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

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Abstract

In this study, it was investigated to increase the lipid yield of the microalgae Schizochytrium sp., by applying different cell disruption methods. Therefore, acid treatment with HCl, osmotic shock, enzyme applications and ultrasonic homogenizer were tried in this algae species combined with the Bligh and Dyer and Soxhlet methods as an alternative to classical lipid extraction methods. As a result of the study, the highest lipid value (21.72 ± 0.74%) was obtained in enzyme application with Bligh and Dyer method (BDE). The cell disruption processes increased the lipid yield compared to the control groups. The highest PUFA DHA was found in the range of 4.58 ± 2.44-19.25 ± 0.09%, and the highest value was observed in the BDE group. Highest SFA was palmitic acid. Effective results were observed in the Bligh and Dyer applied groups in terms of both total lipid and total fatty acids. In cell disruption methods, particularly in enzyme and HCl extraction, good results were obtained in terms of fatty acids. The highest total fatty acids and the highest lipid content were detected in the Bligh and Dyer enzyme (BDE). Enzyme applications are also advantageous because of being environmentally friendly. Lipid health indices such as n-6/n-3, PUFA/SFA, Atherogenicity index (AI), Thrombogenicity index (TI) and hypocholesterolemic/hypercholesterolemic ratios (HH) were almost favorable. With this study, an appropriate lipid extraction methods were determined to provide an economical and environmental friendly suggestion for future studies to be used in areas such as food, feed and cosmetics.

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).

Heterotrophic *Crypthecodinium, Schizochytrium* and *Ulkenia*species are considered as the first commecialized species that has been used for food, feed and biodiesel production, due to their high fat content. *Schizochy-*

trium 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%-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).

Direct lipid extraction from wet biomass is not easy because algal cell walls, are mainly composed of cellulose, are very durable and difficult to break (Lee and Han, 2015). The lipid yield is mainly affected by the rigidity of the microalgal cell wall. 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; 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.

The objective of the present study was to increase the lipid yield of*Schizochytrium sp* ., which has high amounts of valuable fat and fatty acids, but obtained low lipid yield due to the fact that the cell walls are not broken down by conventional lipid extraction methods. In addition to the classical Bligh and Dyer and Soxhlet methods; treatment with HCl as a solvent, osmotic shock as a non-mechanical process, ultrasonic homogenizer as a mechanical process and enzyme applications as a biological process were compared.

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, 4 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 for total crude lipid. Bligh and Dyer (1959) and AOAC (2006) Soxhlet methods applied alone were accepted as 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 in three parallel.

Total (crude) lipid

Bligh and Dyer method

Methanol+chloroform (1:2) (40 mL) mixture was added to the algae sample and homogenized. Then, 20 mL of 0.4% $CaCl_2$ solution was added onto these samples. The samples filtered on a filter paper and then filtered into the tared balloons kept in the oven at 105 °C for 2 hours. 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 at 60 ^{o}C.

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 seperation. Process of immersion for 90 minutes at 180 °C, washing for 120 minutes at 80 @C, and finally recovery for 8 minutes was carried out in the device (AOAC, 2006).

Cell disruption methods

HCl extraction

In the application of HCl as solvent, the algae: HCl (3 N) (1:40) was boiled at reflux for 1 hour (acid hydrolysis step). The sample was passed through filter paper and washed with 500 mL of water and filtered. After filtering, the filter paper was dried for 1 hour in an oven at 103 °C (AOAC, 2005). The dried samples were continued with Bligh and Dyer and Soxhlet methods.

Osmotic shock extraction

In the osmotic shock method, the dry algae sample was kept in a 10% saline solution for 2 days at a ratio of algae:salt water (1:10) (Prabakaran and Ravindran, 2011), lipid extraction was continued with Bligh and Dyer and Soxhlet methods.

Enzyme extraction

In the enzyme application, 10% cellulase enzyme was applied to the dry algae sample with the ratio of 1:10 (algae:enzyme) and kept in the oven at 55 °C for 2 days (Liang et al., 2012). Afterwards, the lipid extraction was continued with Bligh and Dyer and Soxhlet methods.

Ultrasonic homogenizer extraction

Solvent (chloroform:methanol 2:1) was added to the dry algae sample and then homogenized in an ultrasonic homogenizer (Bandelin UW 3200/20 kHz) for 20 minutes (Byreddy et al., 2015). The lipid extraction was continued with Bligh and Dyer and Soxhlet methods.

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 minutes and their final weighings were taken after cooling.

Crude lipid% = (Final Weight - Initial Weight) / Sample Weight x 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 4 mL of 2 M KOH (in methanol) addition. 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.

Gas Chromatography (GC) Conditions: 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 x 0.25 mm x 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 minutes. 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 CH2 Cl2–47885 U, 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 (LNQI)

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 x 14:0 + 16:0) / MUFA + PUFA

Thrombogenicity index

TI = (14:0 + 16:0 + 18:0) / [(0.5 x MUFA +0.5 x n6PUFA+3 x 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

In the present study, lipid yield of the treatment with HCl in the reflux, osmotic shock, enzyme and ultrasonic homogenizer cell disruption methods with Bligh and Dyer and Soxhlet crude lipid methods are shown in the Fig. 1. Bligh and Dyer and Soxhlet lipid extraction methods, which were not applied any disruption processes were accepted as control groups. Lipid ratio of the control groups Bligh Dyer control (BDC) and Soxhlet control (SC) was determined as 18.87 ± 0.4% and 5.54 ± 0.08%, respectively. Lipid ratio of Bligh and Dyer applied groups BDOS, BDE, BDHCl and BDUH was found as 20.92 ± 1.87%, 21.72 ± 0.74%, 19.38 ± 1.20% and 14.52 ± 0.36%, respectively. The lipid yield of Soxhlet method applied groups SOS, SE, SHCl and SUH groups was found as 8.21 ± 1.60%, 11.98 ± 1.41%, 12.26 ± 0.15% and 14.70 ± 0.3%, respectively. The highest lipid value 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 value in the Soxhlet applied groups was found in the SUH group with 14.70 ± 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 ($P< 0.05$).

Fig. 1 Lipid yield of different cell disruption methods

Difficulties arise in obtaining lipid due to the cell walls of microalgae. For this reason, in this study, lipid was obtained from*Schizochytrium sp* . with the HCl treatment, osmotic shock, enzyme and ultrasonic homogenizer cell disruption methods to increase the lipid yield to be used for feed, food and cosmetic raw material for future studies. In our study, it was observed that 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 ± 0.74%). Higher results were obtained in the Bligh and Dyer crude lipid method compared to Soxhlet. Lipid ratio of BDOS (20.92 ± 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 value was found in SUH group (14.70 ± 0.3%), followed by the SHCl (12.26 ± 0.15%). The difference between these two groups was statistically significant ($P < 0.05$). When our results were compared, it was observed that the lipid yield was lower in the Soxhlet applied groups, and the laboratory scale applicability of the Bligh and Dyer method was more appropriate. Araujo et al. (2013) reported that the Soxhlet extraction mechanism is mainly diffusion and the procedure does not involve mechanic disruption to the biomass. Their results showed that simply diffusion of lipids through the

cell membrane is a slow process and results in low yield of lipid. Similar with the study of Araujo et al. (2013), low results were obtained in the Soxhlet method compared with the Bligh and Dyer method, in our study. In the literature studies, 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 in their study. The maximum yield was obtained from the chloroform:methanol (2:1) solvent. Chloroform:methanol (2:1) was used in our study with reference to Byreddy et al. (2015). Liquid nitrogen grinding, osmotic shock, vortexing, water bath and sonication were used in their study to increase the efficiency of lipid extraction. 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.

Araujo et al. (2013) declared the Bligh and Dyer method assisted by ultrasound resulted in the highest extraction of lipid from *C. vulgaris* (52.5%) and lipid yield with Soxhlet method was obtained quite low (1.8%). Lee and Han (2015) applied hydrodynamic cavitation with sulfuric acid for different durations in *Nannochloropsis salina* species to increase lipid yield. While sulfuric acid concentrations were 0.1% and 2% lipid contents were determined as 21.7% and 45.4%, respectively. Lipid yield was increased with the increase of acid concentration and time. In our study, the lipid yield of HCl disruption combined with Soxhlet and Bligh and Dyer methods, was obtained respectively as; 12.26 ± 0.15% and 19.9 ± 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 for use in a microalgal biorefinery because of destroying the activity of valuable cellular components (such as protein and pigment) (Halim et al., 2012). 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%. A single method of extraction cannot be sufficient to reach the maximum yield. Combination of pretreatment methods with different solvents increase lipid yield of the algae (Mubarak et al., 2015). 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 degC, which is the optimum temperature for the cellulase enzyme. Taher et al. (2014) in their 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%). Algal cell disruption methods to increase lipid extraction from microalgae depends on species, age of the culture and composition of cell wall. Only few mechanical methods such as application of enzymes/chemicals either or alone can be considered for industrial applications. Osmotic shock method can also be applied industrially for lipid extraction, because of reducing energy consumption and production cost (Byreddy et al., 2015). Although acid treatment process is efficient in cellulose degradation, sulfuric acid is toxic and corrosive, so this process is not recommended (Taher et al., 2014). As a result in our study, the best 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 acids

The fatty acid results of the sample groups treated with different lipid extraction methods from *Schizochytrium* sp. are shown in Table 1 and Table 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 +- 0.17-74.86 +- 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 +- 0.01-5.55 +- 0.15%. Among the

monounsaturated fatty acids, miristoleic acid (14:1) was found in the highest amount (4.29 +- 0.95-5.86 +- 0.16%). The highest polyunsaturated fatty acid was determined as DHA (22:6) with the range of 4.58 +- 2.44-19.25 +- 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 +- 0.00-0.09 +- 0.00 and the highest PUFA and n-3 was in the BDE group with 20.87 ± 0.04% and 19.53 ± 0.09%, respectively. The lowest PUFA and n-3 was also in the SC group with 4.91 ± 0.23 and 4.58 ± 2.44%, respectively. SFA and MUFA were in the range of 67.08 ± 0.16% - 85.82 ± 3.90% and 7.38 ± 0.01-10.53 ± 0.02%, respectively. The highest PUFAs were obtained in the BD groups, and the highest PUFA in the BDE group was statistically different from the other groups. PUFA/SFA was detected in the range of 0.06 ± 0.03-0.31 ± 0.00 in the lipid extraction groups.

Table 1 Fatty acid results of Bligh and Dyer groups

	BDC	**BDOS**	**BDE**	**BDHCl**	**BDUH**
6:0	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	*
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	*	0.02 ± 0.00	0.02 ± 0.00	*
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	*
16:0	57.10 ± 0.58^{b}	55.78 ± 0.2^{d}	57.68 ± 0.0^{a}	56.31 ± 0.0^{c}	57.43 ± 0.21^{b}
17:0	1.40 ± 0.01^{a}	1.35 ± 0.01^{a}	1.38 ± 0.01^{a}	1.37 ± 0.01^{a}	0.78 ± 0.83^{b}
18:0	1.99 ± 0.01^{b}	2.25 ± 0.04^{a}	1.95 ± 0.11^{b}	2.15 ± 0.03^{ab}	2.26 ± 0.01^{a}
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	*
24:0	3.44 ± 0.06	3.52 ± 0.06	3.61 ± 0.02	3.24 ± 0.00	3.25 ± 0.04
SFA	69.27 ± 0.56^{b}	68.01 ± 0.25^{c}	70.10 ± 0.1^{a}	68.37 ± 0.11^{c}	68.77 ± 0.62^{c}
14:1	5.00 ± 0.00	4.70 ± 0.08	5.06 ± 0.02	4.73 ± 0.01	4.78 ± 0.01
15:1	*	*	*	0.02 ± 0.00^{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 ± 0.01^{c}	3.95 ± 0.08^{b}	1.66 ± 0.03^{d}	4.30 ± 0.01^{a}	4.00 ± 0.01^{b}
20:1n-9	0.19 ± 0.00^{a}	0.25 ± 0.00^{a}	0.10 ± 0.00^{b}	0.26 ± 0.00^{a}	0.27 ± 0.01^{a}
22:1n-9	0.05 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.10 ± 0.02
MUFA	8.68 ± 0.01^{b}	9.68 ± 0.18^{a}	7.38 ± 0.01^{c}	9.52 ± 0.01^{a}	9.85 ± 0.03^{a}
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 ± 0.01^{b}	1.05 ± 0.03^{a}	0.65 ± 0.06^{b}	1.05 ± 0.00^{a}	1.09 ± 0.02^{a}
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	*	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 ± 0.20^{b}	18.52 ± 0.03^{b}	19.25 ± 0.09^{a}	17.50 ± 0.00^{c}	17.29 ± 0.23^{c}
PUFA	20.19 ± 0.23^{b}	20.37 ± 0.03^{b}	20.87 ± 0.00^{a}	19.35 ± 0.00^{c}	19.29 ± 0.30^{c}
n-3	18.69 ± 0.21^{b}	18.80 ± 0.03^{b}	19.53 ± 0.00^{a}	17.75 ± 0.00^{c}	17.55 ± 0.23^{c}
n-6	1.38 ± 0.01^{b}	1.43 ± 0.01^{b}	1.23 ± 0.06^{c}	1.47 ± 0.01^{b}	1.63 ± 0.06^{a}
n-9	3.16 ± 0.01^{b}	4.37 ± 0.08^{a}	1.93 ± 0.02^{c}	4.72 ± 0.01^{a}	4.46 ± 0.01^{a}
n-6/n-3	0.07 ± 0.00^{c}	0.08 ± 0.00^{b}	0.06 ± 0.00^{d}	0.08 ± 0.00^{b}	0.09 ± 0.00^{a}
PUFA/SFA	0.29 ± 0.01^{ab}	0.30 ± 0.01^{a}	0.30 ± 0.01^{a}	0.28 ± 0.00^{b}	0.28 ± 0.01^{b}

*Non-detected

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

Table 2 Fatty acid results of Soxhlet groups

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

*Non-detected

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

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. In the study by Guckert et al. (1988) on lipid solvent system for the analysis of lipid classes in*Chlorella* , Bligh and Dyer provided 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 was detected in the BDE group. The enzymatic hydrolysis was shown to be an effective and nontoxic procedure for improving of extraction efficiency of intracellular compounds (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 C16:0 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. Total fatty acids were 84.3%, 76.9%, 79.7%, 82.2% and 79.0%, in the groups of untreated freeze-dried algae, acid treatment, lysozyme and cellulase enzyme, respectively. Treatments decreased the total fatty acids compared with untreated algae, especially in the acid applied group. 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 autoclaving and microwaving methods, 16:1, 18:0, 18:2 and 18:3 were not obtained when these fatty acids were relatively high in HCl digestion, especially for 18:2 with 28.9 ± 1.4%. 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. Osmotic shock has advantages such as; lower energy consumption, easier scale-up, faster extraction-suitable for all cell types, however it has disadvantages like generation of waste salt water, salt's high cost (Lee et al., 2017) time consuming. Sonication also has disadvantages such as damaging chemical structure of molecules (Byreddy et al., 2015).

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 and up to 10% EPA. Leaño et al. (2003) found the amount of lipid in the range of 13.0-39.1%, in their studies of growth and fatty acid production of *Schizochytrium* sp. at different salinity and temperatures. 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 n-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 n-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; SFA, MUFA and PUFA 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.. PUFA 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 n-3 and n-6 were detected between 4.58 ± 2.44-19.53 ± 0.09% and 0.33 ± 0.07-1.79 ± 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 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 (LNQI)

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).

Table 3 Lipids nutritional quality indices results

	BDC	**SC**	**BDOS**	**SOS**	**BDE**	**SE**	**BDHCl**	**SHCl**	**BDUH**	**SU**
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
TI	0.76±0.01	2.70±1.15	0.74±0.02	1.01±0.14	0.72±0.10	*	0.79±0.00	0.74±0.00	0.82±0.00	0.83
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

*Could not be calculated since n-6 was not found.

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 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

In the study of total crude lipid 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. According to the results, *Schizochytrium* sp. is the best source of DHA 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. Total lipit and fatty acids were found higher than Soxhlet in Bligh and Dyer groups. 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 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.

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