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Enzyme-Assisted Aqueous Extraction of Lipid from Microalgae

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ABSTRACT: An improved lipid extraction process has been established for microalgal using enzyme-assisted aqueous extraction processing (EAEP), which mainly involved in sonication and enzyme treatment. As compared to cellulase, neutral protease and alkaline protease, significantly higher lipid recovery was achieved by snailase and trypsin. The highest lipid recovery of 49.82% was obtained by a combined sonication-enzyme treatment at pH 4. The enhancement mechanism of the EAEP was analyzed in terms of the particle size of cream and zeta potential. In addition, microalgal lipid recovery was also affected by lipid class composition and the type of algae. The present study demonstrates a promising alternative to conventional lipid extraction of microalgae and the quantitative information on EAEP of oleaginous alga can provide valuable data for process design at pilot and industrial scale.

KEYWORDS: *Microalgae, Enzyme-assisted aqueous extraction processing, Lipid extraction, Lipid recovery, Biodiesel*

INTRODUCTION

Microalgae are sunlight-driven cell factories that convert carbon dioxide to potential biofuels such as methane, biodiesel, and biohydrogen.[1] Although some of microalgae have been attempted for oil and biofuels production on a commercial scale,[2] the lipid extraction is still quite restricted as compared with other processes. By far, several extraction methods have been reported, including organic solvent extraction, subcritical water extraction, supercritical fluid extraction and aqueous extraction processing.[3−6] However, none of them are satisfactory. For example, organic solvent extraction is a simple method for extraction of lipids from microalgae, however, this method is gradually abandoned from the environment and safety point of view, since a great amount of organic solvent is used. As alternatives to organic solvent extraction, both subcritical water extraction and supercritical fluid extraction achieve higher selectivity and consume shorter extraction time. However, these methods are impractical for industrial process because of their higher energy consumption and cost.[7,8] Aqueous extraction processing (AEP) is an environmentally friendly approach using water to extract oil from oilseeds, but its low oil recovery is of great concern.[5]

Several assistant treatments have been attempted to increase the oil recovery of AEP. For example, flaking and extruding are adopted to rupture cell walls and facilitate flushing action of water, which is only suitable for dry seeds. In another way, enzyme-assisted aqueous extraction processing (EAEP) together with sonication provides improved efficiency of AEP by the combination of biochemical and mechanical treatments. In one hand, sonication employs high-frequency sound waves to destroy cell walls within a short period of time,[9] whereas on the other hand, the destroyed cell walls and lipid bodies can be further decomposed. EAEP has been employed to extract different compounds from plants, and has been proved to be effective in improving the yield of the target component.[10,11] By using EAEP, improved lipid extraction was observed in many different oil-bearing plant materials including soybean,[12] sunflower seeds,[13] and sesame.[14] In addition, EAEP will make it possible to extract and separate oil directly from algae in the natural aqueous environment of algae cultures, which avoids the collection and drying process of algae biomass. Although the cell wall of algae has similar structure and components as those of land plants, differences to exist. The cell wall of most algae is composed of cellulose, hemicellulose, and polysaccharides.[15] It has been shown that insoluble nonhydrolyzable biopolymers termed algaenans was detected in cell wall of some algae,[16−18] and made cell breakage a challenge. Thus, the EAEP methods established for common terrestrial plants can not be applied directly to the lipid extraction from microalgae and the detailed process should be carefully investigated. Unfortunately, there have been few reports on the lipid extraction from microalgae using EAEP combined with sonication up to now.

In this study, an efficient EAEP technique was developed for microalgae by using *Chlorella vulgaris* as a model system. The process was investigated by considering both the enzyme types and pH values. The underlying mechanism of EAEP was elucidated by cell microstructure and the particle size of cream. Finally, the established EAEP procedure was also verified in some other typical microalgae including *Scenedesmus dimorphus* and *Nannochloropsis* sp.

MATERIALS AND METHODS

Microalgae and Growth. Fresh cell of *Chlorella vulgaris*, *Scenedesmus dimorphus*, and *Nannochloropsis* sp. in paste form with 18% solid content were used in this study. All algae strains were kindly provided by Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences (Qingdao, China). *Scenedesmus dimorphus* was cultivated in BG11 medium,[19] whereas *Chlorella vulgaris* and *Nannochloropsis* sp. were incubated in F/2

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medium,[20] in a 2 L jar with a mixed air/CO_2 (99:1, v/v) gas aerated at constant rate of 120 μmol m^{-2} s^{-1}.

Enzymes. All the enzymes used in EAEP, including cellulose, snailase (a complex of more than 30 enzymes, including cellulose, hemicellulase, galactase, proteolytic enzyme, pectinase, β-glucuronidase, etc.), neutral protease, alkaline protease, and trypsin, were purchased from Xin-jing-ke biotechnology Co. (Beijing, China). The available information on the enzyme formulations used in the experiments was summarized in Table 1.

Table 1. Enzymes Used in Enzyme-Assisted Aqueous Extraction Processing[a]

enzyme type	activity	source	optimum temperature	Optimum pH
cellulase	2.9 u/mg	*Trichoderma*	55 °C	4.8
snailase	>90%[a]	Snail	37 °C	5.8
neutral protease	200 u/mg	*Bacillus subtilis*	50 °C	7.0
alkaline protease	200 u/mg	*Baclicus lincheniformis*	55 °C	8.5
trypsin	40 u/mg	Porcine pancreas	37 °C	8.0

[a]Breakage rate of yeast cells per gram that are hydrolyzed by 30 mg snailase at 37 °C for 1 h.

Quantification of Total Lipid in Microalgae. Algal cells were harvested by centrifugation at 8000 rpm for 15 min and washed three times with distilled water. After dried in a freeze drier, the samples were ultrasonicated for 30 min in 5 mL solution of chloroform/methanol (2:1, v/v). subsequently, 1 mL chloroform and 1.5 mL water were added to the sample.[21] The mixture was centrifuged at 4500 rpm for 10 min, the lower solvent phase was collected and evaporated in a rotary evaporator under vacuum at 60 °C. The resulting lipid was weighed and regarded as total lipid content. Thus, the lipid recovery in the EAEP procedures can be calculated on the basis of the total lipid content determined.

Lipid Fractionation. The lipid extract was fractionated into neutral lipid, glycolipids and phospholipids using a silica cartridges (Waters, Milford, MA), according to Damiani et al.[22] In this method, silica Sep-Pak cartridges (500 mg) were initially equilibrated with 10 mL methanol followed by 30 mL chloroform. Subsequently, 1 mL chloroform solution containing 20 mg lipid was applied to a Sep-Pak cartridge. The cartridge was eluted by 15 mL solution of chloroform/acetic acid (9:1, v: v) to collect neutral lipid, by 20 mL solution of acetone/methanol (9:1, v:v) to collect glycolipids and by 20 mL of methanol to collect phospholipids. Each content of neutral lipid, glycolipids and phospholipids was determined by weighing after dried in a rotary evaporator under vacuum at 60 °C.

Aqueous Enzyme-Assisted Processing. Each microalgae paste (~10 g fresh weight) was exactly weighed and placed in a 50 mL centrifuge tube. The sample was ultrasonicated with an ultrasonic cell disintegrator (JY92-2D, Ningbo Scientz Co,.Ltd., Zhejiang Province, China) at 600 W ultrasonic power, 4 s interval time/2 s ultrasonic time and 15 min total working time. The sample was kept in an ice water bath to avoid overheating. Before the specific enzyme was added, the mixture was preadjusted to the optimum pH value and preheated in a water bath to the optimum temperature, as indicated in Table 1. Various amounts of enzyme were added and incubated for a specific period of time. Subsequently, the reaction was stopped by heating in a water bath at 95 °C for 10 min to make enzyme deactivated. The hydrolysate was then centrifuged (4500 rpm, 15 min) to separate three distinct phases: oil phase, cream phase, and aqueous phase. Subsequently, 5 mL of hexane was added to the supernatant and the upper layer was collected. Finally, the weight of lipid was determined gravimetrically after the solvent was evaporated under vacuum at 60 °C.

pH Treatments. Initially, the pH of the cream (cream sequently treated by snailase and trypsin during the extraction step using the condition described above) was adjusted to the desired value by adding 2 mol/L NaOH or 2 mol/L HCl. Subsequently, the resulted cream was incubated at room temperature for 30 min with constant stirring. Following concentration at 4500 rpm for 15 min, free oil was collected.

Scanning Electron Micrographs. The samples obtained from different treatment methods were observed by scanning electron microscrope (SEM; S-4800, Hitachi High-technologies Corporation, Japan) after freeze-dried, fixed to a specimen holder and sputtered with gold.

Particle Size Distributions and Zeta Potential Measurements. Samples obtained after treatments and before centrifugation were used for droplet size distribution and zeta potential measurements. Both particle size distribution and zeta potential were analyzed by a DelsaNano C instrument (Beckman Coulter, Inc., Brea, CA). The samples were diluted at a 1:50 ratio in distilled water before the analysis. Each measurement was repeated three times at 25 °C. All samples were tested for at least three times and the obtained results were expressed as the average and standard deviation.

Statistical Analysis. All experiments were conducted with triplicate treatments. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's Honeatly Significant Differences Test. P value ≤ 0.05 was regarded as significant, and data were presented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Enzyme-Assisted Extraction of Lipid from *Chlorella vulgaris*. Microalgal cell wall is predominantly composed of cellulose, hemicellulose, and saccharides which hinder the release of intracellular lipids.[23] Microalgal lipid is stored in subcellular compartments called lipid bodies, lipid droplets or oleosomes. Lipid bodies are spherical organelles consisting of a neutral lipid core enclosed by a monolayer lipid membrane coated with proteins.[24] Thus, the extraction of lipid, to some extent, depends on the destruction of cellular and subcellular structure. However, pretreatment using sonication can only achieve 3.97% of the total algal lipid (data not show), indicating that this kind of physical treatment is insufficient. Based on this result, cellulase, snailase, neutral protease, alkaline protease, and trypsin were selected to facilitate the release of lipid from lipid bodies. As shown in Figure 1, when the alga was treated with 0.5% alkaline protease, the lipid recovery increased to 5.27%.

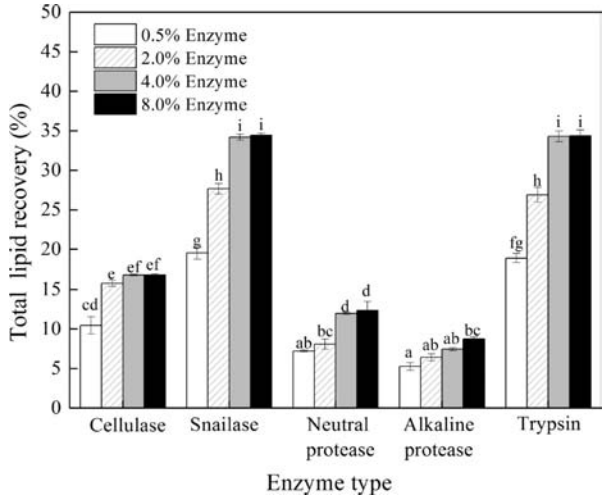

Figure 1. Effect of different enzymes on lipid recovery during enzyme-assisted aqueous extraction processing. Values are represented as mean ± standard deviations of three independent experiments. Identical superscripts indicate nonsignificant ($p \leq 0.05$) differences.

Higher lipid recovery was obtained by snailase and trypsin as compared to the other three types of enzymes. Snailase is a complex of cellulase, hemicellulase, pectinase, and β-glucuronidase, which makes the cellulosic component of cell wall degraded more effectively, whereas proteolytic enzymes could hydrolyze the proteins of membranes and cytoplasmic and modify the emulsifying capacity of some proteins that is related to the aqueous efficiency.[25] Trypsin, that attack basic residues (i.e., arginine and lysine),[26] is very effective in the hydrolysis of protein because these ionizable groups typically exist on the protein surface. As a result, when treated by snailase and trypsin, more lipid was released from microalgae.

Besides the enzyme types, the enzyme dosage and incubation time could also influence the lipid recovery. As shown in Figure 2, the lipid recovery was low when enzyme dosage is lower than

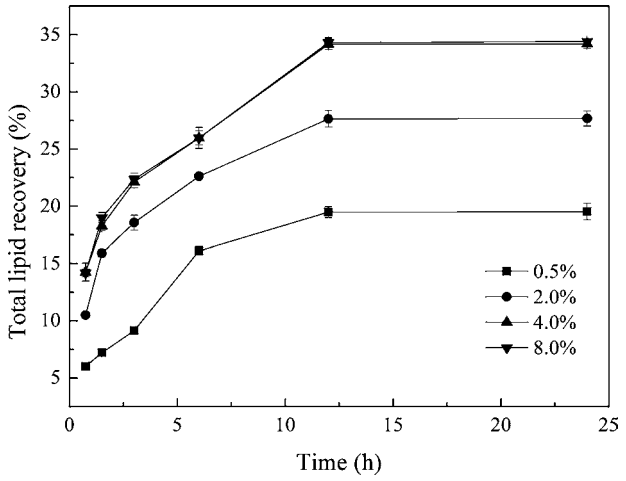

Figure 2. Effect of enzyme dosage on the total lipid recovered from *Chlorella vulgaris*. Values are represented as mean ± standard deviations of three independent experiments.

4%. When the enzyme dosage further increased, no remarkable improvement for lipid recovery could be observed. The lipid recovery also increased with reaction time, and reached a maximum value at 12 h in another aspect, when the enzyme dosage was lower than 2%, significant reduction of lipid recovery was observed irrespective of the incubation time.

In order to get an insight to the EAEP process for algal lipid recovery, the algae materials were analyzed by SEM. Untreated *Chlorella vulgaris* was used as a control, in which fully intact cell wall was observed with no signs of pitting or damage (Figure 3a). The sonicated cells showed breakdown of cell walls (Figure 3b) since the cells were believed to be mechnically broken by the oscillation and collapse of cavitations bubbles created by ultrasound.[27] In previous studies, different cell disruption methods, such as sonication, autoclaving, bead beating (bead diameter 0.1 mm), microwave and 10% NaCl solution treatments, have been tested for lipid extraction from microalgae.[28] It was found that sonication was the best for lipid extraction from microalgae. With the aid of sonication, the algal cells were further disrupted into pieces by snailase treatment (Figure 3c), indicating that the combination of sonication and snailase treatment was more efficient for the degradation of algal cells. Therefore, the effective cracking of the cell wall was a key to increasing the lipid extraction efficiency.

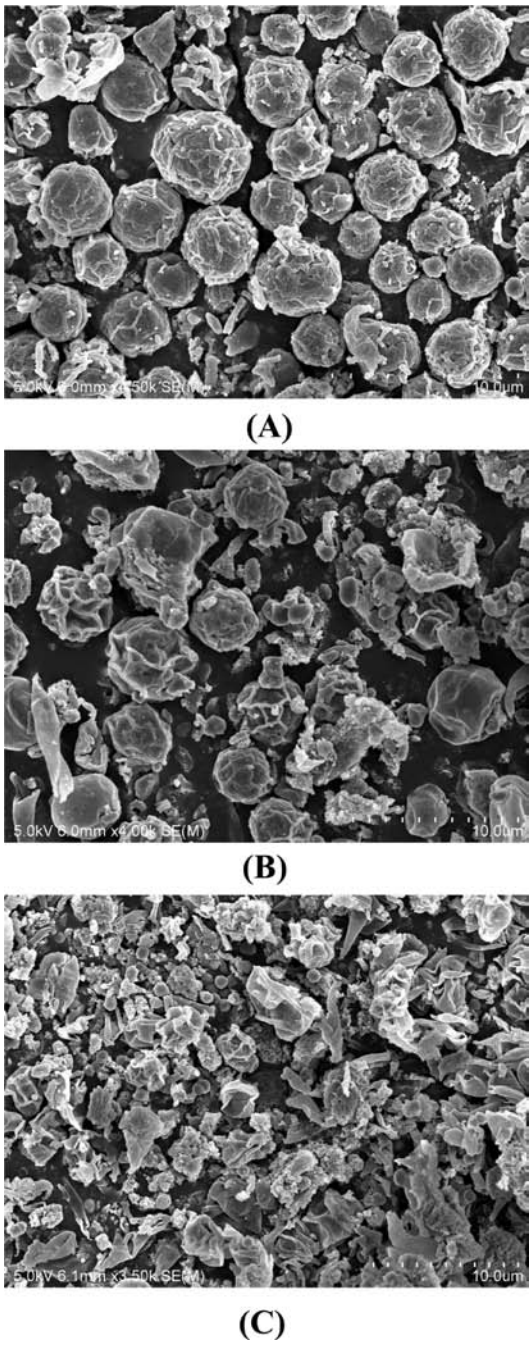

Figure 3. Scanning electron micrographs of *Chlorella vulgaris*. (A) untreated sample; (B) sonication treatment sample; (C) combination of sonication and snaillase treatment sample.

Influence of pH on Algal Lipid Recovery. Lipid recovery was found to be strongly dependent on pH value of the cream layer (Figure 4). By adjustment of pH value of the system, the lipid recovery can be increased from 39.82% at the initial pH of cream (~6.0) to 49.82% at pH 4.0. While further decreasing the pH value, slight decrease of lipid recovery was observed. This phenomenon can be interpreted by the destabilization effect of pH on the emulsion,[29] since electrostatic attraction between protein-stabilized emulsion droplets is particularly sensitive to pH value. The isoelectric point of algal protein was between pH 4.0 and 5.0 (data not shown). At pH near the isoelectric point of proteins the charge on the droplets is low and thus the electrostatic repulsion is weak, so droplets tend to aggregate.[30]

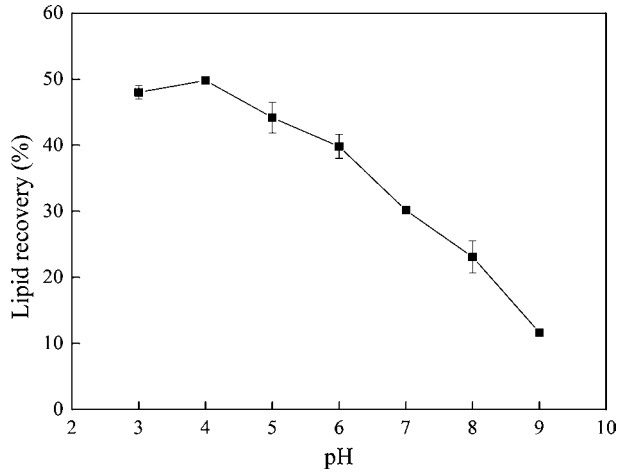

Figure 4. Effect of pH on the total lipid recovered from *Chlorella vulgaris*. Values are represented as mean ± standard deviations of three independent experiments.

Particle size distribution measurements indicated that the majority of particles in the cream were relatively small at pH values far away from the isoelectric point (Figure 5). At pH 9.0,

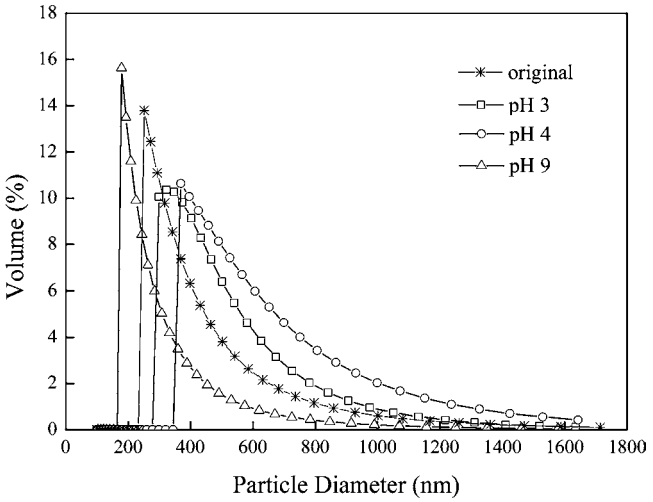

Figure 5. Particle size distribution profile of cream subjected to pH treatment.

the lipid droplet size was in the range from about 170−360 nm, and with the decrease of pH, the proportion of large lipid droplets increased. At pH 4.0, the majority of particles were distributed from 370 to 800 nm. By further decreasing the pH to 3.0, the particle size fell to the range of 300−670 nm. These results showed that larger particles size occurred at pH value closer to the pI of most of the algal proteins.

Application of EAEP to Other Microalgal Species. For *Chlorella vulgaris*, the lipid recovery by EAEP was 49.82% (Table 2), which was lower than that of soybean (90%).[31] This might be the result of different lipid class compositions between *Chlorella vulgaris* and soybean. *Chlorella vulgaris* contains only 66.88% neutral lipids (Table 2) whereas the soybean contains 88%.[32] Neutral lipid bodies are composed of a hydrophobic core of lipids surrounded by a monolayer of phospholipids,[33] and are much more easily extracted by EAEP. In contrast, glycolipids and phospholipids are polar hydrophilic molecules, which are relatively easy to be dispersed in aqueous phases. The levels of polar lipids were lower in the enzyme-extracted oil (Table 2). The lipid recovery from *Scenedesmus dimorphus* was about 46.81%, which was similar to that of *Chlorella vulgaris*. However, the lipid recovery of *Nannochloropsis* sp. was only 11.73% partly due to its lower content of neutral lipid. The difference of the lipid droplet size distribution during EAEP among different algal species was shown in Figure 6. The size of

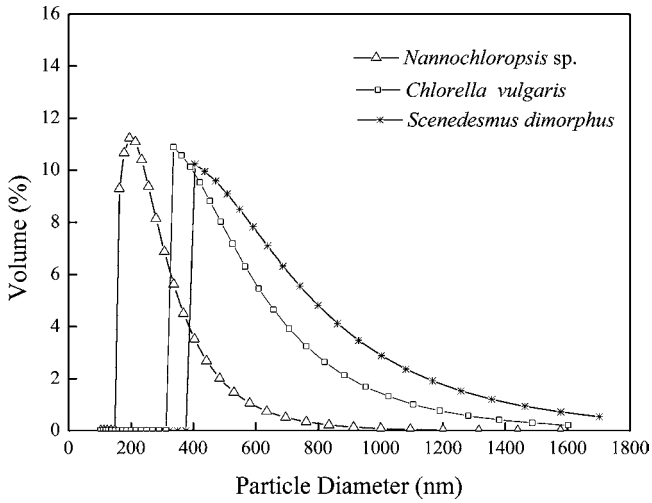

Figure 6. Particle size distribution profile of cream suspensions from different microalgal.

major oil droplet fraction was around 405, 336, and 194 nm for *Chlorella vulgaris*, *Scenedesmus dimorphus* and *Nannochloropsis* sp., respectively. In general, smaller emulsion droplets have greater kinetic stability and offer a more stable emulsion.[34] As per Stokes Law, larger emulsion droplets would have higher terminal velocities in aqueous medium during settling, which could result in rapid formation of a cream layer on the top part of EAEP derived mixture. Once the larger droplets are formed, they are more likely to coalesce.[35]

Table 2. Lipid Content, Classes and Recovery in Different Algal Species[a]

species	lipid content (% dw of cell)	lipid class distribution (% total lipid)			lipid recovery by EAEP (% total lipid)	lipid class distribution from EAEP (% enzyme-extracted lipid)		
		neutral lipids	glycolipids	;hospholipids		neutral lipids	glycolipids	phospholipids
Chlorella vulgaris	15.11	66.88	26.30	6.82	49.82 [a]	72.29	25.28	2.43
Scenedesmus dimorphus	10.62	65.09	31.75	3.16	46.81 [a]	71.51	27.47	1.02
Nannochloropsis sp.	15.98	52.95	44.20	2.85	11.73 [b]	60.57	38.51	0.92

[a]Values are represented as means ± standard deviations of three replicates. Identical superscripts indicate non-significant ($p \leq 0.05$) differences.

The subcritical water extraction method has also been applied to lipid extraction from the wet algal biomass. The yield of 100% was reported for wet algal biomass (70% moisture) at 60 °C, 30 MPa.[36] However, the application of high pressure in SWE method makes it much more complicated for industrial production at higher expense. Although the combination of chloroform and methanol has been reported as the best solvent for lipid extraction from *Synechosystis*,[6] the toxicity of chloroform and methanol is of great concern due to environmental considerations, especially for large scale production. Compared with these methods, less toxic solvent and mild operation conditions such as room temperature and atmospheric pressure were employed in current method. Furthermore, the procedures for ultrasonication prior to EAEP showed high extraction efficiency. Similar results were obtained in other plant materials (e.g., Soybean and *Jatropha curcas* L.[37,38])

In this study, an effective method was developed by the combination of EAEP with sonication for rupturing algae cell microstructure and extracting lipid from microalgae. Microalgal lipid recovery was affected not only by the process conditions such as enzyme type, enzyme dosage, incubation time, and pH but also by the lipid class composition and the type of algae. The highest lipid recovery of 49.82% was achieved by using EAEP. As mild-extraction technique and promising alternative to microalgal lipid extraction, the results obtained in this study are of great importance for the future development. The major drawback in proposed process is the cost of the enzyme. The enzymes may be used in an immobilized form to reuse them. Immobilization can make enzymes more economical since it facilitates separation of enzyme from product, allows reuse, and improves enzyme stability.[39,40] For example, stabilization of multimeric enzymes may be easily achieved using CLEAs (cross-linked aggregates) technology, and the rigidity of the enzyme will be usually higher using multipoint covalent attachment.[41] It should be better to immobilize enzymes on the surface of non porous nanoparticles/other carriers in order to be able to attack no insoluble structures of microalgae. Further work using the immobilized enzymes can be considered in enzyme-assisted aqueous extraction.

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Notes

The authors declare no competing financial interest.

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