Concurrent extraction and reaction for the production of biodiesel from wet microalgae

HanJin Im a, HanSol Lee a, Min S. Park a,b, Ji-Won Yang a, Jae W. Lee a,*

a Department of Chemical and Biomolecular Engineering, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea
b Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA

HIGHLIGHTS
- In situ transesterification was successfully applied to wet microalgae.
- Biodiesel was produced directly from wet microalgae in a single pot process.
- The conversion yield reaches 91% using wet microalgae.

ABSTRACT
This work addresses a reliable in situ transesterification process which integrates lipid extraction from wet microalgae, and its conversion to biodiesel, with a yield higher than 90 wt.%. This process enables single-step production of biodiesel from microalgae by mixing wet microalgal cells with solvent, methanol, and acid catalyst; and then heating them in one pot. The effects of reaction parameters such as reaction temperature, wet cell weight, reaction time, and catalyst volume on the conversion yield are investigated. This simultaneous extraction and transesterification of wet microalgae may enable a significant reduction in energy consumption by eliminating the drying process of algal cells and realize the economic production of biodiesel using wet microalgae.

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1. Introduction
Biofuels have attracted public attention as a source of renewable energy for replacing fossil fuels (Demirbas and Demirbas, 2011). Producing biodiesel from microalgal lipids is feasible since microalgae can accumulate lipid levels that are greater than 50% of their dry cell weight (Xiong et al., 2008). Because microalgae are grown in liquid media, extraction is usually classified as wet extraction or dry extraction depending on whether or not the culture broth was dried before the lipid extraction step. Most of the current extraction processes are carried out after drying the wet microalgae to increase the efficiency of lipid extraction (Rodriguez-Meizoso et al., 2010; Wahlen et al., 2011). However, this drying process is responsible for up to 59% of the total energy consumed during the biodiesel production (Yanfen et al., 2012). To eliminate the drying process, improvement of wet extraction performance by adding other operations such as supercritical, ultrasonic, and microwave processes has emerged as a very important issue (De Boer et al., 2012; Xu et al., 2011; Koberg et al., 2011; Wahlen et al., 2011). However, without the additional operation unit, our current study focuses on high yields of fatty acid methyl ester (FAME) using wet microalgae by combining the extraction and transesterification processes into a single step (so called in situ transesterification). We will show that heating wet microalgae in a mixed solution of methanol, solvent, and acid catalyst leads to production of FAME with a conversion yield around 90 wt.%. This is a significant improvement over in situ transesterification of wet microalgae. This work also investigates the effects of reaction parameters (e.g., amounts of solvent, methanol, and acid catalyst) on conversion yields for one-pot extraction and transesterification of wet microalgae.

2. Methods
2.1. Chemicals and reagents
Extra pure grade sulfuric acid (95%), extra pure grade benzene (99.5%), guaranteed grade chloroform (99%), guaranteed grade toluene (99.5%), and guaranteed grade n-hexane (96%) were obtained.
from JUNSEI Chemical (Japan). HPLC grade methanol (99.8%) was obtained from MERCK (Germany). Carbon tetrachloride (99%) was obtained from Sigma–Aldrich (USA). Heptadecanoic acid methyl ester as a standard material for GC analysis from Sigma–Aldrich (>99%) was also used.

2.2. Strain and cultivation

*Nannochloropsis oceanica* was obtained from the company called NLP (Natural Live Plankton) located in South Korea. The microalgae were grown in a 10-ton raceway pond for 13 days. The artificial water medium used to cultivate the strain contains a mixture of NaNO3 (0.003 wt.%), Na2HPO4 2H2O (0.001 wt.%), Na2SiO3 2H2O (0.003 wt.%), and trace salts. The trace salts consist of MnCl2 4H2O (0.4 ppm), CoCl2 2H2O (0.01 ppm), ZnCl2 (0.04 ppm), and FeCl3 6H2O (0.1 ppm). After 13 days of cultivation, the algal biomass is harvested by continuous centrifugation to maintain the water content of 65%.

2.3. In situ transesterification procedure

In situ transesterification was started by mixing methanol, solvent and sulfuric acid with the wet microalgae in a Teflon-sealed tube (14 ml). Methanol and sulfuric acid were used as transesterification reactant and catalyst. Extraction and reaction were conducted by immersing the tube in a waterbath (CW–05G, JEIO TECH, South Korea). To avoid leakage of methanol from the vapor head, the tube was capped tightly. After the reaction, sodium hydroxide was added to prevent further reaction and the mixture was allowed to settle for 1 h to achieve phase separation. Then, water phase containing methanol, sulfuric acid, and crude glycerol formed an upper layer and chloroform phase containing converted FAME and unconverted lipid formed a lower layer. Reaction parameters including temperature (65–95 °C), methanol and solvent volume (replaced by wet cell weight 0.1–0.8 g), catalyst volume (0.1–0.4 ml), and reaction time (30–120 min) were varied to find optimal conditions for maximizing the FAME yield. All experiments were carried out in duplicates or triplicates to ensure the reproducibility of data.

2.4. FAME analysis and maximum FAME yield

After transesterification, 1 ml of chloroform containing 0.5 mg heptadecanoic methyl ester (C17:0) was added to the reaction tube as a standard agent. The chloroform phase containing both converted and standard FAME was isolated and then filtered by a syringe filter (Minisart RC15 0.2 μm, Sartorius Stedim, Germany) for GC analysis. GC analysis was performed using an Agilent 6890 GC with a HP-INNOWAX column (30 mm × 0.32 mm × 0.5 μm, Agilent, USA) and a FID detector.

To determine the maximum yield of FAME, 10 mg dried cells and 3 ml 2/1 v/v mixture of chloroform and methanol were added to the Teflon-sealed tube and lipids were extracted by vortexing for 10 min. 1 ml methanol and 0.3 ml sulfuric acid were added to tubes and heated to 100 °C for 20 min. After the transesterification reaction, the tube was cooled down to room temperature and left to settle 1 h for phase separation. Then, 1 ml chloroform containing 0.5 mg heptadecanoic methyl ester (C17:0) was added to the tube for GC analysis to determine the maximum FAME conversion yield.

2.5. Control experiments with two-step processes

To compare the performance of in situ transesterification with that of separate, two-step solvent extraction and transesterification, two-step control experiments were carried out: (1) lipid extraction using two systems of single solvent and binary solvent/methanol, and (2) transesterification using sulfuric acid. The experiment was carried out with 0.2 g of wet *N. oceanica*. In the first control experiment, the samples were mixed with 2 ml of solvent. In the second experiment, the samples were mixed with 2 ml solvent and 1 ml methanol. Both samples were heated to 95 °C and kept for 2 h to extract lipids and then cell residue was removed to prevent additional extraction. For the first control experiment, 0.3 ml sulfuric acid and 1 ml methanol were added and then kept at 95 °C for 2 h while the second control experiment used 0.3 ml sulfuric acid for initiating transesterification at 95 °C for 2 h. After reaction, both samples were neutralized by sodium hydroxide solutions. The solvent phase containing FAME and the standard ester was taken to obtain the conversion yields of both samples.

3. Results and discussion

According to the experimental procedure (Section 2.4), the maximum amount of FAME that could be derived from the transesterifiable lipids was determined to be 19.17 ± 0.819 mg FAME/100 mg dry cell. Then, the conversion yield could be obtained by dividing an experimentally determined FAME amount by the maximum FAME amount.

3.1. Solvent selection

Since algal lipids have a high solubility in organic solvents, there were many attempts to use organic solvents for extracting lipids: chloroform was adopted due to its excellent extraction performance while hexane was adopted for its non-toxicity (Halim et al., 2011). Lipids are more viscous than organic solvents and mixing an organic solvent with lipids can improve the performance of transesterification with methanol by reducing the diffusion limit of the reactant to the liquid reaction phase (Lam and Lee, 2013). In this study, experiments to determine the best organic solvent for extracting lipids from *N. oceanica*; leading to high FAME yields, were carried out with benzene, carbon tetrachloride, chloroform, n-hexane, and toluene. Solvents forming sharp phase splits with water were selected for facile liquid–liquid splits. Wet *N. oceanica* paste (0.2 g) was mixed with 0.3 ml sulfuric acid and 3 ml 2/1 v/v mixture of solvent and methanol at room temperature. After mixing, each sample was subjected to 95 °C for 2 h during which the lipids were extracted and converted to FAME. Interestingly, chloroform showed the highest efficiency (90.6% conversion yield) among the solvents chosen. When referring to the polarity of each solvent (Barwick, 1997), there was a positive proportionality between polarity and conversion yield (Table 1). Solvents with higher polarity achieved higher conversion yields. Although the lipids in microalgae were known to be almost non-polar molecules, a non-polar solvent like hexane was found to be too weak for disrupting and extracting lipids from microalgal cells.

Previous studies (Patil et al., 2012, 2013; Wahlen et al., 2011) employing supercritical fluids or microwaves for in situ transesterification, conducted with microalgae containing 50 wt.% moisture, showed a conversion yield of 84% while the chloroform in this

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Polarity</th>
<th>Conversion yield (%)</th>
</tr>
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<tbody>
<tr>
<td>N-hexane</td>
<td>0.1</td>
<td>33.7</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>1.6</td>
<td>50.4</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.4</td>
<td>51.5</td>
</tr>
<tr>
<td>Benzene</td>
<td>2.7</td>
<td>69.3</td>
</tr>
<tr>
<td>1,2–Dichloethane</td>
<td>3.5</td>
<td>61.9</td>
</tr>
<tr>
<td>Chloroform</td>
<td>4.1</td>
<td>90.6</td>
</tr>
</tbody>
</table>
study provided a yield of 90.6% with the microalgae containing 65 wt.% moisture. Given the points that this method has higher extraction and conversion performance even with a high moisture content (65 wt.%) and does not require the extra energy inputs, it is clear that this can reduce the production cost of biodiesel from microalgae. The following section was devoted to carry out optimization experiments by varying reaction parameters to find the most economical way of producing FAME using chloroform solvent.

3.2. Optimization of reaction parameters

Results from previous studies of in situ transesterification showed higher yields of FAME when higher reaction temperatures were used (Patil et al., 2012; Wahlen et al., 2011). In this study, the reaction temperature was varied from 65 °C to 95 °C using 0.2 g of wet cell (equivalent to 0.07 g dry biomass). Then the reaction was allowed to continue in a water bath at a specified temperature. It is clear that the conversion yield increases with rising temperature (Table 2). The difference between the conversion yields at 65 °C and 75 °C was 19.3% while that between 85 °C and 95 °C was 8.5%. The difference of the conversion yield gradually decreased with increasing reaction temperature. Although other studies obtained an optimal reaction temperature near 80 °C (Wahlen et al., 2011), higher temperatures in this study helped chloroform, methanol and sulfuric acid disrupt the cell walls as well as accelerate the transesterification reaction.

Both solvent and reactant (methanol) volumes are dominant parameters that affect conversion yields in biodiesel production (Schafer, 1998). Since the transesterification reaction is reversible, excess methanol is required for higher conversion rates. A ratio of 2:1 (v/v) of chloroform to methanol was chosen for this study (Folch et al., 1957). In order to investigate the effects of chloroform and methanol volumes on the yield, experiments were carried out in which the microalgal mass was increased while the chloroform and methanol volumes were fixed, instead of increasing the respective volumes of chloroform and methanol. There was no difference in conversion yield between 0.1 g and 0.2 g samples (Table 2). A rapid decrease in conversion yield was observed when the algal biomass was greater than 0.2 g. This may be due to the high viscosity of increased microalgal lipids and cells, which gives unfavorable effect on reacting with methanol. The solvent can help microalgal lipids to react with methanol but a high microalgal biomass to reactant ratio may impede the reaction efficiency.

The type of catalyst used also plays an important role in determining the reaction rate. In situ transesterification usually uses acid or base as a reaction catalyst (Velasquez-Orta et al., 2013). When it comes to a base catalyst, additional acid for neutralization process is needed because of saponification. Thus, sulfuric acid was selected as the reaction catalyst because this one has been effective for converting free fatty acids and triglycerides under wet conditions (Sarthish and Sims, 2012). Another experiment was conducted to identify the effect of catalyst volume on conversion yields, with the addition of specified volumes of sulfuric acid. A volume of sulfuric acid ranging from 0.1 to 0.4 ml (in increments of 0.1) was investigated (Table 2). There was almost no increase in the conversion yield at a sulfuric acid volume larger than 0.3 ml. If less than 0.2 ml of catalyst was used with 0.2 g wet microalgae, the conversion yield was around 80% and did not decrease up to a catalyst volume of 0.1 ml. Thus, the conversion yield was not very sensitive to changes in the catalyst volume compared to varying cell weight.

Reaction time affects conversion and the conversion yield is increased by allowing more reaction time. The conversion yield reached a saturated value after a specific time, even if the reaction time increased, which means that the reaction almost approaches reaction equilibrium (Tsige et al., 2012; Wahlen et al., 2011). Reaction time was varied to find the optimal reaction time ranging from 30 to 120 min (increments of 30 min – Table 2). Within 30 min after in situ transesterification, a 65.7% conversion yield was shown. A considerable improvement in the conversion yield was observed from 30 to 90 min reaction time. The equilibrium conversion yield was obtained after a reaction time of 90 min, which can be further reduced using higher reaction temperatures or catalyst holdups.

SEM analyses were carried out to determine whether cell walls were disrupted after in situ transesterification. While uneven cell surfaces indicated that the fresh cell walls had not been disrupted, the lipid-extracted residual biomass obtained after in situ transesterification using chloroform, methanol, and sulfuric acid in a single pot shows that the cell surfaces were even and there were no obvious components that could be confirmed as cytoplasm. Thus, it is clear that the concurrent presence of sulfuric acid, chloroform, and methanol can disrupt cell walls and extract lipids efficiently under heated conditions.

3.3. Control experiments

Chloroform and binary chloroform/methanol were selected as the two solvent systems for the two control experiments. Lipids extracted by both solvent systems were subjected to transesterification using sulfuric acid as a catalyst for both experiments, and methanol as another reactant for the first (chloroform only) control experiment. The first control experiment shows 15.5% conversion yield while the second case was 5.6%. However, when chloroform, methanol, and sulfuric acid were simultaneously added to wet algal samples and heated, the yield jumped to 90.6% as shown in Table 1. Regarding the control experiment yields, chloroform alone was better than mixture of chloroform and methanol.

4. Conclusion

The wet, in situ transesterification process in this work has been demonstrated to achieve conversion yields as high as 91% from wet N. oceanica (65% moisture) using chloroform, methanol, and sulfuric acid in the one pot experiment. This significant improvement of conversion yield is possible without using an energy-intensive drying process before extracting the lipid. The reaction parameters for producing biodiesel via this process have also been investigated.
This in situ approach can be used to produce biodiesel directly from harvested algae and provides a useful step forward for the next generation of renewable biodiesel.

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