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Lipid Extraction from *Scenedesmus* sp. Followed by Purification using Column Chromatography

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Microalgae have been increasingly studied as a source of different bioproducts, from biofuels to valuable food ingredients, due to their potential for a multi-product biorefinery. Moreover, they exhibit many advantages compared to food crops, including high biomass productivity, smaller area, and non-arable land for cultivation. Their lipid fraction can be extracted using different methods, including conventional (hexane, chloroform, methanol) or greener (supercritical CO_2, ethanol, 2-methyltetrahydrofuran, isoamyl alcohol) solvents. Then the oil can be directed for biodiesel production by esterification/transesterification reactions or for consumption of essential fatty acids (omega 3, -6, and -9). Regardless of the application, a cost-efficient lipid extraction and a previous purification step to remove pigments and trace elements are required for catalytic upgrade. Specially chlorophyll and impurities on the lipid extract can significantly reduce process yield. However, purification methods have been scarcely discussed even also being a key step in lipids processing. This study evaluated the extraction of lipids from dried microalga biomass of *Scenedesmus* sp. using ethanol assisted by ultrasound followed by purification using different adsorbent materials (activated carbon, sepiolite, and bentonite) packed in a chromatographic column. The best adsorbent also was tested for the purification of lipids extracted using hexane for comparison. Lipid extracts were evaluated qualitatively by the colour of eluted solvent and lipids mixture, and quantitatively by acylglycerols quantification. Activated carbon showed the least coloured eluted solvent and highest triglycerides concentration (63.1%); however, bentonite showed the highest product's recovery (12%) for a shorter time (100 mL elution in 1 h compared to 8 h). Moreover, when using hexane, lipids purified by bentonite showed higher selectivity towards neutral lipids, as expected, achieving 70.4% triglycerides. These results show an efficient lipid purification method that can be used in microalgae to potentially increase reactions yield.

1. Introduction

Microalgae exhibit several advantages compared to terrestrial plants as they do not compete with conventional food production and are not lignocellulosic biomass. Their biomass consists mainly of three primary metabolites: proteins, carbohydrates, and lipids, which can derive many bioproducts, but the most studied application is biodiesel production from the lipid fraction. However, as their large-scale production is still not competitive for this biofuel, an increasing number of researchers have focused on improving biomass productivity and lowering costs and energy demand in all stages (cultivation, upstream and downstream processes) while seeking to decrease environmental impacts. To that purpose, cultivation coupled with bioremediation, CO_2 sequestration, and biomass valorization (Ferreira et al. 2019), many times using innovative technologies (Rosli et al. 2020; Teng et al. 2020) and greener solvents (de Jesus et al. 2018), are steps towards a cost- and energy-efficient production. Among these techniques, nutrient starvation and CO_2 bubbling are specially known to be effective for lipid accumulation. Additionally, strain selection is key to attend these requirements, being *Scenedesmus* sp. known for their efficient CO_2 capture (Farronan et al. 2021). A study of the metabolic/biological pathways of this microalga growth using extremely high CO_2 concentrations (30-70%) showed they could grow and accumulate lipids while still improving biomass production (Huang et al. 2020). *Scenedesmus* sp. is a freshwater species usually isolated from water bodies, wastewater, or other effluents (Xin, Hong-ying, and Jia 2010; Kumar,

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Banerjee, and Jagadevan 2021). Thus, their cultivation is associated with effluent treatment. high growth rate, and lipid accumulation, but still mostly in laboratory and pilot scales.
Despite lipid compounds having different applications, most catalytic upgrading steps are efficient to process mainly the neutral lipid part (TAGs) and free fatty acids (FFAs) (Ferreira et al. 2021). Chlorophylls are a major technical drawback (Kumar et al., 2015) and can account for a large mass percent of algal lipids, increasing viscosity. Thus, it is essential to quantify FFAs, and acylglycerols: TAGs, diacylglycerols (DAGs), and monoacylglycerols (MAGs) aiming to remove other lipid compounds. Lipid's purification has been timidly explored as conventional methods for biomass purification are not suitable for microalgae. Standard vegetable oil refining steps are degumming, caustic refining, bleaching, and deodorization (Cuellar-Bermudez et al. 2015). Recently, enzyme-assisted alkaline refining has been proposed (Nielsen 2021), achieving 1.8% increase in oil yield from phospholipids hydrolyzed to DAG. However, green refining technologies should be investigated for microalgae oil to remove pigments, polar lipids, and trace elements for example. Efficient lipid extractions could also attenuate these challenges, such as the application of green and clean methods using supercritical CO_2 ($scCO_2$), which is maintain lipid quality (Marino et al. 2021), or nonpolar solvents.
This work provides new information on lipid extraction and purification by comparing different conditions and quantifying acylglycerol groups. Few studies have performed microalgal oil purification using chromatographic columns packed with adsorbent materials. Richmond and Hu (2013) used a column with silicon dioxide, silica gel, and anhydrous sodium sulfate to elute neutral lipids (hydrocarbons, pigments, sterols, triglycerides, waxes, etc.) with chloroform, followed by fatty acids and polar lipids (glycolipids, phospholipids) with methanol. Santillan-Jimenez et al. (2016) used columns packed with silica gel and activated carbon or montmorillonite to elute neutral lipids with diethyl ether. Finally, Lorenzen et al. (2017) also used a column packed with bentonite but with n-hexane. Based on these studies and a vast literature on lipid extraction, this work compared two organic solvents with different polarities for conventional lipid extraction assisted by ultrasound followed by three adsorbent materials for purification, hence a broader approach to this topic.

2. Materials and Methods

2.1 Microalgal biomass

Scenedesmus sp. LF01 microalga was donated as wet biomass after cultivation by Algae Biotecnologia Ltda. Microalgae inoculum was provided by the Federal University of São Carlos (UFSCar) and microalgae cultivation was conducted in an open thin layer cascade (80 ton) in direct contact with air, connected with an underground reservoir with CO_2 injection, from which the culture was recirculated from the tank to the ramp. According to the suppliers, the system was operated semi-continuously, removing biomass periodically (every 2-3 days). Wet biomass was dried using a spray dryer (DR-0,4 AIR SPRAY PROCESS) from Spray Process Ltd.

2.2 Lipid extraction

Lipids were extracted using ethanol or hexane assisted by ultrasound (Unique USC-2800 40 kHz ultrasound device) for 1 h at 60 °C, with dry biomass and organic solvent at the mass: volume ratio of 1:10. Residual biomass was filtered and solvent containing extracted lipids was evaporated in a rotary evaporator (IKA RV 10). The extraction yield was calculated considering the mass of lipids in the dry biomass.

2.3 Lipid purification

After extraction using ethanol and the solvent evaporation, a purification step was conducted using different adsorbent materials (activated carbon, sepiolite, and bentonite) packed in a chromatographic column. Extracts were purified using a typical chromatographic column packed with silica gel and an adsorbent as stationary phase, and chloroform as mobile phase. Firstly, the column was packed with the stopcock closed and chloroform was carefully added to uniformly wet all adsorbent materials. After opening the stopcock, the extracted lipid (around 1-2 g) was dissolved in a few mL of chloroform and added through the top of the column. Chloroform was added dropwise until elution was complete. The end of purification was established when an evident green color reached the bottom of the chromatographic column, which indicates the elution of pigments. Figure 1a shows an example of the column packed with bentonite and in Figure 1b, the same column after elution with chloroform until pigments reached the bottom part. Lastly, for comparison, one adsorbent was selected to test lipids purification after extraction using hexane, instead of ethanol. The reported purification yield in this step is the recovered lipids from the column.

2.4 Lipid extracts characterization

Lipid extracts were evaluated qualitatively by the colour of the eluted solvent and lipids mixture, and quantitatively by acylglycerols quantification. Based on ASTM D6584-17 and EN 14105, the crude lipids extracted from dry biomass were analysed to quantify FFA, MAG, DAG, and TAG.

The purified lipids after purification were also analysed. This quantification method was employed using a GC-FID (Agilent 7890A) and two internal standards (tricaprin and 1,2,4-butanetriol). AOCS Official Method Ca 12-55 (AOCS 2009) was performed to determine phosphorus or the equivalent phosphatide content on microalgae lipids. This method is applicable originally to crude, degummed, and refined vegetable oils, by ashing the sample in the presence of ZnO, followed by the spectrophotometric measurement of P as a blue phosphomolybdic acid complex. A calibration curve was obtained for potassium dihydrogen phosphate.

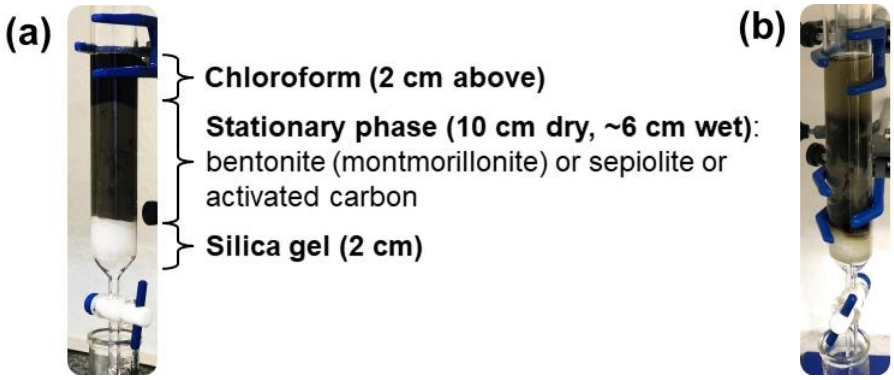

Figure 1: *Chromatographic column packed with bentonite for lipids purification: before (a) and after (b) elution using chloroform.*

3. Results and Discussion

Figure 2 shows the color of approximately the same mass of lipids dissolved in chloroform after the steps of ultrasound-assisted ethanol extraction (Figure 2a), and purification using the three adsorbents studied (bentonite, sepiolite, and activated carbon in Figures 2b, 2c, and 2d, respectively). Also, at the bottom of Figure 2, it is shown the lipid extracts after the chloroform evaporation. Bentonite led to lipid extracts with the most accentuated color, a greenish-yellow. Despite indicating a residual chlorophyll content due to pigmentation, this was the most concentrated solvent eluted. After complete solvent evaporation, sepiolite and activated carbon had the lightest colors of lipid extracts, which could not be seen with dilution. Santillan-Jimenez et al. (2016) also achieved efficient removal of chlorophyll from *Scenedesmus acutus*' crude lipids using K10 montmorillonite or Darco® KBG activated carbon packed in a chromatographic column. Their UV/Vis spectra showed an absence of peaks related to chlorophyll molecules. Lorenzen et al. (2017) were able to obtain clear and liquid oil after processing microalgae lipid extracts with bentonite and hexane, which indicated the removal of pigments.

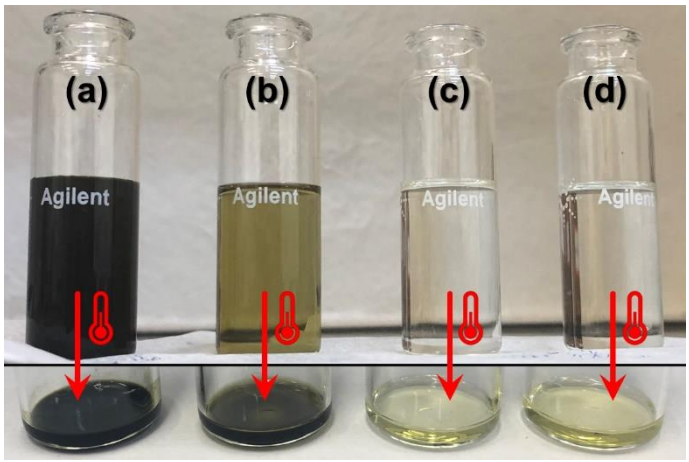

Figure 2: Lipids extracted by ethanol (a) and purified using bentonite (b), sepiolite (c), and activated carbon (d). The bottom image shows lipid extracts after the chloroform evaporation.

Table 1 shows the yields in the extraction and purification steps, together with the acylglycerols quantification, with normalized values to compare the ratio between FFAs and TAGs, DAGs, and MAGs. Considering the lipid purification step after the extraction with ethanol, activated carbon showed the highest triglycerides concentration (63.1%). However, bentonite showed the highest product recovery (12%) for a shorter time (100 mL elution in 1 h compared to 8 h). The purification step still requires optimization to increase yield once around a fifth of *Scenedesmus* sp. identified lipid composition was TAGs (ethanol extraction). The low purification yields reported in Table 1 might be related to processing only a few grams of extracted lipids, thus experiments on a larger scale should be conducted to evaluate the purification efficiency. Additionally, activated carbon showed a clogging problem possibly due to porosity and larger and nonpolar molecules affinity to the adsorbent, which may lead to a much higher elution time. Nonetheless, considering TAGs content and yield, bentonite was chosen for further tests using ultrasound-assisted hexane extraction. This extraction with hexane provided lipids with 44% less FFAs compared to ethanol. It may be related to the lower polarity of hexane. Additionally, lipid extracts obtained with hexane seemed to be less viscous compared to the ethylic extract, though this property was not measured. The purification of the hexane extract using bentonite showed higher selectivity towards neutral lipids, as expected, achieving 70.4% triglycerides.

Table 1: Lipid extracts composition and process parameters for the extraction and purification steps.

	Lipid extraction		Lipid purification			
%m	Ethanol	Hexane	Sepiolite (Ethanol)[1]	Activated carbon (Ethanol)[1]	Bentonite (Ethanol)[1]	Bentonite (Hexane)[2]
FFAs	74.7	41.8	49.0	20.1	42.2	5.2
MAGs	0.5	0.0	0.4	0.0	0.0	0.2
DAGs	6.2	26.1	15.8	16.8	9.1	24.2
TAGs	18.6	32.1	34.7	63.1	48.7	70.4
Yield[3]	13.0	10.0	3.1	5.3	12.0	15.4
Time (h)	2	2	6	8	1	1

[1]Lipid extraction with ethanol. [2]Lipid extraction with hexane. [3]Extraction yield (lipids on dry biomass) and purification yield (recovered lipids from column).

Santillan-Jimenez et al. (2016) determined the total acid number, which increased from 54.8 in crude lipids to 85.6 mg KOH/g in lipids purified using activated carbon. The purified lipids showed a FFA content of 40% for the K10-purified material and 50% for the activated carbon. However, GC-MS results showed an almost identical FA content compared to crude lipids. The main difference in these studies was the proportional reduction of FFAs achieved in this work. This disparity could be associated with the solvent employed and elution time, also shown by the lower extraction yield in this work. As mentioned, lipids were allowed to elute until a green color was noted in the silica gel, stopping the purification process. Also, according to Richmond and Hu (2013), the separation efficiency of column chromatography may be significantly affected by the sample loading size.
Figure 3 shows lipids dissolved in chloroform after extraction with hexane and purification with bentonite. Extracted lipids still had a dark green color compared to Figure 2, but purified lipids showed a clear and concentrated yellow mixture. Although viscosity was not measured, lipids extracted using hexane were closer to a liquid aspect rather than solid at room temperature. Lipids extracted using ethanol were more solid in comparison. Furthermore, after solvent evaporation in an oven at 40 °C, purified lipids in Figure 3b had a clear liquid aspect. These extracts had an intense yellow color and started to solidify at room temperature (around 25 °C), which could be due to fatty acids saturation. Nonetheless, the removal of phosphorous polar lipids is evidenced as the melting point was below 40 °C.

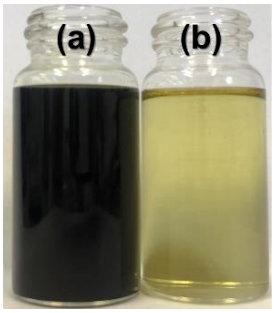

Figure 3: Lipids extracted by hexane (a) and purified using bentonite (b).

Lipids extracted from Scenedesmus sp. using hexane presented 231 ppm of P, which decreased to 40 ppm after purification using bentonite, showing that this purification method was also able to reduce phosphorus content. This reduction would be essential for biodiesel production to attend the specified limit of 10 ppm in quality standards (Moser 2011). Santillan-Jimenez et al. (2016) also showed an efficient removal of phospholipids. Lorenzen et al. (2017) obtained a significant decrease in calcium and phosphor after purification with bentonite, from up to 33 to 1.2% and from 35 to less than 4%, respectively.

Finally, although not approached in this work, previous studies have shown the possibility of recovering the adsorbent by acetone extraction (Santillan-Jimenez et al. 2016). A GC-MS analysis revealed only a trace amount of lipids. Also, thermogravimetric analysis were very similar for the absorbents before and after acetone extraction. Thus, based on the work of Santillan-Jimenez et al. (2016), adsorbents show potential for reuse.

4. Conclusions

This study showed an efficient lipid purification method that can be applied to microalgae crude oil, obtaining a clean product and potentially avoiding catalyst poisoning during the oil conversion into more valuable products. Most results were in accordance with the two previous studies found in the literature regarding pigments and trace elements removal. Comparing to these other studies, one important result reached in this work was the lower content of FFAs reached in this work, which could be advantageous for transesterification. Also, the comparison between the previous lipid extraction using ethanol or hexane showed that hexane is a more selective and efficient solvent. However, greener solvent alternatives should be explored to avoid the use of toxic solvents. Nonetheless, there are still very limited research on this topic, which should be further investigated as microalgae are promising biomass.

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