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Effect of different cell disruption methods on lipid yield of *Schizochytrium* sp.

Mustafa Hac İsa (ORCID) | **Cansu Metin** (ORCID) | **Ertan Ercan** | **Yunus Alparslan**

Faculty of Fisheries, Muğla Sıtkı Koçman University, Kötekli, Muğla, Turkey

Correspondence
Mustafa Hac İsa, Faculty of Fisheries, Muğla Sıtkı Koçman University, 48000 Kötekli, Muğla, Turkey.
Email: mustafahacisa@mu.edu.tr

Abstract

In this study, it was investigated to increase the lipid yield of the microalgae *Schizochytrium* sp., by applying different cell disruption methods; acid treatment with HCl, osmotic shock, enzyme applications and ultrasonic homogenizer were combined with the Bligh and Dyer or Soxhlet methods. In the Soxhlet method, the lipid and fatty acid yields decreased due to the inability of the method to break down the lipid cells sufficiently and the high temperature application. Enzyme application (hemicellulase treatment at 55°C for 2 days) prior to Bligh and Dyer method (BDE) was found more efficient in terms of lipid and DHA yield compared to other methods. BDE process increased the lipid yield to $21.72 \pm 0.74\%$ and DHA content to $19.25 \pm 0.09\%$ from lipid yield of $18.87 \pm 0.4\%$ and DHA content of $18.41 \pm 0.20\%$ by the Bligh and Dyer control (BDC). Major saturated fatty acids were 14:0, 16:0, 18:0, 24:0 and the highest saturated fatty acid was 16:0 (palmitic acid). Lipid health indices such as n-6/n-3, PUFA/SFA, atherogenicity index, thrombogenicity index and hypocholesterolemic/hypercholesterolemic ratios were almost favorable. With this study, appropriate lipid extraction methods were studied to provide an economical and environmental friendly suggestion for future studies to be used in areas such as food, feed and cosmetics grade. It was concluded that the most convenient method among the cell disruption methods was BDE owing to lipid and fatty acid yield.

KEYWORDS
chromatography, fats and oils, lipid chemistry/lipid analysis

INTRODUCTION

Microalgae are photosynthetic microorganisms capable of converting carbon dioxide and water into organic macromolecules such as lipids, polysaccharides and proteins under light conditions (Cho et al., 2013). They can be produced year-round, depending on the climate and solar radiation, grow at very high rates, and in a wide variety of water sources (fresh, brackish water, seawater and wastewater) (Souza Silva et al., 2014). They can also be easily harvested and can be cultivated under various conditions (Ju et al., 2020). Therefore, the microalgae have an extraordinary biotechnological potential as an alternative to non-renewable resources due to production of natural substances and biomaterials that can be used in various industrial applications (Neto et al., 2013). The lipid content of microalgal cells range from 2% to 77% depending on the species' environmental and growth conditions (Souza Silva et al., 2014).

Microalgae have high and good quality lipids, especially long-chain unsaturated fatty acids such as linolenic acid, arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic (DHA), has been a remarkable issue for many years. Adequate DHA intake protects against many noncommunicable diseases such as diabetes, cardiovascular and neurodegenerative diseases, cancer and schizophrenia (Wang et al., 2020). Microalgal lipids can be produced safely and stably using pure cultures, and the obtained products have potential to use in high-value-added industries, such as medicine and cosmetics (Ju et al., 2020). *Schizochytrium* sp. was also accepted as GRAS (generally recognized as safe) by Food and Drug Administration (FDA) (Anonymous, 2020).

Heterotrophic *Crypthecodinium, Schizochytrium* and *Ulkenia* species are considered as the first commercialized species that has been used for food, feed and biodiesel production, due to their high fat content. *Schizochytrium* sp. is a marine algae, which is important in terms of DHA, closely related to diatoms, single-celled, thraustochytrid and commonly found in sea waters, estuaries and sediments (Borowitzka, 2013). *Schizochytrium* sp. is a globose and pale-yellow marine microalga that possesses a thallus thin wall. This is of great industrial interest because of producing metabolites (Ortega-Berlanga et al., 2018). This marine organism has a relatively high fat content. *Schizochytrium* oil that is rich in DHA and EPA, also took its place in the market (Borowitzka, 2013). *Schizochytrium* is considered to be a promising alternative and excellent DHA source (Wang et al., 2020) and it is accumulated intracellular DHA accounts for 30% to 40% of its total lipid content (Chang et al., 2020). There are no reports of this organism producing toxic chemicals or being pathogenic (Fedorova-Dahms et al., 2011).

Microalgal cell wall is predominantly composed of cellulose, hemicellulose, and saccharides which hinder the release of intracellular lipids (Liang et al., 2012). Due to their complex structure, the tensile strength of microalgal cell wall is estimated to be higher than plant's cell wall (Nagappan et al., 2019). Since the thick cell wall of microalgae, which prevents to obtain lipids, it is necessary to use different methods other than the traditional mechanical press to obtain high efficiency in lipid extraction. An ideal solvent plays an important role in lipid extraction (Neto et al., 2013). Solvents including hexane, chloroform, butanol, ethanol, methanol and diethyl ether have been widely reported for lipid extraction from microalgae (Nagappan et al., 2019). Fragmentation methods aim to increase the efficiency of obtaining lipids from microalgae by using mechanical and non-mechanical techniques. Mechanical techniques include compression, high-pressure homogenization, ultrasonic bath, autoclave, bead mill, microwave and magnetic stirring, while non-mechanical techniques include chemical fragmentation and osmotic shock and enzymatic hydrolysis (Neto et al., 2013; Y. Zhang et al., 2018). To get higher lipid yield and quality with lower production costs from microbial cells, a suitable cell disruption method is required.

It is reported that the lipid yield of *Schizochytrium* sp. varies between 50% and 77% in dry weight (Chisti, 2007; Kumar & Sharma, 2014). However, as seen in the literature, it is difficult to reach this rate with conventional lipid extraction methods and additional procedures are required. For example, Sarker et al. (2016) found the lipid value of this algae as 11.9% without applying any disruption process. Also, according to the culture conditions, the lipid content of algae may change periodically. Studies on increasing

lipid yield by cell disruption of this species are very limited. Liang et al. (2012) applied enzyme (cellulase, neutral protease and alkaline protease) and sonication treatment to increase lipid yield of *Chlorella vulgaris*, *Scenedesmus dimorphus* and *Nannochloropsis* sp. Taher et al. (2014) studied enzyme (lysozyme and cellulase) application and Soxhlet method to *Scenedesmus* sp. Yu et al. (2015) investigated the effect of autoclaving, bead-beating, microwaving, sonication, acid (HCl) digestion, and Soxhlet extraction to lipid extraction from *Chlorella sorokiniana*. However, in the literature, the disruption methods that used in our study were not tried together and compared for *Schizochytrium* sp. This species is promising in terms of food, feed and cosmetics grade. Therefore, it was aimed to obtain maximum quality and maximum yield of lipid from *Schizochytrium* sp. by trying classical Bligh and Dyer and Soxhlet methods; with HCl treatment as a solvent, osmotic shock treatment as a non-mechanical process, ultrasonic homogenizer treatment as a mechanical process and enzyme treatment as a biological process and it was supported by lipids nutritional quality indices (LNQI).

MATERIAL AND METHODS

Schizochytrium sp. micro algae species was used as the study material. Spray-dried samples were obtained from Marin Biotechnology Products and Food Industry (Aydın/Turkey).

In order to increase the lipid yield of this algae, four different cell disruption methods were tried; treatment with HCl in the reflux, osmotic shock, enzyme and ultrasonic homogenizer. After each cell disruption method, classical method of Bligh and Dyer (1959) and Soxhlet extraction methods of AOAC (2006) were applied to recover total crude lipid. Bligh and Dyer (1959) or AOAC (2006) Soxhlet methods were applied without cell disruption in control groups. The groups were coded as follows; Bligh and Dyer and Soxhlet groups; control (BDC and SC), osmotic shock (BDOS and SOS), enzyme (BDE and SE), HCl (BDHCl and SHCl), ultrasonic homogenizer (BDUH and SUH); respectively. In each method, 2 g of dry algae samples were used. Each analysis was carried out three times in parallel.

Total (crude) lipid

Bligh and Dyer method

A mixture of methanol and chloroform (1:2, v/v, 40 ml) mixture was added to 2 g of the algae sample (algae/solvent mixture, 1:20, w/v) and homogenized. Then, 20 ml of 0.4% $CaCl_2$ solution was added onto these

samples. The samples were filtered on a filter paper into the tared balloons kept in the oven at 105°C for 2 h. These balloons were kept in a dark environment overnight and the upper layer consisting of methanol water was separated with the help of a separation funnel. Chloroform from the chloroform lipid part in the solution remaining in the flask was evaporated using a rotary evaporator (Heidolph) with the help of a water bath set at 60°C. The resulted lipids were coded as BDC (Bligh and Dyer Control).

Soxhlet method

Samples were placed in cartridges and put into the VELP SCI SER 148-Italy brand automatic soxhlet mechanism and hexane (60 ml) was used for lipid separation. Process of immersion for 90 min at 180°C, washing for 120 min at 80°C, and finally recovery for 8 min was carried out in the device (AOAC, 2006). The resulted lipids were coded as SC (Soxhlet Control).

Cell disruption methods

HCl extraction

The dry algae: HCl (3 mol/L) (1:40, w/v) was boiled at reflux for 1 h (acid hydrolysis step). The sample was passed through filter paper and washed with 500 ml of water and filtered. The filter paper was dried for 1 h in an oven at 103°C (AOAC, 2005). The dried samples were brought to Bligh and Dyer or Soxhlet methods. These groups were coded as BDHCl (Bligh and Dyer HCl) and SHCl (Soxhlet HCl).

Osmotic shock extraction

The dry algae sample was kept in a 10% saline solution for 2 days at a ratio of algae: salt water (1:10, w/v) (Prabakaran & Ravindran, 2011), followed by the lipid extraction by Bligh and Dyer or Soxhlet methods. The resulted lipids were coded as BDOS (Bligh and Dyer Osmotic shock) and SOS (Soxhlet Osmotic shock).

Enzyme extraction

Ten percent hemicellulase enzyme (powder, activity given by the manufacturer, 1338–1636 u/g/Tito/Turkey) was dissolved in distilled water and was applied to the dry algae sample with the ratio of 1:10 w/v (algae: enzyme solution), then kept in the oven at 55°C for 2 days by stirring with magnetic agitation (500 rpm) (Liang et al., 2012; Verdasco-Martín et al., 2020). Afterwards, the lipid extraction was conducted by Bligh and Dyer or Soxhlet methods. These groups were coded as BDE (Bligh and Dyer Enzyme) and SE (Soxhlet Enzyme).

Ultrasonic homogenizer extraction

Solvent (chloroform: methanol, 2:1) was added to the dry algae sample (algae/solvent, 1:20, w/v) and then homogenized in an ultrasonic homogenizer (Bandelin UW, 3200/20 kHz) for 20 min (Byreddy et al., 2015). The lipid extraction was continued with Bligh and Dyer and Soxhlet methods. These groups were coded as BDUH (Bligh and Dyer Ultrasonic homogenizer) and SUH (Soxhlet Ultrasonic homogenizer).

At the end of each disruption method, the beakers in which lipid accumulates, were kept in the oven at 60°C in order to remove the remaining solvent, then they were kept in a desiccator for 30 min and their final weighings were taken after cooling.

$$\text{Yield of crude lipid} = (\text{final weight} - \text{initial weight})/$$
$$\text{sample weight} \times 100.$$

Fatty acids methyl esters (FAME) analysis

The methyl esters of lipid from the samples were prepared by trans methylation according to the method described by Ichihara et al. (1996) with a minor modification. Briefly, 25 mg of extracted oil was dissolved in 2 ml isooctane, followed by adding 4 ml of 2 M KOH in methanol. Then, the tube was vortexed for 2 min at room temperature. After centrifugation at 4000 rpm for 10 min, the isooctane layer was taken for gas chromatography analysis.

The fatty acid methyl esters were analyzed using gas chromatograph of Agilent Technologies model 7820 equipped with a flame ionization detector (FID) and fitted with a HP-88 capillary column (60 m × 0.25 mm × 0.25 μm thickness). Helium was used as the carrier gas at a constant pressure of 16 psi. Injection port was maintained at 220°C, and the sample was injected in split mode with a split ratio of 50:1. Detector temperature was 280°C. Column temperature was started at 175°C, and then programmed at 3°C/min to 220°C, ramped at 1°C/min to 220°C, and held for 10 min. The total running time was 26 min. Helium was used as the makeup gas at a constant flow of 40 ml/min, and hydrogen and dry air were used as detector gases. Identification of fatty acids was carried out by comparing sample FAME peak relative retention times with those obtained for Supelco standards (Supelco 37 Compounds FAME mix 10 mg/ml in $CH_2 Cl_2$-47885U, Supelco 1819-1 Ampule FAME mix C4-C24). Results of each fatty acids were expressed as FID response area

relative percentages of the total fatty acids determined (ISO, 1990).

Lipids nutritional quality indices

The data from fatty acids composition analysis were used to determine the nutritional quality of the lipid fraction by means of three indices using the following calculations (Prato et al., 2019):

Atherogenicity index

$$AI = (12:0 + 4 \times 14:0 + 16:0)/\Sigma MUFA + \Sigma PUFA.$$

Thrombogenicity index

$$TI = (14:0 + 16:0 + 18:0)/[(0.5 \times \Sigma MUFA + 0.5 \\ \times \Sigma n6PUFA + 3 \times \Sigma n3PUFA \\ + (n3/n6)].$$

Fatty acids hypocholesterolemic/hypercholesterolemic ratios

$$HH = (18:1cis9 + 18:2n6 + 20:4n6 + 18:3n3 \\ + 20:5n3 + 22:5n3 + 22:6n3)/(14:0 + 16:0).$$

Statistical analysis

All experiments were carried out in triplicate and the results were reported as the mean and standard deviation of these measurements. Statistics on a completely randomized design were performed with the analysis of variance (ANOVA) procedure in SPSS (Version 21, SPSS Inc., Chicago, IL, USA) software. Tukey's multiple range test ($p < 0.05$) was used to detect differences among mean values of all test intervals.

RESULTS AND DISCUSSION

Crude lipid

The lipid yield of the treatment with HCl in the reflux, osmotic shock, enzyme and ultrasonic homogenizer cell disruption methods combined with Bligh and Dyer and Soxhlet extraction methods are shown in Figure 1. Bligh and Dyer or Soxhlet lipid extraction, without any disruption processes were conducted as control. Lipid yields of the Bligh Dyer control (BDC) and Soxhlet control (SC) groups were $18.87 \pm 0.4\%$ and $5.54 \pm 0.08\%$, respectively. The lipid yield of Bligh and Dyer applied
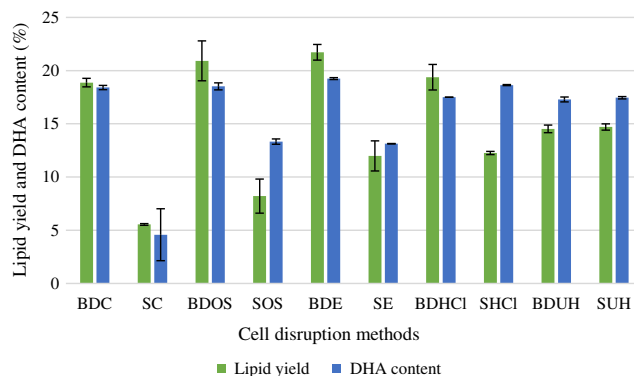
FIGURE 1 Lipid yield and DHA content of different cell disruption methods (%)

groups were $20.92 \pm 1.87\%$ for BDOS, $21.72 \pm 0.74\%$ for BDE, $19.38 \pm 1.20\%$ for BDHCl and $14.52 \pm 0.36\%$ for BDUH, respectively. The lipid yield of Soxhlet method applied groups were $8.21 \pm 1.60\%$ for SOS, $11.98 \pm 1.41\%$ for SE, $12.26 \pm 0.15\%$ for SHCl and $14.70 \pm 0.3\%$ for SUH, respectively. The highest lipid yield was detected in the BDE group ($p < 0.05$) and followed by BDOS, the difference between these groups was found to be statistically insignificant ($p > 0.05$). The lowest value was found in the SC group ($p < 0.05$). The highest lipid yield in the Soxhlet applied groups was found in the SUH group with $14.70 \pm 0.30\%$. It was found statistically significant from other Soxhlet applied groups ($p < 0.05$). Comparing the BDC and SC control groups showed all cell disruption methods were able to disrupt algal cells, although the lipid although lipid yield showed differences ($p < 0.05$).

Difficulties arise in obtaining lipid due to the cell walls of microalgae. For this reason; the HCl treatment, osmotic shock, enzyme and ultrasonic homogenizer cell disruption methods were applied to *Schizochytrium* sp. to increase the lipid yield. It was observed that all of the cell disruption methods increased the lipid yield compared to the control groups. The highest lipid yield was detected in the BDE group ($21.72 \pm 0.74\%$). Bligh and Dyer crude lipid method was better than Soxhlet. Lipid yield of BDOS ($20.92 \pm 1.87\%$) group also followed the BDE group lipid ratio and there was no statistically significant difference ($p > 0.05$). In the Soxhlet groups, the highest lipid was found in SUH group ($14.70 \pm 0.3\%$), followed by the SHCl ($12.26 \pm 0.15\%$). The difference between these two groups was statistically significant ($p < 0.05$). Overall, the lipid yield was lower in the Soxhlet applied groups, and the laboratory scale applicability of the Bligh and Dyer method was more appropriate. In other studies in the literature, different applications such as autoclave, bead-beating, microwave, ultrasonication and osmotic shock were applied to increase the lipid amount of microalgae. Positive results were obtained with different mechanical

applications using different solvents, however environmentally friendly applications such as use of enzymes is promising. Byreddy et al. (2015) studied cell disruption methods to increase the efficiency of lipid extraction of *Schizochytrium* sp. using different solvents. The maximum yield was obtained from the chloroform: methanol (2:1) solvent. The same solvent was therefore used in our study. In the osmotic shock application, the highest efficiency of lipid was obtained for *Schizochytrium* sp. as 48.7%, while lipid yield of the control group was below 10%, in their study. In our study, there was an increase in lipid yield in cell disruption methods compared to the control group.

In our study, the lipid yield of HCl disruption combined with Soxhlet and Bligh and Dyer methods, was obtained respectively as; $12.26 \pm 0.15\%$ and $19.9 \pm 1.20\%$ and an increase occurred compared to the control group. Although its high efficacy for disrupting microalgal cells, sulfuric acid treatment is not appropriate and not recommended for use in a microalgal biorefinery because of destroying the activity of valuable cellular components (such as protein and pigment) (Halim et al., 2012) and being toxic and corrosive (Taher et al., 2014). Liang et al. (2012) studied to increase lipid yield of *Chlorella vulgaris*, *Scenedesmus dimorphus* and *Nannochloropsis* sp. by enzyme (cellulase, neutral protease and alkaline protease) and sonication treatment. The highest lipid yield was obtained as 49.82% in the combined sonication-enzyme treatment. Lipid yield of control group increased from 15.11%. Studies have also demonstrated that lipid extraction efficiency of enzymatic treatment is higher than the mechanical methods including microwave and ultrasonication. The yield of lipid achieved through enzymatic hydrolysis depends on the type of enzyme used, pH, temperature and microalgal type (Nagappan et al., 2019). In our study, the highest amount was observed in enzyme application and in order to get better results, the enzyme was treated at $55°C$, which is the optimum temperature for the cellulase enzyme. Taher et al. (2014) in their study on extracting lipid with enzyme (lysozyme and cellulase) from *Scenedesmus* sp., declared Soxhlet method, the lipid value was 4% in the control group without any application. The highest value was obtained with lysozyme (16.6%) followed by the cellulase enzyme (15.4%). As a result in our study, the best lipid results were obtained in the enzyme applied groups, but also, osmotic shock, ultrasonic homogenizer increased lipid yield compared to the control group, and these methods can be evaluated positively on an industrial scale.

Fatty acid compositions

The fatty acid compositions of the sample groups treated with different lipid extraction methods from *Schizochytrium* sp. are shown in Tables 1 and 2. Palmitic acid (16:0) was the highest fatty acid in the saturated fatty acid group. Palmitic acid was detected in the range of $54.90 \pm 0.17\%$ to $74.86 \pm 2.88\%$ in the sample groups and the highest value was in the SC group ($p < 0.05$). The highest saturated fatty acid after palmitic acid was miristic acid (14:0) in the range of $4.16 \pm 0.01\%$ to $5.55 \pm 0.15\%$. Among the monounsaturated fatty acids, miristoleic acid (14:1) was found in the highest amount ($4.29 \pm 0.95\%$ to $5.86 \pm 0.16\%$). The highest polyunsaturated fatty acid was determined as DHA (22:6) with the range of $4.58 \pm 2.44\%$ to $19.25 \pm 0.09\%$. The highest and lowest values were observed in the BDE and SC groups, respectively ($p < 0.05$). n-6/n-3 was in the range of 0.06 ± 0.00 to 0.09 ± 0.00 and the highest ΣPUFA and Σn-3 was in the BDE group with $20.87 \pm 0.04\%$ and $19.53 \pm 0.09\%$, respectively. The lowest ΣPUFA and Σn-3 was also in the SC group with $4.91 \pm 0.23\%$ and $4.58 \pm 2.44\%$, respectively. ΣSFA and ΣMUFA were in the range of $67.08 \pm 0.16\%$ to $85.82 \pm 3.90\%$ and $7.38 \pm 0.01\%$ to $10.53 \pm 0.02\%$, respectively. The highest ΣPUFAs were obtained in the BD groups, and the highest ΣPUFA in the BDE group was statistically different from the other groups. PUFA/SFA was detected in the range of 0.06 ± 0.03 to 0.31 ± 0.00 in the lipid extraction groups.

According to the results of fatty acids, there were not significant differences in the cell disruption groups compared to the control groups. Among the total crude lipid methods, Bligh and Dyer showed better results in terms of fatty acids. It was thought that the temperature applied in Soxhlet damaged the lipid material. Lower fatty acid amounts were obtained in all Soxhlet applied groups. It also appears that Soxhlet method damaged to DHA. Schmid et al. (2016) investigated effects of food grade solvents (chloroform/methanol, ethanol, hexane, ethanol/hexane) and storage temperature to fatty acids of *Laminaria digitata* and *Palmaria palmata*, in terms of yields of total fatty acids, was in the order: chloroform/methanol > ethanol > hexane > ethanol/ hexane for both species. Also Figueiredo et al. (2019) studied the effects of different extraction methods of lipids from *Nannochloropsis oceanica* on the contents of omega-3 fatty acids. These methods demand the use of safer and food grade solvents. Different solvent systems, including chloroform/methanol (CM), dichloromethane/methanol (DM), dichloromethane/ethanol (DE) and ethanol, were investigated as means of producing EPA-enriched lipid extracts from *Nannochloropsis oceanica* spray-dried biomass. Among the solvent systems the conventional CM and DM gave the highest yields with similar extraction efficiency followed by DE. In the study by Guckert et al. (1988) on lipid solvent system for the analysis of lipid classes in *Chlorella*, Bligh and Dyer provided

TABLE 1 Fatty acid results of Bligh and Dyer groups

	BDC	BDOS	BDE	BDHCI	BDUH
6:0	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	[a]
8:0	0.06 ± 0.00	0.06 ± 0.00	0.08 ± 0.01	0.04 ± 0.00	0.05 ± 0.00
10:0	0.05 ± 0.04	0.08 ± 0.00	0.08 ± 0.00	0.02 ± 0.01	0.05 ± 0.01
11:0	0.02 ± 0.00	[a]	0.02 ± 0.00	0.02 ± 0.00	[a]
12:0	0.20 ± 0.00	0.18 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.18 ± 0.00
13:0	0.17 ± 0.00	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.00	0.16 ± 0.01
14:0	4.37 ± 0.03	4.16 ± 0.01	4.44 ± 0.04	4.37 ± 0.01	4.31 ± 0.01
15:0	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	[a]
16:0	57.10 ± 0.58b	55.78 ± 0.2d	57.68 ± 0.0a	56.31 ± 0.0c	57.43 ± 0.21b
17:0	1.40 ± 0.01a	1.35 ± 0.01a	1.38 ± 0.01a	1.37 ± 0.01a	0.78 ± 0.83b
18:0	1.99 ± 0.01b	2.25 ± 0.04a	1.95 ± 0.11b	2.15 ± 0.03ab	2.26 ± 0.01a
20:0	0.32 ± 0.00	0.33 ± 0.00	0.32 ± 0.00	0.33 ± 0.00	0.32 ± 0.00
22:0	0.10 ± 0.00	0.11 ± 0.02	0.11 ± 0.00	0.11 ± 0.00	[a]
24:0	3.44 ± 0.06	3.52 ± 0.06	3.61 ± 0.02	3.24 ± 0.00	3.25 ± 0.04
ΣSFA	69.27 ± 0.56b	68.01 ± 0.25c	70.10 ± 0.1a	68.37 ± 0.11c	68.77 ± 0.62c
14:1	5.00 ± 0.00	4.70 ± 0.08	5.06 ± 0.02	4.73 ± 0.01	4.78 ± 0.01
15:1	[a]	[a]	[a]	0.02 ± 0.00a	[a]
16:1	0.52 ± 0.00	0.61 ± 0.01	0.40 ± 0.01	0.05 ± 0.01	0.62 ± 0.01
18:1n-9t	0.11 ± 0.00	0.12 ± 0.01	0.11 ± 0.00	0.12 ± 0.00	0.10 ± 0.00
18:1n-9c	2.81 ± 0.01c	3.95 ± 0.08b	1.66 ± 0.03d	4.30 ± 0.01a	4.00 ± 0.01b
20:1n-9	0.19 ± 0.00a	0.25 ± 0.00a	0.10 ± 0.00b	0.26 ± 0.00a	0.27 ± 0.01a
22:1n-9	0.05 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.10 ± 0.02
ΣMUFA	8.68 ± 0.01b	9.68 ± 0.18a	7.38 ± 0.01c	9.52 ± 0.01a	9.85 ± 0.03a
18:2n-6t	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.09 ± 0.01	0.08 ± 0.01
18:2n-6c	0.83 ± 0.01b	1.05 ± 0.03a	0.65 ± 0.06b	1.05 ± 0.00a	1.09 ± 0.02a
20:3n6	0.30 ± 0.00	0.31 ± 0.01	0.31 ± 0.00	0.30 ± 0.00	0.31 ± 0.01
20:4n-6	0.19 ± 0.01	[a]	0.20 ± 0.00	0.03 ± 0.00	0.16 ± 0.04
20:5n-3	0.28 ± 0.01	0.28 ± 0.00	0.28 ± 0.00	0.25 ± 0.00	0.26 ± 0.00
22:2	0.13 ± 0.01	0.14 ± 0.01	0.11 ± 0.00	0.14 ± 0.01	0.12 ± 0.01
22:6n-3	18.41 ± 0.20b	18.52 ± 0.03b	19.25 ± 0.09a	17.50 ± 0.00c	17.29 ± 0.23c
ΣPUFA	20.19 ± 0.23b	20.37 ± 0.03b	20.87 ± 0.00a	19.35 ± 0.00c	19.29 ± 0.30c
Σn-3	18.69 ± 0.21b	18.80 ± 0.03b	19.53 ± 0.00a	17.75 ± 0.00c	17.55 ± 0.23c
Σn-6	1.38 ± 0.01b	1.43 ± 0.01b	1.23 ± 0.06c	1.47 ± 0.01b	1.63 ± 0.06a
Σn-9	3.16 ± 0.01b	4.37 ± 0.08a	1.93 ± 0.02c	4.72 ± 0.01a	4.46 ± 0.01a
n-6/n-3	0.07 ± 0.00c	0.08 ± 0.00b	0.06 ± 0.00d	0.08 ± 0.00b	0.09 ± 0.00a
PUFA/SFA	0.29 ± 0.01ab	0.30 ± 0.01a	0.30 ± 0.01a	0.28 ± 0.00b	0.28 ± 0.01b

Note: Data are expressed as the mean ± SD. The lower case letters show the statistical difference between cell disruption methods ($p < 0.05$).

Abbreviations: BDC, bligh and dyer control; BDE, bligh and dyer enzyme; BDHCI, bligh and dyer HCl; BDOS, bligh and dyer osmotic shock; BDUH, bligh and dyer ultrasonic homogenizer; MUFA, monounsaturated fatty acids; n, Omega; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

[a]Non-detected.

the most quantitative and reproducible recovery of all *Chlorella* lipid classes, also, degradation of the polyunsaturated fatty acids was observed during the Soxhlet procedure, similar with our study. Tang et al. (2011) in their study on supercritical CO_2 and Soxhlet extraction of lipids and enrichment of DHA from oil-rich microalgae *Schizochytrium limacinum*, DHA content was obtained in these groups as 27.5% and 15.4%, respectively. It was concluded supercritical CO_2 extraction exhibits many advantages over the Soxhlet extraction for the DHA enrichment and purity. In our study, cell disruption methods, particularly osmotic shock, enzyme and HCl resulted in high yields of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids in the extracted oil. The highest DHA

T A B L E 2 Fatty acid results of Soxhlet groups

	SC	SOS	SE	SHCl	SUH
10:0	a	a	a	0.03 ± 0.01a	a
11:0	a	a	a	0.03 ± 0.01a	a
12:0	0.23 ± 0.01b	0.70 ± 0.46a	a	0.20 ± 0.00b	0.23 ± 0.01b
13:0	0.23 ± 0.09b	0.56 ± 0.25a	a	0.16 ± 0.00b	0.18 ± 0.01b
14:0	5.55 ± 0.15a	4.29 ± 0.75c	4.91 ± 0.19b	4.33 ± 0.05c	4.60 ± 0.04bc
15:0	a	a	a	0.03 ± 0.00a	a
16:0	74.86 ± 2.88a	60.62 ± 2.16c	62.42 ± 1.29b	54.90 ± 0.17d	60.24 ± 0.23c
17:0	0.99 ± 1.07d	1.23 ± 0.33c	1.40 ± 0.12a	1.34 ± 0.01bc	1.40 ± 0.00a
18:0	2.68 ± 0.05b	2.52 ± 0.58b	3.28 ± 0.39a	2.19 ± 0.03c	2.07 ± 0.05c
20:0	0.41 ± 0.08a	a	a	0.32 ± 0.03a	0.31 ± 0.01a
22:0	a	a	a	0.06 ± 0.03a	a
24:0	0.90 ± 0.42c	2.60 ± 0.08b	3.16 ± 0.79a	3.51 ± 0.02a	3.33 ± 0.01a
ΣSFA	85.82 ± 3.90a	72.51 ± 4.44d	77.56 ± 2.01b	67.08 ± 0.16d	72.34 ± 0.19c
14:1	5.86 ± 0.16a	4.29 ± 0.95d	5.11 ± 0.19b	4.73 ± 0.01c	5.12 ± 0.06b
15:1	a	a	a	0.03 ± 0.01a	a
16:1	0.37 ± 0.02b	0.69 ± 0.34a	a	0.63 ± 0.00a	0.40 ± 0.01b
18:1n-9t	a	a	a	0.12 ± 0.00a	0.11 ± 0.01a
18:1n-9c	1.75 ± 0.11e	3.13 ± 0.69b	2.63 ± 0.74c	4.68 ± 0.01a	2.23 ± 0.14d
20:1n-9	a	a	a	0.28 ± 0.00a	0.14 ± 0.01b
22:1n-9	a	a	a	0.06 ± 0.00a	a
ΣMUFA	7.97 ± 0.03b	8.10 ± 1.98b	7.74 ± 0.55b	10.53 ± 0.02a	7.99 ± 0.08b
18:2n-6t	a	a	a	0.08 ± 0.01b	0.32 ± 0.00a
18:2n-6c	0.33 ± 0.07d	0.98 ± 0.52b	a	1.21 ± 0.01a	0.60 ± 0.02c
20:3n-6	a	a	a	0.31 ± 0.00a	0.32 ± 0.00a
20:4n-6	a	a	a	0.20 ± 0.00a	a
20:5n-3	a	a	a	0.28 ± 0.00a	0.26 ± 0.01a
22:2	a	a	a	0.13 ± 0.00a	a
22:6n-3	4.58 ± 2.44d	13.33 ± 0.25c	13.13 ± 0.02c	18.64 ± 0.05a	17.45 ± 0.11b
ΣPUFA	4.91 ± 0.23e	14.30 ± 0.76c	13.13 ± 0.02d	20.84 ± 0.05a	18.94 ± 0.13b
Σn-3	4.58 ± 2.44d	13.33 ± 0.25c	13.13 ± 0.02c	18.92 ± 0.05a	17.70 ± 0.11b
Σn-6	0.33 ± 0.07d	0.98 ± 0.52c	a	1.79 ± 0.00a	1.24 ± 0.02b
Σn-9	1.75 ± 0.11d	3.13 ± 0.69b	2.63 ± 0.74c	5.14 ± 0.01a	2.48 ± 0.13c
n-6/n-3	0.08 ± 0.03b	0.07 ± 0.04b	a	0.09 ± 0.00a	0.07 ± 0.00b
PUFA/SFA	0.06 ± 0.03d	0.20 ± 0.00c	0.19 ± 0.00c	0.31 ± 0.00a	0.26 ± 0.00a

Note: Data are expressed as the mean ± SD. The lower-case letters show the statistical difference between cell disruption methods ($p < 0.05$).

Abbreviations: MUFA, monounsaturated fatty acids; n, Omega; PUFA, polyunsaturated fatty acids; SC, Soxhlet Control; SE, Soxhlet Enzyme; SFA, Saturated fatty acids; SHCl, Soxhlet HCl; SOS, Soxhlet Osmotic Shock; SUH, Soxhlet Ultrasonic Homogenizer.

[a]Non-detected.

was detected in the BDE group. The enzymatic hydrolysis was shown to be an effective and non-toxic procedure for improving of extraction efficiency of intracellular compounds (R. Zhang et al., 2019). Gomes et al. (2020) reported that the microbial cell disruption using enzymes is a promising and highly energy-efficient technology. Furthermore, enzymatic lysis process is considered to be more environmentally friendly (Lee et al., 2017). Taher et al. (2014) in the study on extracting lipid with enzyme (lysozyme and cellulase) and acid

treatment from *Scenedesmus* sp., reported 16:0 as the major saturated fatty acid which was 16.4 ± 0.08%, 11.9 ± 0.3% and 15.1 ± 0.2% in the enzyme lysozyme and cellulase, and acid treatments, respectively. Yu et al. (2015) in their study investigating the effect of autoclaving, bead-beating, microwaving, sonication, acid (HCl) digestion, and Soxhlet extraction to lipid extraction from *Chlorella sorokiniana*, declared that in Soxhlet method saturated fatty acids 16:0 and 18:0 were affected, and lower results were obtained compared with

other methods. In HCl digestion 16:1, 18:0, 18:2 and 18:3 were relatively high. In our study, most of fatty acids were obtained with HCl digestion. Although HCl that was reported to cause harm in the literature, did not cause any loss in fatty acids in our study. But Taher et al. (2014) reported that using acids requires special materials of construction, which is not economic for large-scale applications. Safety issues, and wastewater treatment are also essential for strong acid treatment (Lee et al., 2017) and it is thought that the method will not be safe in applications such as feed, food and cosmetics. In our study, effective results were obtained in terms of fatty acids in the OS and UH groups with the BD method. In the study by Prabakaran and Ravindran (2011) on cell disruption methods (autoclaving, bead beating, microwave, sonication, 10% NaCl solution) for lipid extraction from *Chlorella* sp., *Nostoc* sp. and *Tolypothrix* sp. sonication was found as the most effective method.

In the present study, palmitic acid and DHA constitute most of the total fatty acids. It is an expected result that palmitic is high in aquatic plants, and the high amount of DHA, as a valuable fatty acid in terms of human health, is an important criterion. High DHA adds value to our product. It is thought that the evaluation of this algae for use in areas such as food and feed will make an important contribution. Ju et al. (2020) in their study the resulting microalgal lipids are mainly docosahexaenoic acid (DHA) and palmitic acid, also they reported palmitic acid is a saturated fatty acid (SFA) that is used as an emollient and diluent in the cosmetics industry. Fedorova-Dahms et al. (2011) reported that *Schizochytrium* sp. oil contains 40%–45% DHA. In the study by Li et al. (2009) on was used *Schizochytrium* as supplements in *Ictalurus punctatus* feed, the highest PUFA in dry algae was determined as DHA with 31.39% and Σn-3 PUFA was 37.08%. It was concluded that addition of 2% dried algae in the diet markedly improves the levels of 22:6 n-3 and Σn-3 LC-PUFAs in the edible tissue of fish. DHA and palmitic acid were high in % total fatty acids similar with our study. In our study; ΣSFA, ΣMUFA and ΣPUFA were obtained in the range of 67.08%–85.82%, 7.38%–10.53%, and 4.91%–20.87%, respectively. In the study of Byreddy et al. (2015) saturated, monounsaturated and polyunsaturated fatty acids were obtained in the range of 49%–57%, 31%–35% and 2%–18%, respectively for *Schizochytrium* sp.. ΣPUFA obtained in our study higher than that reported by Byreddy et al. (2015). n-3 FAs have beneficial effects against chronic metabolic diseases, such as obesity, diabetes, and cardiovascular diseases, different cancers, asthma, inflammatory bowel disease, rheumatoid arthritis, psoriasis and osteoporosis (Prato et al., 2019). Because of their health benefits of long-chain n-3

polyunsaturated fatty acids, especially DHA, consumer preference increased for the products fortified with these desirable nutrients (Liu et al., 2020). n-3, n-6 and n-3/n-6, DHA/EPA, PUFA/SFA, unsaturated (UNS)/SFA ratios are widely used to evaluate the nutritional value of lipid (Prato et al., 2019). In our present study Σn-3 and Σn-6 were detected between 4.58 ± 2.44% to 19.53 ± 0.09% and 0.33 ± 0.07% to 1.79 ± 0.00%, respectively. n-6/n-3 ratio was detected in the range of 0.06 ± 0.00–0.09 ± 0.09. Gonçalves et al. (2021) reported that n-6/n-3 ratio below 4.0 in a diet indicates desirable quantities for human health. In our study, this ratio was below the limits that Gonçalves et al. (2021) reported, in our study. Increasing consumption of fish and fish products rich in n-3 PUFA (polyunsaturated fatty acids), which is important for human health, and poor in n-6 PUFA, should be encouraged (Metin et al., 2021). PUFA/SFA ratio was obtained between 0.06 ± 0.03–0.31 ± 0.00 values. Due to the high content of palmitic acid, this ratio was obtained at low levels. However, the values were close to the minimum limit value of 0.45 specified by Gonçalves et al. (2021) and Liu et al. (2020) except for the SC group.

As a result; as R. Zhang et al. (2019) reported; compared with conventional methods, the use of emerging techniques allowed the recovery bio-molecules avoiding toxic solvent, high temperature and treatment time.

Lipids nutritional quality indices

In the different total crude lipid and cell disruption methods applied groups, atherogenicity index (AI) was detected in the range of 2.31 ± 0.00–7.72 ± 1.76. The highest and lowest values were observed in the SC and SHCl groups, respectively. In the other groups, this value was found to be between 2.42 ± 0.02–3.94 ± 0.21. Thrombogenicity index (TI) was found in the range of 0.74 ± 0.00–2.70 ± 1.15. The lowest value was detected in the BDOS and SHCl and the highest value was in the group SOS. Fatty acids hypocholesterolemic/hypercholesterolemic ratios (HH) were in the range of 0.08 ± 0.04–0.42 ± 0.00, the highest value was found in SHCl and the lowest value was in the group SC (Table 3).

Prato et al. (2019) reported that Atherogenic index (AI), thrombogenicity index (TI) and hypocholesterolemic/hypercholesterolemic fatty acid ratio (HH) provide indications on the dietetic quality of lipids and their potential effect on the development of coronary disease. They found AI and TI below 1 in edible marine bivalves. In the groups of cell disruption methods in our study, TI was detected below 1, except in the group SOS. Hypocholesterolemic/hypercholesterolemic fatty acid ratios (HH) were found between 0.08–0.42. High hypocholesterolemic/hypercholesterolemic fatty acid ratio (HH) is desirable. Prato et al. (2019) was found this index between 0.25–3.23 and qualified as high. In our study

TABLE 3 Lipids nutritional quality indices results

	BDC	SC	BDOS	SOS	BDE	SE	BDHCI	SHCI	BDUH	SUH
AI	2.59 ± 0.04	7.72 ± 1.76	2.42 ± 0.02	3.51 ± 0.18	2.68 ± 0.01	3.94 ± 0.21	2.56 ± 0.00	2.31 ± 0.00	2.57 ± 0.02	2.93 ± 0.04
TI	0.76 ± 0.01	2.70 ± 1.15	0.74 ± 0.02	1.01 ± 0.14	0.72 ± 0.10	[a]	0.79 ± 0.00	0.74 ± 0.00	0.82 ± 0.00	0.83 ± 0.01
HH	0.37 ± 0.01	0.08 ± 0.04	0.40 ± 0.01	0.27 ± 0.01	0.35 ± 0.00	0.23 ± 0.02	0.38 ± 0.00	0.42 ± 0.00	0.37 ± 0.00	0.32 ± 0.01

Abbreviations: AI, atherogenicity index; HH, hypocholesterolemic/hypercholesterolemic ratios; TI, thrombogenicity index.
[a]Could not be calculated since n-6 was not found.

AI was slightly higher, in fact, between 2–3 values, but it was detected as 7.55 only in the SC group. Aussant et al. (2018) in the study of fatty acid composition and nutritional value in eight species of microalgae, found the lowest HI (0.597) in *N. salina*. AI values were between 0.434–1.323, and TI results were 0.189–0.676. In our study, the lowest TI was found in BD groups by associating with DHA values. Concerning HI somewhat low, AI values were average and TI values were also low. Relatively high HI, low AI and TI values are desirable for healthy diet (Aussant et al., 2018; Liu et al., 2020). In our study, it is thought that the relatively low HI and average AI values are caused by high 16:0.

CONCLUSIONS

Schizochytrium sp. is the good source of DHA, the essential fatty acid. Higher amount of DHA that is necessary for human health, is advantageous to be used in many applications such as food, feed and cosmetics. In the study of conventional lipid extraction methods combined with cell disruption applications to increase lipid yield of *Schizochytrium* sp., Bligh and Dyer method was found to be more applicable than Soxhlet. Soxhlet method can be good at lipid extraction, however damages fatty acids. Lipid health indices such as n-6/n-3, PUFA/SFA, AI, TI and HH were almost favorable. To get higher lipid yield and quality from algal cells, a suitable cell disruption method is required. The hemicellulase enzyme application combined with Bligh and Dyer method is thought to give the most efficient result, with being high-efficiency, environmentally friendly, non-toxic and industrial scale feasibility. As a recommendation, combined cell disruption methods can be used in future studies.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
Mustafa Hac İsa, Cansu Metin and Yunus Alparslan carried out the laboratory studies; lipid extraction processes, lipid and fatty acid analyzes and drafted the manuscript. Ertan Ercan designed the experiment and interpreted the results.

ETHICS STATEMENT
This research falls outside of human or animal studies and institutional ethical approval was not required.

ORCID

Mustafa Hac İsa ⓘ https://orcid.org/0000-0002-2221-9981

Cansu Metin ⓘ https://orcid.org/0000-0002-2290-1489

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