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Evaluation of oil productivity of *Chlorella minutissima* cultivated in two homemade photobioreactors (bubble column and tank)

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Abstract*— Several environmental damages caused by the use of fossil fuels, such as the green house effect, have being a motivation for researchers all around the world to develop new green techniques for power generation. Among all the possibilities of clean energy sources, there is the use of microalgae to obtain biodiesel. Many microalgae species, in certain growing conditions, accumulate considerable lipid content within their cells. Therefore, this study aimed to evaluate parameters that could influence the microalgae oil extraction and compare the productivity of the microalgae *Chlorella minutissima* in different photobioreactors' designs (bubble column and tank). For each extraction, the biomass (5 or 10 mg) was mixed in Erlenmeyer flasks (125 or 500 mL) to solvents (methanol and chloroform) and kept for 40 min in ultrasound bath. For each studied condition, extractions were performed in one or two steps. After the extraction, the remaining solvent was removed using a rota-evaporator and oil yield was determined. After the improvement in the extraction process, it was evaluated the efficiency of the microalgae *Chlorella minutissima* cultivation in two different homemade photobiorreactors: 3 bubble column (50 L of capacity, each) and 4 tank photobioreactors (5 L of capacity, each). Regarding to extraction tests, a second extraction step is desirable to increase significantly the amount of oil extracted. The container size used in the extraction process was also a significant factor (extractions showed better results when the 125mL erlenmeyer flask was employed). The biomass amount extracted in each batch was not a contributing factor, especially because the solvents were added in proportional quantities to the biomass amount. It was also observed that cultivation of *Chlorella minutissima* in the bubble column photobioreactor showed lower oil productivity (7.82 $mgL^{-1}day^{-1}$) compared with the tank reactor (14.6 $mgL^{-1}day^{-1}$). The discrepancies of their working volume do not allow a direct comparison between those photobioreactors. However, their good oil productivities show that both can be used for *Chlorella minutissima* cultivation.

Keywords*— *Chlorella minutissima, bubble column photobioreactor, tank photobioreactor, lipid extraction.

I. INTRODUCTION

Microalgae have been considered a promising source of oil for biodiesel production. It has being increasingly clear that human kind cannot depend on petroleum-based fuels as energy source. Especially because of the recent aggravation of environmental issues, such as the global warming, and the depletion of petroleum reserves. Microalgae lipid can be extracted and transesterified to obtain biodiesel. Therefore, many microalgae species have being studied to provide oil for biodiesel production, as a clean power source. Although vegetable oils are widely used in biodiesel production, the use of oil from microalgae has several advantages: no need for arable lands, high growth rate, use less water than terrestrial crops, microalgae lipid is easier to extract, etc [1-2].

Microalgae cultivation can be carried on photobioreactors or open pounds. The main difference between these processes is that when microalgae are cultivated in photobioreactors they do not have a direct contact with the external environment. On the other hand, the cultivation in open pounds allows the cultivation media to be in contact with the atmosphere. Both of these cultivation processes have their advantages and its drawbacks. Open pounds are widely used because they have relatively lower building and operating costs when compared to photobioreactor. On the other hand, cultivation in open pounds presents high contamination risks by the microorganisms from the air. Even though photobioreactors are more expensive, they have lower contamination risk and higher cultivation performance [3-5].

Photobioreactors are made in a wide variety of shapes, including: tubular, flat panel, bubble column and stirred tank. All photobioreactor designs have, more or less, the same goals: to be cheap made (in

order to reduce the cost of biomass production), to provide good illumination (for microalgae photosynthesis), have good mixing process (to avoid cell precipitation), enable good gas exchange (removal of O_2 and introduction of CO_2), have good control of pH and temperature, etc. [6]. Another photobioreactor's parameter to evaluate is the reactors complexity: photobioreactors that are more sophisticated are more versatile, but are generally expensive to built and more complicate to operate nevertheless.

After the cultivation process, in order to produce biodiesel, it is necessary to extract the oil from the microalgae cells. This is an important step in the biodiesel production, however it is still a costly process. Literature shows a high range of studies trying to improve and cheapen the extraction method [7]. From published information, extraction can be, basically, classified in two categories: chemical and mechanical. Examples of mechanical extraction processes are expeller/press, ultrasonic assisted technique, microwave assisted technique. All of these techniques use mechanical effort to disrupt the microalgae cell wall to achieve the stored lipid [7]. Chemical extraction, on the other hand, uses solvents to remove oil from the microalgae cell [8]. Both techniques (chemical and mechanical) have their pros and cons, but a good proposition is to work with both methods together to enhance the extraction efficiency [7-8]. In this proposition, the mechanical procedure disrupts the cell walls allowing a direct contact between the solvent and the oil facilitating mass transfer [7-8].

II. METHODS

A. *Microalgae and cultivation process*

Chlorella minutissima marine microalgae was obtained from the Seaweed Culture Collection of the Oceanographic Institute at the Universidade de São Paulo (São Paulo, Brasil).

Before the cultivation process, the photobioreactors were disinfected with commercial sodium hypochlorite solution (2 to 3% of active chlorine). In order to do so, the reactors were fill with tap water, and then 0.05 mL of the hypochlorite was added to each liter of water. This solution was kept in the reactors for 12 h; meanwhile the reactor lighting was off. After that time, the solution was aerated for 2 h to remove residual chlorine before inoculation [9].

All the cultivation processes, including the inoculum preparation, was carried out using a modified Guillard f/2 medium [10]. The culture medium used in the present work contains the following nutrients concentration (mg L^{-1}): $30*10^3$ NaCl, 65 $NaNO_3$, 5 $NaH_2PO_4.H_2O$, 30 $Na_2SiO_3.9\ H_2O$, $9.8*10^{-3}$ $CuSO_4.5H_2O$, $22.2*10^{-3}$ $ZnSO_4.7H_2O$, $10*10^{-3}$ $CoCl_2.6H_2O$, $180*10^{-3}$ $MnCl_2.4H_2O$, $6.3*10^{-3}$ $Na_2MoO_4.2H_2O$, 3.15 $FeCl_3.6H_2O$, 4.36 Na_2EDTA, $100*10^{-3}$ thiamine, $0{,}5*10^{-3}$ cyanocobalamin, $0{,}5*10^{-3}$ biotin.

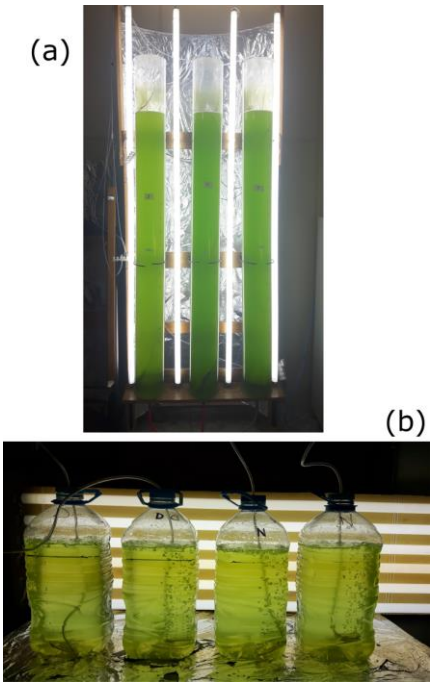

Fig. 1. (a) Bubble column photobioreactors built with acrylic tubes and placed in a wooden support. (b) Tank photobiorreactors built with recycled water bottles. For both reactors, the lighting was generated by fluorescent lamps.

Microalgae were cultivated in three bubble column photobioreactors (each with a capacity of 50 L and working volume of 40 L). The photobioreactors were made by [11] with cheap materials (e.g. wooden support and acrylic tubes) to reduce the final costs of oil production. All three photobioreactors have a cylindrical shape with internal diameter of 184 mm and a total height of 2 m. In operation, the liquid column is, approximately, 1.7 m height. Microalgae were grown with a 150klux continuous white fluorescent light illumination and it was continuously aerated with a 4 L/min rate. Figure 1(a) shows the bubble column photobioreactor already in operation. In order to evaluate the oil and biomass productivity of the *Chlorella minutissima*, it was also cultivated with the same growth parameters in a different photobioreactor design, tank photobioreactor. The tank photobioreactors were made with approximately square shaped bottles with 5 L capacity and 4 L working volume (15 cm wide and 33 cm height), see figure 1(b). When in operation, the culture media reaches 25 cm height.

B. *Lipid extraction tests*

To perform the oil extraction tests, only biomass obtained from the bubble column reactor was used. After cultivation microalgae cells were firstly flocculated with aluminum sulfate ($Al_2(SO_4)_3$) and then filtrated. The extraction tests were performed according to a modified Bligh and Dyer's method [12]. It was used methanol and chloroform as solvents and the cell disruption was made using ultrasonic bath. The extraction process could be summarized in the figure 2.

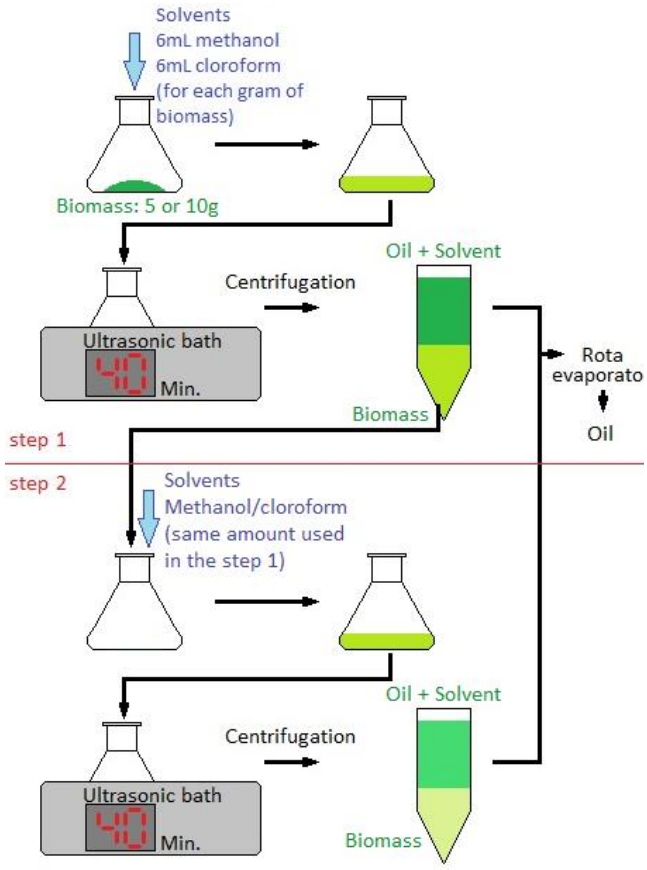

Fig. 2. Extraction process

First, the biomass was put in an erlenmeyer flask, then the solvents were added proportionally to the biomass amount (for each gram of biomass it was added 6mL of methanol and 6mL of chloroform). Then the mixture was placed in an ultrasonic bath for 40min. After that, the biomass was separated from the extracted oil by centrifugation. In order to evaluate the need of a second extraction step, in some of the experiments, the biomass resulted from the first step was extracted again. This second step is very similar to the first step, as it is shown in the figure 2. For the second step it was added the same solvent amount that was used in the first step, with no need to measure again the amount of biomass. When the second extraction step was employed, all the oil extracted in both steps was mixed together. After extraction, the solvents were evaporated in a rota-evaporator, in order to obtain the oil production.

It was measured the influence of some parameters over the extraction efficiency: biomass amount (5 or 10g of wet biomass, obtained after filtration), the size of the erlenmeyer used on the extraction (500 or 125mL) and the use of a second extraction step. All the extraction tests are summarized on the table 1. For a proper comparison between the extractions tests, it was determined the percentage of oil related to the dry biomass (%Oil).

Table 1. Description of the extraction tests.

Exp. number	1	2	3	4	5	6
Number of extraction steps	1	2	1	2	1	2
Erlenmeyer capacity	500mL	500mL	125mL	125mL	125mL	125mL
Amount of biomass	10g	10g	5g (each erlen)	5g (each erlen)	10g	10g

III. Results and Discussion

From the systematic literature review made on Science Direct database, it was obtained 18 publications from 2006 until the search day in May 2016. Among these papers, only 8 had had some empirical results of oil production from *Chlorella minutissima* microalgae. The other documents were not considered in this study.

All the 8 documents obtained from the search are organized in table 2. The second column of the table shows some details of the employed methods in each publication: the used Cultivation medium (CM), the photobioreactor (P) and the oil extraction methods (OE). It was also shown if the publication studied different nutrient compositions in the culture medium (SMD – Studied medium factors). At least, the third column of the table shows some of the results from each publication, especially those results obtained in similar cultivation procedures as the obtained from the present work.

A. Extraction tests

All the results obtained from the extraction tests are shown in figure 3. The number in the graphic are the same as presented in table 1 (see methods). The values obtained for each experiment are presented (%Oil): 1 - 16.6; 2 – 25.1; 3 – 21.7; 4 – 27.7; 5 – 21.3; 6 – 29.3.

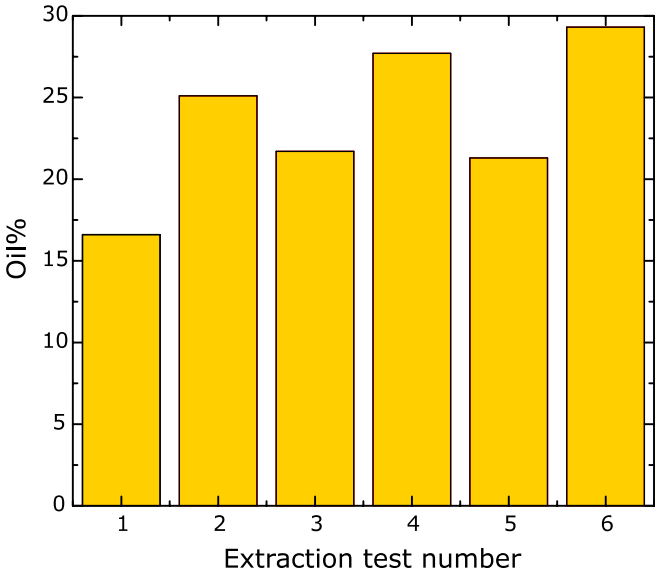

Fig. 3. Oil (%) obtained from each of the extraction test.

Table 2. Main results of the 8 analyzed papers regarding Chlorella minutissima microalgae. Where: CM-Cultivation Medium; P-Photobioreactor, OE- Oil Extraction method, SMF– studied medium factors).

Source	Methods	Results range
[13]	**CM:** BBM (pH 7.2–7.4) with 5% v/v of CO_2 in the aeration system. **SMF:** Nitrate and phosphate concentrations. **P:** 3 L stirred tank **OE:** Modified Bligh and Dyer method.	The maximum oil productivity was 49.2±11.2 $mgL^{-1}d^{-1}$ ($NaNO_3$: 125mg L^{-1}, K_2HP0_4: 75mg L^{-1}). Meanwhile, the lower lipid productivity was 32.4±0.31 $mgL^{-1}d^{-1}$ (with no addition of $NaNO_3$ or K_2HPO_4). This same experiment presented the higher lipid yield 49.09±3%.
[14]	**CM:** Modified Trisacetatephosphate (TAP) medium. **SMF:** Nitrate and phosphate concentration. **P:** 1.2 L flat panel reactors, 1.4 L airlift reactors. **OE:** Bligh and Dyer method.	The higher observed biomass productivity was 193 $mgL^{-1}d^{-1}$ and the lipid content was about 23% w/w (nitrate: 25 mM and phosphate 1.35 mM).
[15]	**CM:** Wastewater **SMF**: Wastewater concentration. **P:** 5 L capacity laboratory grade plastic tray. **OE:** Folch method.	Using pure wastewater, from 2 different sources, they obtained the following oil content and oil productivity: 20.69 and 28.32% w/w, 91.7 and 47.5 $mgL^{-1}d^{-1}$.
[16]	**CM:** Artificial wastewater media. **SMF:** heavy metals concentration. **P:** 500 mL flasks. **OE:** Modified Bligh and Dyer method.	The control oil productivity (cultivation without any addition of heavy metals) was 115 ± 2.84 $mgL^{-1}d^{-1}$. The lipid content was 10.82 ± 0.01% w/w.
[17]	**CM:** Cultivation procedure was in two steps, first using OM medium (for 5 to 7 days), then in a new medium without nitrogen source. **SMF:** NaCl, Fe(III), glycerin, glucose, glycine, mannitol, sodium acetate, sodium bicarbonate concentrations and pH. **P:** 500 mL flasks.	Varying the Fe(III) concentration (from 0 to 0.4mmol/L), the lipid content and productivity varied from 11.25 to 16.78% w/w and from 120 to 139.75 $mgL^{-1}d^{-1}$, respectively.
[18]	**CM:** BG-11. **SMF:** glycerol, glucose, succinate, molasses and press mud. **P:** 500mL Erlenmeyer flasks. **OE:** Total lipids were estimated using a spectrophotometry technique that uses dichromate solution in order to oxidize all the present lipids.	1.5 to 3g of oil in 10 cultivation days.
[19]	**CM:** N8Y. **SMF:** Nitrogen sources were tested (KNO_3, $(NH_4)_2SO_4$, urea and casein) and dextrose, oxalic acid, starch, sucrose, glycine, sodium acetate and glycerin concentrations. **P:** 500 mL flasks. **OE:** Modified Bligh and Dyer method after a hydrothermal acid pretreatment.	The higher oil productivity was 286.76 $mgL^{-1}d^{-1}$, and an oil content of 16.11%w/w (26.37 g L^{-1} of carbon source, 2.61 g L^{-1} of nitrogen source and 0.03 g L^{-1} of phosphorus source)
[20]	**CM:** Seawater from the cooling system of a nuclear power plant enriched with nutrients. **SMF:** Autotrophic and mixotrophic cultivation (glucose addition). **P:** 200 L perfusion type photo-bioreactor (composed of 10 smaller tanks of 20 L each). **OE:** Folch method	With a 2.8 L/h perfusion rate in a mixotrophic medium it was obtained 23.2% w/w of oil after 80days cultivation. For the autotrophic cultivation, the result was 13.1% of oil after 80days.

According to the figure 3, making a comparison with the experiments in which the only difference was the use of one or two extraction steps, it is possible to say that a second extraction step is needed. Looking to the results of the tests number 1 and 2 it is possible to say that the implementation of the second step increased the %Oil in 8.5 %. Between the results 5 and 6, the difference was 8 % and for the experiments 3 and 4 a second step increased 6% on %Oil. On other words, a second extraction step could increase the oil production in, at least 6%.

Besides the use of the second step, the erlenmeyer capacity was an important factor as well. When comparing the results of the tests 2 (%Oil=25.1 %) and 6 (%Oil=29.3 %) it is noticeable that the difference between these tests was of 4.2%. This difference shows that the use of a smaller container for the extraction (125 mL erlenmeyer) is a good proposition, maybe because the smaller container could improve the ultrasound propagation or the cavitation process.

Finally, the amount of biomass in each bath was not an important factor. When comparing the tests 3 (%Oil=29.3 %) and 5 (%Oil=29.3 %) it is clear that the extraction of 10g in a single 125 mL erlenmeyer or the division of the 10 g in two 125 mL erlenmayers (5 g each) did not change %Oil significantly.

B. Oil productivities of Cholorella minutissima cultivated in Bubble column and tank fotobioreactors

An important difference between the two studied homemade photobiorreactors is their complexity. The bubble column reactors are more sophisticated and can operate with larger amount of biomass, nevertheless are more expensive to build and are more complicated to operate. Meanwhile, the tank photobioreactors are cheaper to produce and easier to operate, but can cultivate only 4 L of microalgae each batch. These two homebuilt photobioreactors were chosen aiming to help future researches to understand how sophisticated or cheap their photobioreactor could be. Their choice could be based on the money availability and on the biomass amount, which is need for their studies.

The bubble column reactor, which is a vertical column aerated from its bottom, is a popular kind of photobioreactor design [21-22]. This photobioreactor is compact and can provide a very efficient mixing, and is easy to operate [6, 23]. On the other hand, for lower production rate, the built tank photobioreactors are easier to operate and can, with almost no cost, be replaced.

Oil contents (%Oil) of both of the photobioreactors were 29.3%w/w for the bubble column and 36.7%w/w for the tank photobioreactor. Biomass and the oil daily productivities are shown in figure 4. The biomass productivities were: bubble column: 26.7 $mgL^{-1}d^{-1}$, tank: 39.74 $mgL^{-1}d^{-1}$. Meanwhile, the oil productivities were: bubble column: 7.82 $mgL^{-1}d^{-1}$, tank: 14.6 $mgL^{-1}d^{-1}$.

The obtained results showed bigger oil productivity for the tank photobioreactor (14.6 $mgL^{-1}d^{-1}$), in comparison to the bubble column photobioreactor (7.82 $mgL^{-1}d^{-1}$). The difference between those results could be related to the different sizes of the photobioreactors. Each bubble column photobioreactor is, in terms of volume, more than twelve times bigger than a tank photobioreactor. So, their flow mechanisms and their gas exchange are very different, making difficult a direct comparison between their productivities.

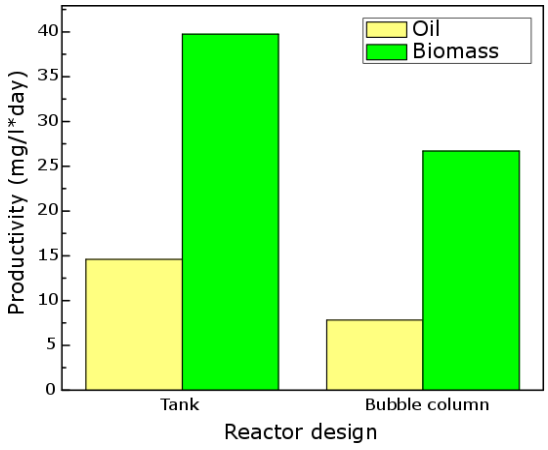

Fig. 4. Biomass and oil daily productivity of two photobioreactor designs (tank and bubble column)

Aiming to organize the comparison between the results obtained from this work with the literature systematic review, each of the 8 analyzed papers is individually discussed below (the presentation order is the same as shown in table 2:

- [13] employs a 3 L stirred tank as photobioreactor. In a certain way, the tank photobioreactor proposed in the present work is a simpler and cheaper version of the photobioreactor employed by [13]. Their photobioreactor have some advantages that can be told: is made of glass, which allows better light absorption; have a temperature control system and a steering system. Meanwhile, the here presented tank photobioreactor is cheaper and easier to be replaced, if necessary. Looking to the oil content results, [13] obtained values between 27.78 ± 2%w/w and 49.09 ± 3%w/w. these results encloses the tank photobioreactor and the bubble column oil yields obtained from this paper. However, their lipid productivity ranged from 49.2 to 32.4 $mgL^{-1}d^{-1}$, higher than the 14.6 $mgL^{-1}d^{-1}$ obtained from the tank photobioreactor. There are some possible explanations to understand the higher oil productivity of [13]. One of them is their use of CO_2, mixed to the aeration system, as carbon source, while this paper did not use concentrated CO_2. In addition, for their experiment with the best oil productivity, the nutrient concentrations were $NaNO_3$: 125mg L^{-1}, K_2HP0_4: 75mg L^{-1}. Meanwhile, the present paper worked with lower nutrient concentrations ($NaNO_3$: 65mg L^{-1} and $NaH_2PO_4.H_2O$: 5mg L^{-1}).

- [14] used a considerably different cultivation media from the f/2, used in this work. Their nitrate concentration was bigger (25mM against 0.76mM) and so was their phosphate concentration (1.35mM against 0.036mM). These differences could explain their much bigger biomass productivity (193 $mgL^{-1}d^{-1}$). However, their oil content was smaller than the obtained in the tank photobioreactor (23%w/w against 36.7%w/w).

- [15] cultivated *Chlorella minutissima* in pretreated wastewater from two different sources and found similar oil contents from the values obtained from the present study (20.69%w/w and 28.32%w/w). On the other hand, their maximum oil productivity was bigger than the obtained from this study: 91.7 and 47.5 $mgL^{-1}d^{-1}$. A possible reason for the discrepancy of the oil productivity could be the differences on the culture media.

- [16] worked with the microalgae absorption of heavy metals. In order to get closer experiment conditions with both studies, for this comparison, it was considered only the oil productivity of their control cultivation (with no addition of heavy metals). Although the use of this precaution, the differences of the cultivation parameters of both works are still huge, especially about the cultivation media. [16] used an artificial wastewater media that included a protein (casein) as nitrogen source, instead of the nitrate. [16] also added 17.5 g L^{-1} of glycerin in their cultivation

media. These cultivation differences should explain the high discrepancy of the oil productivity results (115 $mgL^{-1}d^{-1}$ against 14.6 $mgL^{-1}d^{-1}$ for the tank reactor).

- [17] cultivated the microalgae in two steps, so it is difficult to compare the oil productivities from their work to this work results (that used cultivation in a single step). Also, [17] expressed, in almost all their work, the lipid amount in terms of g L^{-1}, except when they studied the influence of Fe (III) concentration on lipid accumulation. For this reason, these data were chosen to be compared to the results of the present paper. [17] studied Fe (III) concentrations ranging from no added Fe (III) to 0.4mmol/l. Considering all of these experiments the oil content varied from 11.25 to 16.78%w/w, lower in comparison to the data obtained from this paper. Although their oil contents were lower, [17] reached excellent oil productivities, ranging from 120 to 139.75 $mgL^{-1}d^{-1}$.

- [18] obtained from 1.5 to 3g of oil in their cultivation. However, it is very difficult to confront this work's results with [18] results, since they did not informed the working volume of their photobioreactor or the cultivation time (that is probably 10 days, analyzing their results) making impossible to calculate accurately their oil productivity.

- [19] reported a significant influence of the nitrogen source over the oil productivity. When [19] cultivated the microalgae *Chlorella minutissima* with N8Y (nitrate as nitrogen source), with the addition of 1.22 g L^{-1} of glycine, as a carbon source, their oil productivity was 17.86 $mgL^{-1}d^{-1}$ (similar to the productivity of the tank photobioreactor of the present work 14.6 $mgL^{-1}d^{-1}$). On the other hand, the same cultivation parameters, only using casein as nitrogen source, raised the oil productivity to 34.83 $mgL^{-1}d^{-1}$. This results shows that the use of casein as nitrogen source could be a good idea to increase the oil productivity. After using the Box–Behnken Design, [19] found that the optimized culture media had the following nutrient concentrations: 26.37 g L^{-1} of carbon, 2.61 g L^{-1} of nitrogen and 0.03 g L^{-1} of phosphorus (more concentrated than the cultivation media used in this paper). With this cultivation medium, [19] obtained an extremely high oil productivity, 286.76 $mgL^{-1}d^{-1}$, despite of the low oil content of 16.11%w/w (lower than the value obtained in this study using the tank photobioreactor, 36.7%w/w).

- [20] used the seawater from the cooling system of a power plant as the base for making their culture media. This wastewater was originally obtained from sea and its use on the power plant did not change significantly its chemical properties. Since there was no problem using this water source, their strategy allowed them to utilize the heat from the power plant to outdoor microalgae cultivation in a cold weather. [20] made their cultivation system in a way to remove continuously cold old medium and introduce new hot medium (perfusion system) in order to keep warm the cell cultivation for a long-term cultivation (80days). Besides the perfusion system removing continuously medium from their system, [20] always recovered all the microalgae cells using filtration. Hence, due to the heavily different cultivation system, it is difficult to compare their results with the results presented in this work. However, it is still possible to use their oil content to make some correlation with both studies. Besides the use of glucose, their results showed smaller oil content than the result obtained from the tank reactor (23.2%w/w against 36.7%w/w).

Regarding %Oil obtained from the homemade photobioreactors (tank and bubble column), both of them have shown high amounts of oil in relation to the dry biomass, when compared to the literature data. However, their oil productivities are still low. In order to increase their productivities, a good future perspective is to optimize the culture medium. Still according to the literature, it is interesting to increase the nutrient concentrations, specially the nitrate one. This change would decrease the %Oil but is a promising way to increase the oil productivity. Another idea is to change the nitrogen source from the nitrate to a more complex one (e.g. casein). Finally, the use of a carbon source (such as glycerin, glucose, glycine, sodium acetate and sodium bicarbonate) has been shown a good option for oil productivity increasing.

IV. Conclusion

The results show that, for a better extraction process, it is needed a second step of extraction. The size of the Erlenmeyer used is an important factor as well. On the other hand, the amount of biomass did not change %Oil very much, since the amount of solvents was proportional to the biomass content. Therefore, it is concluded that the better extraction was done in a 125 mL erlenmeyer with 10g of biomass (better processing speed) in a two stages process.

Compared to the literature results, both photobioreactors showed high oil contents (29.3 %w/w for the bubble column and 36.7 %w/w for the tank photobioreactor). Meanwhile the oil productivity could be increased. In order to achieve higher lipid amounts a future research step will be related to optimize the nutrient concentrations in the culture medium. These medium improvements will be focused mainly on increasing the nitrogen concentration, experiment more complex nitrogen sources, such as casein, and use a carbon source (e.g. glycerin, glucose, glycine, sodium acetate and sodium bicarbonate)

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