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RESEARCH ARTICLE

EXTRACTION AND EVALUATION OF EDIBLE OIL FROM *SCHIZOCHYTRIUM* SP. USING AN AQUEOUS ENZYMATIC METHOD

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KEYWORDS

antioxidant activity, aqueous enzymatic extraction, edible microalgal oil, fatty acid composition, physicochemical properties

HIGHLIGHTS

- Aqueous enzymatic extraction (AEE) is performed for oil extraction from *Schizochytrium* sp.
- AEE process is optimized by response surface methodology.
- Microalgal oil extracted by AEE has high contents of PUFA, tocopherols and phenolics.
- AEEO exhibits considerable antioxidant activity as compared with SEO.

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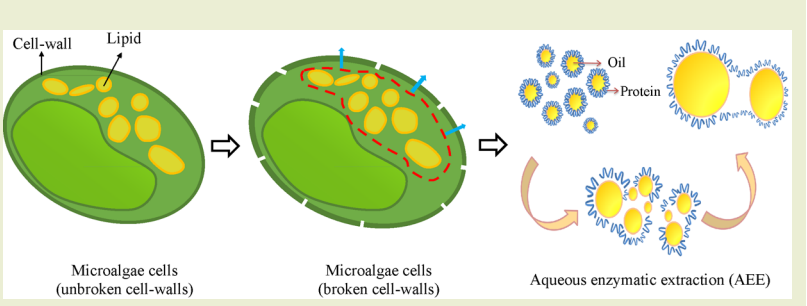
GRAPHICAL ABSTRACT



ABSTRACT

Schizochytrium sp., a marine microalga, is a potential source of edible oil due to its short growth cycle and rapid lipid accumulation, especially of docosahexaenoic acid. An approach to isolate edible microalgal oil from *Schizochytrium* sp. using aqueous enzymatic extraction (AEE) was developed. Parameters were optimized by single-factor experiments followed by Box-Behnken design. Proteases were effective in extracting oil. The maximum free oil recovery (49.7%±0.58%) and total oil recovery (68.1%±0.94%) were obtained under optimum conditions of liquid-to-solid ratio of 4.8:1, a 2.5% enzyme concentration of papain and an extraction time of 2.2 h. There was a significant difference ($P < 0.05$) in polyunsaturated fatty acid composition between microalgal oil obtained by AEE and by Soxhlet extraction, with the former having superior physiochemical properties and higher concentrations of bioactive components including total phenolic compounds and total tocopherols. These findings indicate a potential application of AEE for extraction of oil from *Schizochytrium* sp.

1 INTRODUCTION

Microalgae are photosynthetic microorganisms that convert carbon dioxide into nutrients such as lipids, proteins and carbohydrates[1]. Microalgae have been studied as potential alternatives to agricultural crops for edible oil production due to

their faster growth rate in aquatic environments and higher lipid yield[2,3]. Also, microalgae are potential sources of polyunsaturated fatty acids (PUFAs)[2]. Of the microalgae studied, *Schizochytrium* sp. is a desirable source of functional oil production because it can accumulate lipid exceeding half of the biomass during cultivation. Importantly, numerous studies show that *Schizochytrium* sp. is abundant in PUFAs, especially docosahexaenoic acid (DHA). DHA is an essential ω-3 PUFA that has positive effects on human health such as improving memory, supporting central nervous system development in infants, and preventing cardiovascular diseases associated with aging[4]. *Schizochytrium* sp. is potentially a source of oil with high DHA concentrations. In addition, Sahu et al.[5] found high concentrations of carotenoids, phenolic compounds and tocopherol in microalgae, indicating an enhanced antioxidant capacity of oil if those bioactive constituents can remain in the oil[6]. It is extremely valuable to develop an efficient method to extract oil from *Schizochytrium* sp. in order to obtain maximum lipid recovery while maintaining oil quality with a high oxidative stability.

Several technologies have been employed to extract oil from microalgae such as mechanical processing (e.g., ultrasonication and microwave), chemical extraction (e.g., organic solvents and ionic liquids) and biological methods (e.g., enzymatic treatment). Although organic solvent extraction is a common method for oil production[7], it is still not satisfactory for edible oil extraction because the residues and waste solvent may lead to environmental degradation and human health issues[8]. An approach to extract edible oil using water as the medium needs to be developed to address this problem. Aqueous extraction processing (AEP) seems to be more environmentally-friendly and healthy but it only achieves low oil recoveries due to emulsion formation. Qian et al.[9] reported that there was a significant increase ($P<0.05$) in released soybean oil after enzymatic hydrolysis (92.8%±0.2%) compared to that by AEP (56.7%±0.1%). In recent years there has been growing interest in using aqueous enzymatic extraction (AEE) for oil extraction due to its effectiveness and being an organic solvent-free system[10]. The extraction processing involves crushing, enzymatic hydrolysis and centrifugation[11]. The degumming step is unnecessary because phospholipids are separated from the oil in the AEE processing[12]. In addition, under the mild reaction conditions, oil produced by AEE may have an acceptable oxidative stability[13]. For example, an extraction temperature that is slightly higher than room temperature may protect PUFAs from oxidation and decomposition[11]. Based on these advantages, AEE has been investigated widely in oil production. Furthermore, extraction conditions have been optimized to obtain a higher oil recovery. Some different AEE conditions for various oil sources are listed in Table 1, indicating that the optimum conditions for AEE processing depend on several parameters.

Enzymes are essential for the disruption of lipid complexes (e.g., lipoproteins and lipopolysaccharides) during AEE processing, thereby facilitating lipid release. Studies suggest that protein is the main emulsifier forming an interfacial film on oil droplets to prevent oil release[22]. For example, Nguyen et al.[19] evaluated the activation of proteases and cellulase in the oil extraction process and papain-assisted demulsification was found to be more effective than cellulase, suggesting that protease may be considered a feasible option for demulsification. However, other studies report that carbohydrase (e.g., cellulase and hemicellulose) was also effective in AEE[23,24], indicating that

Table 1 Aqueous enzymatic extraction processing parameters for various plant sources

Plant species	Enzyme type	Water-to-sample ratio ($mL \cdot g^{-1}$)	Enzyme concentration (%, w/w)	Reaction time (h)	Total oil recovery (%, based on SE*)	Reference
Soybean	Protex 51FP	10.00	0.50	1.50	93.0	[14]
Peanut	Alcalase 2.4L	5.00	1.50	5.00	79.3	[15]
Sesame	Alcalase 2.4L	6.00	2.00	2.00	57.4	[16]
Bayberry	Cellulase/neutral protease (1:1, w/w)	4.91	3.17	4.00	49.4	[17]
Pine kernel	Alcalase endo-protease	5.00	1.97	3.00	89.1	[18]
Sacha inchi	Papain	4.45	4.46	4.95	95.6	[19]
Idesia polycarpa fruit	Cellulase/hemicellulose/ pectinase (1:1:1, w/w/w)	7.13	2.00	2.94	79.4	[20]
Yellow mustard flour	Protex 6 L	4.00	2.50	3.00	86.5	[21]

Note: *SE, Soxhlet extraction.

carbohydrase destroyed cell walls and might reduce emulsification to some extent.

The objective of the present study was to develop AEE for oil from *Schizochytrium* sp. The effects of various enzymes on free and total oil recoveries were compared and then extraction conditions of selected enzymes were optimized by response surface methodology (RSM). In addition, the physicochemical properties, bioactive compounds and antioxidant characteristics of *Schizochytrium* sp. oil obtained by AEE were compared with those of oil obtained by Soxhlet extraction (SE)[25].

2 MATERIALS AND METHODS

2.1 Materials

Schizochytrium sp. was provided as a lyophilized powder by Shandong Yuexiang Biology Technology Co., Ltd., Shandong, China; Alcalase 2.4 L (alkaline serine endopeptidase from *Bacillus licheniformis*, 2.0×10^5 U·g^{-1}, optimum pH 8.0, optimum temperature 50°C) was obtained from Novozymes (Novo, Shanghai, China); alkaline protease (2.0×10^5 U·g^{-1}, optimum pH 10.0, optimum temperature 50°C) was purchased from Beijing Solarbio Technology Co., Ltd., Beijing, China; and neutral protease (2.0×10^5 U·g^{-1}, optimum pH 7.0, optimum temperature 50°C), papain (2.0×10^5 U·g^{-1}, optimum pH 6.5, optimum temperature 50°C), and cellulase (2.0×10^5 U·g^{-1}, optimum pH 4.8, optimum temperature 40°C) were obtained from Nanning Pangbo Biological Engineering Co., Ltd., Nanning, China. All enzymes were obtained in powder form and all chemicals and reagents used were of analytical grade.

2.2 Soxhlet extraction procedure

Soxhlet extraction was conducted according to Chen et al.[25] with slight modification. Microalgal powders (5.0 g) were extracted using 100 mL of *n*-hexane in a Soxhlet apparatus fitted with a 0.15-L round-bottom flask and a condenser. The extraction was conducted at 85°C in a water bath for 6 h. After extraction, *n*-hexane was removed at 45°C under vacuum using a rotary evaporator (N-1100, Eyela Instrument Co., Ltd., Shanghai, China) and the oil obtained was stored at −20°C until analysis. An oil yield of 45%±1.3% was obtained by SE and used for comparison with the performance of the developed AEE.

2.3 Influence of different enzymes on oil recovery

The AEE process was conducted according to Yusoff et al.[26] with slight modification. The microalgal powder (2.0 g) was mixed with distilled water at 1:5 (w/v) in a screw-top plastic tube. The cell suspension was processed for 12 min at 630 W with an ultrasonic cell disintegrator (IID, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China). The pH was adjusted to the desired value for each enzyme according to the manufacturer's instructions using 0.1 mol·L^{-1} NaOH and 0.1 mol·L^{-1} HCl. Enzyme screening was carried out with each of five enzymes (alkaline protease, Alcalase 2.4 L, neutral protease, papain and cellulase) separately added to the suspension at concentrations of 3.0% (w/w, based on microalgal powder weight), followed by incubation at the desired temperature of each enzyme for 2.0 h. Extraction without the enzyme was used as a control (pH 6.5, 50°C). After incubation the tubes were heated to 95°C in a water bath for 10 min to terminate the reaction and cooled to room temperature. The hydrolysate was centrifuged at 10,000 r·min^{-1} for 15 min to obtain three distinct phases.

The free oil (upper phase) was carefully collected and the middle emulsion phase was decanted into a screw-top plastic tube, leaving the aqueous phase at the bottom. To separate the emulsified oil the emulsion obtained from each parallel experiment was added to 3.0 mL *n*-hexane followed by stirring for 5 min and centrifugation (4000 r·min^{-1}, 10°C) for 5 min. The upper layer was collected and the solvent was removed with N_2. The recoveries of free and emulsified oil are expressed as percentages (g·g^{-1}, based on extraction yield by SE) and the total oil recovery was computed as the sum of these components.

2.4 Single-factor experiments

Based on the data from preliminary experiments (data not shown), parameters were optimized within the following ranges. The liquid-to-solid ratio was adjusted to 3:1, 4:1, 5:1, 6:1 and 7:1, and the microalgal suspension was added 3.0% enzyme (w/w), which was then allowed to react at 50°C for 3.0 h. To determine the influence of the enzyme concentration on oil extraction a range of enzyme concentrations (1.0%, 1.5%, 2.0%, 2.5% and 3.0%) were added to the microalgal suspension (liquid-to-solid ratio 5:1) and the mixture was incubated at 50°C for 3 h. The effect of reaction time on oil extraction, was determined by mixing the suspension (liquid-to-solid ratio 5:1) with 2.5% enzyme (w/w) and incubating at 50°C for a range of reaction times (1.0, 1.5, 2.0, 2.5 and 3.0 h).

2.5 Response surface methodology

Papain was selected as the optimum enzyme for the RSM experimental design because it provided the highest oil recovery. Based on single-factor tests a three-level and three-factor Box-Behnken design was used to investigate the effects of parameters

(A, liquid-to-solid ratio; B, enzyme concentration; and C, reaction time) on oil extraction in the AEE process. The experiment comprised 17 measuring points including five central points (Table S1). The free and total oil recoveries were the response variables. A regression analysis was conducted to predict the response variable according to the following quadratic polynomial equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

where Y is the response function and β_0, β_1, β_2, β_3, β_{11}, β_{22}, β_{33}, β_{12}, β_{13} and β_{23} are the regression coefficients for intercept, and linear, quadratic and interaction terms, respectively. A, B and C are the independent coded variables. Design-Expert 8.0 (Stat-Ease, Inc., Minneapolis, MN) was used for selection of the optimum reaction conditions. The predicted results were verified by experiments in triplicate at the predicted optimum reaction conditions.

2.6 Physicochemical properties of the oils

Density was estimated by weighing a known volume of oil at 20°C. The acid, peroxide, iodine and saponification values of microalgal oils were determined using Chinese National Standards GB/T 5009.229-2016, GB/T 5538-2005, GB/T 5532-1995, and GB/T 5534-2008, respectively.

2.7 Fatty acid composition

The fatty acid composition of the microalgal oils was determined by gas chromatography. The fatty acid methyl esters (FAMEs) were prepared by base catalysis by the method of Mehanni et al.[27]. The FAMEs were analyzed using a 7890A gas chromatograph (Agilent, Santa Clara, CA) equipped with a flame ionization detector and a CD-2560 capillary column (100 m × 0.25 mm × 0.20 μm, Supelco, Shanghai, China). The split ratio was 10:1 and the injection volume was 1 μL. The carrier gas was helium at a flow rate of 0.5 mL·min^{-1}. The injector and detector temperatures were set at 250% and 260°C, respectively. The oven temperature was initially maintained at 130°C for 5 min, then increased to 240°C at 4°C·min^{-1} and held for 15 min. The FAME peaks were identified by comparing their retention times with those of a mixture of FAME standards. The amount of fatty acids was expressed as the relative percentage.

2.8 Determination of bioactive component concentrations

The concentration of total carotenoids was determined using the method of Franke et al.[28]. Microalgal oils (100 μL) obtained by AEE and SE were diluted in 3.0 mL acetone-petroleum ether (1:1, v/v). The absorbance of each solution was measured at 445 nm by UV-vis spectrophotometry (UV-1800PC, Mapada, Shanghai, China). The solvent without sample was a blank control. The concentration of total carotenoids was calculated as follows:

$$X = \frac{A \times y \times 10^6}{A_{1cm}^{\%} \times m \times 1000} \quad (2)$$

where X is the concentration of total carotenoids (mg·(100 g)$^{-1}$), A is the absorbance of oil at 445 nm, y is the solution volume (mL), $A_{1cm}^{\%}$ is 2500, which is the average absorption coefficient of carotenoids, and m is the weight of oil (g).

The amount of total phenolic compounds was determined using the Folin-Ciocalteu assay as described by Latif and Anwar[16]. Microalgal oils (1.0 g) obtained by AEE and SE were separately mixed with 4.5 mL 80% methanol, vortexed and centrifuged at 4000 r·min^{-1} for 5 min to collect the supernatant. The residue was re-extracted by the same procedure. The two extracted phases were pooled and the final volume was made up to 10 mL with 80% methanol as the test sample, of which 0.25 mL was reacted with equal volumes of Folin-Ciocalteu reagent. The mixture was mixed with 3 mL sodium carbonate solution (10%, w/v) and made up to 10 mL with distilled water. The sample was incubated for 20 min at 40°C. The absorbance was measured at 755 nm by UV-vis spectrophotometry (UV-1800PC, Mapada, Shanghai, China). Gallic acid was used to construct a calibration curve. The result of each sample is expressed as milligram gallic acid equivalent per kilogram oil.

The total tocopherol concentrations of the oils were determined using an HPLC method with UV detection (295 nm) according to the method of Ezeh et al.[29]. Microalgal oil (0.4 g) was accurately weighed and diluted in ethanol. The mixture was centrifuged at 5000 r·min^{-1} for 5 min followed by collection of the upper phase. This was conducted twice and the extracts were dissolved in ethanol to 10 mL. After ultrasonication for 15 min, the solution was filtered through a 0.22-μm organic filter membrane prior to injection. Detection was conducted with an HPLC system (Waters 2695, Waters, Milford, MA) using an Eclipse XDB-C18 (4.6 mm × 150 μm, 0.5 μm) chromatographic column. The mobile phase was methanol at a flow rate of 1 mL·min^{-1}. The column temperature was set at 30°C and the injection volume was 10 μL. The UV detector was set at 295 nm. The results are expressed in mg of α-tocopherol per kg of oil.

2.9 DPPH and ABTS radical scavenging assay

Oils extracted by AEE and SE were diluted to different

concentrations with ethanol. The ability of samples to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined in accordance with Vaisali et al.[30]. Diluted sample (3 mL) was mixed with an equal volume of DPPH solution (0.1 mmol·L^{-1}) and the mixture was incubated in the dark for 30 min at room temperature. After incubation the absorbance was measured at 517 nm using a UV-vis spectrophotometer (UV-1800PC, Mapada, Shanghai, China).

A 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay was conducted. A solution containing ABTS was prepared by mixing equal volumes of ABTS (7 mmol/L) and potassium persulfate (2.45 mmol·L^{-1}) and kept for 16 h in darkness at room temperature. The ABTS stock solution was diluted with ethanol until an absorbance of 0.70±0.02 at 734 nm was obtained. The oil solution (1 mL) was mixed with ABTS dilution (3 mL). After incubation for 30 min the absorbance at 734 nm was measured using a UV-vis spectrophotometer (UV-1800PC, Mapada, Shanghai, China). Percent inhibition of DPPH or ABTS radicals was calculated as follows:

$$\text{Radical scavenging activity}(\%) = \left(1 - \frac{A_i - A_j}{A_c}\right) \times 100\% \quad (3)$$

where A_i is the absorbance of the free radical solution with the diluted oil samples, A_j is the absorbance of the diluted oil samples in ethanol (without free radical solution) and A_c is the absorbance of the reaction without oil samples.

2.10 Statistical analysis

All experiments were conducted in triplicate and the results are expressed as mean±standard deviation. Differences between groups were tested by analysis of variance using the SPSS statistical software package version 22.0 (IBM, Armonk, USA). Pairs of mean values were compared by Duncan's multiple range test at the 5% protection level.

3 RESULTS AND DISCUSSION

3.1 Choice of appropriate enzyme

Five individual enzymes were screened for their effects on oil recovery. The free oil recovery extracted by AEE (Fig. 1(a)) was significantly increased ($P<0.05$) (14%±1.4% to 43%±0.9%) compared to the control (3%±1.0%). Also, the highest free oil recovery was obtained with papain, indicating that proteases were more effective in free oil recovery than cellulase, further suggesting that the protein-based membranes surrounding the oil droplets were the major obstacle limiting lipid release and coalescence[31]. Proteases can catalyze cleavage of peptide bonds, disrupt the viscoelastic interfacial protein film, and facilitate the solubility of proteins in the aqueous phase, thus enhancing oil release[31,32]. Compared with other experimental groups, papain also provided the highest emulsified oil recovery (20%±1.0%) and total oil recovery (62%±1.1%). Similar results were found previously by Niu et al.[33]. The accessibility of protease to hydrolysis sites affects the degree of hydrolysis[34]. Papain is a protease with low specificity and may exert higher accessibility than others examined here, thus hydrolyzing proteins to peptides more effectively[35]. Overall, papain was found to be the best enzyme for further optimization of oil extraction by AEE.

3.2 Effect of process parameters on oil recovery

Selection of an appropriate liquid-to-solid ratio is essential in AEE. The oil recoveries obtained when varying the water-to-microalgae ratio are shown in Fig. 1(b). When the ratio was increased from 3:1 to 5:1 the free oil recovery increased significantly ($P<0.05$) from 31%±1.5% to 43%±1.6% ($P<0.05$) and the emulsified oil recovery also increased significantly ($P<0.05$) from 15%±1.1% to 20%±0.9% ($P<0.05$). Therefore, the total oil recovery achieved a maximum value (63%±1.2%) at a liquid-to-solid ratio of 5:1. Oil recovery was significantly affected by the contact efficiency between papain molecules and protein molecules. The results indicate that viscous mixing hindered the effective penetration of enzymes and decelerated the hydrolytic reaction[36]. Increasing the ratio > 5:1 led to a gradual decrease in free oil recovery because excessive moisture contents reduced the interaction between the enzyme and substrate molecules by changing their concentrations. The optimum liquid-to-solid ratio of 5:1 was therefore selected.

An appropriate concentration of enzyme must be determined for AEE. As shown in Fig. 1(c), the free oil recovery increased with increasing enzyme concentration up to 2.5%. However, a decrease in the emulsified oil recovery was observed. The results may be attributed to a positive effect of the enzyme on the disruption of proteins. The maximum total oil recovery (67%±0.9%) was reached at 2.5% enzyme concentration. Under conditions of low enzyme concentration (< 2.5%) the protein was not hydrolyzed sufficiently, resulting in oil remaining in emulsion or cell debris[37]. However, a high enzyme concentration (> 2.5%) exacerbated the degree of emulsification and decreased the free oil recovery and this would increase production costs. Furthermore, excessive enzyme molecules

can compete with each other for contact sites with substances, leading to a reduction in oil recovery[19]. The optimum enzyme concentration was therefore selected to be 2.5%.

The effect of the reaction time range chosen on the oil recovery of microalgae was investigated (Fig. 1(d)). The free oil recovery increased significantly ($P < 0.05$) during the first 2.0 h, after 2.0 h the free oil recovery decreased slightly with longer reaction time. However, the emulsified oil recovery decreased when extending the reaction time to 2.0 h, with an increase in reaction time beyond 2.5 h, a slight increase in the emulsified oil recovery was observed. The total oil recovery was essentially maintained > 67% within 2.0–3.0 h. These results indicate that the enzymatic-hydrolysis time had a significant impact ($P < 0.05$) on the stability of oil-rich emulsions, leading to weaker stability with increasing reaction time. Wu et al.[38] found similar trends in AEE for soybean oil production. A short reaction time would lead to an insufficient hydrolysis. As the reaction proceeds, proteins are continuously degraded, thus promoting the instability of emulsions[39]. A longer reaction time might cause further emulsification between oil and substrates, decreasing the free oil recovery. In addition, excessive hydrolysis may produce some hydrophobic amino acids and small-molecular peptides, causing bitterness formation[40]. Considering oil recovery and quality, the reaction time range selected as 2.0–2.5 h.

3.3 Response surface analysis

The experimental data and responses for AEE based on RSM designs are presented in Table S1. The analysis of variance of free and total oil recoveries are shown in Table S2 and Table S3, respectively. The P values of both models were < 0.001, indicating that the models had high significance levels. The analysis of variance (Table S2) of the free oil recovery indicates that the coefficient of determination (R^2) of the model was 0.964, and the lack of fit was not significant with a P-value of 0.267 ($P > 0.05$). These data also indicate that all independent variables (A, B and C), one interaction term (AC), and three quadratic

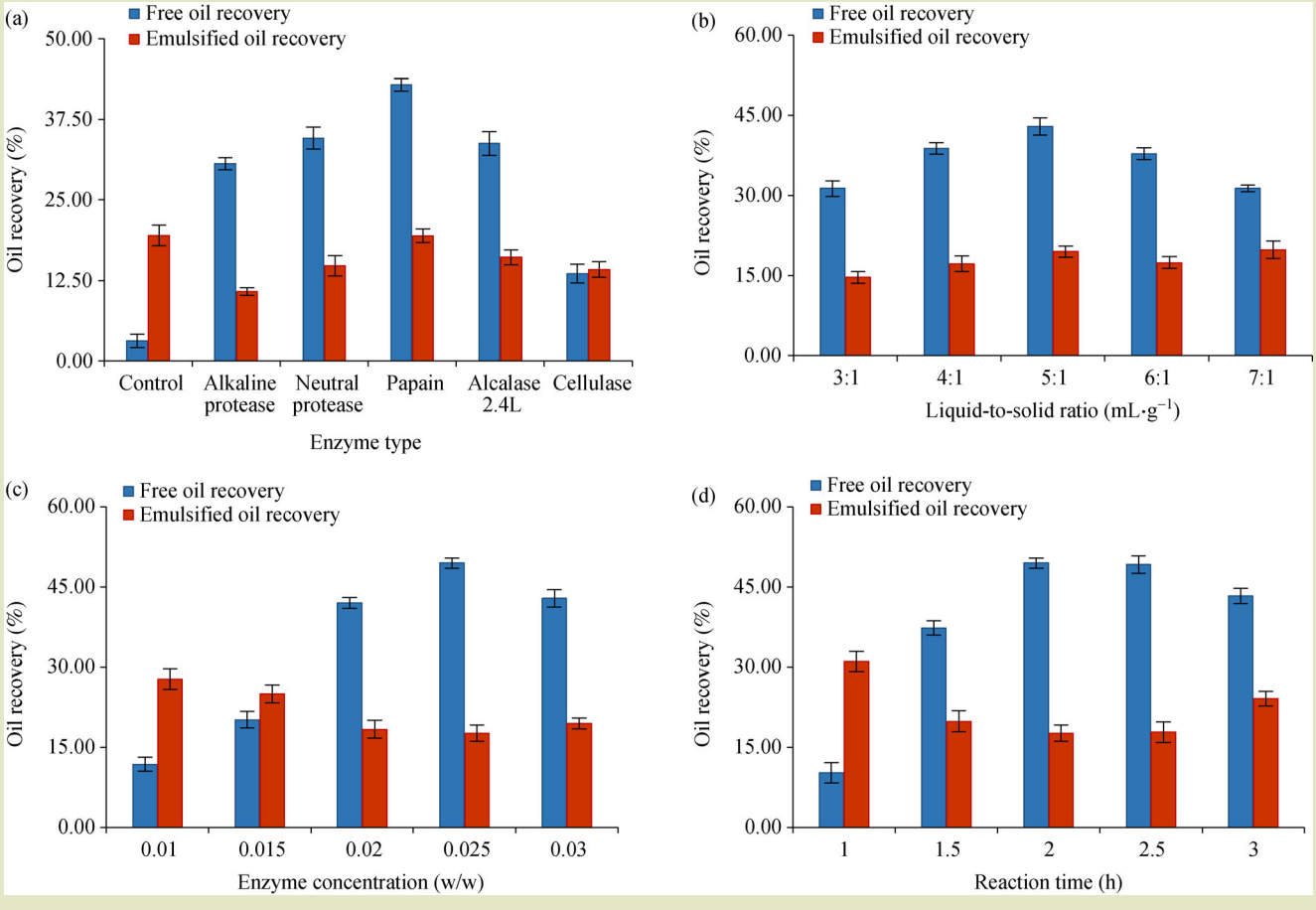


Fig. 1 Effects of (a) individual enzyme treatment, (b) liquid-to-solid ratio, (c) enzyme concentration, and (d) reaction time on the free and emulsified oil recovery by aqueous enzymatic extraction. Different letters above the bars indicate significant differences at $P < 0.05$.

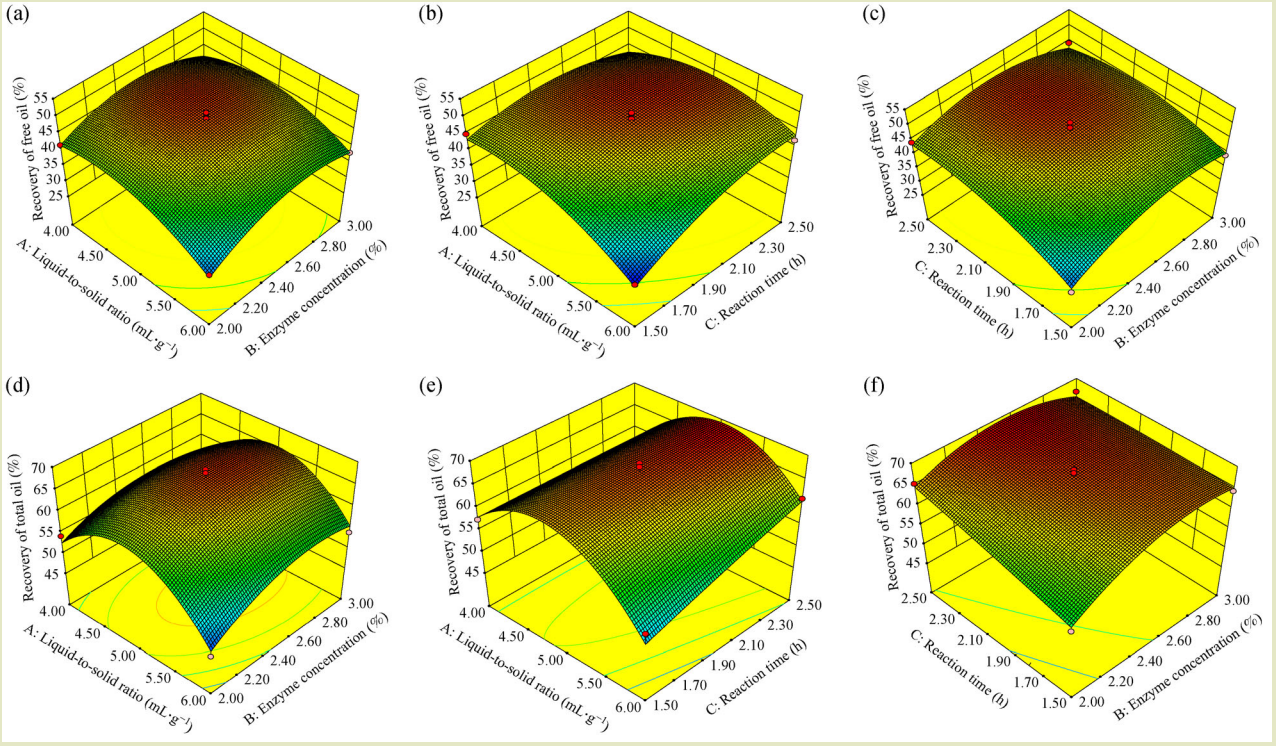


Fig. 2 Response surfaces for free oil recovery by aqueous enzymatic extraction (AEE): (a) varying liquid-to-solid ratio and enzyme concentration, (b) varying liquid-to-solid ratio and reaction time, and (c) varying enzyme concentration and reaction time. Response surfaces for total oil recovery by AEE: (d) varying liquid-to-solid ratio and enzyme concentration, (e) varying liquid-to-solid ratio and reaction time, and (f) varying enzyme concentration and reaction time.

terms (A^2, B^2 and C^2) had significant effects on the free oil recovery ($P < 0.05$). The analysis of variance (Table S3) of the total oil recovery also shows a desirable determination coefficient ($R^2 = 0.960$) and a nonsignificant lack of fit, with a P-value of 0.086 ($P > 0.05$). The quadratic terms of liquid-to-solid ratio (A^2) and enzyme concentration (B^2) had the largest effect on the total oil recovery, followed by the linear terms of enzyme concentration (B) and reaction time (C) and the interaction term of liquid-to-solid ratio and reaction time (AC). The quadratic equations obtained for the coded factors, used to calculate data for free and total oil recoveries, respectively, were as follows:

$$Y_1 = 48.71 - 3.51A + 1.82B + 3.60C + 1.52AB + 3.72AC - 2.12BC - 5.13A^2 - 5.34B^2 - 3.83C^2 \quad (4)$$

$$Y_2 = 67.23 - 1.11A + 2.51B + 2.28C + 1.42AB + 2.76AC - 1.91BC - 10.55A^2 - 4.07B^2 - 0.26C^2 \quad (5)$$

The response surfaces generated by the proposed models are shown in Fig. 2. Each figure presents the effect of interactions between the two independent variables on the recovery by maintaining the other variable at the intermediate level.

The effects of liquid-to-solid ratio and enzyme concentration on the free oil recovery at a constant reaction time (2.0 h) are shown in Fig. 2(a) and indicate that a liquid-to-solid ratio of about 4.8:1 and enzyme concentration of about 2.5% resulted in a high free oil recovery. As shown in Fig. 2(b), the free oil recovery increased with decreasing liquid-to-solid ratio and increasing reaction time, and these two parameters had a significant interaction ($P < 0.05$). As shown in Fig. 2(c) the free oil recovery increased when first increasing the enzyme concentration; however, increasing the enzyme concentration further > 2.5% led to a decrease in the free oil recovery.

As shown in Fig. 2(d), the highest total oil recovery was achieved using a liquid-to-solid ratio of ~ 5:1 and enzyme concentration of ~ 2.5% but the oil recovery was followed by a decrease when the liquid-to-solid ratio exceeded 5:1 and the enzyme concentration exceeded 2.6%. As shown in Fig. 2(e,f), at a given liquid-to-solid ratio or enzyme concentration the total oil recovery increased with extended reaction time initially but the recovery

remained almost stable with a further increase in reaction time.

According to the model the optimum experimental conditions for the predicted maximum free oil recovery (49.8%) were as follows: liquid-to-solid ratio 4.80:1, enzyme concentration 2.54% (w/w), reaction time 2.18 h. The theoretical maximum total oil recovery (69.3%) was obtained in the following conditions: liquid-to-solid ratio 5.08:1, enzyme concentration 2.54% (w/w), reaction time 2.5 h. For convenience of operation the optimal conditions were slightly modified as follows: liquid-to-solid ratio 4.8:1, enzyme concentration 2.5% (w/w), and reaction time 2.2 h. A free oil recovery of 49.7%±0.58% and total oil recovery of 68.1%±0.94% were obtained, confirming the validity of the constructed model.

3.4 Fatty acid composition

The fatty acid composition of microalgal oil extracted by AEE under optimum conditions and SE was determined. Oil prepared by AEE (AEEO) and by SE (SEO) had similar fatty acid composition and the dominant components were saturated fatty acids (SFAs) and PUFAs, indicating that AEE did not alter the fatty acid composition of oil, as shown in Table 2.

Palmitic acid (C16:0) was the highest concentration SFA in the oil, ranging from 27.9%±0.82% in AEEO to 31.1%±0.76% in SEO. Microalgal oil had abundant PUFAs, accounting for > 60% of the total fatty acids and may have an important role in the antioxidant activity of the oil[41]. Previous work also shows a higher concentration of PUFAs than SFAs in algal oil, similar to the findings presented here, indicating the merits of oil production from algae[5]. Notably, the concentration of PUFAs in AEEO was higher than that in SEO, while SFAs extracted by AEE were lower. This may be attributed to SE involving relatively high temperatures, thus leading to the oxidation and decomposition of PUFAs. Therefore, the mild extraction conditions of AEE were more efficient in extracting PUFAs than SE. These results are consistent with a previous study on the fatty acid composition of bayberry oil extracted by AEE[17]. DHA ranged from 60.4%±0.67% (SEO) to 64.2%±0.91% (AEEO) of the total fatty acids, the most prevalent PUFA in the microalgal oil. The results here reveal that AEE may enable the production of microalgal oil with high-concentration DHA. In summary, these results suggest the potential of *Schizochytrium* sp. to be used as a source of DHA oil.

3.5 Physicochemical properties

The physicochemical properties of AEEO and SEO are presented in Table 3. There were significant ($P < 0.05$) differences in the acid value (AV), peroxide value (PV) and iodine value (IV) between AEEO and SEO. However, the density and saponification values were not significantly different ($P > 0.05$). The AV of AEEO (3.26±0.05 mg·g^{-1} KOH, oil) was higher than of SEO

Table 2 Fatty acid composition (g·(100 g)$^{-1}$) of total oil by aqueous enzymatic extraction (AEE) and Soxhlet extraction (SE)

Fatty acid	Extraction method	
	AEE	SE
C14:0	0.77±0.02^{a}	0.81±0.02^{a}
C16:0	27.91±0.82^{b}	31.09±0.76^{a}
C18:0	1.48±0.04^{b}	1.64±0.02^{a}
C20:0	0.68±0.03^{b}	0.77±0.02^{a}
C22:0	0.58±0.03^{b}	0.75±0.01^{a}
Total SFA	31.42±0.94^{b}	35.06±0.75^{a}
C14:1	0.25±0.01^{b}	0.32±0.01^{a}
C18:1n9c	0.55±0.02^{a}	0.56±0.02^{a}
Total MUFA	0.80±0.03^{a}	0.88±0.03^{a}
C20:3n6	0.64±0.03^{a}	0.68±0.02^{a}
C20:3n3	0.84±0.01^{b}	0.92±0.03^{a}
C22:2n6	1.19±0.02^{a}	1.21±0.02^{a}
C20:5n3	0.89±0.02^{a}	0.90±0.02^{a}
C22:6n3	64.22±0.91^{a}	60.35±0.67^{b}
Total PUFA	67.78±0.96^{a}	64.06±0.72^{b}

Note: Values (mean±SD) in the same row with different superscript letters are significantly different ($P < 0.05$).

Table 3 Physicochemical properties, carotenoids, polyphenols, and tocopherols of total oil by aqueous enzymatic extraction (AEE) and Soxhlet extraction (SE)

Property	AEE	SE
Density (20°C) (g·mL^{-1})	0.92±0.01^{a}	0.92±0.01^{a}
Acid value (mg·g^{-1}, oil)	3.26±0.05^{a}	2.82±0.06^{b}
Peroxide value (mmol·kg^{-1}, oil)	0.69±0.03^{b}	4.48±0.14^{a}
Iodine value (g·(100 g)$^{-1}$ I_2, oil)	213.81±0.04^{a}	183.92±0.83^{b}
Saponification value (mg·g^{-1} KOH, oil)	196.84±0.63^{a}	194.35±0.78^{a}
Carotenoids (mg·kg^{-1}, oil)	21.03±1.20^{b}	36.24±1.30^{a}
Phenolic compounds (mg·kg^{-1}, oil)	561.84±7.31^{a}	490.15±7.02^{b}
Tocopherols (mg·kg^{-1}, oil)	219.98±9.84^{a}	203.11±3.61^{a}

Note: Values (mean±SD) in the same row with different superscript letters are significantly different ($P < 0.05$).

(2.82±0.06 mg·g^{-1} KOH, oil). Although the AV indicates that AEEO was more rancid than SE it was within the limits allowed for edible oils. Similarly, Balvardi et al.[42] found that the AV of oil extracted by AEE from wild almond was higher than that extracted with *n*-hexane. However, the opposite was found in a study by Hu et al.[10]. This difference may be due to various factors including crop genotype, enzyme type and process conditions. In contrast, a significantly lower ($P<0.05$) PV (0.69±0.03 mmol·kg^{-1}, oil) was observed of AEEO compared with SEO (4.5±0.14 mmol·kg^{-1}, oil). Microalgal oils rich in PUFAs were more prone to oxidative degradation under conditions of high temperature and long extraction time, so due care should be taken to choose a mild extraction method such as AEE to ensure quality oil. Also, some protein hydrolysate and small peptides obtained by enzymatic hydrolysis have higher antioxidant capacity which may contribute to the inhibition of oil oxidation[43]. Similar results for oils of different origin have been reported[11,12].

The IV of AEEO (213.8±0.04 g·(100 g)$^{-1}$ I_2, oil) was significantly higher ($P<0.05$) than for SEO (183.9±0.83 g·(100 g)$^{-1}$ I_2, oil). The fatty acid composition results presented in Table 2 confirm that the higher IV of AEEO was due to its higher PUFA concentrations, especially DHA. Saponification values of the oils were 194–199 (mg·g^{-1} KOH, oil), slightly lower than those of other vegetable oils such as coconut oil (250–261 mg·g^{-1} KOH, oil)[44] and palm oil (195–220 mg·g^{-1} KOH, oil)[45], indicating that oils here had a higher concentration of long-chain fatty acids than those oils.

Here, AEE were compared with SE for their extraction efficiency and process flow. The oil recovery obtained by AEE (68.1%±0.94%) based on oil yield of SE suggests its potential for edible oil production. It is clear that SE requires large amounts of organic solvents (20 mL·g^{-1}), long operation time (6 h) and high temperature (85°C)[11]. AEE may reduce the use of organic solvents and shorten the extraction time and temperature. These results suggest that AEE is a promising method for the extraction of microalgal oil considering its environmentally-friendly nature and mild conditions.

3.6 Total carotenoids, phenolic compounds and tocopherol concentrations

Carotenoids, phenolic compounds and tocopherols in edible oil are responsible for higher antioxidant activity[6]. As shown in Table 3, the carotenoid concentration of SEO was 36±1.3 mg·kg^{-1}, significantly ($P<0.05$) higher than that of AEEO (21±1.2 mg·kg^{-1}). This may be due to the higher solubility of carotenoids in *n*-hexane than in water. The AEEO was significantly ($P<0.05$) richer in total phenolic compounds (562±7.3 mg·kg^{-1}) than the SEO (490±7.0 mg·kg^{-1}). Furthermore, the total tocopherol concentration of AEEO was 220±9.8 mg·kg^{-1}, which is comparable with that of SEO (203±3.6 mg·kg^{-1}) (Table 3). These results may be attributed to the enzymatic process being beneficial for the release of tocopherols and phenolic compounds[11]. AEE may increase the retention of bioactive constituents in microalgal oil, thus strengthening the oxidation stability of the oil.

3.7 Antioxidant activity of oils

The antioxidant activities of AEEO and SEO were evaluated by

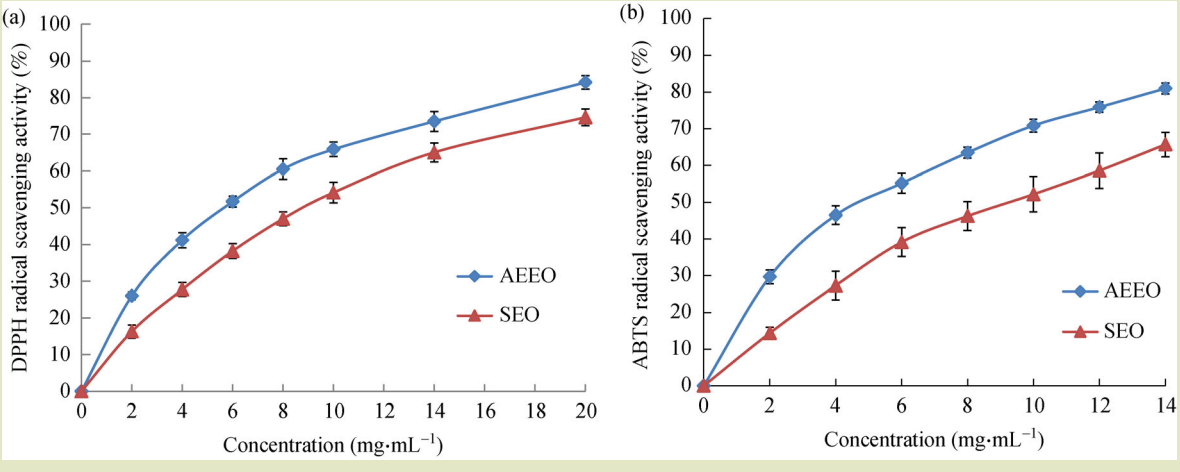


Fig. 3 Antioxidant activity of microalgal oils assessed by (a) DPPH radical scavenging assay and (b) ABTS radical scavenging assay.

DPPH and ABTS radical scavenging assays. As shown in Fig. 3(a), an enhancement in DPPH radical scavenging activity was observed with increasing oil concentration. AEEO had a significantly higher inhibition of DPPH free radicals (IC50 of 5.4±0.10 mg·mL^{-1}) compared to SEO (IC50 of 8.6±0.16 mg·mL^{-1}). As shown in Fig. 3(b), the inhibition of ABTS free radical of both oils appeared to have a dose-dependent relationship. AEEO also had a higher efficacy in scavenging ABTS radicals, resulting in a significantly lower IC50 (4.6±0.12 mg·mL^{-1}) than SEO (IC50 of 8.8±0.17 mg·mL^{-1}) ($P<0.05$). Therefore, AEEO has greater potential antioxidant properties than SEO. Greater release of the phenolic compounds and tocopherols contributed to enhancing the antioxidant activity of AEEO relative to SEO. Based on the results it is concluded that AEE is a promising method for edible oil extraction from microalgae due to the high antioxidant activities of AEEO and may be considered a health-promoting antioxidant in the human diet.

4 CONCLUSIONS

AEE was successfully used to separate oil from *Schizochytrium* sp. Parameters in experiments were optimized as signal-factors and RSM design and the optimum extraction conditions were a liquid-to-solid ratio of 4.8:1, enzyme concentration of 2.5% (w/w) and reaction time of 2.2 h. Under these conditions, the free and total oil recoveries were 49.7%±0.58% and 68.1%±0.94%, respectively. Compared with SE, AEE is more environmentally-friendly by eliminating the need for organic solvents. Furthermore, the mild operational conditions in AEE ensured higher retention of valuable PUFAs, tocopherols and phenolic compounds, resulting in a higher oil quality and a greater oxidative stability. Also, the physiochemical characteristics of AEEO indicate its suitability for human consumption. In summary, this study has shown that AEE may be a valuable method for edible oil extraction from microalgae such as *Schizochytrium* sp.

Supplementary materials

The online version of this article at https://doi.org/10.15302/J-FASE-2021400 contains supplementary materials (Tables S1–S3).

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Compliance with ethics guidelines

Zhaohui Xue, Fang Wan, Xin Gao, Wancong Yu, Zhijun Zhang, Jing Liu, and Xiaohong Kou declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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