Research Article

Biomass Production and Ester Synthesis by *In Situ* Transesterification/Esterification Using the Microalga *Spirulina platensis*

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The increasing energy demand and reduction in the availability of nonrenewable energy sources, allied with an increase in public environmental awareness, have stimulated a search for alternative energy sources. The present study was aimed at producing biomass from the microalga *Spirulina platensis* and at assessing *in situ* synthesis of alkyl esters via acid transesterification/esterification of biomass to produce biodiesel. Two alcohols (ethanol and methanol) and two cosolvents (hexane and chloroform) were tested, at different temperatures (30, 45, 60, 75, and 90°C) and reaction times (10, 20, 30, 60, and 120 min). The factorial analysis of variance detected an interaction between the factors ($P < 0.05$): temperature, reaction time, alcohol, and cosolvent. The best yields were obtained with the combination ethanol and chloroform at 60°C, after 30 min of reaction, and with hexane at 45°C, after 10 min of reaction. *In situ* transesterification/esterification of alga biomass to form esters for biodiesel production adds unconventional dynamics to the use of this feedstock.

1. Introduction

The global concern about future availability of energy comes from national security, economic stability, and environmental sustainability issues [1, 2]. In this context, the demand for petroleum derivatives has increased, but the current concern about reduction in pollution and the energy crisis has stimulated the global biofuel market. The global economy keeps increasing and so does the need for clean, renewable energy sources [3].

In the sector of automotive fuels, the inclusion of renewable fuels, such as biodiesel, has several advantages, such as a reduction in pollution caused by exhaust emissions. Furthermore, biodiesel is an alternative to petroleum and contributes to regional development and social security, mainly in developing countries [4, 5].

A wide variety of feedstocks are currently used for biodiesel production, and microalgae have been considered a potentially useful and promising biodiesel source, because they are photosynthesizing organisms very efficient in the process of converting light into chemical energy [6–8]. In addition, microalgae stand out for presenting high productivity, exceeding that of any commercially produced plant in the world [9–11]. Microalgae have a life cycle of a few days and they may use CO₂ from polluting companies as an input for photosynthesis, which helps decrease the emission of greenhouse gases; hence, microalgae may contribute significantly to the global energy matrix [9–11].

Microalgae contain lipids and fatty acids as membrane components, storage products, metabolites, and energy sources. Each microalga species produces different proportions of lipids, carbohydrates, and protein. Algal lipids are typically

composed of glycerol, sugars or esterified bases, and fatty acids, which may be saturated or mono/polyunsaturated. Fatty acids correspond to the highest proportion of lipids, and in some species, polyunsaturated fatty acids (PUFA) represent between 25% and 60% of the total lipids [12, 13]. The oil from microalgae is similar to fish and plant oils.

One of the challenges of using microalgae is the complexity of lipid extraction with organic solvents, whose extract can be used in the process of transesterification to obtain biodiesel [14, 15]. *In situ* (direct) transesterification has been used with success to produce biodiesel from materials for which traditional extraction with solvents is not efficient [15–18].

In situ transesterification/esterification refers to the conversion of alkyl esters from free fatty acids and triacylglycerides present in biological material. *In situ* transesterification not only simplifies the production process but also improves the yield of alkyl esters compared to conventional extraction [17]. Reactions conducted in this way are simple and comprise the addition of one alcohol, one catalyst, and a lipid at moderate atmospheric pressure and temperature. In these reactions, the produced esters diffuse into the liquid phase, from which they can be easily recovered [18].

Biofuels are a viable alternative to fossil fuels and were consolidated by an intensive search for new feedstock in the past years. Microalgae biomass is among the feedstocks considered as promising energy sources. However, considering the current technological development, biodiesel production from algae biomass has several disadvantages in comparison to conventional feedstocks, such as soy [19]. Taking into account the technological development needed for an efficient and sustainable biofuel production from algae biomass, it is necessary to develop and control a technological route for this promising feedstock.

Hence, the present study was aimed at producing biomass from the microalga *Spirulina platensis* and at assessing the synthesis of alkyl esters via *in situ* acid transesterification/esterification of the biomass. Two alcohols (ethanol and methanol) and two cosolvents (hexane and chloroform) were tested, at different temperatures and reaction times.

2. Material and Methods

2.1. Kinetics of Growth and Recovery of Biomass. The microalga *Spirulina platensis* was grown in a standard synthetic medium (Zarrouk) for this type of microalga [9, 20–22]. Cultivation was carried out in laboratory at controlled temperature (25°C), in a photoperiod of 24 h and illuminance of 5,000 Lux. The volume ratio of medium:inoculum:water was 1:1:8 (v/v/v).

The increase in biomass or cell density in the alga culture was monitored through cell concentration, and a predetermined correlation (standard-curve) was used between biomass and absorbance at 670 nm [12, 23]. Measurements were taken in a Shimadzu UV-vis 1203 spectrophotometer. Population growth was calculated as the exponential growth rate (r), following the equation

$$r = \frac{\ln(N/N_0)}{\Delta t} \quad (1)$$

Since microalgae growth in cultures results from the binary division of mother cells, the increase in the microalga population was expressed in log base 2. Hence, the growth rate was converted into duplications per day (k):

$$k = \frac{r}{\ln 2} = \frac{r}{0.6931} \quad (2)$$

Biomass productivity (P) was calculated with the formula

$$P = \frac{(X_t - X_0)}{(t - t_0)} \quad (3)$$

Recovery of biomass from the culture was made with the addition of the cationic flocculant in order to promote the decantation of biomass, which was then filtered and dried in an oven at 60°C for 24 h. The dry biomass was ground and stored at –8°C, until being used.

2.2. Determination of the Acidity Index. Two grams of the oil sample was diluted in 25 mL of an ether: alcohol solution at the volumetric proportion of 2:1 (v/v). Next, 2 drops of the indicator phenolphthalein 1% were added, and the sample was titrated with a solution of sodium hydroxide 0.01 mol L^{–1} until it turned pink, a coloration that persisted for approximately 30 s. Hence, the acidity value (mg KOH g^{–1}) was given by

$$\text{Acid Value} = \frac{Vf5.61}{m} \quad (4)$$

2.3. Fatty Acid Profile of the Oil. Since microalgae oil has high acidity content, an acid esterification of lipids was made to determine the fatty acid profile [24]. In a test tube, 100 mg of oil was weighed, and 4 mL of NaOH solution in methanol (0.5 eq L^{–1}) was added. The tube was closed and heated in water bath until the oil globules were dissolved, and the solution became transparent. Then, the tube was cooled, and 5 mL of the esterifying reagent (methanol) was added. Next, the tube was heated again in water bath, and 4 mL of saturated solution of sodium chloride and 5 mL of the solvent (n-heptane) were added. The tube was stirred for 30 s, and then it was left to stand for approximately 90 min at 10°C. The supernatant was used for the analysis of the oil through gas chromatography.

2.4. Molecular Mass of the Oil. With the formation of the triglyceride molecule facilitated by the combination of fatty acid molecules and a molecule of glycerol with the condensation of three molecules of water, the average molecular mass of the microalgae oil (MM_{oil}) can be calculated using [25, 26]

$$MM_{\text{oil}} = 3 \times (\%MM_{\text{fatty acids}}) + MM_{\text{glycerol}} - 3 \times MM_{\text{water}} \quad (5)$$

2.5. Ester Synthesis via In Situ Transesterification/Esterification. The synthesis of alkyl esters from the biomass of the alga *Spirulina platensis* was performed as proposed by Lewis et al. [17]. Hence, 20 mg of dry biomass was weighed

TABLE 1: Productivity, growth rate, and duplications per day in the microalgae culture.

Interval (day)	Productivity (mg L ⁻¹ day ⁻¹)	r (day ⁻¹)	k (day ⁻¹)
1-2	35.52 ± 8.57	0.451	0.65
2-4	91.98 ± 2.93	0.703	1.01
4-7	4.91 ± 13.07	0.020	0.03
7-9	67.99 ± 43.71	0.211	0.31
9-11	18.99 ± 11.34	0.046	0.07
11-14	40.41 ± 15.41	0.082	0.12
14-16	48.76 ± 15.87	0.081	0.12

in 10 mL stoppered test tubes. Next, 3 mL of a solution containing alcohol, a catalyst (HCl at 37%), and the cosolvent at the volume ratio of 10:1:1 (v/v/v) was added to the tube. The biomass was suspended in the solution through manual stirring and immediately placed in water bath, at a predetermined temperature. After the appropriate reaction time, tube was removed from the water bath and cooled at ambient temperature in a cold water bath, and 1 mL of deionized water was added to it.

Next, 2 mL of a hexane and chloroform mixture at the proportion 4:1 (v/v) was added. After the separation of phases, the supernatant was collected with another tube. This process was repeated three times to assure the complete removal of the esters formed. Then, the solvent was evaporated and the produced esters were diluted in a solution of hexane and tricosanoic acid methyl ester, used as an internal pattern (IP). The sample was stored in a freezer at -8°C, until being analyzed.

In the study of the *in situ* reaction, two alcohols were assessed, ethanol and methanol, and two cosolvents, hexane and chloroform. Do not miss the reaction was performed at five temperatures: 30, 45, 60, 75, and 90°C, and the reaction times analyzed were 10, 20, 30, 60, and 120 min. All reactions were performed in triplicate.

2.6. Chromatographic Analysis. The esters produced were determined in a Varian chromatograph, model CP-3800, using a flame ionization detector (FID) with a capillary column BP-X70-SGE of 30 m × 0.25 mm and using helium as a carrying gas at a split ratio of 1:10. The temperatures in the detector and injector were 220 and 260°C, respectively. The initial temperature in the column was 140°C and was warmed at a rate of 5°C min⁻¹ until reaching 250°C.

The esters were identified by external comparison with the pattern FAME Mix (fatty acid methyl ester), and the tricosanoic acid methyl ester was quantified with the internal pattern (IP) 99%. Then, the mass of produced ester was calculated per gram of dry biomass (mg of ester/g biomass) [27].

2.7. Data Analysis. A repeated measures analysis of variance (ANOVA) was applied to the culture parameters, followed by a Tukey post hoc test. To the process of ester synthesis, a factorial ANOVA was applied, being the factors: (a) type of alcohol (ethyl, methyl), (b) cosolvent used (hexane, chloroform), (c) reaction time (10, 20, 30, 60, and 120 min), and (d) reaction

TABLE 2: Fatty acid profile of the *Spirulina platensis* microalga oil.

Fatty acids	Composition* (%)
C4:0	6.52
C8:0	3.40
C10:0	38.7
C16:0	11.5
C18:0	2.66
C18:1	14.0
C18:2	13.7
C18:3	9.56

* Oil composition in mass percentage.

temperature (30, 45, 60, 75, and 90°C), followed by a Tukey post hoc test. The statistical analysis was performed in the program Statistic 7.1 [28].

3. Results and Discussion

3.1. Microalgae Cultivation. The productivity, growth rate, and duplications per day obtained in the culture of *Spirulina platensis* are presented in Table 1. A growth induction phase (1-2 days) was observed, which is characterized by cell adaptation to the new environment, and during this phase, the increase in biomass may be imperceptible.

After the adaptation phase, an increase in biomass concentration was observed, phase of exponential growth. The highest productivity occurred on 2-4 days as well as 7-9 days. It was possible to notice that the growth rate and, consequently, the number of duplications decreased with time, until reaching a minimum value.

Whenever a component of the culture medium becomes limiting, the exponential growth phase ends and a growth at reduced rate takes place. This reduced growth is frequently provided by the consumption of cell reserves (lipids and fatty acids). Hence, it is important to recover the biomass at the exponential growth phase [9, 12].

In the culture of *Spirulina platensis*, it was possible to obtain good biomass productivity in a relatively short time; these values are close to those reported in the literature, using the medium Zarrouk [21].

3.2. Analysis of the Microalga Oil. The fatty acid profile of the *Spirulina platensis* microalga oil is presented in Table 2.

FIGURE 1: Mass of methyl ester obtained in the *in situ* reaction with chloroform (a) and hexane (b).

TABLE 3: Characteristics of the of the *Spirulina platensis* microalga oil.

Characterization	Result
Average molecular mass of fatty acids	235 g mol ⁻¹
Average molecular mass of the oil	742 g mol ⁻¹
Acidity	14.3 mg KOH g ⁻¹ , 7.2%

There is a predominance of saturated fatty acids; among those; a high concentration of C10:0 stands out. Therefore, we expect the biodiesel produced from the oil of this microalga to present high oxidative stability and cetane number [29], as well as low viscosity. Regarding cold flow properties, it is Difficult to define the tendency, since on the oil is saturated but has larger Also contains Amounts of short-chain fatty acids.

Based on the results of the fatty acid profile, the average molar mass of the fatty acids obtained and the average molar mass of the oil were calculated (Table 3). The values obtained corroborated the data found in the literature [23, 30].

The acidity value of the oil is important to define the route to obtain biodiesel, and the acidity of the oil is higher than 0.5% (m/m). This characteristic indicates that the best process for the production of esters aiming at biodiesel production is via acid transesterification/esterification [17].

3.3. Ester Synthesis. The mass of methyl ester obtained in the *in situ* reaction in relation to the reaction time for the

cosolvents chloroform and hexane at different temperatures is presented in Figure 1.

Apparently, the reactions with methanol were more stable throughout time than the reactions with ethanol, with similar ester yields at different reaction times. In the methanol and chloroform mixture, the esters had the best yields at 60 and 90°C (Figure 1(a)). When the cosolvent hexane was used, the best yields were obtained at 45 and 75°C (Figure 1(b)).

The mass of ethyl ester obtained in the *in situ* reaction in relation to the reaction time for the cosolvents chloroform and hexane at different temperatures is presented in Figure 2.

In the reactions carried out with ethanol and chloroform, there was no effect of temperature. However, the best yields in esters were observed at 60°C (Figure 2(a)). In addition, it was also possible to observe high yields at 30°C after only 10 min of reaction. When ethanol and hexane were used, there was an increase in the ester yield with time, except for the reaction at 45°C (Figure 2(b)), which presented the best yields at shorter reaction times.

The factorial analysis of variance detected an interaction among temperature, reaction time, alcohol, and cosolvent ($P < 0.05$). Hence, interaction among factors was used for the Tukey test. Based on the Tukey test applied to the ester yield, the best yields were obtained with the combinations of ethanol and chloroform at 60°C, for 30 min, and ethanol and hexane at 45°C, during the first 10 min of reaction. Although there was no significant difference between the two conditions, the reaction with the combination ethanol and hexane at 45°C, for 10 min, seems to be better in terms of sustainable energy production, since it is carried out at

FIGURE 2: Mass of ethyl ester obtained in the *in situ* reaction with chloroform (a) and hexane (b).

a milder temperature and for a shorter time, consequently leading to a reduction in costs.

The mixability of triacylglycerides in the cosolvent allows an efficient extraction of oil from the microalga biomass, and the mixability of the solvent in alcohol favors the occurrence of *in situ* reaction in a homogeneous environment and also increases the production of esters during *in situ* reaction [15, 31].

Alcohol plays an important role in the *in situ* transesterification process, acting in the extraction of lipids from the biomass and also in the conversion of esters from fatty acids [32]. When ethanol was used in the *in situ* reactions, the kinetics of reaction was slower, and this result corroborates the literature [5, 33]. This result may be explained by a higher reactivity of methoxide radicals compared to ethoxide radicals, since the carbon chain length is larger for ethanol and the nucleophilicity of the alkoxide anion decreases, leading to a decrease in reactivity [32].

In the *in situ* reactions with alga biomass (Figures 1 and 2), ester production reaches a peak followed by a decrease and stabilization with time. This result suggests that, in addition to acid transesterification/esterification, other reactions may occur. It is known that ester formation via acid catalysis is reversible, but the amount of alcohol in excess tends to limit the reverse reaction, moving the direction of reaction to products. However, under these reaction conditions, in which there are triacylglycerides, water alcohol, and an acid catalyzer in the same system, secondary reactions, such as triacylglyceride hydrolysis combined with the hydrolysis of the esters produced, may occur. In the reaction of triacylglyceride

hydrolysis, diacylglycerols, monoacylglycerols, and free fatty acids are formed, whereas in the reaction of ester hydrolysis, carboxylic acid and alcohol are produced. Hence, until a balance is reached in the system, the kinetics of esterification undergoes changes in time, as observed in the behavior of the reactions obtained (Figures 1 and 2) [33, 34].

4. Conclusion

The production of biomass from microalgae is relatively simple, requires light and a nutrient source, and reaches high productivity in a short time. Microalgae oil may be converted into esters through *in situ* transesterification/esterification of the biomass. In the present study, ester synthesis from the biomass of *Spirulina platensis* showed the best result when ethanol was used as alcohol and hexane was used as a cosolvent. *In situ* transesterification/esterification for ester formation, aiming at producing biodiesel, adds an unconventional dynamics to the use of this feedstock, in particular due to the reduction in reaction time and volume of solvents used in the process.

Nomenclature

- f : Correction factor of the solution of NaOH (0.01 mol L^{-1})
- k : Duplications per day (day^{-1})
- m : Mass of the sample (g)
- N : Cell density or biomass (g mL^{-1}) at any time t

N_0 :	Initial cell density or biomass (g mL^{-1}) at time $t = 0$
P :	Productivity ($\text{mg L}^{-1} \text{ day}^{-1}$)
r :	Exponential growth rate
t :	Time (day)
t_0 :	Initial time (day)
V :	Volume of the solution of NaOH 0.01 mol L^{-1} used in titration (mL)
X_t :	Cell density at time t (g L^{-1})
X_0 :	Cell density at time t_0 (g L^{-1})
$\text{MM}_{\text{fatty acids}}$:	Molecular mass of fatty acids (g gmol^{-1})
$\text{MM}_{\text{glycerol}}$:	Molecular mass of glycerol and water (g gmol^{-1})
MM_{water} :	Molecular mass of water (g gmol^{-1}).

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