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Optimization of lipid extraction from marine green macro-algae as biofuel resources

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Abstract−The extraction of lipid from *Enteromorpha intestinalis* was investigated. Among 13 types of organic solvent, the chloroform/methanol (2 : 1, v/v) system obtained the highest content. Considering the lipid extraction procedure and extracted amount, the optimal solid/liquid ratio was 1 : 50 (w/v). With increasing extraction temperature, the content was enhanced until 45 °C, after which it increased only slightly. Correspondingly, with increasing extraction time, the content linearly increased until 2.5 hr, after which it held steady. From a fatty acid compositional analysis of the lipid extracted from *E. intestinalis*, the content of palmitic acid and stearic acid was 39.85±1.19% and 14.61±0.80%, respectively. The unsaturated fatty acids (C18:1, C18:2 and C18:3) concentrations, meanwhile, were 0.3-3.2%.

Keywords: *Enteromorpha intestinalis*, Lipids, Bioenergy, Extraction, Operation Factor, Marine Macro-algae

INTRODUCTION

Marine macro-algae, seaweed, are categorized into three classes: red, brown and green. All grow at a faster rate and are more productive than land plants. Each differ in carbohydrate and lipid content and composition according to species, harvesting point, and time [1,2]. From marine macro-algae, many natural products are derived and utilized in the pharmaceutical and food industries [3,4]. In recent years, the research into macro-algae as a bioenergy resource has consistently expanded [1,2,5,6].

Enteromorpha intestinalis, a green-algae, is a genus in the family *Ulvaceae* within the order *Ulvales*. It is distributed over rocky coasts around the world. Included in the same genus are *E. compressa*, *E. prolifera*, *E. linza*, and others [4]. They are all used as food and feed in Korea, Japan, and other Asian countries due to their high carbohydrate, lipid, protein, vitamin, and inorganic-compound contents [3,4].

Useful functional compounds from macro-algae can be separated by hot water, chemicals (acid, alkali), organic solvents or an enzyme-treatment process [5,7,8]. Recently, several studies have investigated the separation from macro-algae of sugars and lipids for use as feedstocks in the production of bioenergy such as bioethanol, biobutanol, and biodiesel [1,2,5-8].

Lipid extraction focuses mainly on microalgae derived from fresh and sea water [1,5,6,9]. Traditionally, it is by soxhlet extraction using an organic solvent such as hexane, though recently, supercritical fluid has been introduced to the process. Another recent efficiency-enhancing innovation in lipid extraction is micro-algae pretreatment, either by autoclaving, bead-beating, sonication, osmotic shock, microwave, or other method [10-12]. Whereas many lipid extraction methods have been investigated for micro-algae applications [10, 11,13-16], relatively few have been evaluated for use with macro-algae [12,14,17]. Suganya and Renganthan [12] conducted kinetic studies on algal oil extraction from *Ulva lactuca* using autoclaving, bead-beating, ultra-sound, osmotic shock, and microwave pretreatment modalities. Kumari et al. [14] reported the results of a comparative evaluation and selection of a method for lipid and fatty acid extraction from macro-algae. Suganya et al. [17] obtained lipid by microwave pretreatment and soxhlet extraction with 1% diethyl ether and 10% methylene chloride in an n-hexane solvent system.

In this study, the effects of solvent kind, solid/liquid ratio, temperature, and time on lipid extraction from *E. intestinalis* were investigated to apply bioenergy resources. Additionally, the fatty acid compositions of the extracted lipids by gas chromatography were analyzed.

MATERIALS AND METHODS

1. Materials

Dried *E. intestinalis* was obtained from Wando (Jonnam, Korea). The dried biomass was ground and screened to a smaller than 100 μm size through a 140 mesh and kept in a sealed bag at room temperature. The extraction solvents were of analytical grade. Every chemical was used without further treatment.

2. Extraction of Lipids from *E. intestinalis*

Effect of extraction solvent: The effects of acetone, butanol, chloroform, diethyl ether, ethyl acetate, heptane, hexane, isopropanol, methylene chloride, pentane, petrolium ether, toluene, and chloroform/methanol (2 : 1, v/v), as solvent systems, were evaluated. The extraction procedure was performed for each solvent as follows. A 500 mg dry-powder sample and 10 mL extraction solvent were added to an extraction bottle and mixed at 500 rpm for 2 hr at 45 °C; the extract was separated by 10 min of 4,000 rpm centrifugation; the supernatant residue was dried and then weighted, preparatory to calculation of the lipid content.

The effect of the solid/liquid (S/L) ratio on lipid extraction was

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evaluated for each ratio as follows. A dry-powder sample (100, 200, 300, 500, 750, 1,000 mg) (S/L ratio range (w/v): 1 : 100-1 : 10) and 10 mL extraction solvent (chloroform/methanol (2 : 1, v/v)) were added to an extraction bottle and mixed at 500 rpm for 2 hr at 45 °C; the extract was separated by 10 min of 4,000 rpm centrifugation; the supernatant residue was dried and then weighted, preparatory to calculation of the lipid content.

The effect of extraction temperature was evaluated as follows. A 500 mg dry-powder sample and 10 mL extraction solvent (chloroform/methanol (2 : 1, v/v)) were added to an extraction bottle and mixed at 500 rpm for 2 hr at temperatures ranging from 35 to 65 °C; the extract was separated by 10 min of 4,000 rpm centrifugation; the supernatant residue was dried and then weighted, preparatory to calculation of the lipid content.

The effect of extraction time was evaluated as follows. A 500 mg dry-powder sample and 10 mL extraction solvent (chloroform/methanol (2 : 1, v/v)) were added to an extraction bottle and mixed at 500 rpm for times ranging from 0.5 to 6 hr, at 45 °C; the extract was separated by 10 min of 4,000 rpm centrifugation; the supernatant residue was dried and then weighted, preparatory to calculation of the lipid content.

3. Calculation of Lipid Content

The quantity of lipid extract in the biomass was calculated by the following equation:

Lipid content (%)=Dry weight of extracted lipid (g)
/Dry weight of biomass (g)×100.

4. Analysis of Fatty Acid Composition of Lipids

A compositional analysis of the extracted lipids was performed by acid-catalyzed esterification as follows: 10 mg of lipid sample and 1 mL of BF_3/methanol were inserted into a screw-cap glass tube, which was purged by nitrogen gas, and its cap replaced; the reactant in the tube was reacted in a heating block (100 °C) for 90 min and subsequently cooled to room temperature; 1 mL of distilled water and 2 mL of pentane were added to the reactant, which was vigorously vortexed for 1 min; after 2,000 rpm centrifugation, the supernatant sample was dried by nitrogen gas to remove the pentane, after which it was mixed with 50 μL of n-hexane, preparatory to gas chromatography analysis. The analyses, on standard lipid compounds C14:0, C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, and C22:0 (Sigma-Aldrich Co. Ltd. USA), were conducted on the YL6100 gas chromatograph (YoungLin Inc., Korea) using a fused silica capillary column (HP-INNOWAX (Polyethylene glycol, 30 m×0.32 mm×0.5 μm), Agilent Technologies, USA) and a flame-ionization detector with an injector temperature of 250 °C, an oven temperature of 210 °C, and a detector temperature of 250 °C [9]. The analysis of fatty acid composition of lipid was repeated three times. Also, results were stated as average value±standard deviation.

RESULTS AND DISCUSSION

Lipid extraction is influenced by both of the solubility of lipid compounds in solvent and the characteristics of the biomass [14]. Also, lipid extraction varies by solvent characteristics, the S/L ratio, extraction temperature, and extraction time [12], according to which parameters we evaluated lipid extraction from green macro-algae *E. intestinalis* in the present work.

1. Effect of Extraction Solvent

The solvent type is instrumental to the lipid extraction process. Extraction solvents are required to have certain properties such as high extraction capacity, low viscosity, high solubility with oil, and cost-effectiveness [12]. Hexane is the organic solvent most widely used to extract oil from micro-algae, macro-algae, heterotrophic microorganisms, and oil plants [11,12,14,17,18]. To obtain high lipid-recovery yields from biomass/solvent mixtures, the solvent should have a polarity sufficiently high for recovery of lipids from the relevant cell components; a too-high polarity of solvents, however, limits the solubilization of triacylglycerols and non-polar lipids [14,19].

In this study, the lipid content of *E. intestinalis* by the soxhlet extraction using hexane was 1.96±0.40%. Data on the effects of solvent on lipid extraction from *E. intestinalis* are presented in Fig. 1. As can be seen, 13 solvent types, including acetone, butanol, chloroform, diethyl ether, ethyl acetate, heptane, hexane, isopropanol, methylene chloride, pentane, petrolium ether, toluene, and chloroform/methanol (2 : 1, v/v), were evaluated. As a result of 500 rpm mixing for 2 hr at 45 °C, the highest lipid content, 2.39%, was achieved in the chloroform/methanol (2 : 1, v/v) solvent system. The other 12 solvents showed only low, 0.39-1.66%, content. The lipid content for acetone, for example, was approximately 1.43%; those for butanol, chloroform, isopropanol, and methylene chloride, meanwhile, were 1.66, 1.10, 1.47, and 1.05%, respectively; diethyl ether and ethyl acetate, 0.84 and 0.90%; heptane, pentane, toluene and petrolium ether, 0.39, 0.57, 0.67 and 0.47%; hexane, notwithstanding its wide utilization as an oil extraction solvent, showed the second lowest lipid content, 0.46%.

The lipid extraction yield or lipid content determined by solvent extraction depends on the properties of solvents. The polar-

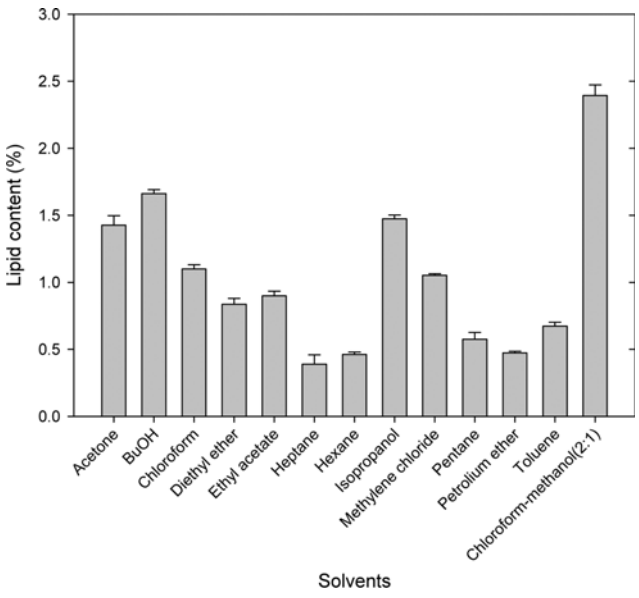

Fig. 1. Effect of solvent on lipid extraction from *E. intestinalis*. A 500 mg dry-powder sample and 10 mL extraction solvent were added to an extraction bottle and mixed at 500 rpm for 2 hr at 45 °C.

ity indices (or dielectric constant) of solvents are relative to that of water [20]. The lower polarity indices (or dielectric constant) indicate the higher non-polarities. The polarity indices (or dielectric constant) of hexane, chloroform and methanol are 0.1 (1.88), 4.1 (4.18) and 5.1 (33), respectively [20,21]. The highest content was obtained in the chloroform/methanol (2 : 1, v/v) solvent system due to their polarity index (or dielectric constant) of solvent. This solvent mixture presents the intermediate polarity. From the principle of solvent extraction, which is known as "like dissolves like," the lower extraction yield (or lipid content) of hexane indicated that the biomass contained small amount of non-polar lipids [20]. By the way, high yield (or lipid content) of chloroform/methanol (2 : 1, v/v) solvent system indicated the higher amount of polar and neutral lipids [20].

Comparable works, Suganya and Renganathan [12], by contrast, reported that in algal oil extraction from *U. lactuca*, an 8.53% content was obtained using n-hexane, a higher yield. Kumari et al. [14], having conducted a comparative evaluation of the Bligh and Dyer, Folch, and Cequier-Sanchez methods for quantitative determination of the total lipid and fatty acid contents of *U. fasciata*, *Gracilaria corticata*, and *Sargassum tenerrimum*, found that Folch's buffered solvent system achieved the highest total lipids yield from *U. fasciata* and *G. corticata*, while Bligh and Dyer's buffered system was optimal for *S. tenerrimum*. All of these results indicate that the selection of the proper solvent will accord with the species and its optimal extraction conditions. In the present study, because the highest extraction yield was obtained in the chloroform/methanol (2 : 1, v/v) solvent system, this was used as the extraction solvent in subsequent evaluations.

2. Effect of Solid/Liquid Ratio

High S/L ratios make for high viscosity, which hinders homogeneous mixing of the reactant as well as its handling [12,14]. Fig. 2 illustrates the effect of the S/L ratio on lipid extraction from *E. intestinalis*. The evaluation was conducted in the chloroform/methanol (2 : 1, v/v) solvent system for different S/L ratios (1 : 10-1 : 100 (w/v)) under the 500 rpm, 2 hr, 45 °C conditions. The lipid content was slightly increased by the increase of the S/L ratio. At the 1 : 10 S/L ratio, the lipid content was 1.96%; at the 1 : 100 S/L ratio, it was 2.64%. In consideration of both the extraction procedure and the extracted lipid amount, the 1 : 50 S/L ratio was selected for further analysis. Comparably, Suganya and Renganathan [12], evaluating algal oil extraction from *U. lactuca* for S/L ratios ranging from 1 : 3 to 1 : 7, found that by increasing the S/L ratio from 1 : 3 to 1 : 6, they could increase the yield from 9% to 10.88%. Above 1 : 6 (the optimum ratio), the increase was insignificant. Direct comparison among those studies is difficult, because lipid contents differ significantly by species, habitant, and season [1,2].

3. Effect of Extraction Temperature

In the oil extraction process, temperature is an important factor, as higher extraction temperatures incur higher operation costs [12,14]. Nonetheless, higher temperature enhances the dissolution capacity of solvents as well as the solubility and diffusion rate of solids [12,22]. Fig. 3 plots the effect of temperature on lipid extraction from *E. intestinalis*. In this evaluation, extraction was conducted again in the chloroform/methanol (2 : 1, v/v) solvent system for temperatures ranging from 35 to 65 °C under the 500 rpm, 2 hr conditions. By increasing the temperature to 45 °C, the lipid content was linearly increased, whereas after 45 °C, only slight increases were observed. Comparably, Suganya and Renganathan [12], evaluating algal oil extraction from marine macro-algae *U. lactuca* for temperatures ranging from 35 to 65 °C, found that by increasing the temperature from 35 to 55 °C, they could improve the extraction yield from 7% to 9.75%.

4. Effect of Extraction Time

In the oil extraction process, time is another important factor, as long reaction durations incur higher operation costs [12,14]. Fig. 4 plots the effects of time on lipid extraction from *E. intestinalis*. In the evaluation, extraction was performed once more in the chloroform/methanol (2 : 1, v/v) solvent system, but this time for the 0.5-

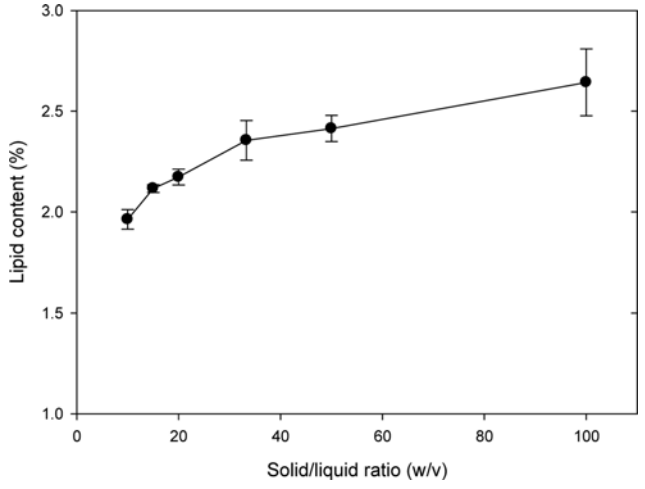

Fig. 2. Effect of solid/liquid ratio on lipid extraction from *E. intestinalis*. A dry-powder sample (100-1,000 mg) and 10 mL extraction solvent (chloroform/methanol, 2 : 1) were added to an extraction bottle and mixed at 500 rpm for 2 hr at 45 °C.

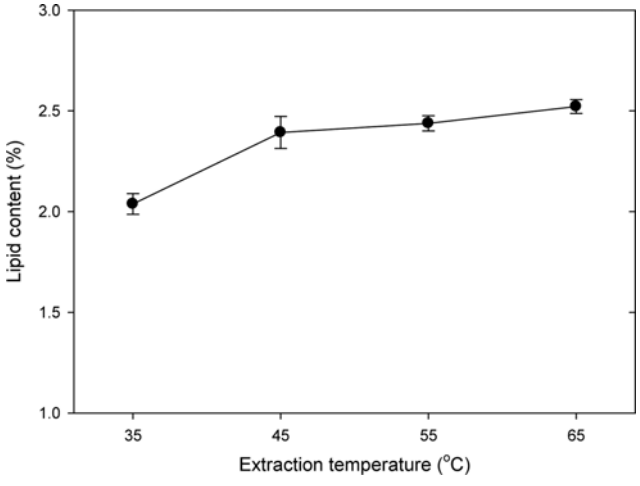

Fig. 3. Effect of extraction temperature on lipid extraction from *E. intestinalis*. A 500 mg dry-powder sample and 10 mL extraction solvent (chloroform/methanol, 2 : 1) were added to an extraction bottle and mixed at 500 rpm for 2 hr at temperatures ranging from 35 to 65 °C.

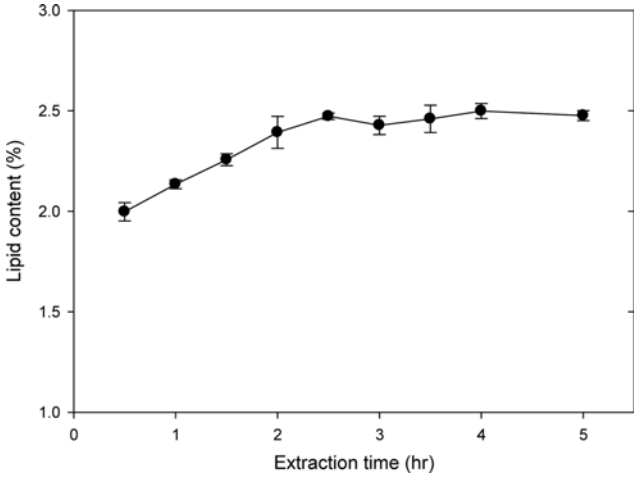

Fig. 4. Effect of extraction time on lipid extraction from *E. intestinalis*. A 500 mg dry-powder sample and 10 mL extraction solvent (chloroform/methanol, 2 : 1) were added to an extraction bottle and mixed at 500 rpm for times ranging from 0.5 to 6 hr, at 45 °C.

6 hr time range, under the 500 rpm, 45 °C conditions. The lipid content was linearly increased until 2.5 hr, at which point it was 2.47%. For durations over 2.5 hr, the lipid content was just maintained at the 2.4-2.5% level. Comparably, Suganya and Renganathan [12], evaluating algal oil extraction from *U. lactuca* within the 20-160 min time range, found that the yield increased as the reaction proceeded, but that beyond 140 min (the optimum extraction time), there was no significant increase.

Overall, in this study, the highest lipid content (2.47%) was obtained under the optimum condition of S/L ratio of 1 : 50 at 45 °C for 2.5 hr in chloroform/methanol (2 : 1, v/v) solvent system from lipid extraction of *E. intestinalis*. This may be due to the increase in the reactivity and solubility of chloroform/methanol (2 : 1, v/v) solvent system to enhance the extraction yield of polar and neutral lipids in biomass [12,20]. Also, due to high viscosity and density of reactant, which hinders homogeneous mixing, caused by higher S/L ratios [12,14], S/L ratio of 1 : 50 is optimized. Moreover, higher temperature enhances the solubility and diffusion rate of solvent into solids [12,22]. By the way, the decrease of extraction yield may be due to the evaporation of solvent in higher temperature. Comparably, Suganya and Renganathan [12], evaluating algal oil extraction from marine macro-algae *U. lactuca*, found optimum extraction condition of 5% moisture content, 0.12 mm particle size, 500 rpm stirrer speed, 55 °C extraction temperature, 140 min and S/L ratio 1 : 6 in 1% diethyl ether and 10% methylene chloride in an n-hexane solvent system.

5. Fatty Acid Composition of Lipid Extracted from *E. intestinalis*

The fatty acid compositions of the extracts of *E. intestinalis* extracted using chloroform/methanol (2 : 1, v/v) solvent system were analyzed by gas chromatography after acid-catalyzed esterification. As the Table 1 results indicate, the C16:0 (palmitic acid) and C18:0 (stearic acid) fatty acid concentrations were 39.85±1.19% and 14.61± 0.80%, respectively. The unsaturated fatty acid (C18:1, C18:2 and C18:3) concentrations, meanwhile, were low. Comparably, Suganya and Renganathan [12], on the basis of their fatty acid composition analysis of the algal oil extracted from *U. lactuca* after ultrasound pretreatment, reported saturated and unsaturated fatty acid content of 79.82% and 20.18%, respectively. Palmitic acid, notably, contained a maximum of 50.16% saturated fatty acid. Another study in this context is that of Martins et al. [15], who compared the fatty acid contents and compositions of algal oil extracted from *U. lactuca* by reference to different extraction methods. The highest oil extraction yields were specifically 2.49 mg/g-dw saturated fatty acid (mainly palmitic acid), 0.79 mg/g-dw mono-unsaturated fatty acid (mainly oleic acid), and 1.09 mg/g polyunsaturated fatty acid. Direct comparison among such studies is always problematic, because lipid contents and compositions differ significantly by species, habitant, and season. Nevertheless, this present work, like those that came before it and helped to guide it, can be considered to add appreciably to the knowledge base on the process of lipid extraction from marine resources for application of bioenergy production.

Table 1. Fatty acid compositions of lipids extracted from *E. intestinalis* by gas chromatography analysis

Fatty acid	Content (%)
C14:0	2.80±0.09
C16:0	39.85±1.19
C18:0	14.61±0.80
C18:1	3.22±0.86
C18:2	0.29±0.0
C18:3	1.08±0.06
C20:0	1.94±0.10
C22:0	3.12±1.12

Data are shown as content (%)±SD

CONCLUSIONS

The extraction condition of lipid from *E. intestinalis* was evaluated. The chloroform/methanol (2 : 1, v/v) solvent system enabled the highest lipid content. In the optimal condition of S/L ratio of 1 : 50 (w/v) at 45 °C for 2.5 hr, 2.47% lipid content was obtained. In the results on the lipid extracts' fatty acid compositions, the palmitic acid and stearic acid concentration was 39.85±1.19% and 14.61± 0.80%, respectively. Meanwhile, the unsaturated fatty acids (C18:1, C18:2 and C18:3) concentrations were low (0.3-3.2%). These results support marine macro-algae's valuable application to bioenergy production processes.

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