Food Science and Technology

Tetradesmus obliquus microalgae: solvent extraction of lipids under different process conditions

Neuza Liliana Calvo Meroiço[1] , Maurício de Oliveira Leite[2] , César Augusto Sodré da Silva[1] , Marcio Arêdes Martins[2] ,
Monique Ellen Torres Resende[3] , Eduardo Basílio de Oliveira[1] , Jane Sélia dos Reis Coimbra[1]*

[1]Universidade Federal de Viçosa – Depto. de Tecnologia de Alimentos, Av. Peter Henry Rolfs, s/n – 36570-900 – Viçosa, MG – Brasil.
[2]Universidade Federal de Viçosa – Depto. de Engenharia Agrícola, Av. Peter Henry Rolfs, s/n – 36570-900 – Viçosa, MG – Brasil.
[3]Universidade Federal de Juiz de Fora – Depto. de Farmácia, R. Israel Pinheiro, 2000 – 35020-220 – Governador Valadares, MG – Brasil.
*Corresponding author <jcoimbra@ufv.br>

Edited by: Erick Sigisfredo Scheuermann Salinas

Received December 29, 2022
Accepted August 10, 2023

ABSTRACT: Microalgal lipids exhibit physical-chemical characteristics similar to vegetable oils, representing alternative sources of long-chain fatty acids for food supplements. However, separating intracellular lipids from microalgae biomass is still challenging due to downstream processing technology limitations that depend on the particular microalgal species and its cultivation conditions. Thus, this work evaluated a multistep process for lipid extraction from *Tetradesmus obliquus* biomass instead of conventional single extraction methods to improve the lipid yield. The wet biomass was freeze-dried, the cells of the dried biomass were disrupted in a ball mill, and the milled biomass was suspended in water to obtain solid contents of 5 % and 10 % w v^{-1}. Lipids were extracted from the aqueous suspension using the ethanol polar solvent and the hexane apolar solvent with ethanol:hexane ratios of 1:1, 1:2, and 3:5 v v^{-1}. Tests were also carried out on the deproteinized biomass to evaluate the effect of proteins on emulsion formation since deproteinization can avoid emulsion formation. Thus, lipid downstream processing could be designed without an emulsion removal stage. The biomass was deproteinized before lipid extraction with NaOH at pH 10. The highest lipid content was verified for the 1:2 ethanol:hexane ratio, in which up to 42 % of the total lipids were extracted from the biomass. With the 3:5 ratio, the organic and aqueous phases emulsified due to the presence of lipoproteins, which hindered the lipid extraction process. Under the studied conditions, no increase in lipid extraction yield was observed with increases in the solid content or biomass deproteinization.

Keywords: ethanol, hexane, cell disruption, ball mill, lipoproteins

Introduction

Microalgae are a renewable energy source with promising prospects for the supply of bioproducts with economic value, such as proteins, pigments, polysaccharides, and lipids, for different technological fields. Cultivating microalgae cells under stressful conditions can increase bioproduct production and alter their properties and associated uses (Estevam et al., 2023; Liang et al., 2018). For some green microalgae, for example, the nutritional stress of phosphorus, nitrogen, and iron supplementation deprivation increased lipid accumulation at the expense of additional glyceride synthesis (Hu et al., 2008). Nevertheless, cell stress may not always be reflected in higher lipid productivity, as increasing the lipid is to the detriment of the growth and division of the cells (Halim et al., 2012; Rodolfi et al., 2008).

Microalgal lipids are used in the production of biofuels, food, pharmaceuticals, biofilms, and soil biofertilizers (Amorim et al., 2021) on account of the following characteristics: (i) they consist of glycerol or esterified bases of fatty acids; (ii) they can be saturated or unsaturated (Neofotis et al., 2016); (iii) they are classified as neutral lipids (mono-, di-, triglycerides, and free fatty acids) and polar lipids (phospholipids and glycolipids); (iv) they exhibit high fatty acid content with chains of 14-22 carbons and physical-chemical characteristics similar to vegetable oils (Gunstone et al.,

2007); and (v) they contain fatty acids belonging to the omega-3 and omega-6 families, which are attractive to human nutrition (Wong et al., 2022).

Since microalgal lipid extraction is still challenging (Grossmann et al., 2018; Silva et al., 2021a, b), it is necessary to develop efficient separation processes to elaborate strategies to reduce the formation and stability of emulsions. Most lipids are withdrawn from microalgae, mainly using hexane as the solvent. However, a very stable emulsion is formed due to the protein in the biomass, making lipid extraction difficult by using only hexane as an extraction agent. Thus, the present work evaluated lipid extraction from *Tetradesmus obliquus* (Turpin) Wayne (Chlorophyta) at different solvent:biomass ratios. *T. obliquus* was previously named *Scenedesmus obliquus* (Wynne and Hallan, 2016). Two types of biomasses, protein, and deproteinized biomasses, were tested. The microalgal cell wall was broken using a ball mill to achieve high lipid recovery. The process variables evaluated were the solid content of the rehydrated biomass suspensions, the solvent ratios, and the presence or absence of proteins during lipid extraction.

Materials and Methods

Six steps were taken in the experimental procedure of lipid extraction from microalgal biomass: (1) processing of chemicals and *T. obliquus* biomass; (2) determination

of the *T. obliquus* composition; (3) disruption of cell biomass; (4) determination of the influence of the solids content on lipid extraction; (5) extraction of lipids from the biomass; and (6) deproteinization of biomass and extraction of lipids from deproteinized biomass.

Chemicals and processing of *Tetradesmus obliquus* biomass

The chemicals used were ethanol (≥ 99.5 % purity, Sigma-Aldrich), hexane (Neon), methanol (99.8 % purity, Sigma-Aldrich), and chloroform (≥ 99 % purity, Sigma-Aldrich). All other chemicals were PA grade.

The strain of the microalgae *T. obliquus* had been previously isolated from freshwater reservoirs, following Rocha et al. (2017), and cultivated in BG11 culture medium as described by Covell et al. (2020). The microalgal biomass was separated from the growing medium, according to Cesário et al. (2021). *T. obliquus* biomass (6 % w v^{-1}) was frozen and stored at –10 °C and was a gentle gift from the Laboratory of Biofuels at the Universidade Federal de Viçosa, Brazil. The frozen biomass was thawed for fractionation, washed with deionized water, filtered in an organza fabric filter medium to remove impurities, packed in containers, and frozen (GE freezer) at –80 °C. The frozen biomass was freeze-dried (LS 3000, Terroni), giving rise to dry biomass, reaching a final moisture content of 8 % w v^{-1}.

Confocal microscopy of the biomass

A sample of the *T. obliquus* culture collected in the exponential growth phase was centrifuged (Eppendorf, 5430) at 4000 × g for 5 min, and the cells were washed twice with phosphate-buffered saline (PBS) (0.1 M and pH 7.2). They were then examined in a laser scanning confocal microscope (Zeiss LSM 510 Meta) with argon laser excitation at 488 nm and emission at 650 nm for autofluorescence.

Proximate composition of the *Tetradesmus obliquus* microalgae

The lyophilized biomass was characterized for moisture (AOAC, 2005), ashes (AOAC, 2005), lipids by the Folch method (Folch et al., 1957) before cell rupture, lipids by ethanol and hexane solvents (Bermudez-Sierra, 2018) after cell rupture, and protein by the Kjeldahl method (AOAC, 2005) with a nitrogen factor of 5.89 (Afify et al., 2018). The carbohydrate content was calculated by difference (Carbohydrate % = 100 % – ashes % – lipids % – proteins %).

Quantification of lipids

Two lipid-determining methods were applied according to the cell structure condition, e.g., whole or disrupted cells.

The Folch method (Folch et al., 1957) was used for biomass containing whole cells, that is, before cell disruption. Thus, methanol and chloroform solvents were associated with acid hydrolysis (Bermudez-Sierra, 2018) to quantify lipids in biomass with whole cells. Acid hydrolysis is a destructive method utilized in the determination of the total lipid content.

A gravimetric method based on the Schmid-Bondzynski-Ratzlaff (SBR) principle (IDF, 1988) was applied to quantify lipids in microalgal biomass after cell disruption with a ball mill.

Hexane and ethanol (Viegas et al., 2020; Vieira et al., 2021) are alternatives to chloroform and methanol, solvents commonly proposed for lipid extraction.

Hexane was used because of its greater selectivity for neutral lipid fractions and minimal affinity for nonlipid contaminants, according to Shin et al. (2018). These authors investigated the extraction of lipids from *Tetraselmis* sp. using mixtures of hexane and methanol. The literature (Khoo et al., 2023; Vasistha et al., 2021) also reported other solvent systems for microalgal lipid extraction: (i) combinations of chloroform-methanol, chloroform-methanol-water, chloroform-ethyl acetate, methanol-sulfuric acid, hexane-isopropanol, water-(propan-2-ol)-cyclohexane, methanol-ethyl acetate-light petroleum, water-ethanol-hexane, and hexane-ethanol-acid; (ii) biobased solvents such as ethyl acetate-ethyl lactate, cyclopentyl methyl ether, and 2-methyl-tetrahydrofuran to replace hexane; and (iii) single solvents such as acetone, dimethyl sulfoxide (DMSO) ethanol, ether, ethyl acetate, hexane, liquefied dimethyl ether, and methanol.

In our work, ethanol was selected as the polar solvent due to its green, environmentally friendly, and low toxicity characteristics, allowing it to extract lipids to be applied in human and animal nutritional supplements. Methanol is a toxic material that causes several adverse effects on human health.

Folch method associated with acid hydrolysis for lipid quantification in whole-cell biomass

Methanol (10 mL) and HCl (5 mL, 3 M) were added to the freeze-dried biomass (1 g) for acid hydrolysis in Falcon tubes. The Falcon tubes were placed in a 65 °C water bath (Solab, SL-153), withdrawn after 10 min, cooled to room temperature, and added to chloroform (20 mL). Next, the Folch method was applied to the mixture (Bermudez-Sierra, 2018).

Schmid-Bondzynski-Ratzlaff (SBR) method for lipid quantification in disrupted cell biomass

The SBR method, modified according to Vieira et al. (2021), used ethanol and hexane solvents to quantify the lipid content in the disrupted and rehydrated biomass suspensions containing 5 %, 10 %, and 36 % w v^{-1} solids.

Biomass cell disruption

Mechanical disruption by ball milling was used to disrupt *T. obliquus* cells (Vieira et al., 2021). The lyophilized biomass was disrupted using a ball mill (Retsch, Model MM 400) at a frequency of 30 kHz and a processing time of 90 s, making the cell compounds accessible for separation. The freeze-dried biomass was characterized before and after cell disruption for moisture, ash, lipids, and protein, according to proximate composition of the *T. obliquus* microalgae.

Influence of solids content

The influence of solid content on lipid quantification and extraction was studied using suspensions of freeze-dried biomass diluted in water at proportions of 5 %, 10 %, and 36 % (w v^{-1}). Water was carefully added with even distribution over the biomass, then manually stirred for 5 min at room temperature to complete the hydration.

Biomass lipid extraction

Lipid extraction tests were conducted in disrupted and rehydrated biomass suspensions containing 5 % and 10 % w v^{-1} solids. Extraction tests were also performed on the protein and deproteinized biomass to define the levels of extracted lipids in both cases. The solvents used in SBR lipid quantification after cell rupture are the same as those used in the lipid extraction of disrupted and rehydrated biomass (DRB).

Ethanol was added to DRB in a Falcon tube and manually shaken for 1 min. Then, hexane was added, and the system was shaken until the mixture was complete. The proportions (v v^{-1}) of ethanol (polar solvent) and hexane (nonpolar solvent) were 1:1 (15:15 mL of ethanol: hexane), 1:2 (10:20 mL of ethanol: hexane), and 3:5 (11:25:18.75 mL of ethanol: hexane) a total of 30 mL of ethanol and hexane. The solvents were added in a 3:1 ratio of solvent:microalgal biomass. The mixture was centrifuged (Eppendorf, 5430) to separate the organic upper phase (hexane phase rich in lipids) and the aqueous phases (rich in proteins and carbohydrates). The hexane phase was removed with a volumetric pipette, placed in an Erlenmeyer flask, dried in an oven at 102 °C, cooled in a desiccator, and weighed. This procedure was repeated four times. At each repetition, the hexane volume corresponding to each proportion was added to the mixtures of DRB and ethanol. The volumes were 15 mL in a 1:1 ratio, 20 mL in a 1:2 ratio, and 18.75 mL in a 3:5 ratio. The volumes of the organic phase were collected in an Erlenmeyer flask, and the hexane was evaporated at 70 °C. After evaporation of the solvent, the amounts of extracted lipids were obtained by weighing the lipids in an Erlenmeyer flask. This entire procedure was performed in triplicate. The percentage of lipids extracted from the biomass or yield was determined as described by Patel and Kannan (2021) using Eq. (1):

$$\text{Extraction efficiency}(\%) = \frac{\text{Lipids recovered by the experimental method (g)}}{\text{Lipids recovered by the analytical method (g)}} \times 100\% \quad (1)$$

Biomass deproteinization and lipid extraction

The influence of protein removal from biomass on lipid extraction was evaluated after cell disruption since proteins can emulsify in solvents for lipid extraction (Amorim et al., 2021). Before and after each test condition of lipid extraction, the contents of proteins and lipids were determined. Thus, the influence of deproteinization on lipid extraction could be evaluated.

The methodology proposed by Silva et al. (2021a) was used for protein separation before lipid extraction. An amount of dried biomass, already broken in a ball mill (30 kHz and 90 min), was weighed, and diluted to obtain the desired percentage of solids (5 % or 10 % w v^{-1}). The pH was adjusted to 10 with 1 M NaOH; the biomass was shaken manually (1 min) and then centrifuged (5430, Eppendorf) at 7500 × g for 10 min at 20 °C. The first centrifugate (first residual biomass), containing lipids and insoluble proteins, was separated from the first supernatant, containing soluble proteins, polar lipids, pigments, and other soluble bioproducts. The first supernatant was stored for later extraction of the proteins. The pH of the first centrifugate was adjusted to 10 with 1 M NaOH (99 % purity, ACS científica). The mixture was shaken manually (1 min) and centrifuged again (7500 g, 10 min, 20 °C). The second centrifugate (second residual biomass) was separated from the second supernatant, which was stored. This process was similarly applied to obtain the third centrifugate (third residual biomass), assumed to be the control sample. The third supernatant was also stored.

After all protein separation steps, (1) the lipid content was determined in the third residual biomass (control sample) with the ethanol:hexane method. The insoluble protein present in the third residual biomass is difficult to separate from it (Silva et al., 2021a). (2) The soluble protein content was determined in the mixture of the stored supernatants (1, 2, 3). Soluble proteins were extracted at their isoelectric point (PI) of 2.55 after adding 1 M HCl, manual stirring, and rest for three days for protein precipitation. After protein precipitation, centrifugation (7500 g, 10 min, 20 °C) was used to separate the soluble proteins.

Statistical analysis

The results of the proximate composition were submitted to analysis of variance (ANOVA), and then the Tukey test of means was performed at 5 % probability. The results are represented as the means ± standard deviations. The statistical analysis was performed using the licensed SAS software (Statistical Analysis System, version 9.2, 1999).

Results

The fluorescence micrograph of the cells of *T. obliquus* in the division can be observed in Figure 1A-F. Cells are grouped into four to eight elements, with autofluorescence occupying most of the cells in addition to prominent spherical central pyrenoids.

The proximate composition of the *T. obliquus* biomass was 40.64 ± 0.563 % proteins, 21.66 ± 0.11 % ashes, 6.01 ± 0.30 % moisture, 15.77 ± 1.09 % lipids, and 15.85 % carbohydrates. The lipid content from microalgal biomass with different solid fractions using the 1:2 (ethanol:hexane) solvent ratio is described in Table 1. The protein content in residual biomass after lipid extraction is presented in Table 2. The effect of biomass deproteinization on lipid extraction under different solid proportions and solvent ratios is featured in Table 3.

Table 1 – Lipid extraction from microalgal biomass with different solid contents using a solvent proportion 1:2 (ethanol:hexane).

% Microalgal biomass	% Extracted lipids based on 0.1 kg of biomass	% Extracted lipids based on the lipid of total biomass
5	5.70 ± 0.63[a]	36.14
10	6.62 ± 0.28[a]	41.98
36	5.10 ± 0.37[a]	32.34
100	6.54 ± 0.68[a]	41.47

The data are presented as the mean ± standard deviation. Means followed by the same letter do not differ statistically by Tukey's test at 5 % probability.

Table 2 – Protein content in residual biomass after lipid extraction.

	% Proteins	
	NEF	WEF
Deproteinized biomass	6.73 ± 0.45[b]	
Biomass with proteins	9.85 ± 0.19[a]	8.02 ± 0.25[b] (organic phase + emulsified interface)
		12.51 ± 0.5[a] (aqueous phase + emulsified interface)

NEF = no emulsification formation; WEF = with emulsification formation at the system interface; Data are represented by mean ± standard deviation; Means followed by the same letter do not differ statistically by the Tukey test at 5 % probability.

Table 3 – Influence of biomass deproteinization on lipid extraction under different proportions of solids and solvent ratios.

	% Lipids			
Ethanol:hexane	Deproteinized biomass		Biomass without deproteinization	
Proportion	Standard deviation			
	5 %	10 %	5 %	10 %
1:1	3.48 ± 0.56[bA]	3.37 ± 0.25[bA]	3.25 ± 0.60[bA]	3.49 ± 0.21[bA]
1:2	5.01 ± 0.68[aA]	4.55 ± 0.26[aA]	4.97 ± 0.40[aA]	4.91 ± 0.38[aA]
3:5	5.15 ± 0.76[aA]	4.82 ± 0.26[bA]	4.87 ± 0.61[aA]	4.91 ± 1.03[aA]

Means followed by the same letter (lowercase in columns and uppercase in rows) do not differ statistically by the Tukey test at 5 % probability.
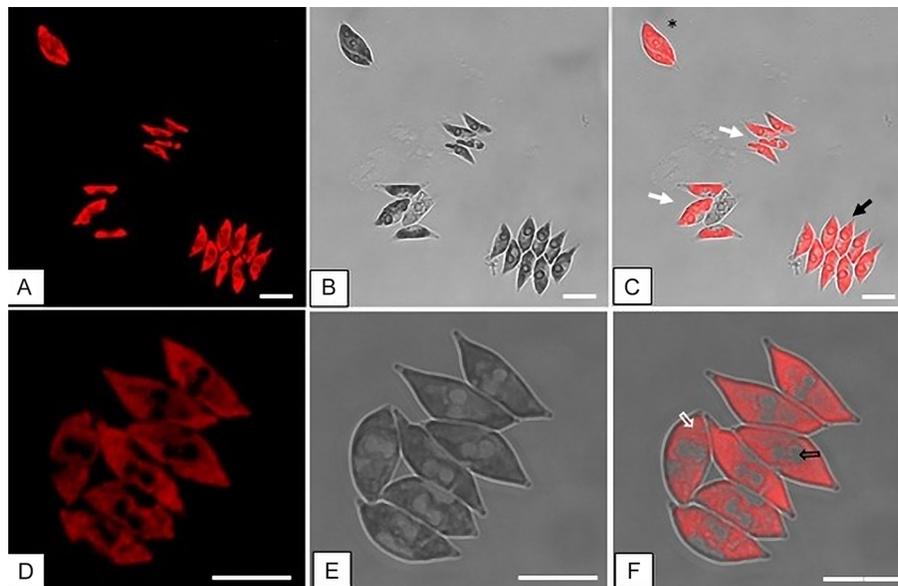

Figure 1 – Fluorescence micrograph of *Tetradesmus obliquus* showing cells in division (*), grouped into four (closed white arrow) or eight cells (closed black arrow), with autofluorescence occupying most of the cells (open white arrow) in addition to prominent spherical central pyrenoids (black open arrow). Autofluorescence (A, D), brightfield (B, E), and superimposition (C, F). Bar = 10 μm.

Discussion

Lipid extraction from microalgae is still a challenge (Amorim et al., 2020; Ebert et al., 2019; Grossmann et al., 2018; Law et al., 2018; Leal et al., 2021; Silva et al., 2021a, b; Vieira et al., 2021) since a stable emulsion is formed during lipid removal from biomass by nonpolar solvents. The emulsion stability can be due to microalgal compounds such as proteins.

Tetradesmus obliquus (formerly *Scenedesmus obliquus*) can form and stabilize emulsions, even at low protein concentrations (Lima et al., 2023; Silva et al., 2021a). Emulsions containing *T. obliquus* proteins showed greater stability in systems containing high NaCl concentrations and resistance to pH variation than emulsions with commercial whey and soy proteins (Silva et al., 2021a). This type of emulsion is desirable in the food, cosmetic and pharmaceutical industries; nevertheless, its formation is undesirable if the aim is to obtain lipid fractions without proteins and pigments for biofuel production, for example (Amorim et al., 2020; Ebert et al., 2019; Grossmann et al., 2018; Law et al., 2018; Leal et al., 2021; Silva et al., 2021a; Vieira et al., 2021).

Emulsion formation in microalgal lipid extraction can be avoided by using different solvents (ethyl acetate, hexane, petroleum ether, chloroform, methanol, ethanol) and mixtures of polar and nonpolar solvents in different proportions during lipid separation. This procedure can also promote system demulsification instead of using processes of high energy consumption such as centrifugation (Amorim et al., 2020; Ebert et al., 2019; Grossmann et al., 2018; Law et al., 2018; Leal et al., 2021; Silva et al., 2021a, b; Vieira et al., 2021). Therefore, it is necessary to elaborate strategies to reduce emulsion formation and stability to develop efficient lipid extraction processes.

In this context, the results of the present work are discussed regarding lipid extraction from the microalgal species *T. obliquus* evaluated at different solvent:biomass ratios and using two types of biomasses: protein and deproteinized biomasses.

The results of the proximate composition of the *T. obliquus* biomass for protein, ash, moisture, lipids, and carbohydrates are consistent with destructive methodologies used for their quantification.

The protein content was 40.64 ± 0.563 % before cell disruption and 40.54 ± 0.677 % after that, indicating that using a mechanical method (ball milling) to disrupt cells does not affect the protein content. These results are in the range for *T. obliquus*, as reported by Vieira et al. (2021), between 32.1 ± 0.3 % and 58.6 ± 0.5 %. The microalgal protein content varies according to the species and cultivation conditions, such as nitrogen and light availability. In connection with this, *Acutodesmus obliquus* ranges from 36 to 56 % protein content in its biomass, *Chlorella pyrenoidosa* and *Dunaliella salina* may comprise up to 57 %, and Spirulina platensis up to 63 % (Amorim et al., 2021; Renuka et al., 2017).

The ash content (21.66 ± 0.11 %) is within the range for *T. obliquus*, according to Miranda et al. (2018), reporting 18.02 %, and Vieira et al. (2021), 36.2 ± 0.4 %. The ash content also depends on the microalgae cultivation conditions because culturing with agricultural fertilizers, which lack analytical purity and contain significant insoluble compounds increases ash percentages (Vieira et al., 2021).

The high ash content of biomass can be attributed to the inorganic residues (oxides) formed after the calcination of organic matter. It can compromise the quality of the extracts obtained (e.g., pro-oxidant metals in oils) and the application of these extracts, for instance, for biodiesel production since they cause damage in engines through abrasiveness (Domingos et al., 2007). High amounts of ash in biomass reduce the calorific value of biomass since mineral materials do not participate in the thermodegradation process (Fakayode et al., 2023). A suggestion to reduce ash contents in the biomass is washing it before processing.

Lipid extraction may decrease at moisture contents higher than 64 % since water can promote the formation of a polar barrier between the solvent and the lipid, reducing mass transfer (Escorsim et al., 2019; Zorn et al., 2017). However, in our work, moisture more than 64 % facilitated lipid extraction, probably due to the better differentiation in the physical-chemical properties between the aqueous and organic phases, favoring lipid extraction with ethanol and hexane solvents.

The lipid content of the lyophilized biomass determined before cell disruption was obtained by using acid hydrolysis of dried biomass with HCl (destructive analysis technique) followed by the Folch method (methanol:chloroform ratio of 1:2). The lipid content (15.77 ± 1.09 %) determined by the Folch method was higher than that attained for ruptured and rehydrated biomass with an ethanol:hexane ratio of 1:2 (between 5.10 ± 0.37 % and 6.54 ± 0.68 %) for solid contents of 5 %, 10 %, 36 %, and 100 %, as shown in Table 1. Thus, the higher percentage of lipids obtained with a destructive cell technique was not associated with the water content of microalgal biomass (wet or freeze-dried) but with the type of solvent that enabled total cell wall destruction, such as HCl (Bermudez-Sierra, 2018; Martins et al., 2020).

The maximum percentage of total lipids in the biomass was 15.77 %. Therefore, this value was used to calculate the extracted lipid content in the assays. Consequently, the percentages of extracted lipids ranged from 32.34 to 41.98 % (Table 1), indicating an extraction capacity of up to 42 % for a solids content of 10 %. The lipid contents observed after cell disruption of biomass suspensions with 5 %, 10 %, 36 %, and 100 % solids did not vary. Such behavior can contribute to mitigating the high cost of energy consumption used to promote the drying of microalgal biomass and extract its lipids (Amorim et al., 2020).

High lipid contents extracted from (1) freeze-dried biomass of *Nannochloropsis oculata* with ethanol

were reported by Baumgardt et al. (2016) (11.33 % w w^{-1} of fatty acid methyl esters) and (2) from *Chlorella minutissima* biomass with 36 % w v^{-1} of solids were reported by Zorn et al. (2017) (20 % of lipids).

Compositions between 4.4 and 12.3 % w w^{-1} of concentrated and broken biomass to extract wet lipids efficiently were reported (Law et al., 2018). The authors found that the hexane recovery increased as the solids concentration decreased (12.3 % to 4.4 %). This promoted a decrease in the critical centrifugal force needed to coalesce the droplets from 200 g to 75 g. This behavior could be due to the lower amount of surface-active agents at lower solids concentrations, promoting the system viscosity.

The cellular walls reveal greater rigidity when dehydrated, thereby hindering solvent cell entrance and the release of the lipid-solvent complex. Since water can intensify cellular disruption and dissolve some cell polysaccharides, the solvent permeability through the wall can be increased, thereby providing efficient lipid extraction (Cohen, 1999; Zorn et al., 2017), most likely due to the dissolution of polar lipids in the aqueous phase. However, our results indicated that the lipid contents were statistically similar using freeze-dried biomass with 5 %, 10 %, and 36 % solids (w v^{-1}). Such behavioral distinctions could be imputable to different microalgal species, culture conditions, and solvents used by the authors and us. Thus, the solid quantity used to enable a high percentage of lipid extraction was selected using few amounts of biomass and low volumes of organic solvents (economic selection). Accordingly, biomasses with 5 % and 10 % solids (w v^{-1}) were used in the subsequent analyses.

The carbohydrate contents obtained using the difference between 100 % and the other compound contents (proteins, moisture, ash, and lipids) were calculated by Eq. (2) and are shown in Tables 1 and 2. The carbohydrate content includes fiber and glycogen:

% carbohydrate = 100 % – (% protein + % moisture + % ash + % lipids)   (2)

The dried biomass protein contents were approximately 40 %. After lipid extraction, the microalgal protein contents in the third residual biomass were 6.73 ± 0.45 % (for deproteinized biomass) and 9.85 ± 0.19 % (without deproteinization).

Emulsion formation did not occur during the extraction of lipids with deproteinized biomass, while emulsion formation was found in biomass without deproteinization (Table 2). Two possible reasons can explain the low quantity of proteins in the residual biomass: the dragging of apolar compounds (as apolar proteins) by apolar solvents (such as hexane) and the protein partitioning from the aqueous to the organic phase. Emulsion formation was also verified at the interface of some systems (without deproteinization) during the protein quantification experiments, indicating

the presence of proteins at the end of the lipid extraction process. Therefore, deproteinization before lipid extraction can potentially prevent emulsion formation, and thus facilitate lipid extraction.

The ability and stability of emulsions formed with *T. obliquus* proteins (Tobl-P) were studied by Silva et al. (2021a). The authors found that the proteins, even at low concentrations (0.3-0.5 %), could form more stable emulsions than those of isolated soy protein. The authors also evaluated the resistance capacity of the Tobl-P emulsions to high concentrations of NaCl (≤ 500 mM), verifying their superior performance to the salt concentration resistance than emulsions formed with whey protein isolated (WPI). Thus, after these emulsions are formed, one of the ways to break them is to use unit operations such as centrifugation to extract the oil from them, which would require a high amount of energy.

The behavior of four fractions of broken biomass of microalgae, e.g., cell debris, delipidated debris, lipids, and serum, was studied by Law et al. (2018). The authors reported that emulsions were formed after adding a nonpolar solvent (hexane or hexadecane) to each fraction. The emulsions were broken down using centrifugation to evaluate the system's stability. Four other distinct layers were formed after centrifugation: a cellular sediment at the bottom, a supernatant aqueous layer, an emulsion layer, and a hexane-lipid top layer. The behavior of the interfacial tension and rheology of the emulsions indicated that each fraction contributed to the system's stabilities in different ways. The authors found that the protein-rich serum fraction stabilizes the emulsion by forming rigid interfacial films.

A similar result was observed in our study, as shown in Table 2, with emulsion formation in the nondeproteinized systems. Thus, these results emphasize the importance of protein extraction before lipid extraction and how emulsion stability is critical to wet lipid extraction. Thus, developing strategies to attenuate emulsion stability is fundamental to defining an efficient process for wet lipid extraction, which can only be achieved by processes that require high energy consumption, such as centrifugation (Law et al., 2018).

The highest protein content in the residual biomass was 12.51 ± 0.5 %, as shown in Table 2. This result was reached when the emulsion formed during the lipid extraction was not collected along with the organic phase and for biomass without deproteinization (biomass with polar proteins). The protein content reached the lowest value (6.73 ± 0.45 %) for the residual deproteinized biomass (biomass without polar proteins) and when the emulsion was collected with the organic phase containing the extracted lipids. This behavior indicates that the emulsification presented proteins and lipids, agreeing with Tavoni et al. (2020).

The ash contents of freeze-dried biomass before and after disruption were higher (21 %) than the percentage of ash in the residual biomass after lipid extraction with ethanol:hexane solvent. The ash results

were 4.68 $\pm$ 0.50 % in the residual deproteinized biomass and 4.520 $\pm$ 0.682 % in the residual biomass without deproteinization, which exhibits statistical equality. The reduction in the ash content may have been due to the rehydration of biomass to lipid extraction. The water added to the biomass solubilized part of the ash since there are soluble and insoluble ashes in water. After lipid extraction, the residual biomass was dried, and the ashes were quantified. Thus, a large part of the water with soluble ash was removed to facilitate biomass drying in the muffle calcination. This behavior can explain the decrease in ash content after lipid extraction.

Full access to microalgal metabolites can be complex due to the metabolite location inside the cells and the composition and morphology of structurally diverse cell walls. The resistance of the cell wall to breakdown hinders the efficient extraction of intracellular metabolites and can interfere with the accuracy of compound quantification (Schüler et al., 2020). For this reason, when using some unit operations for cell wall rupture, it is necessary to allow the solvent to come into intimate contact with the microalgae target metabolites. The techniques are classified into mechanical and nonmechanical methods, such as chemical and/or enzymatic procedures.

Nonmechanical methods promote cell disruption through enzymatic agents, chemical products, or heat caused by transformations in membrane permeability and cellular appearance. The most applied mechanical methods are high-pressure homogenization, grinding, ultrasonication, and microwaves (Goh et al., 2019).

The different disruption methods exhibited both advantages and disadvantages in terms of cost, cellular compound degradation, and process efficiency. For example, the cost-benefit for cell lysis and, consequently, for biocompound separation is a parameter that needs improvement in microalgae processing.

Disruption methods in wet processing routes of microalgal biomass are already being used, such as sonicators and high-pressure homogenizers, specific enzymes for cell wall breakage, chemicals, and ionic liquids (Amorim et al., 2020; Dixon and Wilken, 2018; Lee et al., 2017; Silva et al., 2021a, b).

Regarding dry biomass routes, mechanical disruption by ball milling has been widely used (Amorim et al., 2020; Vieira et al., 2021). Grinding in a ball mill is a unitary operation considered to have high breaking efficiency, capable of processing a large volume of biomass in batches and efficiently separating the broken biomass by gravity (D'Hondt et al., 2017). Since our experiment used dry biomass, we chose mechanical disruption by ball milling because of its low cost and demonstrated efficiency in disrupting *T. obliquus* cells (Amorim et al., 2020; Vieira et al., 2021). Thus, the lyophilized biomass was disrupted using a ball mill (Retsch, Model MM 400). The freeze-dried biomass was characterized before and after cell disruption for moisture, ash, lipids, and protein.

Extraction of lipids using the organic solvents ethanol and hexane is intensified when associated with a physical method to break the microalgal cell walls before lipid extraction (Bermudez-Sierra, 2018).

Solvents solubilize target compounds if they match the compounds' polarities and weaken specific intermolecular interactions, such as hydrogen and van der Waals interactions, to permeate the cellular structure (Amaro et al., 2015; Grossmann et al., 2018). Solvents with low polarities, such as hexane, cannot fully access compounds of hydrated cells due to their low polarity (Grossman et al., 2018). Thus, these types of solvents dissolve only certain cellular compounds, such as nonpolar lipids, compared to more polar solvents that can partially solubilize other compounds, such as polar lipids, proteins, and pigments (Grossmann et al., 2018; Halim et al., 2012). If the extraction of polar lipids is the goal, extracting proteins and pigments is not desirable.

In microalgal cells, not all compounds are freely accessible, and some are bound to different structures, such as membranes and cell walls or chloroplasts; therefore, the solvent must fully permeate the cells and cell debris. Thus, it is necessary to use solvents with intermediate polarity or mixtures of solvents to allow cellular permeabilization and effective extraction of the target compounds. Within this context, ethanol (εr = 25.3) dissolves mainly polar lipids, such as membrane-associated lipids and polyunsaturated fatty acids (Amaro et al., 2015). In contrast, hexane acts more in extracting nonpolar and neutral lipids. In the present study, mixtures with different concentrations of polar:nonpolar solvents were tested to extract microalgal lipids, and the results showed a difference in the solvent proportion used (Table 3).

The results of lipid extraction under the different experimental conditions pertaining to the ethanol:hexane ratios (1:1, 1:2, 3:5 w v^{-1}), solid contents of biomass (5 % and 10 % w v^{-1}), and biomass deproteinization are shown in Table 3. The lowest percentage of lipid extraction was obtained at a 1:1 ethanol:hexane solvent ratio.

The statistical analysis showed that the lipid content was unaffected by the solids percentages (5 % and 10 % w v^{-1}) and biomass deproteinization; however, the polar:nonpolar solvent ratio significantly affected the lipid extraction.

The 1:2 and 3:5 solvent proportions showed no difference between the lipid content extracted, as seen in Table 3. Both 1:2 and 3:5 solvent ratios also exhibit higher lipid extractions than the 1:1 solvent ratio since the neutral lipids have a greater affinity for nonpolar solvents, requiring their presence in larger quantities than polar solvents.

The use of a polar solvent in microalgal lipid extraction is necessary because this solvent allows for greater permeabilization within the cell and facilitates the movement of the compounds to be extracted. Since organic solvents are expensive to recycle on a large scale, it is critical to determine the minimum volume of solvent required to provide the highest yield in lipid

extraction. Thus, our results indicate that the solvents and their proportions are crucial for lipid extraction. The solvents ethanol, acetone, ethyl lactate, and a mixture (3:2) of hexane/isopropanol were used to extract lipids from *Scenedesmus obliquus* (actually *T. obliquus*) and *Gloeothece* sp. (Amaro et al., 2015). Ethanol promoted higher extraction of polyunsaturated fatty acids (PUFAs).

In conclusion, the combined use of a polar and apolar solvent allows the separation of up to 42 % of the total lipids from the microalgal biomass. The highest lipid extraction values were observed using a solvent ratio (ethanol:hexane) of 1:2. In contrast, the use of a lower amount of the apolar solvent hexane in the solvent ratio of 3:5 hindered the intended extraction due to the formation of an emulsion between the organic and aqueous phases. This emulsion made it difficult to collect samples to obtain the intended lipid extraction. Emulsion formation was avoided by prior biomass deproteinization. The solid content (5 and 10 % w v^{-1}) did not significantly affect the amount of lipids extracted, indicating the possibility of using low-concentration biomass in the disruption stage. This condition can reduce energy consumption in the drying stage.

In this context, the results found in the present work can help to define a multistep process strategy for extracting a high quantity of lipids from the microalgae *T. obliquus*, suggesting novelties to traditional single extraction by applying (1) a combination a polar and a nonpolar solvent; (2) a reduced number of stages before cell wall breaking, since low-concentrate biomass suspensions can be used as the solid content in the evaluated range did not affect lipid extraction; (3) a reduced number of unit operations before solvent lipid extraction, since the step of emulsion extraction can be eliminated if the biomass is previously deproteinized; and (4) a reduced amount of energy due to the possibility of using low-concentrate biomass suspensions in the breaking step. In addition, our results indicated the feasibility of testing other types of polar and nonpolar solvents to extract lipids from the broken biomass of *T. obliquus*.

Accordingly, future studies using combinations of disruption and extraction techniques may be a way to optimize the extraction of lipids from microalgae at high levels. Notably, the estimated growth for food, whether of animal or plant origin, is approximately 70 % in the next 30 years (United Nations, 2019) due to the projected increase in the global population to nearly 9.7 billion by 2050. Thus, the interest in sustainable and ecological technologies to obtain human food in large quantities such as lipid support research on lipid extraction. Given this context, microalgae stand out as a food source.

Authors' Contributions

References

Afify AEMR, El Baroty GS, El Baz FK, El Baky HHA, Murad SA. 2018. *Scenedesmus obliquus*: Antioxidant and antiviral activity of proteins hydrolyzed by three enzymes. Journal of Genetic Engineering and Biotechnology 16: 399-408. https://doi.org/10.1016/j.jgeb.2018.01.002

Amaro HM, Fernandes F, Valentão P, Andrade PB, Sousa-Pinto I, Malcata FX, et al. 2015. Effect of solvent system on extractability of lipidic components of *Scenedesmus obliquus* (M2-1) and *Gloeothece* sp. on antioxidant scavenging capacity thereof. Marine Drugs 13: 6453-6471. https://doi.org/10.3390/md13106453

Amorim ML, Soares J, Vieira BB, Batista-Silva W, Martins MA. 2020. Extraction of proteins from the microalga *Scenedesmus obliquus* BR003 followed by lipid extraction of the wet deproteinized biomass using hexane and ethyl acetate. Bioresource Technology 307: 123190. https://doi.org/10.1016/j.biortech.2020.123190

Amorim ML, Soares J, Coimbra JSR, Leite MO, Albino LFT, Martins MA. 2021. Microalgae proteins: production, separation, isolation, quantification, and application in food and feed. Critical Reviews in Food Science and Nutrition 61: 1976-2002. https://doi.org/10.1080/10408398.2020.1768046

Association of Official Analytical Chemists - International [AOAC]. 2005. Official Methods of Analysis. 18 ed. AOAC, Gaithersburg, MD, USA.

Baumgardt FJL, Zandoná Filho A, Brandalize MV, Costa DC, Antoniosi Filho NR, Abreu PCOV, et al. 2016. Lipid content and fatty acid profile of *Nannochloropsis oculata* before and after extraction with conventional solvents and/or compressed fluids. The Journal of Supercritical Fluids 108: 89-95. https://doi.org/10.1016/j.supflu.2015.11.003

Bermudez-Sierra JJ. 2018. Lipid extraction from the microalgae *Scenedesmus* sp. with different mixtures of organic solvents. Biotecnología en el Sector Agropecuario y Agroindustrial 16: 88-98 (in Portuguese, with abstract in English).

Cesário CC, Soares J, Cossolin JFS, Almeida AVM, Bermudez-Sierra JJ, Leite MO, et al. 2021. Biochemical and morphological characterization of freshwater microalga *Tetradesmus obliquus* (Chlorophyta: Chlorophyceae). Protoplasma 259: 937-948. https://doi.org/10.1007/s00709-021-01712-3

Cohen Z. eds. 1999. Chemicals from Microalgae. CRC Press, London, UK. https://doi.org/10.1201/9781482295306

Covell L, Machado M, Vaz MGMV, Soares J, Batista AD, Araújo WL, et al. 2020. Alternative fertilizer-based growth media support high lipid contents without growth impairment in *Scenedesmus obliquus* BR003. Bioprocess and Biosystems Engineering 43: 1123-1131. https://doi.org/10.1007/s00449-020-02301-z

D'Hondt E, Martín-Juárez J, Bolado S, Kasperoviciene J, Koreiviene J, Sulcius S, et al. 2017. Cell disruption technologies. p. 133-154. In: Gonzalez-Fernandez C, Muñoz R. eds. Microalgae-based biofuels and bioproducts: from feedstock cultivation to end products. Woodhead, Oxford, UK. https://doi.org/10.1016/B978-0-08-101023-5.00006-6

Dixon C, Wilken L R. 2018. Green microalgae biomolecule separations and recovery. Bioresources and Bioprocessing 5: 14. https://doi.org/10.1186/s40643-018-0199-3

Domingos AK, Saad EB, Vechiatto WWD, Wilhelm HM, Ramos LP. 2007. The influence of BHA, BHT and TBHQ on the oxidation stability of soybean oil ethyl esters (biodiesel). Journal of the Brazilian Chemical Society 18: 416423. https://doi.org/10.1590/S0103-50532007000200026

Ebert S, Grossmann L, Hinrichs J, Weiss J. 2019. Emulsifying properties of water-soluble proteins extracted from the microalgae *Chlorella sorokiniana* and *Phaeodactylum tricornutum*. Food & function 10: 754-764. https://doi.org/10.1039/C8FO02197J

Escorsim AM, Hamerski F, Ramos LP, Corazza ML, Cordeiro CS. 2019. Multifunctionality of zinc carboxylate to produce acylglycerols, free fatty acids and fatty acids methyl esters. Fuel 244: 569-579. https://doi.org/10.1016/j.fuel.2019.01.178

Estevam BR, Pinto LFR, Filho RM, Fregolente LV. 2023. Effects of cultivation systems and nutrient limitation on the growth and metabolite biosynthesis of *Botryococcus terribilis*. Biomass Conversion and Biorefinery. https://doi.org/10.1007/s13399-023-03805-w

Fakayode OA, Wahia H, Zhang L, Zhou C, Ma H. 2023. State-of-the-art co-pyrolysis of lignocellulosic and macroalgae biomass feedstocks for improved bio-oil production- A review. Fuel 332: 126071. https://doi.org/10.1016/j.fuel.2022.126071

Folch J, Less M, Stanley GHS. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226: 497-509. https://doi.org/10.1016/S0021-9258(18)64849-5

Goh BHH, Ong HC, Cheah MY, Chen W, Yu KL, Mahlia TMI. 2019. Sustainability of direct biodiesel synthesis from microalgae biomass: A critical review. Renewable and Sustainable Energy Reviews 107: 59-74. https://doi.org/10.1016/j.rser.2019.02.012

Grossmann L, Ebert S, Hinrichs J, Weiss J. 2018. Effect of precipitation, lyophilization, and organic solvent extraction on preparation of protein-rich powders from the microalgae *Chlorella protothecoides*. Algal Research 29: 266-276. https://doi.org/10.1016/j.algal.2017.11.019

Gunstone FD, Harwood JL, Dijkstra AJ. 2007. The Lipid Handbook with CD-ROM. 3ed. CRC Press, Boca Raton, USA. https://doi.org/10.1201/9781420009675

Halim R, Danquah MK, Webley PA. 2012. Extraction of oil from microalgae for biodiesel production: A review. Biotechnology Advances 30: 709-732. https://doi.org/10.1016/j.biotechadv.2012.01.001

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, et al. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant Journal 54: 621-639. https://doi.org/10.1111/j.1365-313X.2008.03492.x

International Dairy Federation [IDF]. 1988. Caseins and caseinates: determination of fat content - 127A - International Dairy Federation Standard - Schmid-Bondzynski-Ratzlaff gravimetric method (Reference Method). Available at: https://www.iso.org/obp/ui/#iso:std:iso:5543:ed-2:v1:en [Accessed Jan 22, 2023]

Khoo KS, Ahmad I, Chew KW, Iwamoto K, Bhatnagar A, Show PL. 2023. Enhanced microalgal lipid production for biofuel using different strategies including genetic modification of microalgae: A review. Progress in Energy and Combustion Science 96: 101071. https://doi.org/10.1016/j.pecs.2023.101071

Law SQK, Mettu S, Ashokkumar M, Scales PJ, Martin GJO. 2018. Emulsifying properties of ruptured microalgae cells: Barriers to lipid extraction or promising biosurfactants? Colloids and Surfaces B: Biointerfaces 170: 438-446. https://doi.org/10.1016/j.colsurfb.2018.06.047

Leal MA, Monteiro S, Silva MET, Rodrigues FA, Martins MA, Sousa RCS, et al. 2021. Extraction of microalgae oil by organic solvents: experimental determination and modeling of liquid-liquid equilibria using vegetable oils mixture as a model system. Brazilian Journal of Chemical Engineering 38: 629-638. https://doi.org/10.1007/s43153-021-00118-1

Lee SY, Cho JM, Chang YK, Oh Y. 2017. Cell disruption and lipid extraction for microalgal biorefineries: A review. Bioresource Technology 244: 1317-1328. https://doi.org/10.1016/j.biortech.2017.06.038

Liang M, Wang L, Wang Q, Zhu J, Jiang J. 2018. High-value bioproducts from microalgae: Strategies and progress. Critical Reviews in Food Science and Nutrition 59: 2423-2441. https://doi.org/10.1080/10408398.2018.1455030

Lima VS, Oliveira DRB, Silva CAS, Santana RC, Soares NFF, Oliveira EB, et al. 2023. Stabilization of oil-water emulsions with protein concentrates from the microalga *Tetradesmus obliquus*. Journal of Food Science and Technology 60: 797-808. https://doi.org/10.1007/s13197-023-05666-7

Martins LB, Soares J, Silveira WB, Sousa RDCS, Martins MA. 2020. Dilute sulfuric acid hydrolysis of *Chlorella vulgaris* biomass improves the multistage liquid-liquid extraction of lipids. Biomass Conversion and Biorefinery 11: 2485-2497. https://doi.org/10.1007/s13399-020-00661-w

Miranda MT, Sepúlveda FJ, Arranz JI, Montero I, Rojas CV. 2018. Physical-energy characterization of microalgae *Scenedesmus* and experimental pellets. Fuel 226: 121-126. https://doi.org/10.1016/j.fuel.2018.03.097

Neofotis P, Huang A, Sury K, Chang W, Joseph F, Gabr A, et al. 2016. Characterization and classification of highly productive microalgae strains discovered for biofuel and bioproduct generation. Algal Research 15: 164-178. https://doi.org/10.1016/j.algal.2016.01.007

Patel S, Kannan DC. 2021. A method of wet algal lipid recovery for biofuel production. Algal Research 55: 102237. https://doi.org/10.1016/j.algal.2021.102237

Renuka N, Guldhe A, Singh P, Ansari FA, Rawat I, Bux F. 2017. Evaluating the potential of cytokinins for biomass and lipid enhancement in microalga *Acutodesmus obliquus* under nitrogen stress. Energy Conversion and Management 140: 14-23. https://doi.org/10.1016/j.enconman.2017.02.065

Rocha RP, Machado M, Vaz MGMV, Vinson CC, Leite M, Richard R, et al. 2017. Exploring the metabolic and physiological diversity of native microalgal strains (Chlorophyta) isolated from tropical freshwater reservoirs. Algal Research 28: 139-150. https://doi.org/10.1016/j.algal.2017.10.021

Rodolfi L, Zittelli GC, Bassi N, Padovani G, Biondi N, Bonini G, et al. 2008. Microalgae for oil: Strain selection induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering 102: 100-112. https://doi.org/10.1002/bit.22033

Schüler LM, Gangadhar KN, Duarte P, Placines C, Molina-Márquez AM, Léon-Bañares R, et al. 2020. Improvement of carotenoid extraction from a recently isolated, robust microalga, *Tetraselmis* sp. CTP4 (Chlorophyta). Bioprocess and Biosystems Engineering 43: 785-796. https://doi.org/10.1007/s00449-019-02273-9

Shin H, Shim S, Ryu Y, Yang J, Lim S, Lee C. 2018. Lipid extraction from *Tetraselmis* sp. microalgae for biodiesel production using hexane-based solvent mixtures. Biotechnology and Bioprocess Engineering 23: 16-22. https://doi.org/10.1007/s12257-017-0392-9

Silva MET, Leal MA, Resende MO, Martins MA, Coimbra JSR. 2021a. *Scenedesmus obliquus* protein concentrate: A sustainable alternative emulsifier for the food industry. Algal Research 59: 102468. https://doi.org/10.1016/j.algal.2021.102468

Silva MET, Martins MA, Leite MO, Milião GL, Coimbra JSR. 2021b. Microalga *Scenedesmus obliquus*: extraction of bioactive compounds and antioxidant activity. Revista Ciência Agronômica 52: e20196848.

Tavoni TM, Sprandel MCO, Laverdy OG, Strunz CMC, Ramires JAF, Kalil-Filho R, et al. 2020. Lipid transfer to HDL, CETP and HDL composition in coronary artery disease patients with or without type 2 diabetes mellitus. European Journal of Preventive Cardiology 27: 2223-2225. https://doi.org/10.1177/2047487319880364

United Nations. 2019. WPP: world population prospects; the 2019 revision. United Nations Population Division, New York, NY, USA. (Technical Report). Available at: https://population.un.org/wpp/ [Accessed Mar 29, 2022]

Vasistha S, Khanra A, Clifford M, Rai MP. 2021. Current advances in microalgae harvesting and lipid extraction processes for improved biodiesel production: A review. Renewable and Sustainable Energy Reviews 137: 110498. https://doi.org/10.1016/j.rser.2020.110498

Viegas CV, Díaz GC, Cruz YR, Mendes LBB. 2020. Characterization of the lipid components in *Desmodesmus* and *Scenedesmus* strains: lipid content, lipid classes and fatty acid profile. American Journal of Plant Sciences 11: 2103-2121. https://doi.org/10.4236/ajps.2020.1112148

Vieira BB, Soares J, Amorim ML, Bittencourt PVQ, Superbi RC, Oliveira EB, et al. 2021. Optimized extraction of neutral carbohydrates, crude lipids and photosynthetic pigments from the wet biomass of the microalga *Scenedesmus obliquus* BR003. Separation and Purification Technology 269: 118711. https://doi.org/10.1016/j.seppur.2021.118711

Wynne MJ, Hallan JK. 2016. Reinstatement of *Tetradesmus* G. M. Smith (Sphaeropleales, Chlorophyta). Feddes Repertorium 126:83-86. https://doi.org/10.1002/fedr.201500021

Wong JF, Hong HJ, Foo SC, Yap MKK, Tan JW. 2022. A review on current and future advancements for commercialized microalgae species. Food Science and Human Wellness 11: 1156-1170. https://doi.org/10.1016/j.fshw.2022.04.007

Zorn SMFE, Pedro GA, Amaral MS, Loures CCA, Silva MB. 2017. Evaluation of the factors involved in the extraction of lipids from the biomass of the microalgae *Chlorella minutissima* via solvents. Holos 2: 66-78 (in Portuguese, with abstract in English). https://doi.org/10.15628/holos.2017.5655