Evaluation of lipid yield for biodiesel production extracted from microalgae Scenedesmus sp. submitted to different homogenization times and physicochemical changes

Avaliação do rendimento lipídico para produção de biodiesel extraído de microalgas Scenedesmus sp. submetidos a diferentes tempos de homogeneização e alterações físico-químicas

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ABSTRACT
Due to the growing demand for fuels and the consequent concern with the problems related to the burning of these products, the interest arises in developing new technologies linked to the use of natural resources to generate renewable energy1,2, replacing conventional sources. Based on this assumption, biomass is an excellent alternative for biofuel production.3 Microalgae have shown great advantages compared to traditional agricultural crops. Microalgae have a high production capacity of
oil per unit area; are adaptable crops, including using brackish or wastewater in their cultivation and which would be unsuitable for traditional agricultural crops, provided they have satisfactory heat and radiation conditions. In addition, microalgae have high atmospheric CO2 fixation capacity. In this context, an evaluation of the yield of lipids extracted from biomass is presented microalgae Scenedesmus sp. following various treatments (homogenization and physicochemical variations of the environment: such as temperature and/or pH), which allow the selection of a technological route for the extraction of lipids of interest, aiming the production of biodiesel.

**Keywords:** Microalgae, lipids, homogenization, biomass, emulsion.

**RESUMO**
Devido à crescente demanda por combustíveis e à consequente preocupação com os problemas relacionados à queima desses produtos, surge o interesse em desenvolver novas tecnologias ligadas ao uso de recursos naturais para gerar energia renovável, substituindo as fontes convencionais. Com base nesse pressuposto, a biomassa é uma excelente alternativa para a produção de biocombustíveis. As microalgas têm mostrado grandes vantagens em comparação com as culturas agrícolas tradicionais. As microalgas possuem alta capacidade de produção de petróleo por unidade de área; são culturas adaptáveis, incluindo o uso de água salobra ou efluente em seu cultivo e que seriam inadequadas para culturas agrícolas tradicionais, desde que tenham condições satisfatórias de calor e radiação. Além disso, as microalgas têm alta capacidade de fixação atmosférica de CO2. Nesse contexto, uma avaliação do rendimento de lipídios extraídos da biomassa é apresentado microalgas Scenedesmus sp. seguindo diversos tratamentos (homogeneização e variações físico-químicas do ambiente: temperatura e/ou pH), que permitem a seleção de uma rota tecnológica para a extração de lipídios de interesse, visando a produção de biodiesel.

**Palavras chave:** Microalgas, lipídios, homogeneização, biomassa, emulsão.

**1 INTRODUCTION**
With the energy sector crisis that is beginning to affect many parts of the world due to rapid industrialization and population growth, one of the main focuses of the studies has been the search for new energy sources, especially those from renewable sources, aiming to develop and use more sustainable energy.

Biodiesel is miscible and physical-chemically similar to petroleum diesel and can be used in diesel engines without the need for adaptations.

Preferably, in biodiesel production, raw materials that do not compete with the food industry should be used. Thus, microalgae have been the source of several researches in the last decade for synthesizing biomass that can be used in the production of biodiesel and other biofuels, such as renewable hydrocarbons and second generation ethanol.

Microalgae are known to use carbon compounds such as carbon dioxide (CO2) as a source of nutrients for carbohydrate, protein, lipid and pigment production. Thus, in addition to being a renewable source of biodiesel, microalgae help capture CO2 from the environment, mitigating the damage caused by the greenhouse effect.
The most common process for obtaining biodiesel is the transesterification of vegetable and animal oils and fats. In the field of production from microalgae, the processes of hydroesterification and saponification are being studied followed by acidulation and esterification, which allow the use of any raw material, regardless of the moisture content and acidity.\textsuperscript{14}

Regarding biomass disruption, as stated by\textsuperscript{15}, it is known that the extraction efficiency of lipids from microalgae biomass depends on binomial disruption method and cultivated microalgae species. Although all steps of microalgae production are essential for biodiesel production, the choice of lipid extraction method through cell rupture, is fundamental to increase process efficiency\textsuperscript{16,17}.

There are several types of methods to perform this disruption, such as mechanical compression, grinding, extraction with supercritical fluid, enzymatic extraction, microwave extraction, osmotic shock, homogenization, solvent extraction and ultrasound-assisted extraction, among others.\textsuperscript{18}

The method of cell disruption is to pump a suspension of cells at high pressure through the narrow orifice of a valve which is then released into a low pressure chamber.\textsuperscript{5}

The fall through the orifice produces a very high speed and, the resulting expansion, generates a hydraulic shear force that, combined with the impact generated by the high pressure, results in high mechanical stress, being the twisted and deformed cells.\textsuperscript{19,20,21} Although the configuration of the homogenization valve is apparently simple, the fluid dynamics involved is quite complex, as there are several energy changes in the valve when the liquid passes from the high pressure and low speed zone to the low pressure and high speed zone.\textsuperscript{21}

According to HALIM\textsuperscript{5}, the kinetics involved in the rupture of the cell wall is known to follow a first order model. Thus, it can be deduced that the rate of cell breakdown decreases as the number of passes through the homogenizer valve increases.

This method has been increasingly used to extract substances internal to the cell. According to KIM\textsuperscript{19}, some of the advantages of this method involve low heat generation, low degradation of the products, without dead volume in the homogenizer and easily scaled.

The major challenge for making biodiesel production via microalgae viable is to achieve a cultivation method that produces an oil-rich biomass, an effective breakdown of the cell wall and an efficient extraction of the lipids produced by the microalgae with the least number of unit operations, also taking advantage of the by-products to add value to the process.\textsuperscript{22,2}

In view of the great interest in the use of microalgae for the production of biofuels, there is a need for a production route that combines the aspects of cultivation, extraction and obtaining of microalgal oil in a profitable way.\textsuperscript{23}
In this sense, the present work allows the selection of a technological route for the extraction of the lipids of interest from the wet biomass of microalgae, aiming at the production of biodiesel.

2 MATERIALS AND METHODS

For the development tests of the cell disruption procedure in the homogenizer, the wet biomass of the microalgae *Scenedesmus* sp., cultivated in the pilot plant of the Federal University of Rio Grande do Norte (figure 1), using cultivators the sky open raceway type. The biomass was thawed and sieved three times, then distilled water was added until it obtained sufficient consistency to be processed in the homogenizer (93% moisture).

![Figure 1. (A) Cultivation system Raceway ponds - UFRN/Natal; (B) Flocculation tanks; (C) Wet biomass of Microalga *Scenedesmus* sp., used as raw material](image)

2.1 DETERMINATION OF MOISTURE CONTENT:

It is necessary to determine the moisture content of the biomass used in the breaking tests. This determination was performed on a Shimatzu moisture analyzer, model MOC63u. For this, approximately 2 g of microalgal biomass were used. The analysis was performed in triplicate. The measurement temperature value of 120 ºC, which is the standard, has been adjusted to 160 ºC and the analyzer level to 0,05 %, so the analysis is finished when the humidity changes below 0,05 % in 30 seconds.

The sample was spread on the measuring plate in a thin layer, increasing its measuring surface, generating a more accurate and faster stabilization measurement. Whenever the initial moisture content of the biomass was less than 93 %, water was added to the biomass in order to increase the humidity, thus avoiding clogging of the homogenization system.

2.2 HOMOGENIZER OPERATING CONDITIONS:

To facilitate access to the lipid content that is inside the microalgae biomass cells, a homogenizer was used, designed by a research group from the Federal University of Viçosa that
works in partnership with the Green Technology Laboratory (GreenTec) of the School of Chemistry / UFRJ. The system consists of a high pressure pneumatic pump and a cell extrusion valve, built in stainless steel by the company Serafim Ferreira ME in Minas Gerais.

This homogenizer can be seen in figure 2, and is based on two associated principles that cause effective cell disruption, pressure and shear.

![Greentec Homogenizer](image)

**Figure 2. Greentec Homogenizer**

### 2.3 SIEVING

To prepare the microalgal biomass, this was passed four times through domestic steel mesh, as shown in figure 3C, to eliminate lumps that could cause the homogenizer to clog.

### 2.4 HOMOGENIZATION

On this route, the fact of being able to break the cell and extract the nonpolar fraction of interest without using high temperatures is a differential. In this way, it is possible to guarantee the integrity of the compounds of interest and also that of other high value added co-products in the residual biomass. The proposed route was evaluated through preliminary tests where the biomasses were processed in a homogenizer at 100 bar pressure, for 5 and 10 minutes, approximately 5 to 10 passes, respectively (figura3). A control sample did not undergo homogenization and did not even undergo any variation in temperature and pH.
2.5 TREATMENT OF HOMOGENIZED BIOMASSES:

The homogenized biomasses were subjected to variations in pH, between acid and basic (pH 2 and pH 10), and in temperature (25 and 70 °C). The content of lipids extracted from the biomasses submitted to the different treatment conditions was compared with the extract yield of the control samples (figura 4).

For all tests, 10 g of biomass were weighed in falcon tubes. For the pH 2 treatment, 500 µl of H₃PO₄ were added, vortexed for complete mixing and the pH was checked with a pH tape. In the case of a change in pH 10, 50 mg of KOH was added, the samples were vortexed for complete mixing and the pH was checked with a pH tape. For treatment with temperature, the weighed samples were placed in a heating bath at a temperature close to 70 °C for 1 hour.
2.6 EXTRACTION OF LIPIDS

After each of these treatments, 10 mL of ethyl acetate and 10 mL of hexane were added, and vortexed vigorously for 3 minutes for complete and effective mixing of the biomass with the solvents. Stirring times shorter than this were not effective, since the solvent phase did not mix with biomass. After stirring, the samples were centrifuged at 1500 rpm (F = 314 G) for 4 min. The upper phase was poured into 100 mL flasks, previously dried and weighed, and evaporated in a heating plate at 120 °C, until the solvent was completely evaporated. Then, the flasks were kept in an oven at 102 °C until constant weight. The amount of lipids extracted in each treatment was determined by gravimetry, in relation to the initial dry mass of microalgae.

2.7 LIPID EXTRACTION J METHOD: SCHMID-BONDZYNSKI-RATZLAFF

Using this methodology the lipid fraction was extracted from the wet biomass of the microalgae Scenedesmus sp. The lipid extracts were analyzed by Thin layer chromatography (TLC) in order to compare the lipid classes present in these extracts with those identified in the lipid samples extracted under the different treatment conditions (homogenization, pH and temperature variation).

The extraction was performed starting from 1g dry biomass - in a 50 mL - 10 mL HCl 8M falcon tube; Hydrolysis: 10 minutes water bath at 60 °C; 1st Extraction: 10 mL absolute ethanol; 25 mL ethyl ether; 25 mL petroleum ether; Separation of the phases in a funnel. 2nd and 3rd Extraction: 10 mL absolute ethanol; 25 mL ethyl ether; 25 mL petroleum ether; Washing of the “solvent” phase with distilled water until pH of the water = 7 (to remove HCl residues); Evaporation of the solvent; Kiln drying at 60 °C. The experiments were conducted in duplicate. (samples A and B).

2.8 IDENTIFICATION OF THE LIPID CLASSES

In order to assist in the selection of the procedure that allows a more selective extraction of the fatty components, the lipid classes of interest, present in the extracts of the microalgae Scenedesmus sp., were identified, using the modified method of Thin layer chromatography (TLC).

In this analysis, 0,001 g of the extracted lipid fraction was dissolved in 600 µl of chloroform. The equivalent of 0,03 µl of the solution was applied to a 60 TLC (Merck) silica gel plate with the aid of an automatic pipette. In addition to the sample to be analyzed, equal amounts of the triolein, diolein, monoolein, fatty acid, ergosterol and cholesteryl oleate were applied to the plates for comparison and quantification.

To ensure efficient separation and obtain acute bands of nonpolar lipids, the separation was performed as described below. First, the solvent mixture composed of petroleum ether / diethyl ether / acetic acid (70:30:2 v/v) was used to separate the lipids, until reaching 2/3 the height of the silica
plate (7 cm). After drying the plate, the separation continues in the same direction using the solvent mixture composed of petroleum ether / diethyl ether (100:2 v/v) until it reaches the top of the plate (9 cm) (figura 5).

![Figure 5. Example of marking the plate TLC 10X10 cm.](image)

The chromatographic plate after elution was revealed with iodine vapor and the stain retention (Rf) factor of the standards and sample components was determined.

To achieve an irreversible staining of nonpolar lipids and sterols in the TLC plate, it was kept in a solution of 0.63 g of MnCl₂·4H₂O, 60 mL of water, 60 mL of methanol and 4 mL of sulfuric acid for 10 seconds, followed by heating to 105 °C. The intensity of the color depends on the heating time, which must be a minimum of 30 min.

### 3 RESULTS

![Figure 6. Initial microalgae biomass. 1:10 dilution. Magnifying 100x.](image)
Figure 7. HPH microalgae biomass: 5 min. 1:10 dilution. Magnifying 100x.

Figure 8. HPH microalgae biomass: 10 min. 1:10 dilution. Magnifying 100x.
Table 1. Means and standard deviation of the percentage of extracted lipids (dry basis) in each treatment for biomass at different times of homogenization.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time</th>
<th>Treatments</th>
<th>Extracted lipids dry base (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 min</td>
<td>control</td>
<td>1,65 ± 0,002</td>
</tr>
<tr>
<td>2</td>
<td>pH10</td>
<td>1,64 ± 0,006</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>pH2</td>
<td>1,77 ± 0,017</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>temperature</td>
<td>2,45 ± 0,006</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0 min</td>
<td>control</td>
<td>1,23 ± 0,007</td>
</tr>
<tr>
<td>6</td>
<td>pH10</td>
<td>1,63 ± 0,003</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>pH2</td>
<td>0,85 ± 0,003</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>temperature</td>
<td>3,18 ± 0,006</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0 min</td>
<td>control</td>
<td>0,71 ± 0,003</td>
</tr>
<tr>
<td>10</td>
<td>pH10</td>
<td>1,42 ± 0,003</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>pH2</td>
<td>0,59 ± 0,003</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>temperature</td>
<td>3,50 ± 0,002</td>
<td></td>
</tr>
</tbody>
</table>

Figure 9. Thin layer chromatography of extracts (1-12) vs Ratzlaff extracts (A and B). TG: Triglycerides; Di: Diglycerides; AG: Fatty acids; LP: Polar lipids.
4 DISCUSSION

Through the tests performed, it was possible to observe in the processed samples the action of the cold mechanical rupture promoted in the cell wall due to the hydraulic shear and the impact that the pressure generates.

The biomass aliquots removed from the homogenizer after 0, 5, and 10 minutes were observed under the microscope in order to identify the occurrence of cell disruption. The images obtained, after 1:10 dilution and using a 100x magnifying, can be viewed in figures 6, 7 and 8, indicating a greater cell disruption in the homogenized biomass for 10 min. However, this result did not contribute to an increase in the content of extracted lipids. As can be seen in table 1, the content of extracted lipids decreased as the biomass homogenization time increased, as a result of the formation of a more stable emulsion that interfered with the release of lipids. The formation of emulsion during homogenization is directly related to proteins, the greater the cell disruption, the greater the content of proteins released. It is also observed that the pH variation in non-homogenized samples (time = 0 min) was not significant (samples 2 and 3), when compared to sample 1 - control. In homogenized samples (time = 5 and 10 min), where there was an emulsion formation, it was possible to verify an increase in the content of lipids extracted in samples submitted to pH 10. The increase in pH influenced the isoelectric point of some proteins, helping in their precipitation and facilitating the extraction of lipids. The percentage of lipids extracted in sample 6 exceeded the control sample (sample 5). The treatment of biomass with temperature, even before adding the extracting solvents, proved to be the most efficient treatment. The temperature directly influences the functional properties of proteins, which are the main emulsifying agents, thus facilitating the access of the solvent to the lipids of interest (figure 9), even before the biomass is processed in the homogenizer. This difference is very evident when observing the color in the extracts of each of the treatments applied to the biomass in their respective homogenization times.

In the chromatographic plate of figure 9, the samples extracted with ethyl acetate: hexane were compared, with previous variation in the physical-chemical conditions of the homogenized biomass (samples 1 - 12), with two samples extracted by the Ratzlaff method (samples A and B).25 Once again, the low selectivity of the latter methodology was observed. Most of the extract from samples A and B remained at the base, with a low concentration of fatty acids and few triglycerides. In the samples extracted with variation of the physical-chemical conditions, the behavior was very similar in relation to the identified lipid classes, with emphasis on a higher extract yield in samples 4, 8 and 12, treated with temperature. As previously treated in terms of cell disruption, it is known that the longer the samples passed through the homogenizer, the greater the disintegration of the cells. However, in terms of lipid extraction, as the time in the homogenizer increases, the amount of the
fraction of interest extracted decreases, which confirms what has been found in the literature on the stability of the emulsion.26,27,28,29

As it is an equipment that aims to form emulsions, the homogenizer can be efficient for cell disruption, but it must be considered that a new question arises: destabilize the emulsion formed by proteins and carbohydrates that are in large quantities in the microalgae and which are natural emulsifiers, as well as extracting this oil, which is now in the form of nanoparticles, due to the homogenization process. The treatments with pH 2 proved to be less efficient even than the control treatment, while the increase in pH was more significant than the control treatment. This is probably due to functional changes in protein solubility due to the pH change of the medium.

The need for vigorous stirring to promote contact between the solvent and biomass phases, and, consequently, the formation of an emulsion, especially in cases where there was a physical-chemical modification of the biomass, as well as greater emulsion formation as the it increased the biomass time in the homogenizer, explain the lower yields in these cases.

Proteins, as well as amino acids, are compounds that can undergo protonation and deprotonation, that is, the addition or removal of H ions, being positively charged, electrically neutral or negatively charged, depending on the pH conditions of the medium. The electrically neutral form can only exist in a pH condition intermediate to the predominant existence of the positive and negative form of the protein, that is, this charge distribution can be altered depending on the pH of the medium. In this study, it is observed that there was a change in these loads and, consequently, in the interaction between solute and solvent, since in the treatment at pH 10 with 10 min of homogenization an increase in the amount of lipids extracted was observed in relation to the control treatment of 10 min homogenization. However, for the same homogenization time, there was a decrease in extraction with pH 2, indicating that the isoelectric point of the proteins in this case is closer to basic pH than acidic. PH values close to the isoelectric point, result in a balance between the number of positive and negative charges, that is, the repulsion forces between the protein molecules and the interaction forces with the solvent are minimal. Thus, the proteins form clusters that, each time larger, tend to precipitate, facilitating the reduction of the emulsion. It is worth mentioning that the decrease in solubility varies from protein to protein.

BENELHADJ30, for example, when studying the emulsifying capacity of the Spirulina platensis protein isolate, they found that at pH close to the isoelectric point, less protein solubility occurred, with a reduction in the formation of the oil / water interface with the protein network, decreasing the capacity of emulsion formation. Protein denaturation, in general, occurs in the range of 65 - 80 °C, in which case the protein loses its secondary and / or tertiary structure, that is, the three-dimensional arrangement of the polypeptide chain is disrupted, making it, almost always, lose its
characteristic biological activity. In our specific case, this loss of biological activity is beneficial, since the emulsifying activity of the protein is reduced.

5 CONCLUSIONS

Physico-chemical changes such as an increase in pH and an increase in temperature positively influenced the increase in the yield of the lipid fraction. The increase in temperature in the extraction showed better results than the other treatments. There was no increase in lipid extraction with the use of homogenization under the applied conditions, most likely due to the increase in the stability of the emulsion. In the treatment that combines homogenization with increased temperature, the highest lipid yields were obtained. From the results of this study, it is possible to select a technological route that enables the extraction of lipids from the wet biomass of microalgae, aiming at the production of biodiesel. This technology uses solvents that can be evaporated and recycled within the process itself, with steps similar to those already used for conventional raw materials.

The extraction of lipids, including the cell disruption step with the use of a homogenizer, is extremely advantageous, as it preserves all by-products and can commercialize them.

The designed homogenizer meets the desired cell disruption requirements. It was possible to determine its operating conditions, testing and monitoring different times, and establishing the passage of the sample for 10 minutes with a pressure generated of 100 bar.

The use of the ethyl acetate: hexane solvent mixture was more selective for the extraction of the lipid classes of interest (glycerides and fatty acids) than the solvent mixture used in the J: Schmid-Bondzynski-Ratzlaff methodology.

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ABBREVIATIONS

HPH, High pressure homogenization system; TLC, Thin layer chromatography.

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