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Evaluation of Fatty Acid Extraction Methods for *Thraustochytrium* sp. ONC-T18

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Various extraction methods were assessed in their capacity to extract fatty acids from a dried biomass of *Thraustochytrium* sp. ONC-T18. Direct saponification using KOH in ethanol or in hexane:ethanol was one of the most efficient techniques to extract lipids (697 mg g^{-1}). The highest amount of fatty acids (714 mg g^{-1}) was extracted using a miniaturized Bligh and Dyer extraction technique. The use of ultrasonics to break down cell walls while extracting with solvents (methanol:chloroform) also offered high extraction yields of fatty acids (609 mg g^{-1}). Moreover, when the transesterification mixture used for a direct transesterification method was doubled, the extraction of fatty acids increased approximately 77% (from 392 to 696 mg g^{-1}). This work showed that *Thraustochytrium* sp. ONC-T18 has the ability to produce over 700 mg g^{-1} of lipids, including more than 165 mg g^{-1} of docosahexaenoic acid, which makes this microorganism a potential candidate for the commercial production of polyunsaturated fatty acids. Finally, other lipids, such as myristic, palmitic, palmitoleic, and oleic acids, were also produced and recovered in significant amounts (54, 196, 123, and 81 mg g^{-1}), respectively.

KEYWORDS: Docosahexaenoic acid; eicosapentaenoic acid; polyunsaturated fatty acids; ω-3 fatty acids; *Thraustochytrium* sp. ONC-T18, lipids; extractions; ultrasonics

INTRODUCTION

Polyunsaturated fatty acids (PUFAs), particularly the ω-3 fatty acids DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid), have been reported to provide several beneficial health effects. Covington (*1*) mentioned the following health benefits: lowering of cholesterol levels and mitigating the effects of hypertension, stroke, diabetes, osteoporosis, depression, schizophrenia, asthma, macular degeneration, colon cancer, prostate cancer, and rheumatoid arthritis. Recently, a mass influx of ω-3-fortified functional foods has made their way onto the world market. Currently, fish oils are the only concentrated source of these ω-3 fatty acids (*2*). Consequently, marine fish stocks are in decline, and these sources of fish oil are also in decline, limiting the protective role of long-chain PUFAs in human health (*3*). This has prompted research into possible alternative, renewable, microbial sources of PUFAs including species of thraustochytrids, a marine protist, that can produce large quantities of DHA and some EPA (*4*, *5*) and can potentially be tailored to enhance the production rates of specific fatty acids (*6*).

Various methods for the extraction of fatty acids from microbial biomass have been reported, with extraction yields dependent upon the nature of the microbial cell structures and the extraction techniques used (*7*−*11*). The method of Bligh and Dyer (*12*) is one of the most commonly used methods for the extraction of lipids (over 23500 journal citations currently), which is a quantitative extraction technique that uses a monophasic ternary system of chloroform:methanol:water (1:2:0.25, v/v/v). After the extraction, the system is converted to a biphasic state by dilution with additional chloroform and water yielding a chloroform layer containing lipids and a methanolic layer containing nonlipids. Modifications of the Bligh and Dyer method, including the addition of water to the solvent mixture when extracting lipids from dry (*13*) or lyophilized samples (*14*), the use of various disruption techniques, such as sonication (*15*), or elevated temperature (*9*), have also been used. Also, several methods have tried to optimize this process by varying solvent ratios or adding acid or buffers to the water fraction (e.g., trichloroacetic acid and phosphate buffer) (*10*, *11*, *16*). In one instance, a Bligh and Dyer method (added with 5% of trichloroacetic) for the extraction of polar lipids from the archaebacterium *Methanobacterium thermoautotrophicum* reported a six times increase in lipids extracted as compared to the classical Bligh and Dyer procedure (*10*).

A method reported by Lewis et al. (*9*) combined lipid extraction with transesterification (90 °C for 15−120 min),

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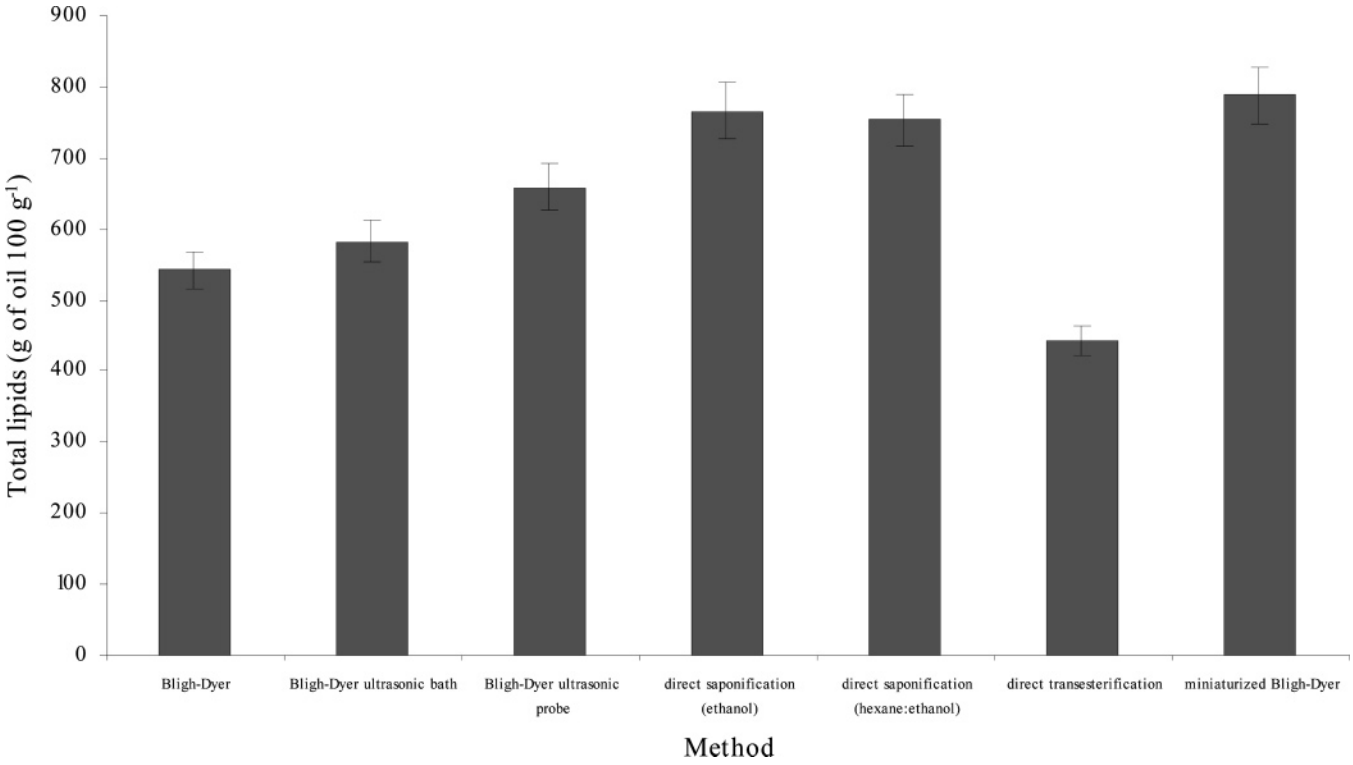

Figure 1. Gravimetric values of oil (as per equation 1) extracted using different methods ($n \geq 3$).

followed by recovery of the fraction of fatty acid methyl esters (FAMES) using a biphasic solvent system of hexane:chloroform (4:1). Other methods, such as saponification, have reported significant lipid recoveries from several types of microalgae (e.g., *Isochyrsis galbana*, *Spirulina platensis*, *Phaeodactylum tricornutum*, and *Porphyridium cruentum*) (*17*−*20*). Cartens et al. (*7*) used a direct saponification of biomass method with two solvents that contained KOH for lipid saponification: ethanol (96%) and hexane:ethanol (96%) (1:2.5, v/v). Other solvent systems and methods have also been evaluated for microbial use. For example, while an hexane−isopropanol solvent system was found to be effective for the bacterium *Pseudomonas atlantica* (*21*), this system gave a lower recovery of lipids in the green alga *Chlorella* sp. (*22*).

The Bligh and Dyer method works well for extracting lipids from fish tissue; however, it may not necessarily work similarly for lipid extraction from microbial biomass. Microbial lipids contain either saturated or monounsaturated fatty acids, with a few notable exceptions such as deep sea organisms (*23*), cyanobacteria (*24*), and protists (*25*). An effective oil extraction method for an oleaginous microbe such as *Thraustochytrium* sp. ONC-T18 can be used to accurately determine fatty acid yields and fatty acid compositions of the extracts. Therefore, to achieve optimal fatty acid yields from freeze-dried biomasses of this microbe, the Bligh and Dyer method, including variations to the biocompatible solvent mixtures and sonication techniques, along with a direct saponification and a direct transesterification procedure, were evaluated for total oil, total fatty acids, and DHA and EPA extraction efficiencies.

MATERIALS AND METHODS

Materials. The microbial biomass of *Thraustochytrium* sp. ONC-T18 for all extraction experiments was prepared using a single fermentation [Biostat Bplus Twin 5L Bioreactor (Sartorius BBI Systems Inc., Bethlehem, PA)] and was freeze-dried prior to sample processing. Specifically, liquid medium was prepared in artificial seawater containing 2 g L^{-1} yeast extract (BD, Franklin Lanes, NJ) and 8 g L^{-1} monosodium glutamate, which was sterilized by autoclaving, followed by the addition of 20 g L^{-1} and 0.2 μm filter-sterilized glucose. A 100 mL volume inoculum culture of *Thraustochytrium* sp. ONC-T18 was prepared from an agar plate and grown for 24 h at 25 °C on a shaker at 120 RPM. This inoculum was then used to inoculate 4.9 L of medium in the bioreactor. More details of the fermentative process were previously described by Burja et al. (*6*).

Bligh−Dyer. The Bligh and Dyer (*12*) method and four modifications of it (described below) were performed in triplicate or more. The Bligh−Dyer tenchnique used approximately 4 g of *Thraustochytrium* sp. ONC-T18. Ten milliliters of distilled water was added, along with 120 mL of methanol:chloroform (2:1, v/v), and blended for 2 min using a polytron homogenizer (model 75D, Rose Scientific Ltd., Darmstadt, Germany). Forty milliliters of chloroform was added, the mix was blended for another 30 s, and the extract was filtered through a Whatman #4 filter paper. The filtrate was subsequently mixed with 62 mL of distilled water using a mixing cylinder and inverted once, the layers were separated overnight, and the bottom chloroform layer was recovered. Fifteen milliliters of the chloroform layer was then transferred to a preweighed aluminum dish, and the solvent was evaporated for 30 min in a drying oven (Equatherm, Curtin Matheson Scientific Inc., Melrose Park, IL) set at 80 °C. After it was cooled, the dish was reweighed, and the total lipid extract values were determined gravimetrically (weight of oil extracted) using the following equation:

$$\text{total lipids (g of oil 100 g}^{-1}\text{ sample)} = \frac{(W_L - W_D) \times V_C \times 100}{V_P \times W_S} \quad (1)$$

where W_D is the weight of an empty aluminum dish (g); W_L is the weight of an aluminum dish with dried lipid residue (g); W_S is the weight of sample (g); V_C is the total volume of the chloroform layer in a graduated cylinder (mL); and V_P is the volume of chloroform transferred to an aluminum dish (mL).

Bligh−Dyer Ultrasonics (Bath and Probe). The Bligh−Dyer method was modified to incorporate the use of an ultrasonic bath and an ultrasonic probe. For the Bligh−Dyer ultrasonic bath method, 15 and 30 min of ultrasonics were applied in the place of polytron cellular disruption. The ultrasonic bath (Aquasonic model 75D, VWR Scientific

Table 1. Profile of FAMEs Recovered (mg g^{-1}) from *Thraustochytrium* sp. ONC-T18 Using Different Methods[a]

method	C14:0	C14:1	C15:0	C16:0	C16:1	C18:0	C18:1	C18:2^6	C20:0	C20:2^6
Bligh–Dyer	41.39 a	0.43	6.61	128.45 b	87.83 a	5.49	50.25 a	0.00	1.26	0.63
Bligh–Dyer ultrasonic bath	44.69 b	0.45	7.14	138.13 b	95.04 b	5.87	54.46 a	0.00	1.35	0.64
Bligh–Dyer ultrasonic probe	51.37 b	1.54	8.18	157.63 b	108.32 b	6.85	62.03 b	0.00	1.29	0.00
direct saponification (ethanol)	48.35 b	1.15	9.03	196.36 c	117.95 b	9.64	87.61 c	0.00	2.31	0.00
direct saponification (hexane:ethanol)	50.08 b	0.61	9.16	194.74 c	115.80 b	9.84	81.05 b	0.00	2.06	0.00
direct transesterification	34.81 a	1.12	5.60	103.04 a	73.43 a	5.56	42.85 a	0.43	0.98	1.10
miniaturized Bligh–Dyer	64.35 c	1.40	10.32	196.06 c	134.25 c	7.82	74.60 b	0.03	1.65	0.35

method	C20:3^6	C20:4^6	C20:4^3	C20:5^3 (EPA)	C22:0	C22:4^6	C22:5^6	C22:5^3	C22:6^3 (DHA)	total
Bligh–Dyer	0.19	0.81	1.12	4.85 a	0.20	0.21	30.76	2.63	120.57 b	483.67 b
Bligh–Dyer ultrasonic bath	0.17	0.89	1.23	5.23 a	0.18	0.41	33.48	2.86	131.34 b	523.56 b
Bligh–Dyer ultrasonic probe	0.00	1.91	1.47	5.54 a	0.00	0.00	38.14	3.33	150.07 c	597.67 c
direct saponification (ethanol)	0.25	1.21	1.60	5.83 a	1.74	0.39	49.79	4.04	166.43 c	703.69 d
direct saponification (hexane:ethanol)	0.42	1.18	1.47	5.86 a	0.69	2.28	47.03	4.04	163.59 c	689.90 d
direct transesterification	0.32	0.72	1.15	4.54 a	0.28	0.38	23.16	2.19	89.83 a	391.51 a
miniaturized Bligh–Dyer	0.22	1.28	1.73	4.34 a	0.20	0.23	42.69	3.77	168.26 b	713.53 e

[a] Notes: Values in each column with different letters (a–e) were shown to be significantly different ($p < 0.05$, $n \geq 3$). A superscript 3 denotes a fatty acid with a double bond located in the n-3 position (ω-3 fatty acid). A superscript 6 denotes a fatty acid with a double bond located in the n-6 position (ω-6 fatty acid).

Products, Westchester, PA) was used at a frequency of 35 kHz. For the Bligh−Dyer ultrasonic probe method, a 30 s pulse of ultrasonics was applied instead of the polytron. The ultrasonic probe (model CPX 130PB, Cole Parmer, Vernon Hills, IL) applied a focused energy burst of 20 kHz.

Acid Bligh−Dyer. Upon the basis of the Bligh−Dyer method, this technique referred by Nishihara et al. (*10*) was performed using 10 mL of H_2O with 5% (0.5 mL) trichloroacetic acid to rehydrate the biomass.

Miniaturized Bligh−Dyer and Collection of Fractions. This method used 95% less solvent than the amounts used by Pinkart et al. (*11*). The next procedure was as follows: 0.25 g of ONC-T18 was placed in tinted screw cap test tubes, and 12.5 mL of chloroform, 25 mL of methanol, and 10 mL of a 50 mM K_2HPO_4 buffer solution (pH 7.4) were added. The sample was then agitated using a wrist action shaker (Burrell model 75, Pittsburgh, PA) at a speed setting of 3 for 1 h. The sample was then placed in a stoppered graduated cylinder, and 12.5 mL of chloroform and 12.5 mL of buffer were added. The cylinder was inverted 30 times and allowed to settled for 1 h recovering the bottom layer (approximately 25 mL) to determine total lipids gravimetrically, which were carried out, for all Bligh−Dyer modified methods, as described above for the Bligh−Dyer procedure (eq 1).

Using the 10 mL of the final recovered bottom layer of the miniaturized Bligh−Dyer method, neutral lipids were separated into mono-, di-, and triglycerides using aminopropylsilyl columns [500 mg Ultra-Clean NH_2 solid-phase extraction (SPE) colums, Alltech, Deerfiled, IL], and following the procedures of Pinkart et al. (*11*), the mono-, di-, and triglycerides fractions (miniaturized Bligh−Dyer fractions I, II, and III, respectively) were collected for analysis by using high-pressure liquid chromatography with an evaporative light-scattering detector (HPLC-ELSD) and gas chromatography with a flame ionization detector (GC-FID).

The methylation of fat collected from all extraction methods based on Bligh−Dyer was performed in accordance to the method used by Lewis et al. (*9*) (described below), where 3 mL of transesterification reaction mixture [methanol:hydrochloric acid:chloroform (10:1:1, v/v/v)] was added to 20 mg of fat instead of 20 mg of freeze-dried cells. The fat recovered was also analyzed by GC-FID to determine its cholesterol content (see analysis of lipids).

Direct Saponification. Two direct saponifications of biomass techniques (*7*) were carried out in triplicate or more, using KOH dissolved in ethanol and hexane:ethanol for the saponification step. The direct saponification with ethanol was performed by weighing out 0.5 g of freeze-dried ONC-T18 cells, adding 38 mL of 3 mM KOH in ethanol (96%) into a 150 mL Erlenmeyer flask, evacuating the headspace of the flask by replacing it with nitrogen, and shaking at 100 RPM for 1 h in a water bath set at 60 °C (BS-21, Lab Corp., Korea). Samples were cooled to room temperature and filtered through a glass fiber filter paper under vacuum. The biomass was washed with 10 mL of ethanol and transferred to a graduated mixing cylinder, and 10 mL of water was added. Unsaponifiables were extracted via the adding of 20 mL of hexane and gentle mixing twice. After layers were separated, the pH was adjusted to 1 (from pH 13−14) by addition of $HCl:H_2O$ (1:1, v/v). The top layer, containing the fatty acid fraction, was recovered by two rounds of the addition of 10 mL of hexane and gentle mixing. The second direct saponification method used a solution of KOH in hexane:ethanol (96%) (1:2.5, v/v) instead of KOH in ethanol. Gravimetric total lipid determinations for the organic layers recovered were carried out as described for the Bligh−Dyer method (equation 1). Twenty mg of recovered fat (instead of 20 mg of freeze-dried cells) was transesterified by adding 3 mL of methanol:hydrochloric acid:chloroform (10:1:1, v/v/v) using a transesterification process described below (direct transesterification method).

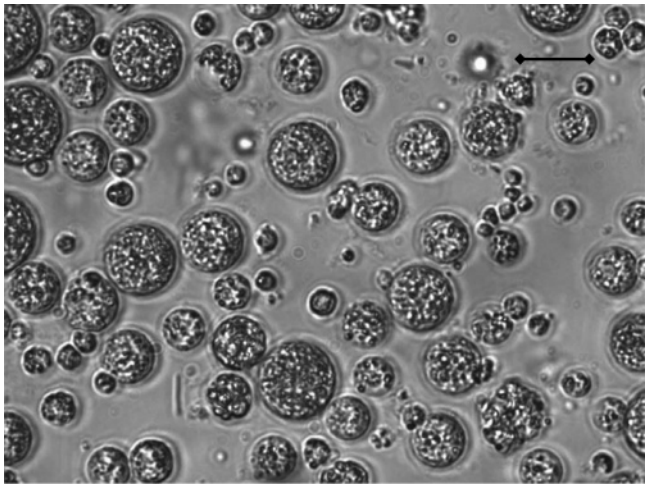

Figure 2. Phase contrast 400× light micrograph of actively dividing vegetative cells of *Thraustochytrium* sp. ONC-T18 that contain oil micelles. Scale bar, 10 μm.

Direct Transesterification. This method, referred by Lewis et al. (*9*), was performed (in triplicate or more) by adding 3 mL of transesterification reaction mixture [methanol:hydrochloric acid:chloroform (10:1:1, v/v/v)] to 20 mg of freeze-dried cells. One hundred microliters of a 100 mg mL^{-1} C23:0 standard solution was also added to the mix. The sample was vortexed for 5 s and placed in a preheated water bath at 90 °C for 120 min. Once heating was completed, samples were allowed to cool to room temperature, 1 mL of H_2O was added, and the sample tube was inverted 2−3 times. FAMEs were then

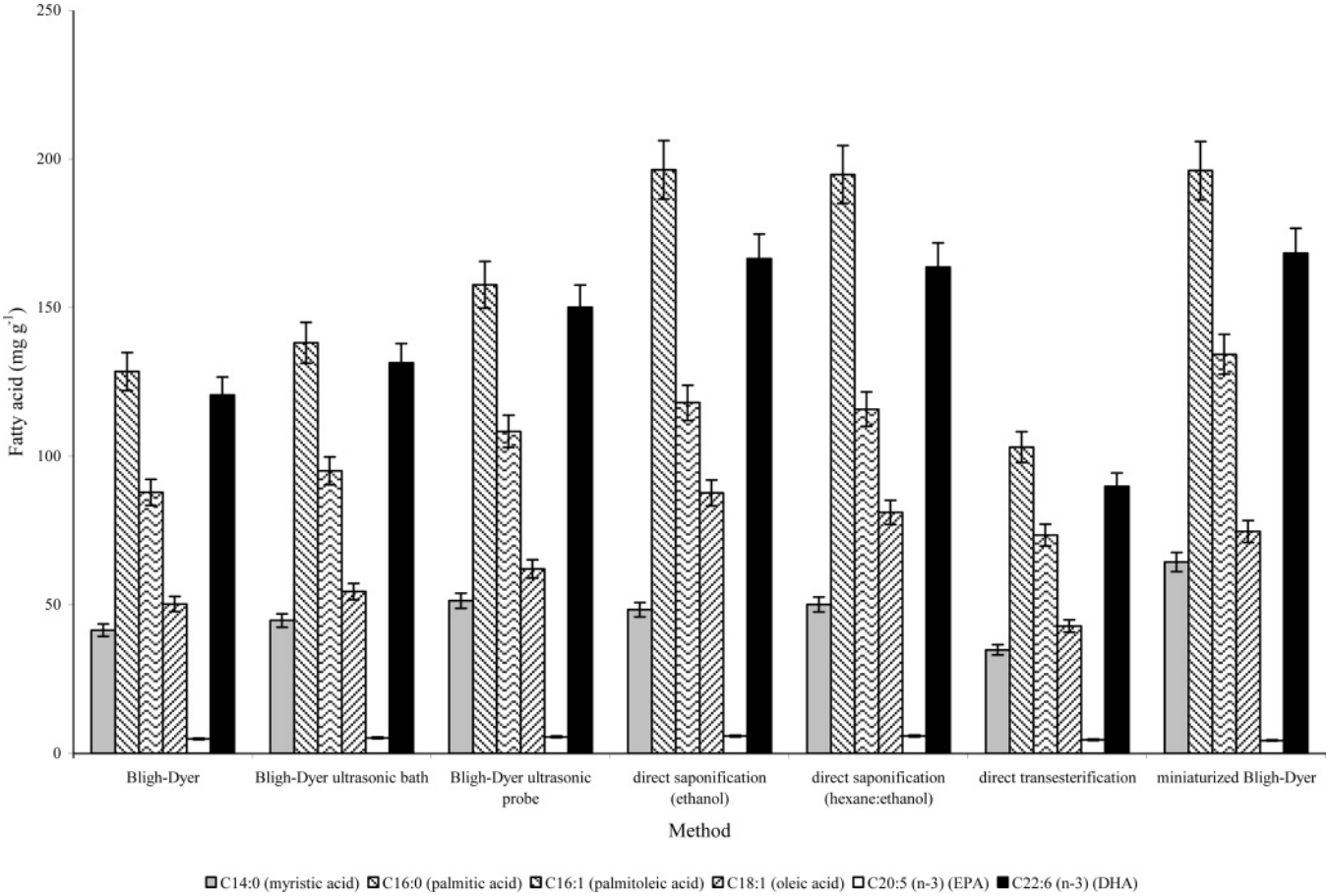

Figure 3. Recovered DHA and EPA, myristic, palmitic, palmitoleic, and oleic acids ($n \geq 3$).

extracted via the addition of 3 × 2 mL aliquots of hexane:chloroform (4:1, v/v) and vortexed for 10 s, and the layers were allowed to separate and were recovered, and the organic layers recovered in a clean tube were pooled. A 0.5 g amount of anhydrous Na_2SO_4 was added to eliminate traces of H_2O or acid that may be present and that could damage the GC column. The sample was then transferred to a new clean tube, and 100 μL of C19:0 (from a 100 mg mL^{-1} solution) was added to calculate the concentration of FAMES. The sample was then ready for GC-FID analysis. A 0.4 g amount of freeze-dried biomass and its respective increased amount of solvents (with no addition of standards) were used to determine total lipids gravimetrically (eq 1) in a direct transesterificated extract.

The effect of duration of acid transesterification (between 10 and 240 min) when incubated at 90 °C was determined (in triplicate or more) for direct transesterification to see at what time the major production of FAMEs first occurred. Finally, the effect of increasing the transesterification mixture (from 3 to 6, 9, and 12 mL) was also assessed in triplicate or more.

Analysis of Lipids. GC analysis of FAMEs was carried out using two internal standards. Specifically, tricosaenoic acid (C23:0) (for calculating % recovery) was added prior to the transesterification (as per direct transesterification method) and nonadecaenoic acid (C19:0) was added directly before the analysis to determine concentration of FAMEs. Analyses were performed using an Agilent 6890 GC (Agilent Technologies) equipped with a 30 m × 0.32 mm i.d. (0.25 μm film thickness) OMEGAWAX 320 fused-silica capillary column (Sigma-Aldrich, St. Louis, MO) and a FID (injection volume, 1 μL; carrier H_2 gas with a constant flow of 5.0 mL min^{-1}; set to 250 °C; split ratio, 50:1 to FID detector at 275 °C). Confirmation of FAMEs identity was performed using a Trace GC-DSQ mass spectrometer (Thermo Electron, Boston, MA) and comparison of retention times to laboratory standards (NuChek Prep Inc., Elysian, MI).

An Agilent HPLC with ELSD detector was used to analyze the lipid classes of the miniaturized Bligh−Dyer fractions I, II, and III. This instrument was equipped with a Waters Spherisorb S3CN 150 mm × 2 mm column (Waters, Milford, MA). A flow rate of 0.6 mL min^{-1} during the 10 min of analysis was used. The mobile phase gradient employed was hexane:methyl terbutyl ether (98:2, v/v) for the first 5 min, then ramped to 80:20 over the next 2 min, and finally ramped to 0:100 for the remaining 3 min. The sample injection volume and the column compartment temperature were 1 μL and 30 °C, respectively. The cholesterol content in oils recovered was analyzed by GC-FID following AOAC Official Method 970.51 (*26*).

All extractions methods were performed in triplicate or more and analyzed for significant difference by analysis of variance. Statistical analyses of data were performed using the Duncan Multiple Range Test (SPSS 13.0, SPSS Inc., Chicago, IL). Differences between individual means were deemed to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

The most efficient extractive methods in terms of g of oil extracted per 100 g of freeze-dried biomass of *Thraustochytrium* sp. ONC-T18 were both direct saponifications (with ethanol and hexabe:ethanol) and the miniaturized Bligh−Dyer methods, extracting 720 g of oil per 100 g of biomass (**Figure 1**).

In general, the percentage of each fatty acid (as FAMEs) found within these extracts was consistent; nevertheless, it was interesting to note the variations in total extracted fatty acid fractions from identical biomass samples (**Table 1**). For example, the direct transesterification technique [found to be an efficient method to recover fatty acids from thraustochytrids in accordance to Lewis et al. (*9*)] produced the lowest amount ($p < 0.05$) of total FAMEs at 391.51 mg g^{-1} of dried biomass, as compared to the standard Bligh−Dyer method of 483.57 mg g^{-1} (23.5% increase). Further analysis of the extract results

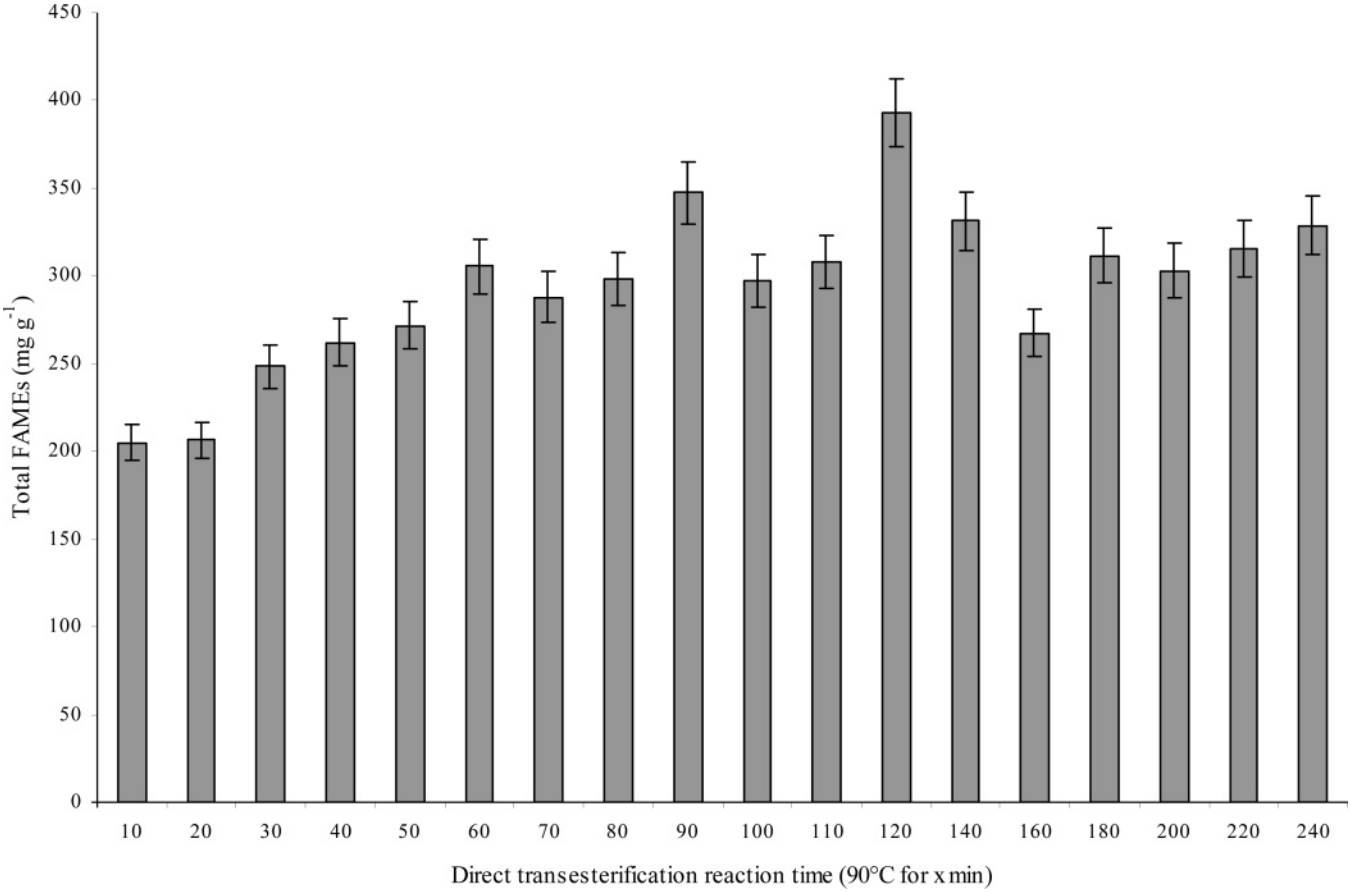

Figure 4. Recovery efficiencies of fatty acids during the duration of the direct transesterification method ($n \geq 3$).

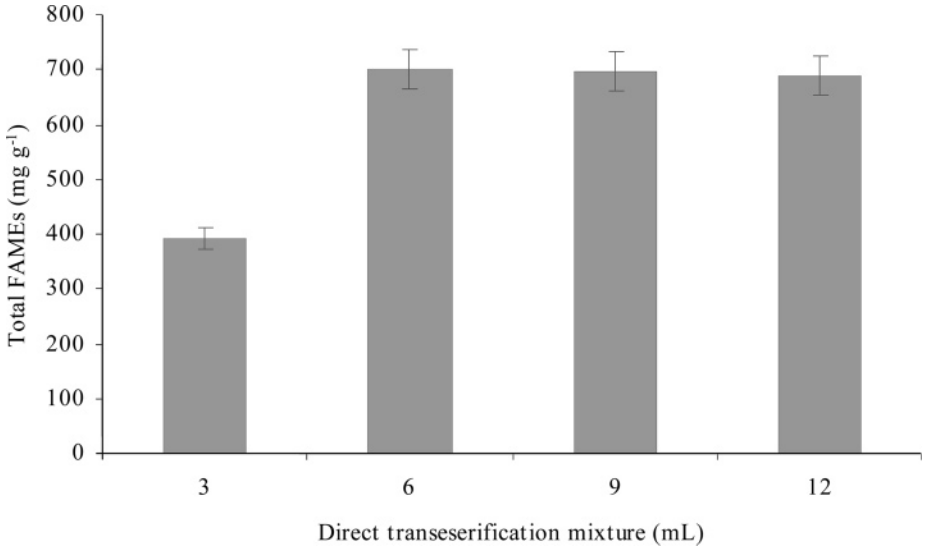

Figure 5. Effect of the transesterification mixture volume in the recovery efficiencies of fatty acids ($n \geq 3$).

(**Table 1**) shows that fatty acid recovery was further increased ($p < 0.05$) using the Bligh−Dyer technique when coupled with an ultrasonic bath or probe cell disruption process, resulting in recoveries of 523.56 mg g^{-1} (8.2% increase) and 609.08 mg g^{-1} (23.6% increase), respectively. Mason and Lorimer (*27*) stated that an effective ultrasonic treatment to release cellular contents can be achieved when a maximum breakdown of cell walls is balanced with a minimal degradation, from the power ultrasound effect (cavitational collapse and bulk heating), of the natural product of interest.

When direct saponification was attempted (a method that produces fatty acids bound to potassium salts), using the solvents ethanol (703.69 mg g^{-1}) or hexane:ethanol (689.9 mg g^{-1}), a further significant increase (with respect to the Bligh−Dyer ultrasonic probe, 597.67 mg g^{-1}) in fatty acid recovery (17.7 and 15.4%, respectively) was noted ($p < 0.05$). Thus, direct saponification techniques increased the extraction of fatty acid significantly ($p < 0.05$) by 45.5 and 42.6%, respectively, when compared with the Bligh−Dyer method. These yield increases may be attributed to the greater ability of these fatty acids to form potassium salts and precipitate, rather than crude oils. Furthermore, the step of separating the biomass from the soap solution before adding hexane for extraction of unsaponifiables avoids problems associated with emulsion, thus aiding the

extraction efficiencies (*7*). A significant difference ($p < 0.05$) in fatty acid recovery was not observed among the two treatments of direct saponification (with ethanol and hexane:ethanol).

Finally, the miniaturized Bligh−Dyer method recovered the greatest amount ($p < 0.05$) of fatty acid material (713.53 mg g^{-1}, an increase of 47.5% with respect to the Bligh−Dyer method). Here, we reduced 95% of the amount of chloroform and methanol used by Pinkart et al. (*11*), who used 1 and 2 mL of chloroform and methanol, respectively, per mg of dried biomass, while in this study 0.05 and 0.1 mL of chloroform and methanol, respectively, per mg of biomass were used. The miniaturized Bligh−Dyer method reduced the amount of biomass used for the extraction when compared to the Bligh−Dyer method (from 4 to 0.25 g). However, the miniaturized Bligh−Dyer used a total of 100 mL of chloroform and 100 mL of methanol per g of biomass, whereas the regular Bligh−Dyer used 20 mL of chloroform and 20 mL of methanol per g of biomass. The fact that the miniaturized Bligh−Dyer extracted 47.5% more fatty acids than the regular Bligh−Dyer may have occurred because in the latter method the solvent was saturated with oil.

The miniaturized Bligh−Dyer extracts were further subjected to separation of lipid classes using solid-phase aminopropylsilyl columns since a previous study (*11*) found that these columns, when used with suitable solvent systems, offered a rapid, efficient, and quantitative separation of microbial lipid classes (mono-, di-, and triglycerides). Analysis of the resultant fractions using a HPLC-ELSD confirmed miniaturized Bligh−Dyer fractions I, II, and III as 7.8% monoglyceride, 8.9% diglyceride, and 83.3% triglyceride (83.3%), respectively. Interestingly, transesterified miniaturized Bligh−Dyer Fractions were found to total 815.22 mg g^{-1} of fatty acids. The difference of 101.69 mg g^{-1} with the miniaturized Bligh−Dyer extract was possibly because the oil was further eluted with solvents (minimizing saturation) used to recover the miniaturized Bligh−Dyer fractions; thus, the transesterification of oil was slightly more efficient. Previously, several species of thraustochytrids were found to produce more than 50% of weight as lipids and more than 30% of DHA from the total fatty acids (*28*). Therefore, at >70% of biomass as lipids, *Thraustochytrium* sp. ONC-T18 has an impressive ability to not only produce prolific amounts of PUFAs but also sequester these fatty acids. **Figure 2** shows oil miscelles within the cells of *Thraustochytrium* sp. ONC-T18.

Furthermore, analysis of the major fatty acid components of the miniaturized Bligh−Dyer fractions (I, II, and III, as referred to mono-, di-, and triglyceride fractions, respectively) found that C14:0 (myristic acid), C18:0 (stearic acid), C18:1 (oleic acid), and C22:6 (n-3) (DHA) were to be triglycerides in their native form. C16:0 (palmitic acid), C16:1 (palmitoleic acid), and C20:5 (n-3) (EPA) were found to be dispersed throughout the three fractions. C20:3 (n-6) (eicosatrienoic acid), C20:2 (n-6) (eicosadienoic acid), C22:5 (n-3) (docosapentaenoic acid), and several other smaller components were found to be present in two of the three miniaturized Bligh−Dyer fractions (I and II). Interestingly, previous reports have shown that triglycerides constitute the final form of storage fatty acids present within these microorganisms, while mono- and diglycerides represent fatty acids currently in the process of being either formed or degraded (*6*). On average, both direct saponification methods (with ethanol and hexane:ethanol) and the miniaturized Bligh−Dyer method recovered the most DHA (**Figure 3**), at between 163.43 and 168.26 mg g^{-1} of dried biomass, with EPA recoveries consistent throughout between 4.34 and 5.86 mg g^{-1} (**Figure 3**). In addition, high amounts of myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), and oleic (C18:1) acids were extracted (54, 196, 123, and 81 mg g^{-1}, respectively) (**Figure 3**). In general, the miniaturized Bligh−Dyer extraction method proved to be one of the most efficient and simplest procedures to extract lipids from strain ONC-T18.

The lipid fraction from the acid Bligh−Dyer method was not recovered due to the consistent production and interference of a black residue. The production of this residue may in part have been due to the 5% TCA used in the initial solvent mix. A possible reason may have been that the protein content of strain ONC-T18 was enough to react with the TCA and promote precipitation. A previous report of a successful use of the acidified Bligh−Dyer method (*10*) was carried out using the archaebacterium *M. thermoautotrophicum*, which likely had a lower fatty acid and protein content that did not react with TCA. However, when experiments with less TCA (2 and 3%) were performed, the black precipitate continued to be formed. Therefore, results of total lipids from this method were deemed unreliable.

Results of this study highlighted the limitations in the direct transesterification technique. Specifically, direct transesterification was found to be 88% less effective in fatty acid recovery than both the direct saponification methods and the miniaturized Bligh−Dyer technique. The direct transesterification method involves a combined lipid extraction and acid transesterification process consisting of treatment with 3 mL of MeOH:HCl:$CHCl_3$ (10:1:1, v/v/v) at 90 °C for 120 min (*9*). Subsequently, to determine whether recovery efficiencies for direct transesterification could be increased, several variables were investigated. First, the effect of duration of acid transesterification incubated at 90 °C for between 10 and 240 min showed that the maximum production of FAMEs (392.88 mg g^{-1}) occurred at 120 min (**Figure 4**). Second, the possibility that solvent saturation with oil affected the recovery was investigated and found that indeed the total fatty acid recovery improved when the transesterification mixture increased from 3 to either 6, 9, or 12 mL (**Figure 5**). This indicates that the direct transesterification method extracted approximattley 78% more fatty acids when doubling the 3 mL of transeserification mixture that Lewis et al. (*9*) used. Further improvement after 6 mL did not occur. This change makes the direct transesterification an efficient method to extract fatty acids from *Thraustochytrium* sp. ONC-T18 (with the advantage of transesterifying fatty acids while being extracted from the biomass) with similar results to those observed with the direct saponification methods.

The cholesterol content in oils extracted from *Thraustochytrium sp.* ONC-T18 was approximately 1.3 ± 0.04 mg g^{-1} of oil ($n \geq 3$) , which is significantly lower when compared to fish oil (5−8 mg g^{-1}), egg yolk (16−17.5 mg g^{-1}), milk fat (3.4 mg g^{-1}), and butter (2.8 mg g^{-1}) and significantly high when compared to soybean oil (0.0079 mg g^{-1}) and olive oil (0.006−0.0075 mg g^{-1}) (*29*).

This research showed that *Thraustochytrium* sp. ONC-T18 produces high amounts of lipids (>700 mg g^{-1}) and DHA (>165 mg g^{-1}). Specifically, the direct saponifactions, modified direct transesterification (with 6 mL of transesterification mixture) and the miniaturized Bligh−Dyer methods, were found to be the most efficient procedures for extracting fatty acids from *Thraustochytrium* sp. ONC-T18. Overall, the miniaturized Bligh−Dyer extraction was found to be an easier method to perform with no requirement to drastically reduce the pH during the extraction. However, its use of a chlorinated solvent

(chloroform) may be inconvenient. Extraction outcomes also showed that ONC-T18, besides producing high amounts of DHA, produced significant amounts of myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), and oleic (C18:1) acids (54, 196, 123, and 81 mg g^{-1}, respectively). Furthermore, it was found that ultrasonic energy improved 24% of the fatty acid recovery over the traditional Bligh−Dyer method. Moreover, a procedure to separate mono-, di-, and tri-glyceride fractions from an extract of *Thraustochytrium* sp. ONC-T18, using aminopropylsilyl SPE columns, was evaluated and found to separate these lipid fractions effectively.

Finally, the significance of this work relates to the maximization of lipid extractions from *Thraustochytrium* sp. ONC-T18, which has the potential to be used as a source of oil with high concentrations of DHA (>165 mg g^{-1}). This oil, together with the fact that it contains relatively low amounts of cholesterol (1.3 mg g^{-1} of oil), may represent an alternative vegetarian source of PUFAs that could be used to fortify food products and provide beneficial human health effects (*1*) attributed to DHA consumption.

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