation Stability (OS) (hours)	6.3	10.5
Higher Heating Value (HHV)	37.1	35.7

As shown in Table 4, *Trichormus* sp. lipids previous optimization [5] generated the highest ratio of saturated fatty acids to unsaturated ones, and the highest LCSF and CP in contrast to the lowest values for DU and IV. *Trichormus* sp. lipids [5] also had the highest CN value, but the lowest IV and, consequently, the highest CFPP. Therefore, the biodiesel obtained from *Trichormus* sp. lipids [5] could be recommended to be used in hot regions due to a couple of reasons: having the lowest IV and, as a result, the least degree of biodiesel oxidation and degradation. In contrast, the lowest CP value (0.8) obtained for biodiesel from *Trichormus* sp. lipids cultured under the optimized conditions of the present work (culture medium enriched with Na_2CO_3 and high light intensity-150 $\mu mol.m^{-2}.s^{-1}$) favors its use in cold regions. In addition to increasing lipids productivity, the establishment of *Trichormus* sp. culture conditions also makes this feedstock a better candidate for biofuel synthesis, since the predicted biodiesel properties are in accordance with ASTM D6751 and EN 14214.

3.4 FAEE Synthesis under Microwave Irradiation

The acid value of the microbial oil was 15.7 mg KOH g^{-1}, with high free fatty acid (FFA) content of 9.0%. Lipids with FFA content over 5 wt % are not suitable for alkaline-catalyzed transesterification, as the FFA will tend to consume the catalyst and form soap, thus leading to serious separation problems. Hence, the lipase Novozym® 435 was used in the present study to mediate the simultaneous esterification and transesterification reactions of the microbial oil. To overcome such slow rate, the reaction was conducted under microwave irradiation [14,16,29].

Enzymatic FAEE synthesis from microbial oil was performed under high frequency electromagnetic fields (microwaves), according to the conditions described in 2.6. Macaw palm oil was used as reaction control medium since this vegetable oil has similar fatty acid profile in relation to the microbial oil, and most of it has high free fatty acid level (acid index = 16.1 mg KOH g^{-1}). The reaction progress for each tested feedstock (*Trichormus* sp. and macaw palm oils) was carried out under microwave irradiation, which is displayed in Fig. 5. Similar kinetic profiles were achieved for both feedstocks, though the reaction rates and yields were dependent on the oil source. The reaction performed with microbial oil was found to have the lowest performance and attained maximum ethyl esters (FAEE) yield of about 80.0% in 12 h. For the control reaction, higher reaction rate was verified, which allowed attaining FAEE yield up to 98.5% in 10 h of reaction. Such differences may be attributed to a few features of the microbial feedstock that was

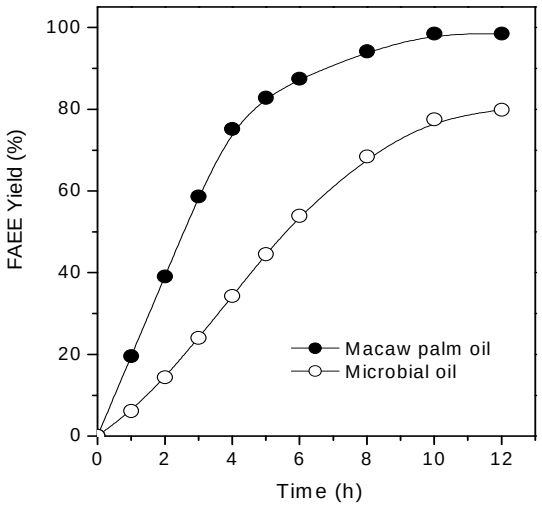


Fig. 5. Transesterification progress of microbial and vegetable oils under microwave irradiation catalyzed by Novozym® 435. The reactions were performed at a molar ratio of oil to ethanol 1:12 using iso-octane as solvent at 50ºC. The macaw palm reaction was used as means of control

used without prior treatment to eliminate impurities, such as dyes and pigments. Typical microbial oils from cyanobacteria are green or brown due to the presence of pigments such as chlorophyll *a*, phycocyanin and phycoerythrin [30]. Although the Novozym 435® is considered to be a robust biocatalyst, the presence of non-lipid compounds may have affected the activity of lipase preparation. It is probable that the adsorption of dyes or pigments onto the immobilizing support causes diffusion limitations and lipase inhibition, on account of the formation of a layer around the enzyme, which may impose restriction of substrate access onto the enzyme active site, thus affecting its efficiency.

To elucidate this hypothesis, the original and recovered biocatalysts were submitted to a specific analysis to quantify any changes that might have occurred in the biocatalyst after using it in the ethyl ester synthesis from the microbial oil, as described in 3.6. It is important to mention that for the control reaction, such behavior was not noted.

3.5 Analysis of Purified Fatty Acid Ethyl Ester (FAEE) Samples

The viscosity values for the microbial and macaw palm oils were, respectively, 51.9 and 29.5 $mm^2.s^{-1}$, and sharply decreased upon the transesterification reaction. The lowest viscosity value (3.8 $mm^2.s^{-1}$) was found for the sample obtained from macaw palm oil transesterification, which also confirmed the highest conversion rates of triacylglycerols into ethyl esters, as it was determined by the GC analysis (98.5%) and the lowest levels of monoacylglycerols (0.4%) and diacylglycerols (1.2%), as determined by the HPLC (Table 5). On the other hand, the sample obtained from the microbial oil transesterification showed high amounts of monoacylglycerols (17.2%) and diacylglycerols (2.8%), which is in agreement with the viscosity value (10.7 $mm^2.s^{-1}$) and ester contents (80.0%).

3.6 Changes on the Recovered Biocatalyst from the Transesterification Reaction of Microbial Oil under Microwave Irradiation

In this section, attention was given to clarifying the lowest performance attained by the Novozym® 435 in the transesterification reaction of the microbial oil under microwave irradiation. For such a purpose, samples of the original and recovered biocatalysts were analyzed in terms of their catalytic activity, FT-IR and morphological (SEM) analyses. The recovered biocatalyst from the transesterification of macaw palm oil was also assessed as a control parameter.

Table 5. Properties of fatty acid ethyl ester (FAEE) samples obtained from the transesterification reaction of microbial (*Trichormus* sp.) and vegetable (macaw palm) oils catalyzed by Novozym® 435 under microwave irradiation

Properties	Microbial oil	Macaw palm oil
Ester content (%)	80.0	98.4
Monoacylglycerols (%)	17.2	0.4
Diacylglycerols (%)	2.8	1.2
Triacylglycerols (%)	0	0
Absolute viscosity (cP)	10.7	3.8

The esterification activities of the biocatalysts (original and recovered) were achieved according to the methodology described in 2.9. By factoring this in, the esterification activities were calculated and displayed in Table 6.

Table 6. Comparison of esterification activities of the original and recovered biocatalysts

Biocatalyst (Novozym® 435)	Esterification activity (U g^{-1})
Original	1624.5 ± 3.3
Recovered from the transesterification of microbial oil	887.9± 4.7
Recovered from the transesterification of vegetable oil	1119.3 ± 2.8

Results clearly show that, regardless of the feedstock being used, the activity of the recovered Novozym® 435 decreased in comparison with the original biocatalyst. However, the activity dropped for the biocatalyst recovered from microbial oil transesterification was much higher, about 45%, whereas it was 30% for the macaw palm oil. This behavior of Novozym® 435 has already been reported when it was being used in the transesterification reaction of a vegetable oil [31,32]. It appears that the phenomenon involved in such activity

loss can be attributed to the formation of a shell around the enzyme, which limits the diffusion of the substrate to it.

According to Xu et al. [31], the glycerol formed from biodiesel synthesis by using Novozym® 435 can slight reduce the reaction rate due to a hydrophilic layer around the catalyst, thereby limiting substrate mass transfer into the enzyme. In addition to the glycerol effect, Jose et al. [32] reported that ethyl esters are also able to diffuse across the Novozym®435 beads, leading to their swelling, modifying their internal texture and also dissolving the polymeric matrix. Moreover, alcohol is rapidly diffused inside the biocatalyst beads, thus affecting polymer integrity.

Therefore, in the case of the biocatalyst recovered from the vegetable oil transesterification reaction, the activity loss can be associated with the expected behavior described by both research groups [31,32]. On the other hand, for the biocatalyst recovered from microbial oil transesterification, the presence of additional inhibitors (dyes and pigments) in the feedstock greatly affects biocatalyst performance.

Additional information on the recovered biocatalysts was assessed by FT-IR. Fig. 6 displays the infrared spectra for the original Novozym® 435 and the recovered biocatalysts from microbial and vegetable oils transesterification reactions.

The infrared spectrum of the original Novozym® 435 shows intense bands corresponding to Amide I and Amide II signals centered at 1653 cm^{-1} and 1540 cm^{-1}, respectively [32], a typical spectrum of proteins with band resulting from CO double bond stretching, C–N stretching and NH bending [33]. These signals were also observed in the case of the lipase recovered from both reactions, thus confirming the integrity of the protein after biodiesel production. It should be mentioned that the biocatalysts were not washed with a solvent after the transesterification reactions. Therefore, the infrared spectra show a slight increase on peak at 1700 cm^{-1} for the recovered lipase, which is ascribed to the stretching vibration of carbonyl species (CO) of the ester linkage COOR that belongs to either the free fatty acids or the FAEE that may be adsorbed on the biocatalysts beads.

The presence of a broad band in the 3100-3500 cm^{-1} region related to axial deformation characteristic of OH groups suggests that the original and recovered biocatalysts had water contents (for original lipase) or remaining ethanol from the transesterification reaction (for recovered lipase). All samples showed a spectrum with vibration band at 3006 cm^{-1} related to CH stretching.

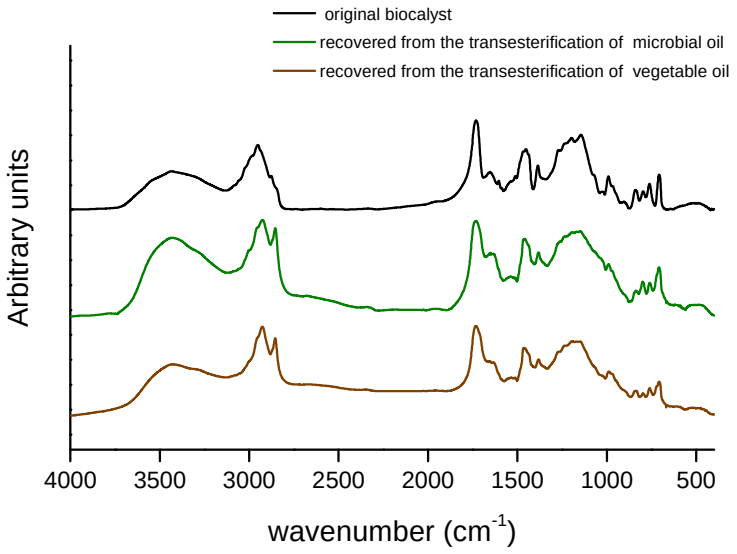
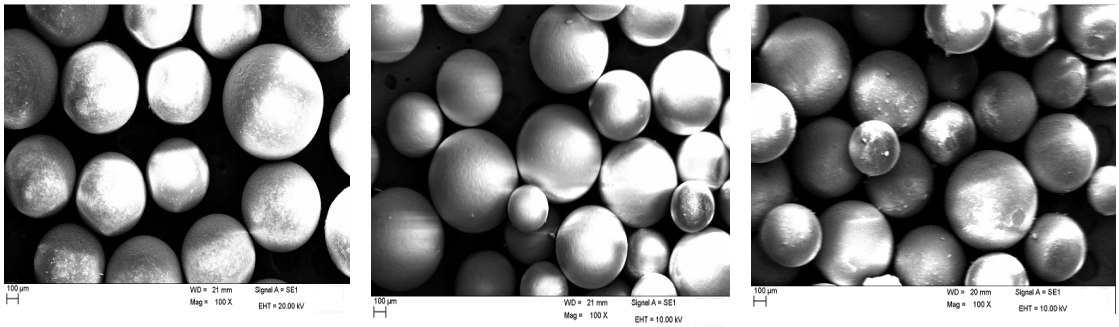


Fig. 6. Infrared spectra for the Novozym® 435 original biocatalyst (black line); biocatalyst recovered from the transesterification of vegetable oil (brown line) and biocatalyst recovered from the transesterification of microbial oil (green line)



(a) (b) (c)

Fig. 7. SEM microphotography of Novozym® 435 original biocatalyst (a) biocatalyst recovered from the transesterification of vegetable oil (b) and biocatalyst recovered from the transesterification of microbial oil (c)

Scanning electron microscopy (SEM) was also used to analyze the modification of the external texture of biocatalyst recovered from the microbial and macaw palm oil reactions. Original Novozym® 435 was assessed as a control parameter.

SEM microphotography shows the similarity between the recovered biocatalyst from the macaw palm oil transesterification (Fig. 7 b) and original Novozym® 435 (Fig. 7 a). However, modifications on the beads of the recovered biocatalyst from microbial oil transesterification (Fig. 7 c) were observed, such as small encrusted residues on the support surface, probably due to the impurities present in the microbial feedstock.

4. CONCLUSION

Suitable conditions to culture the *Trichormus* sp. were established by the factorial design. Within the studied range, lipid productivity was significantly increased, attaining values that were 6 times greater than what had been previously reported. Under the established culture conditions (1.5 g L^{-1} of Na_2CO_3 and 150 µmol photon $m^{-2}s^{-1}$), biomass productivity and lipid productivity were nine times and six times greater, respectively, if compared to previous studies. In addition, the efficiency of lipid extraction by Folch's method can be increased in approximately 48% by using highly hydrated biomass samples (about 45% of moisture) instead of dry biomass samples. The qualitative analysis of the fatty acids demonstrates a large amount of unsaturated fatty acids (82.7%), which adjusted the predicted biodiesel properties in accordance with ASTM D6751 and EN 14214. In addition to increasing lipids productivity, the established culture conditions for *Trichormus* sp also makes this feedstock a better candidate for biofuel synthesis. Nevertheless, further studies should be performed in order to assess a more robust catalyst that can tolerate the non-lipids impurities present in this microbial oil.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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