Organic solvent-free lipid extraction from wet *Aurantiochytrium* sp. biomass for co-production of biodiesel and value-added products

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**Abstract** Increasing demand for renewable energy has led to the production of biodiesel from microalgae. Microalgae have been regarded as one of the best feedstocks for biodiesel due to their high growth rate and lipid content as compared to other crops and plants. However, use of microalgae is still hindered by technical barriers and high processing costs. The economic viability and environmentally friendly aspects of biodiesel production will be significantly improved by co-producing value-added chemicals and extracting lipids directly from wet biomass without involvement of organic solvents, respectively. In this study, organic solvent-free lipid extraction from wet *Aurantiochytrium* sp. biomass was developed to co-produce biodiesel and value-added products such as docosahexaenoic acid. Organic solvent-free lipid extraction was performed by applying alkali and heat treatments to wet biomass. Key parameters for the extraction from *Aurantiochytrium* sp. were optimized to increase extraction yields. A ratio of 15 mg/mL of biomass to alkaline solution and an alkaline solution concentration of 1% w/w were determined to be the optimal conditions. Both heating temperature and treatment time were associated with positive effects on lipid recovery, up to a certain level. The maximum extraction yield (77.37% of total lipid) was obtained by heating at 150 °C for 30 min. Additionally, microwaves were employed to the extraction system and could further reduce the reaction time. Our study could be expanded to other types of microalgal biomass and will aid in establishing a protocol for organic solvent-free lipid extraction directly from wet biomass.

**Keywords** Biodiesel · Microalgae · Microwave · Organic solvent-free extraction

**Introduction**

Biodiesel is a fuel derived from the transesterification of biological sources such as animal fats and vegetable oils [1, 2]. Owing to limited availability of fossil fuels and increasing demands for energy, biodiesel has received considerable attention in recent years. Biodiesel is one of the most promising renewable energy sources due to relatively high biodegradability, low toxicity, and low emission profiles, as compared with petroleum diesel [1]. Microalgae are ubiquitous single cell organisms that are present in various habitats, from fresh water to marine and hypersaline environments [3]. Microalgae have been considered to be one of the best non-edible feedstocks due to the following distinct advantages: high oil productivity,
fast reproduction, sequestration of CO₂, wastewater treatment (uptake of nitrates and phosphates), high content of valuable co-products, and less land area use [4–7]. However, biodiesel production from microalgae is currently limited by several technical barriers and high processing costs. Particularly, autotrophic microalgae typically generate low biomass concentrations (0.1–1 g/L), leading to difficulties in harvesting cells [8]. In addition, the lipid content of autotrophs is relatively low as compared with that of heterotrophs. As an alternative to autotrophs, heterotrophic microalgae are regarded as a promising feedstock for biodiesel due to their high cell density and lipid content. As an example, *Chlorella protothecoides* was reported to have a cell density of 51.2 g/L and a lipid content of more than 50% of the dry biomass in a fed-batch heterotrophic culture system [9]. Recently, the heterotrophic thraustochytrids have been investigated for the potential of producing value-added products such as long-chain polyunsaturated fatty acid (PUFA). Many strains of thraustochytrids accumulate large quantities of fatty acids (50–80% of dry biomass) with a high proportion of PUFAs, particularly docosahexaenoic acid (DHA; 22:6n-3) and docosapentaenoic acid (DPA; 22:5n-6) [10]. According to Nagano et al. [11], the thraustochytrid *Aurantiuchytrium limacina* mh0186 has a high fatty acid content (466.5 mg/g dry cells), biomass yield (23.1 g/L), and DHA yield (4.3 g/L). DHA is an essential fatty acid for the normal functional development of the retina and brain, and therefore, it has been commercially utilized in dietary supplements for human health [12]. In addition to DHA production, *Aurantiuchytrium* sp. are considered a promising feedstock for biodiesel production due to their high level of fatty acids. Palmitic acid (C16:0), accounting for 46% of the total fatty acid content of *A. limacina*, has positive effects on the quality of microalgal biodiesel due to its high cetane number, low iodine concentration, and high oxidation stability [11, 1]. As a result, the thraustochytrid *Aurantiuchytrium* sp. have great potential for the co-production of biodiesel and value-added products. The co-production of high-value fatty acids will increase the economic viability of biodiesel production.

In order to utilize microalgal biomass (e.g., *Aurantiuchytrium* sp.) in biodiesel production, dewatering of microalgae must be performed, which is one of the main bottlenecks in algal harvesting [13], and 90% of the processing energy is consumed by oil extraction [14]. To improve the overall energy usage, it is necessary to decrease the energy consumption for drying the biomass. Furthermore, one of the conventional methods for oil extraction is to dissolve the intracellular lipids of a dry biomass using organic solvents. The Bligh–Dyer and Folch methods, which use chloroform and methanol solvents, are commonly used for organic solvent extraction as they have high extraction yields [15, 16]. However, recent concerns about the hazardous nature and environmental dangers of organic solvents have led to the urgent search for extraction techniques that are less toxic and use lower amounts of organic solvents, including supercritical fluid extraction and microwave-assisted extraction (MAE) [17–19]. In addition, cell disruption techniques have been applied to extraction processes, since during oil extraction, it is critical to break the rigid cell walls of the microalgae. Various methods such as microwaves, sonication, and bead-beating have been used to disrupt cell walls and increase extraction efficiency [20–22]. However, there has been no study that has established an efficient wet extraction method without using organic solvents with either autotrophic or heterotrophic microalgae.

In this study, lipid extraction, without using an organic solvent, from a wet *Aurantiuchytrium* sp. biomass, was thoroughly assessed for the co-production of biodiesel and value-added products. To the best of our knowledge, there has been no report on organic solvent-free extraction in the research areas of lipid extraction for biodiesel production. This organic solvent-free extraction system will significantly contribute to eco-friendly and cost-effective biodiesel production.

In this study, organic solvent-free lipid extraction from a wet *Aurantiuchytrium* sp. biomass was developed for the co-production of biodiesel and value-added products. This extraction system without an organic solvent will significantly contribute to eco-friendly and cost-effective biodiesel production. In addition, direct usage of wet cells eliminates the step for generating a dry biomass and, therefore, increases cost-effectiveness [14]. This organic solvent-free lipid extraction was performed using an alkali and heat treatment, which is a conventional method for protein hydrolysis. It has been reported that thraustochytrids such as *Schizochytrium aggregatum* and *Thraustochytrium* sp. contain 30–43% of their protein content within their cell walls, on a dry weight basis, while this value for green microalgae such as *Chlorella, Monoraphidium, Ankistrodesmus, and Scenedesmus* is 2–16% [23, 24]. Therefore, it appears that cell wall disruption using protein hydrolysis might be advantageous, especially for the thraustochytrids, which contain protein-rich cell walls. This study focuses on the optimization of extraction conditions, while investigating the following key parameters: biomass/solvent ratios, alkaline solution concentrations, heating temperature/time for conventional heating, and heating pressure/time for microwave heating.

**Materials and methods**

**Preparation of biomass**

The *Aurantiuchytrium* sp. strain KRS101, which was previously isolated from a mangrove ecosystem in Malaysia [25],
was obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea. The strain was maintained on agar plates containing B1 medium (1 g/L yeast extract, 1 g/L peptone, and 10 g/L agar in 1 L natural seawater containing 0.3 g/L penicillin G and 0.5 g/L streptomycin sulfate) [26], and colonies were cultured for 3 days in a 250 mL culture flask containing 50 mL of basal medium (60 g/L glucose, 10 g/L yeast extract, 9 g/L KH₂PO₄, and 15 g/L sea salt) at 25 °C with reciprocal shaking at 125 rpm. A 0.5-mL aliquot of each culture was incubated in 50 mL of the same basal medium at 25 °C with shaking at 125 rpm for 3 days. These incubated cells were then used as seed cultures for all cultures in this study. A 5-mL aliquot of each seed culture was inoculated into a 500-mL baffled flask with vented cap containing 195 mL of medium (40 g/L glucose, 10 g/L yeast extract, 9 g/L KH₂PO₄, and 2 g/L sea salt). Batch cultures were grown for 2 days at room temperature on a shaker at 200 rpm. The lipid productivities of the cultivated cells were summarized in Table 1.

Organic solvent-free extraction from wet biomass

Organic solvent-free lipid extraction from *Aurantiochytrium* sp. biomass was investigated using sodium hydroxide for cell wall disruption and lipid extraction. The harvested wet biomass was suspended in 10 mL of a sodium hydroxide solution, and the cell suspension was vigorously mixed with Vortexer for 5 min. The suspension was then transferred to a Teflon-capped glass tube and heated using a conventional heating block (Wise Therm HP-48, Wisd, Witeg, Germany). After heating, reactions were stopped using water quenching. The concentrations of sodium hydroxide were varied from 0 to 3% w/w to test the effects of sodium hydroxide concentration on the lipid extraction. The biomass to solvent ratio was assessed by varying the amount of biomass employed, while the volume of the sodium hydroxide solution was consistently 10 mL for all experiments. The effects of heating temperature and reaction time were investigated at various temperatures (90, 120, and 150 °C) over a period of 30 min. Phase separation was performed using centrifugation at 5000 rpm for 5 min. Extracted lipids were located in the upper portion of the solution, which separated from the aqueous alkaline solution. Disrupted cells were located between the thin lipid layer and the aqueous phase, and undisrupted cells were pelleted at the bottom. The lipid and aqueous phases were separated from the undisrupted cells and transferred to a new glass tube. This was done to avoid further extracting lipids from the undisrupted cells during the subsequent lipid isolation procedure. The extracted lipid phase was separated using cyclohexane and acetic acid. The homogenate was centrifuged at 4000 rpm for 5 min, and the upper cyclohexane phase was transferred to a pre-weighed tube. Organic solvents were evaporated under a stream of nitrogen gas, and the lipid residues were further dried in an oven at 80 °C for 30 min. After cooling to room temperature, the extracted lipids were weighed, and extracted fatty acids were quantified by fatty acid analysis. Lipid and fatty acid recoveries for each extraction condition were calculated using the following equations, respectively:

Lipid recovery (% total lipid) = \( \frac{\text{Extracted lipid (g dry cell weight)}}{\text{Total lipid (g dry cell weight)}} \times 100 \)

Fatty acid recovery (% total fatty acid) = \( \frac{\text{Fatty acid of extracted lipid (g dry cell weight)}}{\text{Fatty acid of total lipid (g dry cell weight)}} \times 100 \)

Additionally, MAE was performed using an MDS-2100 microwave system (CEM, Matthews, NC, USA), and the pressure was monitored inside PTFE microwave vessels. Cell suspensions, 100 mL in alkaline solution, were introduced into PTFE vessels and then subjected to the microwave system with 70% power (maximum power of 950 W). To determine the optimal parameters for MAE, pressure and heating time were varied. Mixtures were heated by microwaves at the given pressures and time periods.

Analysis of total lipid content

The harvested biomass was washed twice with phosphate-buffered saline, followed by freeze-drying for 2 days in

| Initial glucose (g/L) | Cultivation period (days) | Lipid productivity (g/L) | Lipid productivity (g/L/day) | Lipid productivity (g/L/day/g glucose) |
|----------------------|---------------------------|--------------------------|------------------------------|---------------------------------------|
| 40                   | 2                         | 9.95                     | 4.98                         | 0.124                                 |
| 60                   | 2                         | 11.53                    | 5.77                         | 0.096                                 |
| 80                   | 4                         | 14.15                    | 3.54                         | 0.044                                 |
pre-weighed tubes. The tubes were weighed, and the dried cell weight was calculated.

The total lipid content was determined using a modified Folch method [15, 16]. Chloroform–methanol (2:1 v/v) was added to the lyophilized biomass to extract lipid. The organic phase containing the extracted lipid was transferred to a pre-weighed tube, and the organic solvent was evaporated under a stream of nitrogen gas. The residual lipid was further dried in an oven at 80 °C for 30 min for complete removal of the organic solvent. After cooling to room temperature, the lipid residues were weighed to estimate total lipid content. The total lipid content was determined using the following equation [25]:

\[
\text{Total lipid (% dry cell weight)} = \frac{(W_2 - W_1) \times V_1}{W_b \times V_2} \times 100
\]

where \( W_1 \) is the weight of an empty tube (g), \( W_2 \) is the weight of a tube with dried lipid residue (g), \( W_b \) is the weight of the biomass sample (g), \( V_1 \) is the total volume of chloroform added (mL), and \( V_2 \) is the volume of chloroform transferred to the empty tube (mL).

**Fatty acid analysis**

The extracted lipids were transesterified to yield fatty acid methyl esters (FAMEs). Methanol and sulfuric acid were added to each extracted lipid sample, and transesterification was allowed to proceed at 105 °C for 20 min. After the reaction, 2 mL of deionized water was added, and the organic phase was separated by centrifugation at 4000 rpm for 5 min. FAMEs in the organic phase were analyzed using a gas chromatograph (HP5890, Agilent, Santa Clara, USA) equipped with a flame ionized detector (FID) and INNOWAX capillary column (Agilent, 30 m × 0.32 mm × 0.5 μm).

The temperatures of the injection port and detector were 250 and 280 °C, respectively. The GC column temperature profile was as follows: (1) initial temperature 50 °C for 1 min; (2) increased to 200 °C at 15 °C/min, held for 9 min; and (3) increased to 250 °C at 2 °C/min; held for 2 min. The fatty acids were identified by comparing the retention times with those of standard fatty acids (37 Component FAME Mix, Supelco) and quantified using the peak areas on the chromatogram with C19:0 fatty acid (nonadecanoic acid) as an internal standard.

**Results and discussion**

**Organic solvent-free lipid extraction using sodium hydroxide**

The ability to use sodium hydroxide for lipid extraction from a biomass by disrupting biomass cell walls was assessed. The sodium hydroxide solution plays a role not only as a reactant for cell wall disruption but also as a solvent for extracted lipid in this organic solvent-free extraction. The effect of sodium hydroxide concentration on lipid extraction was examined using a 15 mg/mL biomass/solvent ratio and heating at 90 °C for 20 min (Fig. 1). Concentrations of sodium hydroxide were varied from 0 to 3% w/w. When sodium hydroxide was not added (0% w/w), approximately 7% of the total biomass lipid was extracted. This indicates that heat treatment, in the absence of alkali, could extract lipid from the biomass, but the effect was insignificant for cell disruption and lipid extraction. However, the addition of sodium hydroxide increased the lipid extraction yield drastically, and a lipid extraction yield of approximately 50% was obtained using the solution with 1% w/w sodium hydroxide. The extraction yields of fatty acids followed the same trends as lipid extraction. However, recovery of fatty acids was lower than that of lipid, which was clearly evident for DHA. The decreased yield of DHA might be the result of using low polarity cyclohexane during the separation step for fatty acid quantification, which is not a part of the extraction process itself. Cyclohexane might be too hydrophobic to dissolve polyunsaturated fatty acids such as DHA from the sodium hydroxide solution.

The lipid extraction yields increased with increasing sodium hydroxide concentration (up to 1%), while they decreased at higher alkaline levels (over 1% w/w). Low quantities of sodium hydroxide might not efficiently hydrolyze the protein of cell walls because sodium hydroxide is not sufficient to react with whole protein. On
the other hand, the lower extraction yields at higher alkaline levels are most likely caused by emulsions and soaps that are formed by the interaction between base and organic matter, which could prevent oil extraction from cells [27, 28]. It has been reported that surfactant agents such as soaps and proteins facilitate and stabilize emulsions [27]. The formation of emulsions was observed during experiments, and the amount of emulsions increased as the concentration of the basic solution increased (at alkaline levels of 2–3% w/w). Additionally, the hydrolysis reaction and lipid extraction can be hindered by the presence of various organic compounds extracted from cells at higher alkaline conditions [29]. Thus, optimal concentrations of alkaline solution are advised to supply sufficient alkali for advancing the hydrolysis reaction and lipid extraction, while forming minimal emulsions or soaps.

**Effect of the biomass to solvent ratio**

For large-scale production, increasing the amount of biomass per the amount of required solvent is beneficial for solvent separation and recovery during downstream processes [30, 31]. However, a high biomass to solvent ratio might also have a negative impact on lipid extraction due to insufficient supply of sodium hydroxide, which might lead to low hydrolysis and extraction yields. Therefore, the effect of the biomass to solvent ratio on lipid extraction was examined using three ratios of dry biomass to sodium hydroxide solution: 10, 15, and 20 mg/mL biomass/solvent. The lipid extraction reaction was consistently conducted at 90 °C over a period of 30 min.

Figure 2 illustrates the lipid recoveries at different biomass to sodium hydroxide solution ratios. The lipid extraction yields increased with incubation for 20 min. After heating for 20 min, lipid recoveries for the 10, 15, and 20 mg/mL biomass/solvent ratios reached maximum values of 49.32, 50.56, and 47.12% of the total lipid, respectively. The biomass to solvent ratio affected lipid recovery, particularly at the early stage of the reaction. After 5 min, lipid recovery was higher at the lower biomass/solvent ratio. However, the effect of the biomass to solvent ratio was minimized after 20 min, when the highest lipid extraction yields were observed for all biomass/solvent ratios. A lower biomass to solvent ratio could increase the contact area between the sodium hydroxide solution and the biomass, which would lead to a faster reaction and a higher lipid yield at the beginning of the reaction. On the other hand, the higher biomass to solvent ratio decreased the reaction between the biomass and solvent. The higher biomass to solvent ratio could also increase the contact within the biomass itself, which might result in the retardation of hydrolysis reactions at the early stage of the reaction. However, as the reaction time increased, there was a greater increase in lipid recovery for the higher biomass to solvent ratio, which was observed between 5 and 10 min. Overall, a similar lipid recovery was obtained for all biomass/solvent ratios after 20 min. In the previous section, a low concentration of sodium hydroxide affected the lipid yield from the biomass due to an insufficient reaction. The amount of sodium hydroxide (1% w/w) appeared to be sufficient for all biomass/solvent ratios used in this study. Therefore, the biomass to solvent ratio would not affect the reaction rate when sufficient amounts of reagent and heating energy are applied to the system.

**Effect of temperature**

The effect of temperature on lipid extraction was studied using a 1% w/w sodium hydroxide solution and a 15 mg/mL biomass to solvent ratio, as identified in the previous results. The biomass solution was heated at various temperatures (90, 120, and 150 °C) over a period of 30 min. Lipid recovery increased as heating time increased, and higher temperature yielded higher lipid recovery (Fig. 3). The highest lipid extraction yield (77.4%) was obtained at 150 °C after 30 min.

We compared our results with previous publications to evaluate the feasibility of this system. Direct comparison was not possible, since to our knowledge, no studies have been published on organic solvent-free lipid extraction
from microalgae, particularly with thraustochytrid species. Instead, the comparison was made to a report on saponification reactions with ethanol, and our lipid extraction yield was quite comparable to this of study: a yield of 75% from lyophilized microalga *Porphyridium cruentum* bio-

mass using direct saponification with ethanol (96% v/v) containing potassium hydroxide and 87% from wet microalga *Phaeodactylum tricornutum* [27]. The maximum fatty acid yield of this study is at a competitive level when taking into account the cost and toxicity of chemicals. Although further analysis will be required to establish the economic feasibility of the extraction process, the largest lipid yield, identified with heating at 150 °C for 30 min, indicates there is great potential in using bulk wet biomass to develop alternatives to conventional lipid extraction methods.

**Comparison between conventional and microwave heating**

A microwave system was applied to the process of organic solvent-free extraction to compare extraction yields between conventional (heating block) and microwave heating. To determine the optimal conditions of the microwave system, pressures and heating times in the microwave system were varied in the range of 10–30 psi and 1–10 min, respectively. Shorter heating times were selected for microwave irradiation than those for conventional heating (5–30 min), since microwaves could reduce the reaction times and consequently increase heating rates compared with the conventional heating process. Other than the pressure and time period, the other extraction conditions for MAE were set to be equal to those for conventional heating. Specifically, the optimal extraction conditions, a 15 mg/mL biomass to solvent ratio and 1% w/w of sodium hydroxide solution, were consistently utilized as with the conventionally heated extraction.

Figure 4 shows lipid, fatty acid, palmitic acid, and DHA recoveries using microwave system at different pressures and heating time periods. Palmitic acid recoveries were slightly higher than DHA recoveries for all pressures and heating times. On the other hand, contrary to conventional extraction, extraction yields decreased as the pressure applied increased, for the majority of the heating times. These results indicate that the higher pressure of the microwave system has negative impacts on lipid extraction, which was not observed in conventional organic solvent-free extraction. Additionally, the heating time of microwaves appeared to have modest effects on lipid yields, while those of conventional heating showed positive impacts on lipid yields for all temperatures. Consequently, using microwaves could be advantageous, since maximum results can be obtained within a minute. The highest lipid, fatty acid, palmitic acid, and DHA recoveries for the microwave system (57.09% total lipid, 55.99% TFA, 57.18% total PA, and 50.97% total DHA, respectively) were obtained with heating at 20 psi for 6 min. Maximal lipid yields with microwaves were lower than those for conventional heating (77.37% total lipid, 75.11% TFA, 75.43% total PA, and 68.98% total DHA). However, maximum extraction levels using microwaves were comparable to the extraction yields of using a heating block at temperatures below 150 °C (e.g., 61.43% total lipid with conventional heating at 120 °C for 30 min). It is evident that the microwave system can reduce the extraction time and increase extraction rates, since microwave extraction at 10 psi reached a maximal lipid recovery (50.29% total lipid) at 3 min, whereas the conventional system at 90 °C achieved the highest recovery (53.75% total lipid) at 30 min. Therefore, the heating time was reduced using microwaves instead of a heating block, although using a heating block resulted in a higher maximal extraction yield (77.37% total lipid) than using microwaves (57.09% total lipid). Considering that the reduction in heating time results in decreased energy consumption, the use of microwaves during the extraction process showed great potential as a heating source for organic solvent-free lipid extraction. Further analysis of the economic viability of using the microwave system will be required for optimal biodiesel production.
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Fig. 4 Effect of pressure and heating time on lipid, fatty acid, palmitic acid, and DHA recoveries using conventional microwave system. Extraction was performed in microwave system with a 15 mg/mL biomass to solvent ratio and a 1% w/w sodium hydroxide solution.
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