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# Production of levulinic acid from wet microalgae in a biphasic one-pot reaction process

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**Abstract**−This work addresses the conversion of wet microalgae to levulinic acid (LA) using a one-pot reaction system. Utilizing moisture in microalgae forms a biphasic system with an organic solvent of 1, 2-dichloroethane (DCE) is formed. This system enhances the LA yield by making an acidic environment through the decomposition of DCE in a small quantity and the recovery of products in each aqueous and organic phase. With lipid-rich *Nannochloropsis gaditana* and carbohydrate-rich *Chlorella* species, the effects of reaction variables of temperature, water content, and DCE dosage on the LA production were investigated. The LA yield was 30.13 wt% and 28.15 wt% based on the mass of total hexoses (43-47 wt% of convertible hexoses) for the two types of microalgae at 160 °C, while the yield of free fatty acids reached 90.13 w/w% at 180 °C based on the esterifiable lipid. This biphasic system facilitates the forward reaction and the product recovery for concurrent reaction and separation.

Keywords: Microalgae, Levulinic Acid (LA), Biphasic System, One-pot Reaction

## INTRODUCTION

Worldwide attention on alternative resources to petroleum has increased with the depletion of fossil fuels and the concern over environmental issues such as global warming. Biomass, which refers to all living sources such as plants, crops, and algae, has several advantages as a renewable source such as reduced air pollution and flexible conversion to other chemicals [1]. Therefore, many types of biomass such as spent coffee grounds, plants, and sewage sludge have been widely studied to produce various alternative products such as biodiesel and platform chemicals [2-6]. In particular, microalgae, a form of marine biomass, are a high potential resource for producing bioproducts [7-10]. Based on the advantages of fast growth rate, carbon neutrality, and high lipid content, previous studies have researched the production of various bioproducts using microalgae via hydrothermal liquefaction, microwave, pyrolysis, etc. [11-14].

Levulinic acid (LA), a platform chemical, can be converted to useful organic compounds such as plasticizers, fuel additives, and chemicals [15]. It has two functional groups of ketone and carboxylic acid. LA consequently can have various reaction routes by a versatile building block. Having these advantages, LA was selected by the US Department of Energy as a key biomass-derived chemical [16]. The production of LA and its derivatives in intensified processes was studied using various types of biomass such as corn stover, waste biomass, and lignocellulosic biomass [17-23]. While this lignocellulose biomass has a large portion of carbohydrates and is usually used for the production of LA, it contains lignin. Lignin usually makes the cell wall of plants rigid and it hinders hydrolysis of carbohydrates. Therefore, a pretreatment step is required for weakening bonds between hemicellulose and lignin or removing lignin, for making easier hydrolysis of hemicellulose to monomers [24,25]. However, this step inevitably employs a solvent and an acid catalyst, thereby increasing costs [26,27]. Marine biomass has an advantage of a negligible portion of lignin and thus a pretreatment step is unnecessary for the LA production [28]. Several articles have reported conversion of LA from macroalgae utilizing this advantage [29,30]. However, even if a chemical conversion route from microalgae was proposed for the production of ethyl levulinate, an ethanol-esterified form of LA, as a byproduct in biodiesel production [31-32], LA production utilizing microalgae which has both lipid and carbohydrate has rarely been studied.

A well-known mechanism of LA production is acid hydrolysis of carbohydrates. C6 sugars, mainly glucose, are isomerized to fructose which is converted to 5-hydroxymethylfurfural (5-HMF), a dehydrated intermediate product. The intermediate is converted to LA by rehydration. Theoretically, the maximum yield of LA from totally converted glucose is 64.5 wt% [33]. However, the LA yield commonly reaches about 66% of the theoretical yield because of a side reaction causing the formation of humin [34,35]. Therefore, many studies have been carried out to obtain a high yield of LA by using various types of catalysts. Mineral acid catalysts such as hydrochloric acid, sulfuric acid, and phosphoric acid have high catalytic activity and thus are conventionally used for the production of LA [36,37]. However, it has disadvantages of equipment corrosion and difficulty in separating the desired product of LA. In response, heterogeneous catalysts such as zeolites, Amberlyst, were investigated to recover the reactant and products from the catalysts, but the produced LA had low yield owing to its low activity [38].

However, this study introduced a facile, hydrothermal route that was applied for the conversion of non-treated wet microalgae (80 wt% moisture) to LA in the biphasic phase. This route utilizes moisture in

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microalgae as subcritical water (SCW) at high temperature, which induces hydrolysis of chlorine-containing organic solvent. The acidic environment is created by $H^+$ released from the hydrolyzed organic solvent and, thus, the organic solvent plays the role of an acid catalyst as well as an organic agent for forming a biphasic system [39,40]. This work also investigates the biphasic reaction system so that facile separation of the desired product of LA by recovering it from the aqueous phase. For the production of LA, the main target product, various effects such as reaction temperature, solvent amount, and moisture content were investigated. Since microalgae have species specificity, two microalgae species, the lipid-rich microalgae *Nannochloropsis gaditana* and the carbohydrate-rich microalgae *Chlorella* species were examined. Response surface methodology (RSM) was employed to obtain the optimum condition and maximum LA yield based on the experimental data. By using directly wet microalgae without any pretreatment in a biphasic system that facilitates the recovery of desired product, this work offers an economically feasible manner to produce the platform chemical of LA.

## MATERIALS AND METHODS

### 1. Chemical Reagents

The main species studied in this study are *Nannochloropsis gaditana* (*N. gaditana*) and *Chlorella* species (*Chlorella* sp.). Dried *N. gaditana* was purchased from AlgaSpring (Netherlands). To investigate the hydrothermal reaction in the biphasic system using microalgal species with a high carbohydrate portion, dried *Chlorella* sp. was acquired from Daesang Company (South Korea). Both cells were stored at −10 °C and saturated with deionized water (DIW, Milli-Q 18.2 MΩ $cm^{-1}$).

Guaranteed reagent grade 1, 2-dichloroethane (DCE) was purchased from Junsei Chemical (Japan). For the quantitative analysis of products by high performance liquid chromatography (HPLC), levulinic acid (>97.0%, LA, Tokyo Chemical Industry Co., Ltd., Japan) was used as an internal standard. Glucose, fructose, rhamnose, galactose, and mannose (ACS reagent grade, Sigma Aldrich) were also used as standard chemicals in HPAEC and HPLC to calibrate the amount of each carbohydrate. Guaranteed grade $H_2SO_4$ was purchased from Junsei Chemical (Japan) to analyze the monosaccharides in microalgae. Guaranteed reagent grade chloroform was acquired from Junsei Chemical (Japan) and extra pure grade methanol was purchased from DAEJUNG (Korea) to analyze the esterifiable lipid in microalgae. Ethyl heptadecanoate (>97.0%, Tokyo Chemical Industry Co., Ltd., Japan) was used as internal standard to analyze GC.

### 2. Determination of Convertible Monosaccharides and Esterifiable Lipid in Microalgae

To quantify the monosaccharides in microalgae, the NREL method [41] was executed. For hydrolysis, 3 mL of 72 wt% $H_2SO_4$ was added to 0.03 g of microalgae and reacted at 30 °C for 1 h. To dilute the mixture by 4 wt% $H_2SO_4$, 8.4 mL of DIW was added and the mixture was heated at 121 °C for 1 h. After the mixture was slowly cooled to room temperature, $CaCO_3$ was added until the pH became neutral followed by filtering through a 0.2 μm filter. The amounts of monosaccharides from hydrolyzed microalgae were quantified by high performance anion-exchange chromatography (HPAEC, Dionex, ICS-5000) equipped with an amperometric detector using 18 mM NaOH solution as the mobile phase.

To determine maximum esterifiable lipid in microalgae, the Folch method [40] was adopted. For lipid extraction, 10 mg of dried microalgae and 1.34 mL of chloroform and 0.66 mL of methanol were added to Teflon sealed glass tube. After sonication for 30 min, 1 mL of methanol and 0.3 mL of $H_2SO_4$ were added and heated at 100 °C for 20 min. The mixture was cooled to 25 °C. To neutralize and analyze for mixture, 1 mL of 0.3 M NaOH and 1 mL of internal standard which is 0.5 mg of ethyl heptadecanoate with 1 mL of chloroform were added and then analyzed by GC.

### 3. Experimental Method to the Conversion of Levulinic Acid from Wet Microalgae

For the conversion of LA production from wet microalgae, the experimental procedure in previous studies was modified in this research [39,40]. All experiments were done in triplet and the average value was reported. As harvested microalgae have an average 80 wt% of moisture based on the total weight, 2 g of the dried microalgae paste was saturated in 8 mL of deionized water via 30 min sonication to ensure the paste contained 80 wt% of moisture. After saturation, wet microalgae were loaded into a 100 mL Teflon liner with additional amounts of DIW and DCE. The liner was then inserted into a 100 mL SUS360 reactor designed to endure high temperature and pressure. At the desired temperature of 140-200 °C, the reactor was heated for 5 h in a thermostat bath excluding heating-up time of 30 min. After the reaction, the reactor was immediately cooled to inhibit further reaction. To clearly separate the two layers of the upper aqueous and the bottom organic phases of the reaction medium, 3 mL of DIW and 2.5 mL of DCE were further added. To analyze LA and monosaccharides in the aqueous phase and fatty acids and cellulose derived products dissolved in the organic phase, the collected sample was centrifuged at 5,000 rpm for 10 min for easy phase separation. After centrifuging, the sample was filtered by a 0.2 μm filter and analyzed.

### 4. Analysis

#### 4-1. HPLC Analysis

High performance liquid chromatography (HPLC, Thermo Scientific, Dionex Ultimate 3000) equipped with an RI detector was used for the analysis of the aqueous phase. Aminex HPX-87H ion exclusion column (300×7.8 mm) was used and operated at 65 °C. As a mobile solution, 5 mM $H_2SO_4$ solution was used with a flow rate of 0.6 mL/min. The concentration of product was calculated by the calibration curve of an external LA standard ranging from 0.1 to 50 mg/mL. Yield (wt%), which was the ponderal yield of LA produced from microalgae, was calculated by the following equation:

$$\text{Yield (wt\%)} = \frac{\text{The amount of LA obtained from the experiment (g)}}{\text{The amount of total hexoses in microalgae (g)}} \times 100 \quad (1)$$

Percent yield of LA from microalgae was calculated by the following equation, based on the maximum theoretical amount (convertible amount) of LA production:

$$\text{Yield (w/w\%)} = \frac{\text{The amount of LA obtained from the experiment (g)}}{\text{The amount of convertible hexoses in microalgae (g)}} \times 100 \quad (2)$$

4-2. GC-MS Analysis

To investigate the amount and composition of the free fatty acid dissolved in the organic phase, gas chromatography-mass spectroscopy (GC-MS, Thermo Fisher, ISQ QD300) equipped with TG-5MS column (30.0 m×0.2 mm×0.25 μm) was used by referring to our previous studies [39,40]. He gas was used as a carrier gas for the GC-MS analysis. The oven temperature was maintained initially at 30 °C for 4 min and held for 1 min after being increased to 50 °C at a rate of 25 °C/min. The temperature was thereafter raised from 50 °C to 175 °C at a rate of 25 °C/min and from 175 °C to 240 °C at a rate of 4 °C/min and held for 8 min.

4-3. Ionic, Thermal and Microscopic Analyses

CHONS analysis was carried out with CHONS elemental analyzer (Thermo Scientific, FLASH 2000 series) equipment. Thermogravimetric analysis (TGA) was conducted to determine the content of cellulose and lignin in high resolution TGA (NETZSCH, TG209 F1 Libra) equipment. Temperature was increased from 25 to 900 °C at a rate of 10 °C/min under a $N_2$ condition. To investigate the degree of DCE hydrolysis, pH and $Cl^-$ were measured via pH meter (Mettler Toledo, USA) and ion chromatography (Metrohm, 881 Compact IC pro, Swiss) equipped with Metrosep A Supp5 150 (150×4.0 mm I.D.) column. The column temperature was maintained at 30 °C and the data recording time was 20 min with MagIC Net version 2.4. The ion concentration was obtained in mg/kg. Scanning electron microscopy (SEM, Hitachi, SU500) was employed to observe the humin component, which is a solid residue after the one-pot reaction.

## RESULTS AND DISCUSSION

### 1. Characteristics of Microalgae

The compositions of the two microalgae species are shown in Table 1. The existence of cellulose, hemicellulose, and fatty acids in the algal cells was confirmed by the TGA data and percentages are shown in Table 1. As shown in Fig. 1(a), the main peak appeared at 200 to 400 °C, which denotes the degradation of hemicellulose and cellulose. Also, the degradation of protein overlaps this region.

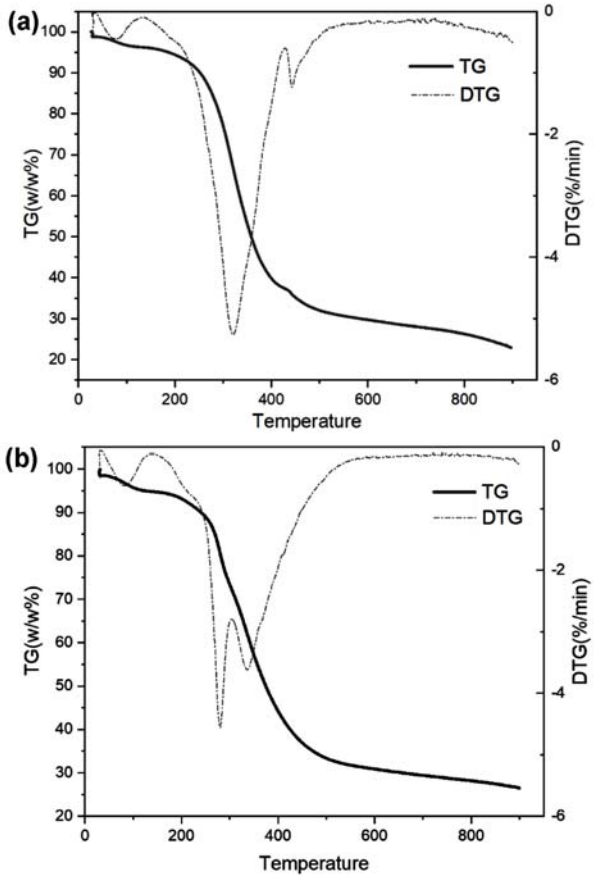


**Fig. 1. Thermogravimetric (TG) and derivative thermogravimetric (DTG) graphs of (a) *N. gaditana* and (b) *Chlorella* sp. microalgae. The first DTG peak of both graphs is shown at about 80 °C, which indicates the degradation of moisture and other volatile components.**

Additionally, a third peak is observed between 420 and 450 °C. This indicates decomposition of fatty acid since *N. gaditana* is a lipid rich microalgae. In the case of *Chlorella* sp., the main peak

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**Table 1. Characteristics of carbohydrates and compositions of each species**

| Proximate analysis (wt%) | *N. gaditana* | *Chlorella* sp. | Ultimate analysis (wt%) | *N. gaditana* | *Chlorella* sp. |
|---|---|---|---|---|---|
| Glucose | 6.36 | 17.76 | C | 50.56 | 49.06 |
| Galactose | 2.22 | 4.89 | H | 7.29 | 7.24 |
| Mannose | 0.58 | 0.74 | O | 24.84 | 28.36 |
| Rhamnose | 0.67 | 1.15 | N | 7.65 | 8.78 |
| Xylose | 0 | 0.42 | S | 0.38 | 0.36 |
| Arabinose | 0.086 | 0.083 | | | |
| Total carbohydrate | 12.31 | 29.53 | | | |
| Total lipid | 29.22 | 8.54 | | | |
| Protein | 45.40 | 52.83 | | | |
| Ash | 11.22 | 3.84 | | | |
| Esterifiable lipid[a] | 9.29 | 7.06 | | | |

[a]Esterified fatty acid which has 12 to 22 carbon number based on the Folch method.

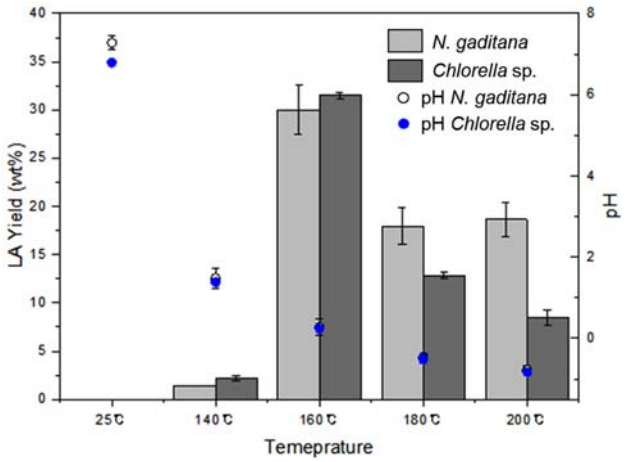


**Fig. 2. Effect of temperature on the LA yield of each species via one-pot conversion with 5 mL of DIW and 2.5 mL of DCE per gram dried microalgae.**

showing the decomposition of hemicellulose and cellulose is also observed between 200 to 450 °C as shown in Fig. 1(b). This peak was split into two peaks, the former is the degradation of hemicellulose and the latter is cellulose. However, there is no peak at 470 °C in both graphs, which means that there is no degradation of lignin. Thus, lignin was found to be absent in the two microalgae.

Based on the ultimate analysis, the two species have similar amounts of elements. However, *N. gaditana* has a larger esterifiable lipid portion than *Chlorella* sp. and the carbohydrate portion shows the opposite trend. Therefore, the total amount of LA produced is higher in *Chlorella* sp. than in *N. gaditana*, as shown in Fig. 2. The total convertible sugars are 9.83 wt% of the dried cell weight of *N. gaditana* and 24.53 wt% of *Chlorella* sp. Both microalgae have the largest portion of glucose, 6.36 wt% and 17.76 wt% for each. Also, the other hexoses such as galactose, mannose, and rhamnose can be a source for the production of LA [42]. Since glucose is the main hexose for producing LA by acid hydrolysis, the composition analysis shows that lignin-free microalgae can be a promising biomass for the production of LA.

**2. RSM Analysis**

Before determining the optimum conditions for LA production with wet microalgae in a biphasic system, preliminary tests were conducted to determine the range and set of reaction variables. Temperature, which is an important factor for LA production, was first investigated and the amounts of organic solvent (1, 2-dichloroethane, DCE) and distilled water (DIW) were varied to understand the effect of each variable.

2-1. Effect of Temperature on Levulinic Acid Production

LA conversion of wet microalgae was investigated at various temperatures from 140 °C to 200 °C with a fixed amount of 5 mL DIW and 2.5 mL DCE per gram dried microalgae, to determine the effect of temperature on the LA yield. As shown in Fig. 2, the LA yield rapidly increased when the temperature was increased from 140 °C to 160 °C. At 160 °C, the maximum LA yield (29.99 wt%, 50.27 w/w% for *N. gaditana* and 31.48 wt%, 48.81 w/w% for *Chlorella* sp.) was obtained and this yield gradually decreased when the temperature was further increased. This tendency appeared in both species identically.

To convert LA from wet microalgae, the cellulosic portion should first be hydrolyzed to monosaccharides under high temperature and acidic conditions. Cellulose consists of hexose monomers that are combined by glycosidic bonds. This hydrogen bond weakens as the temperature rises, making acidic protons more accessible to the bonds [43]. Therefore, increasing temperature facilitates the hydrolysis of carbohydrates. Also, previous studies showed that water decomposition to hydrogen and hydroxide ions at the subcritical region induced the DCE decomposition to $H^+$ and $Cl^-$. The ions from DCE decomposition provide acidic conditions without any additional acid catalyst by acting like Lewis and

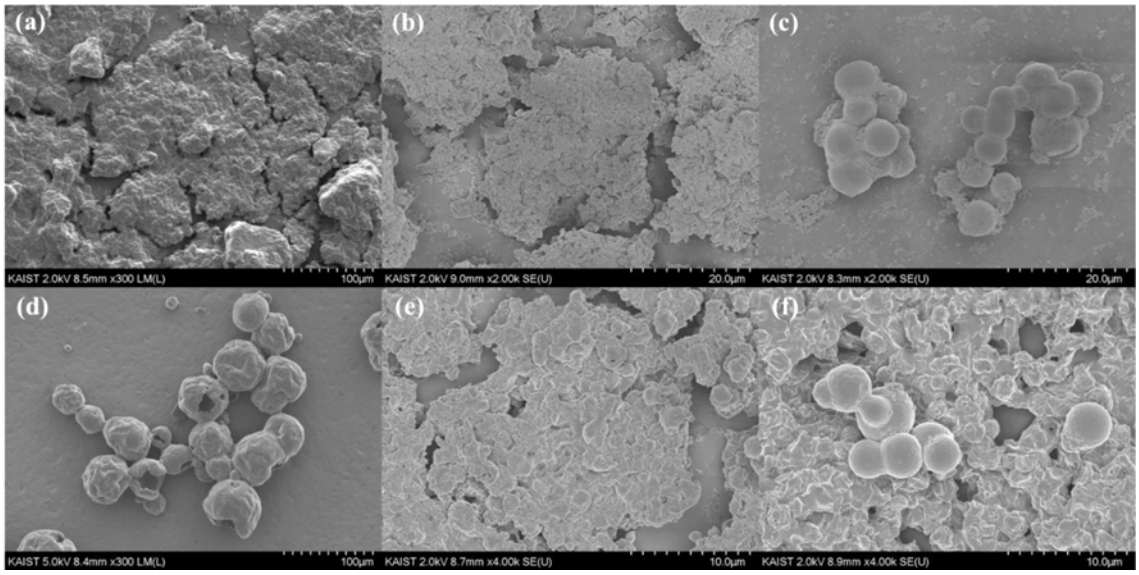


**Fig. 3. SEM images of solid residue after the reaction at various temperature. (a), (b), (c) were *N. gaditana* and (d), (e), (f) were *Chlorella* sp. (a) and (d) of raw microalgae, (b) and (e) of reaction at 160 °C, and (c) and (f) of reaction at 180 °C.**

Brønsted acids. Fig. 2 shows the pH for each temperature condition after the one-pot reaction. The pH decreased when the temperature was raised. This supports that acidic conditions were successfully realized for the LA conversion above 140 °C (pH=1.4 to −1.0). The LA conversion yield was highest at 160 °C and the amount of decomposed $Cl^-$ ions was 5.8 to 7.5 mmol for each microalgal species (Fig. S1). However, overly high temperature induces unwanted side products of humin, as reported in a prior study [44]. The LA yield decreased above 180 °C because the formation of a side product of spherical humin was observed in Fig. 3 (SEM images) after the reaction. Overall, the optimal reaction temperature for the production of LA from both wet microalgae is 160 °C.

2-2. Effect of Solvent and Distilled Water (DIW) on Levulinic Acid Production

In addition to 1, 2-dichloroethane (DCE) serving as an acid catalyst as described in the previous section, it also forms a water-DCE biphasic system in these reaction conditions. The effect of the solvent and DIW on the yield of LA was determined with different amounts of DCE and DIW at 160 °C. The results are shown in Fig. 4. The LA yield of *N. gaditana* and *Chlorella* sp. rapidly increased from 2.05 wt% and 3.14 wt% to 29.99 wt% and 31.48 wt%, respectively, upon raising the amount of DCE from 0 mL to 2.5 mL per gram dried microalgae, as seen in Fig. 4(a). This means that the formation of the water-DCE biphasic system effectively promotes the conversion of LA. Since 5-HMF, which is an intermediate in the conversion from glucose to LA, is more soluble in the organic phase than the water phase, it can be extracted into the organic phase when the two phases exist [45].

On the other hand, LA is more miscible in the water phase. Therefore, upon the conversion of 5-HMF to LA, LA is extracted from the organic phase to the aqueous phase and, by Le Chatelier's principle, 5-HMF conversion to LA is promoted. As a result, the overall yield of LA was increased. However, further increasing the amount of DCE from 2.5 to 5 mL per gram dried microalgae slightly decreased the LA yield. The increased amount of DCE means a decreased proportion of water in the overall system, and thus the polarity of the system is lowered, resulting in weak hydrolysis of cellulose [46].

Fig. 4(b) shows the yield of LA with various amounts of additional DIW. The yield of LA increased for both species when the amount of water increased until a certain point, 7.5 mL and 5 mL per gram dried microalgae of *N. gaditana* and *Chlorella* sp., respectively, and thereafter decreased. Water is a reactant for the rehydration of 5-HMF to LA and thus increasing water content causes a

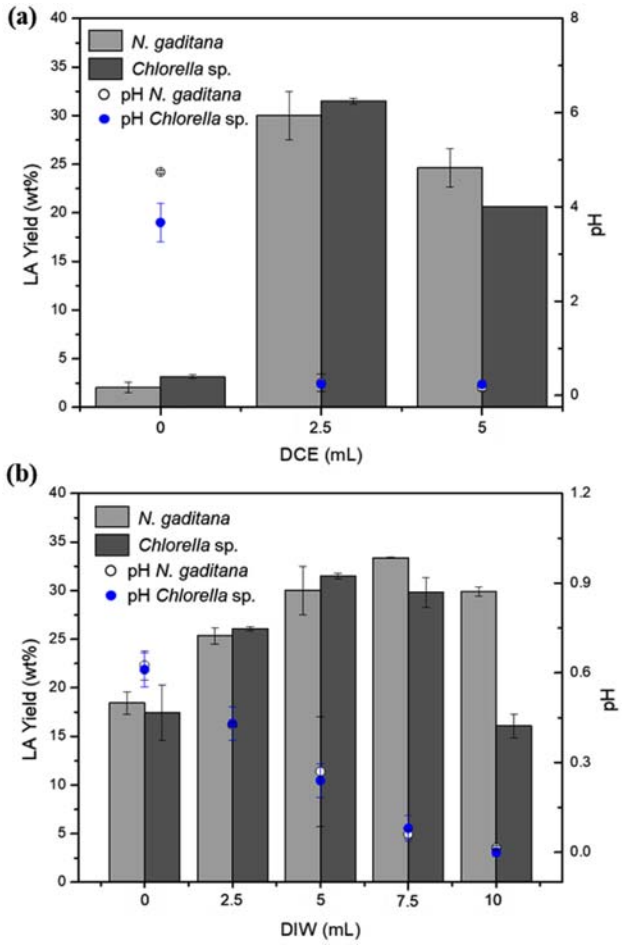


**Fig. 4. Effect of solvent (DCE) and distilled water (DIW) on the LA yield of each species via one-pot non-catalytic conversion with (a) 160 °C and 5 mL of DIW per gram dried microalgae and (b) 160 °C and 2.5 mL of DCE per gram dried microalgae.**

**Table 2. Box-Behnken experimental conditions and corresponding data of LA production from *N. gaditana* and *Chlorella* sp. used in RSM optimization**

| Run | Coded variables | | | *N. gaditana* | *Chlorella* sp. |
|---|---|---|---|---|---|
| | Temp. (A)[a] | DIW (B)[b] | DCE (C)[c] | Yield (wt%) | Yield (wt%) |
| 1 | 0 | 0 | 0 | 13.76±2.22 | 23.96±0.65 |
| 2 | −1 | 0 | 1 | 25.73±2.07 | 1.39±0.05 |
| 3 | 0 | 1 | −1 | 7.97±0.56 | 3.14±0.14 |
| 4 | 0 | 0 | 0 | 13.74±0.09 | 26.16±0.04 |
| 5 | 0 | 1 | 1 | 20.61±0.53 | 20.65±0.01 |
| 6 | −1 | −1 | 0 | 18.43±1.13 | 1.05±0.28 |
| 7 | 1 | −1 | 0 | 13.27±0.08 | 11.07±0.26 |
| 8 | 0 | 0 | 0 | 16.12±3.51 | 28.15±0.18 |
| 9 | 1 | 1 | 0 | 18.63±1.76 | 12.85±0.29 |
| 10 | 0 | 0 | 0 | 16.59±3.93 | 28.40±0.01 |
| 11 | 0 | 0 | 0 | 16.96±1.02 | 27.58±0.18 |
| 12 | 1 | 0 | 1 | 14.54±0.84 | 13.50±0.79 |
| 13 | −1 | 0 | −1 | 3.07±0.02 | 0.91±0.24 |
| 14 | 1 | 0 | −1 | 8.41±0.21 | 4.68±0.06 |
| 15 | 0 | −1 | 1 | 12.31±1.17 | 16.23±1.06 |
| 16 | −1 | 1 | 0 | 29.99±2.52 | 2.21±0.19 |
| 17 | 0 | −1 | −1 | 4.91±0.14 | 1.93±0.19 |

[a]Range of reaction temperature 160 °C (−1)~200 °C (1) in *N. gaditana* and 140 °C (−1)~180 °C (1) in *Chlorella* sp.
[b]Range of the amount of additional distilled water 0 mL (−1)~5 mL (1) per gram dried microalgae in both species
[c]Range of the amount of 1, 2-dichloroethane 0 mL (−1)~5 mL (1) per gram dried microalgae in both species

further forward reaction. However, the formation of 5-HMF from glucose is a dehydration step and excessive water hinders the conversion from glucose to 5-HMF. Also, SCW induces DCE hydrolysis, which leads to a highly acidic environment for the reaction. The pH of each condition decreased steadily upon increasing hydrolysis of DCE with excessive water, and thus an unwanted side reaction of humin formation could occur at low pH [47].

2-3. Optimization of the Reaction Variables on LA Production

Table 2 shows the parameters of RSM and the corresponding results of the experiment. The RSM analysis was conducted to investigate the effect of reaction variables on the LA yield. The Box-Behnken quadratic model was applied to RSM and the optimum condition of reaction variables for the maximum LA yield was obtained. Design-Expert 12.0 software was employed for the RSM analysis. The following statistical equations were obtained for the yield of LA based on the quadratic model:

$$\text{LA (wt\%)} = 15.43 - 2.80A + 3.53B + 6.10C - 1.55AB - 4.13AC + 1.31BC + 3.07A^2 + 1.58B^2 - 5.56C^2 \quad (3)$$

$$\text{LA (wt\%)} = 26.82 + 4.57A + 1.07B + 5.14C + 0.1554AB + 2.09AC + 0.8012BC - 12.70A^2 - 7.33B^2 - 9.00C^2 \quad (4)$$

**Table 3. Statistical analysis of predicted quadratic polynomial model of experimental data of LA production from *N. gaditana* and *Chlorella* sp.**

| | *N. gaditana* | *Chlorella* sp. |
|---|---|---|
| | Yield (wt%) | Yield (wt%) |
| $R^2$ | 0.9503 | 0.9568 |
| F-value | 14.87 | 17.24 |
| P-value | 0.0009 | 0.0006 |

where A, B, and C are temperature (°C), the amount of DIW (mL), and the amount of DCE (mL), respectively.

Eq. (3) is the statistical equation of *N. gaiditana* and Eq. (4) is that of *Chlorella* sp. case. Table 3, Fig. 5(d) and Fig. 6(d) show the fitness of each model via ANOVA test. When the F-value is higher than 0.05 and the P-value is less than 0.05, the reaction variables are meaningful to the RSM model. Therefore, the higher F-value (14.87, 17.24 of *N. gaditana*, and *Chlorella* sp. respectively) and lower P-value (0.0009, 0.0006 of *N. gaditana*, and *Chlorella* sp. respectively) with the value of $R^2$ (0.9503, 0.9568 of *N. gaditana*,

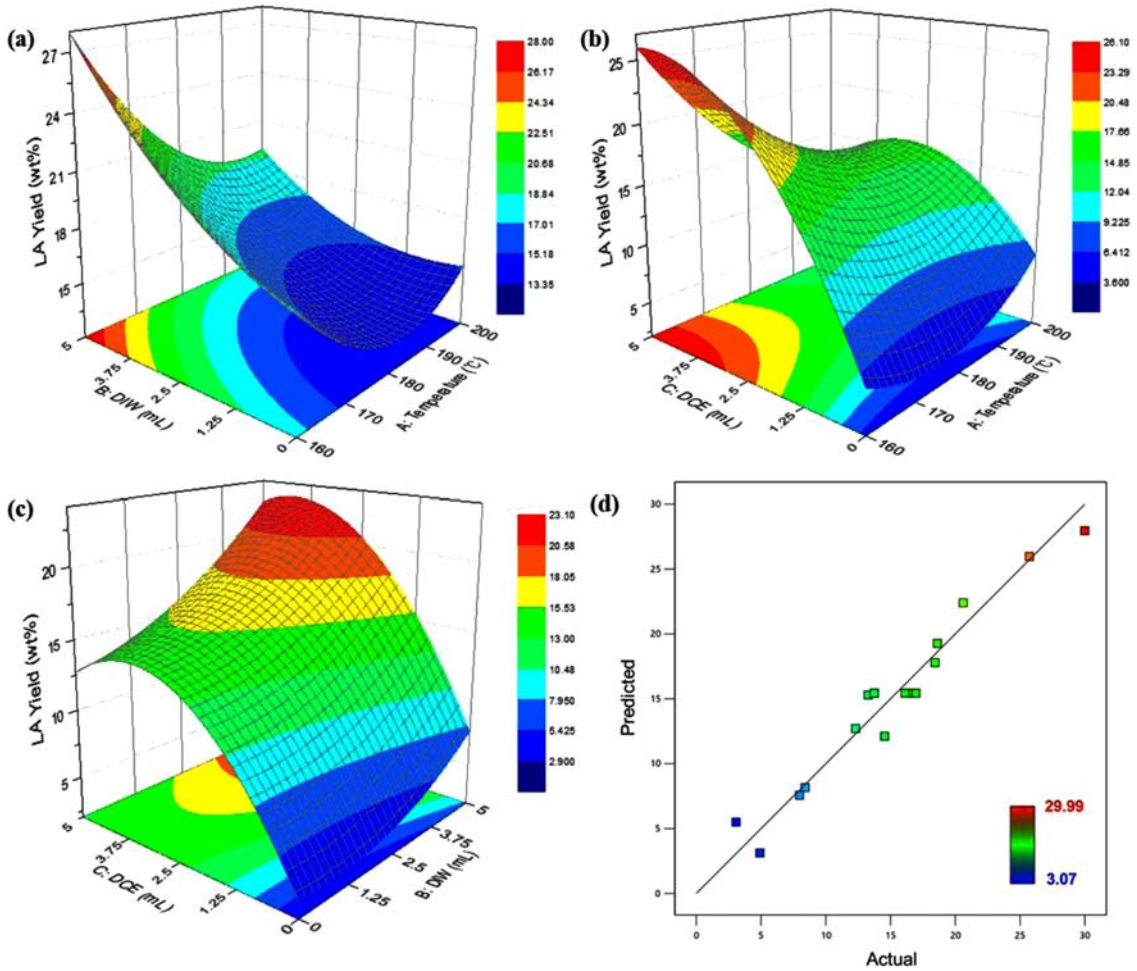


**Fig. 5. RSM results of *N. gaditana* of the LA yield with reaction variables (a) temperature vs. DIW (the amount of DCE was fixed as 2.5 mL) (b) temperature vs. DCE (the amount of DIW was fixed as 2.5 mL) (c) DIW vs. DCE (Temperature was fixed at 180 °C) and (d) predicted vs. actual plot of the surface response.**

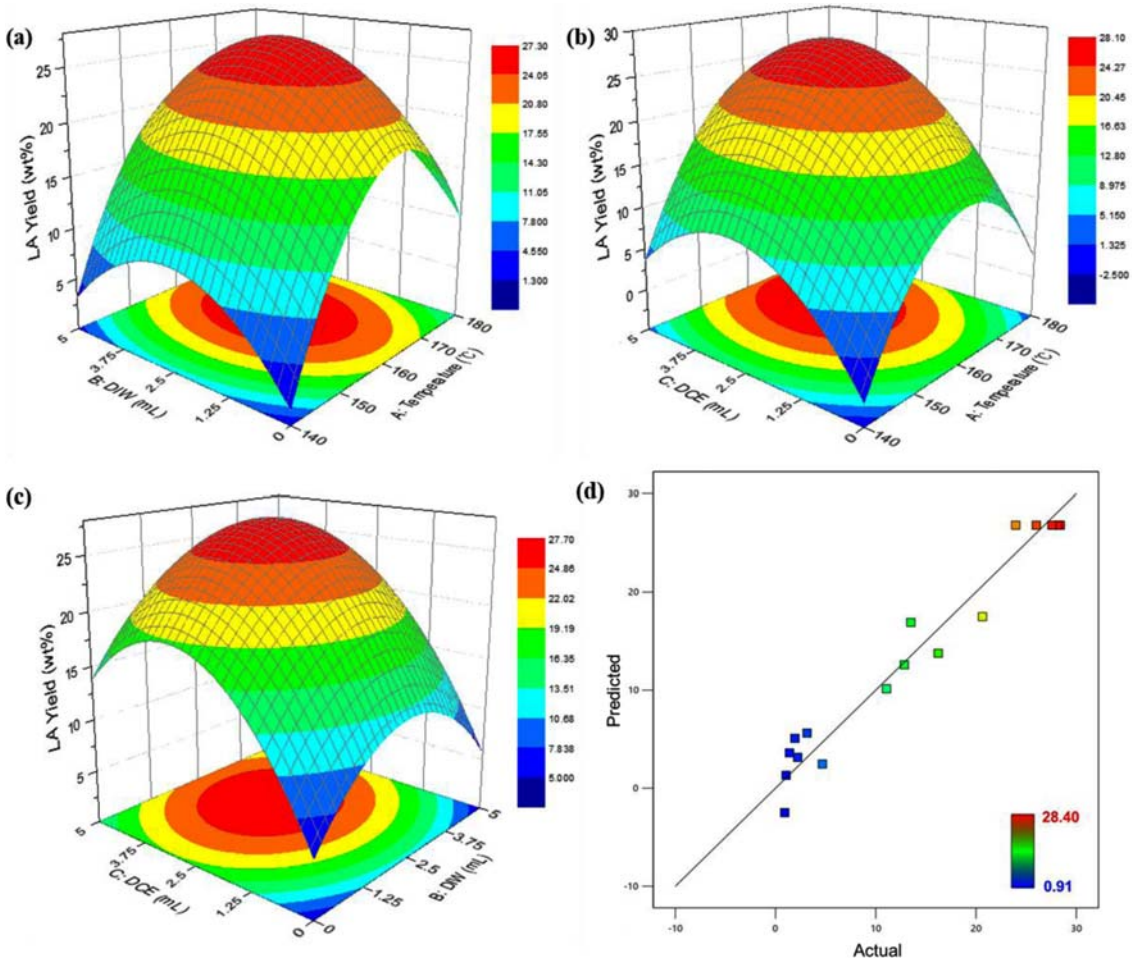


**Fig. 6. RSM results of *Chlorella* sp. of the LA yield with reaction variables (a) temperature vs. DIW (the amount of DCE was fixed as 2.5 mL) (b) temperature vs. DCE (the amount of DIW was fixed as 2.5 mL) (c) DIW vs. DCE (Temperature was fixed at 160 °C) and (d) predicted vs. actual plot of the surface response.**

and *Chlorella* sp. respectively) indicate the model is well fitted. Fig. 5 and Fig. 6 show the relation of variables associated with the yield of LA from each microalgae. As shown in (a), (b) in Figs. 5 and 6, when the temperature was near 160 °C, the yield of LA was less affected by the amount of DIW and DCE. This shows a similar trend of LA conversion as described in section 2-1, because temperature more significantly affected the LA yield than DIW and DCE. The obtained optimum condition of *N. gaditana* was 161.64 °C, 4.53 mL of DIW and 3.95 mL of DCE per gram dried microalgae with 30.13 wt% of LA yield (46.71 w/w%). The optimum condition of *Chlorella* sp. was 164.12 °C, 2.73 mL of DIW and 3.28 mL of DCE per gram dried microalgae with 28.15 wt% LA yield (43.64 w/w%). Actual experiments at each set of the optimal condition provide LA yields of 25.93 wt% (40.20 w/w%) and 24.17 wt% (37.48 w/w%), respectively.

The optimum conditions were not the same between the two species. *N. gaditana* requires more DIW than *Chlorella* sp. for a similar amount of DCE because of species specificity such as characteristics of the cell wall. The cell wall of *N. gaditana* comprises two layers where the outer hydrophobic rigid alganan layer protects the inner cellulose-based wall. To break the thick cellulosic wall, more DIW is need for the hydrolysis of the wall. In contrast, *Chlorella* sp. have a thin alganan outer wall surrounding a polysaccharide inner wall [48]. Therefore, *N. gaditana* requires more DIW than *Chlorella* sp. for the hydrolysis of the cellulosic wall.

**3. Fatty acids in the Organic Phase**

The components of biocrude oil in the organic phase after the reaction were analyzed. The major components of the organic phase are free fatty acids (Table S1). Lipids in microalgae are hydrolyzed to free fatty acids at subcritical water condition [49]. More free fatty acids were converted from the hydrolysis of *N. gaditana* than *Chlorella* sp. because the former has more esterifiable lipids than the latter as shown in Table 1. The maximum yield of free fatty acids was obtained at 180 °C as 90.13 w/w% from *N. gaditana* and 52.47 w/w% from *Chlorella* sp. based on the maximum esterifiable lipids. Especially, yield of useful free fatty acids for biodiesel which have carbon number from 14 to 22 was 87.19 wt% and 62.2 wt%, respectively, based on maximum yield of free fatty acids of each microalgae. Compared to the LA production, free fatty acids require a higher temperature for obtaining the maximum yield [40]. The major components of free fatty acids were palmitic acid (C16:0) and palmitoleic acid (C16:1) from *N. gadi-*

*tana* and linoleic acid (C18:2) from *Chlorella* sp. These products are also valuable for industrial usage. Not only was LA produced but also organic phase components were valuable products in the one-pot non-catalytic process.

## CONCLUSION

This work has demonstrated LA production from raw microalgae in the water-DCE biphasic system. This biphasic system enhances the LA yield by providing an acidic environment by hydrolyzing DCE with SCW. The optimum points for the LA production were determined by investigating the effect of temperature and the amounts of DIW and DCE on the LA yield. The maximum yields were 30.13 wt% of *N. gaditana* and 28.15 wt% of *Chlorella* sp. at the optimum point. Useful products of free fatty acids were also obtained. In the biphasic system, the one-pot reaction of raw microalgae provides a sustainable biorefinery for microalgal cells.

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## SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

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# Supporting Information

# Production of levulinic acid from wet microalgae in a biphasic one-pot reaction process

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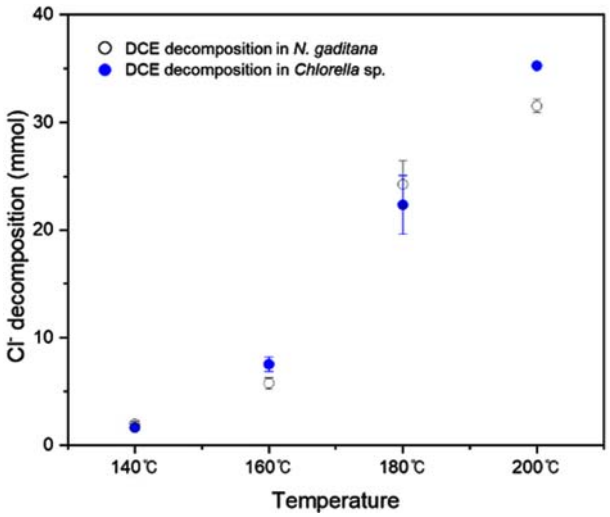


**Fig. S1. $Cl^-$ decomposition of the aqueous medium after conversion of LA from both species with a fixed amount of 5 mL of DIW and 2.5 mL of DCE per gram dried microalgae at various temperatures.**

**Table S1. Products distribution of the organic phase from microalgae via one-pot non-catalytic conversion in GC-MS**

| *N. gaditana* | | *Chlorella* sp. | |
|---|---|---|---|
| Name | Structure | Name | Structure |
| Myristic acid (C14:0) | | Palmitic acid (C16:0) | |
| Palmitic acid (C16:0) | | Oleic acid (C18:1) | |
| Palmitoleic acid (C16:1) | | Linoleic acid (C18:2) | |
| Stearic acid (C18:0) | | | |
| Oleic acid (C18:1) | | | |
| Eicosapentaenoic acid (C20:5) | | | |

Based on GC-MS data, minor components which have low concentration below the internal standard (5 g/L) were not included.