Enhanced Biomass and Lipid Production Capacity of *Chlamydomonas reinhardtii* under Mixotrophic Cultivation using Sewage Effluent and Waste Molasses

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**Abstract**

Microalgae are recognized as a renewable feedstock for biofuel production. However, low lipid productivity and high cultivation costs are the major bottlenecks for the practical application of microalgal biofuel production. This study aimed to investigate the benefits of a mixotrophic cultivation of *Chlamydomonas reinhardtii* using organic wastes and wastewater effluent for enhanced biomass and lipid production and reducing cultivation cost. *C. reinhardtii* was cultured under mixotrophic conditions in a synthetic C medium or sewage effluent supplemented with a commercial organic carbon source (glucose, fructose, sucrose, or acetate) or organic wastes (molasses or corn steep liquor). *C. reinhardtii* grew and accumulated lipids in all cultivation conditions using C medium or sewage effluent. Sewage effluent could be an alternative culture medium for *C. reinhardtii*. Supplementing a commercial organic carbon or organic wastes enhanced *C. reinhardtii*’s biomass production. The highest biomass (86 mg–dry cell/L/d) and lipid (34 mg/L/d) production by *C. reinhardtii* was observed in the C medium with 1 g/L molasses. Cultivation using sewage effluent with 1 g/L of molasses also provided a relatively high biomass (73 mg–dry cell/L/d) and lipid (27 mg/L/d) production; these rates were significantly higher than those under photoautotrophic cultivation and mixotrophic cultivation in C medium supplemented with other commercial carbon sources except for acetate. Molasses was thus found to enhance growth and lipid synthesis of *C. reinhardtii*. These results indicate that mixotrophic cultivation using sewage effluent and molasses can be a promising strategy for a cost-effective and highly efficient biofuel production by *C. reinhardtii*.

**Keywords:** *Chlamydomonas reinhardtii*, lipid production, mixotrophy, sewage effluent, organic waste.

**INTRODUCTION**

Microalgae have been recognized as an alternative and renewable feedstock for biofuel production due to their rapid growth, short harvesting cycle, and high lipid accumulation ability¹,². However, the microalgal biofuel production is still in its infancy with respect to its commercialization and social implementation. Lipid productivity and the costs of microalgal culture are the two major bottlenecks that need to be addressed in order to expand the practical application of microalgal biofuel production.

There are three different microalgal cultivation types based on the nutrition
modes: photoautotrophic, heterotrophic, and mixotrophic\(^{1,3}\). In photoautotrophic cultivation, microalgae can grow via photosynthesis using carbon dioxide (CO\(_2\)) and light. Photoautotrophic cultivation is commonly used for microalgal biomass production; however, a low biomass productivity due to the light penetration is a major limitation of this method of cultivation\(^{1,2}\). In heterotrophic cultivation, microalgae can grow using organic carbon source as the sole carbon/energy source, under dark conditions. Heterotrophic cultivation offers a high biomass productivity; however, this method has some limitations including a very limited number of microalgal species that can grow under heterotrophic conditions and increased costs due to the addition of organic carbon compounds\(^{6,4}\). Finally, mixotrophic cultivation has combined advantages of photoautotrophic and heterotrophic modes in the presence of light, i.e., microalgae can grow utilizing CO\(_2\) and organic carbon. Hence, the mixotrophic cultivation is useful for overcoming the limitations of photoautotrophic and heterotrophic cultivation of microalgae\(^{3,5,6}\).

Some studies have demonstrated an enhanced biomass and lipid production by mixotrophic microalgal cultivation using several organic carbons such as glucose, xylose, galactose, fructose, sucrose, glycerol, and acetate\(^{10-14}\). However, supplementing these commercial organic compounds increases the costs of microalgal cultivation. Organic wastes and byproducts in agricultural and food industries may be an ideal organic carbon source, with high cost effectiveness for microalgal mixotrophic cultivation\(^{10-14}\). In addition, the use of wastewater, which contains nutrients for microalgal growth, as a culture medium could also reduce the cost of microalgal cultivation\(^{15,16}\). Mixotrophic microalgal cultivation using organic waste and wastewater can improve the productivity and reduce the costs of microalgal lipid productions. However, there is a limited number of studies on the effects of organic wastes or, specifically, of both organic wastes and wastewater, on microalgal growth and lipid production under mixotrophic conditions. Further, there is no study that quantitatively compares the biomass and lipid production under mixotrophic conditions using organic waste with those using commercial carbon sources.

The objectives of this study were, therefore, to demonstrate the enhanced lipid production by the mixotrophic microalgal cultivation using organic waste and wastewater effluent. We used *Chlamydomonas reinhardtii* because it is widely used as model species for basic microalgal studies, as well as for biofuel production\(^{17,18}\). The wasted molasses and corn steep liquor (CSL) were used as organic wastes. Molasses is produced as a byproduct from a sugarcane factory or raw sugar refinery, and CSL is a byproduct from corn wet-milling. We cultured *C. reinhardtii* under mixotrophic conditions in the synthetic C medium supplemented with one of the commercial organic carbon sources (glucose, fructose, sucrose, or acetate) or organic waste (molasses or CSL). Then, we determined the effects of organic waste on the biomass and lipid productivities of *C. reinhardtii* compared to the commercial organic compounds. Further, we determined the usefulness of the sewage effluent in the biomass and lipid production by *C. reinhardtii*, compared to synthetic C medium. Finally, we discussed the enhanced lipid production by *C. reinhardtii* under mixotrophic conditions in the sewage effluent supplemented with organic waste.

**MATERIALS AND METHODS**

*C. reinhardtii* and its subculture conditions

Axenic *C. reinhardtii* (NIES-2235) was obtained from the Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan, and cultured in C medium (Table 1). The axenic *C. reinhardtii* culture was incubated in a flask containing C medium in a growth room at 28 ± 1°C with fluorescent lamps at a photosynthetic photon flux density of 80 µmol/m\(^2\)·s and a 12:12-h light–dark cycle for one week. Every week thereafter, cells were sub-cultured via routine transfer into fresh C medium.

Commercial organic carbon compounds and organic wastes

D-glucose, fructose, sucrose, and acetate were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Molasses
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was purchased from TMR Co., Ltd., (Tokyo, Japan). CSL was purchased from Oji Cornstarch Co., Ltd., (Tokyo, Japan). Before the use of molasses and CSL, their chemical composition was analyzed. The chemical properties are presented in Table 2.

**Sewage effluent** Sewage effluent was collected from the final settling tank of a conventional activated sludge sewage treatment plant in Kofu City, Yamanashi, Japan. The effluent sample was first passed through a glass microfiber filter (pore size, 1 µm; GF/B grade; GE Healthcare UK Ltd, Buckinghamshire, England) to remove suspended solids and organisms larger than bacteria, including microalgae from the effluent sample. Before the use of sewage effluent, its chemical composition was analyzed. The chemical properties are presented in Table 3.

*C. reinhardtii* cultivation experiments under photoautotrophic and mixotrophic conditions

For photoautotrophic cultivation, 150 mL C medium without any supplementation with organic carbon was prepared and poured into 200-mL baffled flask. All flasks were autoclaved (121 °C, 20 min). *C. reinhardtii* was harvested through centrifugation (6000 × g, room temperature, 5 min) from the above subculture, and washed with sterile C medium. The *C. reinhardtii* cells were inoculated into all the 200-mL baffled flasks. The

| Table 1 Composition of C medium |
| Composition | Concentration (mg/L) |
|---------------|---------------------|
| Ca(NO₃)_2 · 4H₂O | 150 |
| KNO₃ | 100 |
| β-Na₂glycerophosphate · 5H₂O | 50 |
| MgSO₄ · 7H₂O | 40 |
| Tris(hydroxymethyl)aminomethane | 500 |
| Vitamin B₁₂ | 0.0001 |
| Biotin | 0.0001 |
| Thiamine HCl | 0.01 |
| PIV metals |
| Na₂EDTA · H₂O | 3 |
| FeCl₃ · 6H₂O | 0.588 |
| MnCl₂ · 4H₂O | 0.108 |
| ZnCl₂ | 0.0312 |
| CoCl₂ · 6H₂O | 0.012 |
| Na₂MoO₄ · H₂O | 0.0075 |

| Table 2 The chemical properties of molasses and corn steep liquor |
| Composition | Molasses | Corn steep liquor |
|---------------|----------|-----------------|
| Moisture (g/100 g) | 36.9 | 52.9 |
| Energy (kcal/100 g) | 203 | 159 |
| Total sugars (g/100 g) | 40.9 | 6.5 |
| Total proteins (g/100 g) | 5.0 | 21.6 |
| Total lipids (g/100 g) | 0.5 | 0.1 |
| Other organics (g/100 g) | 9.8 | 11.4 |
| Ash (g/100 g) | 6.9 | 7.5 |
| Total nitrogen (g/100 g) | 0.8 | 3.5 |
| Phosphorus (g/100 g) | 0.13 | 0.40 |
| Minerals (mg/100 g) |
| Na⁺ | 87 | 4.0 |
| K⁺ | 6900 | 3100 |
| Ca²⁺ | 840 | 9.4 |
| Mg²⁺ | 480 | 820 |
| Fe³⁺ | 41 | 9.7 |
| Cu²⁺ | 1.7 | 0.20 |
| Zn²⁺ | 1.6 | 14.0 |

| Table 3 Composition of sewage effluent |
| Composition | Concentration (mg/L) |
|---------------|---------------------|
| TOC | 13.2 |
| NH₄-N | 6.52 |
| NO₂-N | 0.08 |
| NO₃-N | 4.64 |
| PO₄-P | 1.71 |
| Minerals |
| Na⁺ | 52.1 |
| K⁺ | 9.7 |
| Ca²⁺ | 19.0 |
| Mg²⁺ | 5.6 |
| Fe³⁺ | 0.03 |
| Cu²⁺ | < 0.01 |
| Zn²⁺ | 0.07 |
initial \(C. \textit{reinhardtii}\) dry cell concentration was about 50 mg–dry cell/L. Then, \(C. \textit{reinhardtii}\) was cultured under phototrophic and mixotrophic conditions in a growth room, at 28 ± 1°C, photosynthetic photon flux density of 80 \(\mu\text{mol/m}^2\cdot\text{s}\) and a 12:12-h light-dark cycle, for 7 days. All cultivation experiments were performed in sterile conditions and in triplicates (\(n = 3\)). During the cultivation experiments, the dry cell weight and lipid content of \(C. \textit{reinhardtii}\) and total organic carbon (TOC) concentration in medium were monitored.

**Analyses of organic wastes and sewage effluent** For organic wastes, moisture (water content), energy value (calorific value), total sugar content, total protein content, total lipid content, ash content, and minerals were measured according to the test methods of the Standard Tables of Food Composition in Japan with minor modifications. Moisture was calculated using wet and dried weight (at 90°C for 24 h). Energy value was measured by using an auto-calculating bomb calorimeter (CA-4Ad; Shimadzu Co. Ltd., Kyoto, Japan). Total sugar content was measured by the phenol-sulfuric acid method. Total protein was calculated by the nitrogen–protein conversion factor (total protein = 6.25 × total nitrogen)\(^{19}\); the total nitrogen was measured using an automatic combustion–elemental analyzer isotope ratio mass spectrometer (ANCA-GSL with Hydra20-20; Sercon Ltd., UK). Total lipids were measured by chloroform-methanol (2:1) lipid extraction method. Ash was measured by the dry ashing method at 550°C. Other organic compounds were calculated as original organic waste – (water + sugars + proteins + lipids + ash). Minerals were measured using inductively coupled plasma-optical emission spectrometry (ICP–OES) (Varian Vista MPX; Varian, Palo Alto, CA, USA) and Atomic Absorption Spectroscopy (AAS) (Hitachi Z–2000, Hitachi; Tokyo, Japan).

For sewage effluent, TOC, ammonium-nitrogen (\(\text{NH}_4^-N\)), nitrite–N (\(\text{NO}_2^-N\)), nitrate–N (\(\text{NO}_3^-N\)), phosphate (\(\text{PO}_4^{3-}-P\)), and minerals concentrations were measured according to Standard Methods for the Examination of Water and Wastewater as follows. TOC was measured by using TOC–LCSH (Shimadzu). The indophenol method was used for \(\text{NH}_4^-N\); the \(N\)-(1-naphthyl) ethylenediamine method was used for \(\text{NO}_3^-N\); the reduction–\(N\)-(1-naphthyl) ethylenediamine method and UV adsorption (at 220 and 275 nm) method was used for \(\text{NO}_2^-N\); and the molybdenum blue method was used for \(\text{PO}_4^{3-}-P\). Minerals were measured using ICP-OES and AAS.

For TOC measurement in \(C. \textit{reinhardtii}\) cultivation experiments, the culture medium was centrifuged (6,000 × g, room temperature, 5 min), and then the supernatant was filtrated using a membrane filter (polypropylene, pore size = 0.45 \(\mu\text{m}\); Membrane Solutions Co. Ltd., Tokyo, Japan). The filtrated sample was diluted using pure water. The samples were subjected to TOC–LCSH (Shimadzu).

**Analyses of dry biomass and lipids of \(C. \textit{reinhardtii}\)** The dry weight (mg/L) of \(C. \textit{reinhardtii}\) was measured as follows. Culture medium (20 mL) was collected from each flask into a tube, and the \(C. \textit{reinhardtii}\) cells in the medium were collected on a pre­weighed GF/B filter (pore size, 1 \(\mu\text{m}\)), dried (70°C, 1 d), and then weighed.

The lipid content (dry weight %) of \(C. \textit{reinhardtii}\) was quantified in terms of the percentage of the lipids in dry biomass as follows. Culture medium (50 mL) was collected from each flask into a centrifuge tube, and the \(C. \textit{reinhardtii}\) cells were then collected via centrifugation (6,000 × g, room temperature, 5 min). The cell pellet was harvested and dried at 70°C, for 1 d. The dried cells were ground using a BioMasher (Takara Bio, Shiga, Japan). Total lipids were extracted from the \(C. \textit{reinhardtii}\) cells powder using \(n\)-hexane/isopropanol as follows\(^{20}\). The \(C. \textit{reinhardtii}\) powder (20 mg) was re-crushed with 1 mL of \(n\)-hexane in a BioMasher. The \(C. \textit{reinhardtii}\) sample was transferred into a 50–mL glass tube, and 9 mL of \(n\)-hexane and 6 mL of isopropanol were added to the tube. The tube was agitated at 225 rpm, for 24 h. Thereafter, 33 mL of distilled water was added to the tube. The tube was agitated for 1 min and then centrifuged (10,000 × g, 5 min). The \(n\)-hexane layer containing the lipids was harvested on a pre-weighed aluminum tray, dried at room temperature.
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Biomass production rate (mg/L/d) and lipid production rate (mg/L/d) of *C. reinhardtii* were calculated as follows:

Biomass production rate (mg/L/d) = [final dry cell weight (mg/L) - initial dry cell weight (mg/L)] / 7 (d)

Lipid production rate (mg/L/d) = [final dry cell weight (mg/L) × final lipid content (%) - initial dry cell weight (mg/L) × initial lipid content (%)] / 7 (d)

**RESULTS**

Growth and lipid production of *C. reinhardtii* in synthetic C medium supplemented with different carbon sources

During the 7-day mixotrophic cultivation in C medium supplemented with organic carbons, TOC concentration decreased gradually under all mixotrophic conditions (Fig. 1). The TOC removal rate and efficiency were 13–21 mg/L/d and 23–57%, respectively (Table 4). TOC removal by *C. reinhardtii* from C medium with acetate or molasses was relatively higher than that in other mixotrophic conditions. On the other hand, TOC concentration in C

| Cultivation mode | Organic carbon source | TOC removal rate (mg/L/d) | TOC removal efficiency (%) | Biomass concentration (mg/L) | Biomass production rate (mg/L/d) | Lipid content (%) | Lipid production rate (mg/L/d) |
|------------------|-----------------------|--------------------------|----------------------------|-----------------------------|---------------------------------|------------------|-------------------------------|
| Photoautotrophic cultivation (C medium) | — | — | — | 298 ± 19 | 36 ± 3 | 25.7 ± 1.2 | 9 ± 0 |
| Mixotrophic cultivation (C medium supplemented with 1 g/L organic compound) | Glucose | 15 ± 3 | 25 ± 4 | 531 ± 18 | 69 ± 2 | (1.9) | 9.8 ± 0.6 | 7 ± 0 | (0.7) |
| | Fructose | 15 ± 3 | 25 ± 4 | 619 ± 27 | 82 ± 4 | (2.3) | 20.9 ± 1.2 | 17 ± 1 | (1.7) |
| | Sucrose | 13 ± 1 | 23 ± 2 | 524 ± 13 | 68 ± 1 | (1.9) | 12.4 ± 0.8 | 8 ± 1 | (0.9) |
| | Acetate | 19 ± 1 | 41 ± 3 | 627 ± 9 | 83 ± 1 | (2.3) | 37.9 ± 2.8 | 31 ± 2 | (3.4) |
| | Molasses | 21 ± 1 | 57 ± 3 | 687 ± 5 | 86 ± 1 | (2.4) | 40.0 ± 1.3 | 34 ± 2 | (3.7) |
| | CSL | 13 ± 1 | 38 ± 2 | 533 ± 24 | 64 ± 3 | (1.8) | 33.0 ± 2.3 | 21 ± 1 | (2.1) |

Values are mean ± SD (n = 3).

* shows the significant difference compared with photoautotrophic conditions (P<0.05).

* The number in a parenthesis indicates the ratio of each value to photoautotrophic cultivation experimental value.
medium without organic carbon (photoautotrophic condition) increased from 4.2 to 20.7 mg/L over 7 days. The increased TOC might be due to the exudates released from *C. reinhardtii*\(^\text{21}\).

Dry cell weight of *C. reinhardtii* increased in the C medium with and without supplementing organic carbon, over 7 days (Fig. 1). The dry cell weight in all C medium supplemented with organic carbon at the end of the 7-day period was significantly higher than that in C medium without supplementing organic carbon (Table 4). Biomass production rates of *C. reinhardtii* in the C medium supplemented with organic carbon were 1.9 to 2.4 times higher than the rate of *C. reinhardtii* in photoautotrophic conditions (Table 4).

The lipid content (%) of *C. reinhardtii* in the C medium supplemented with acetate, molasses, or CSL and without organic carbon increased greatly (Fig. 1). Lipid content of *C. reinhardtii* at the 7th day in the C medium supplemented with acetate, molasses or CSL was significantly higher than that in the photoautotrophic conditions. Lipid production rate of *C. reinhardtii* in the C medium supplemented with fructose, acetate, molasses or CSL were 1.7 to 3.7 times higher than that in the photoautotrophic conditions. In contrast, the lipid content and lipid production rate of *C. reinhardtii* in the C medium supplemented with glucose or sucrose was lower than that in the photoautotrophic conditions (Table 4).

**Biomass and lipid production of *C. reinhardtii* in sewage effluent supplemented with different carbon sources**

TOC concentration in the sewage effluent supplemented with organic compounds decreased gradually during the 7-day cultivation (Fig. 2). The TOC removal rate and efficiency were 4–18 mg/L/d or 9–50%, respectively (Table 5). TOC removal by *C. reinhardtii* from the sewage effluent with acetate or molasses was relatively higher than that in other mixotrophic conditions. On the other hand, TOC increased in sewage effluent without supplementing organic carbon from 14.6 to 29.3 mg/L over 7 days.

Dry cell weight of *C. reinhardtii* increased in all sewage effluents with and without supplementing organic carbon (Fig. 2). Biomass production rates of *C. reinhardtii* in the sewage effluent supplemented with organic carbon were 1.1 to 1.8 times higher than those in the sewage effluent without supplementing organic carbon (Table 5). Specifically, biomass production rate of *C. reinhardtii* in the sewage effluent supplemented with acetate or molasses was relatively higher than that under other conditions (Table 5).

The lipid content of *C. reinhardtii* in all sewage effluents, with and without supplementing commercial organic carbon, increased over 7 days (Fig. 2). Lipid contents of *C. reinhardtii* at the 7th day in the sewage effluent supplemented with acetate, molasses
or CSL was significantly higher than that in other conditions. Lipid production rates of *C. reinhardtii* in the sewage effluent supplemented with acetate, molasses or CSL were 1.6 to 2.8 times higher than those in the sewage effluent without supplementing organic carbon (Table 5). However, lipid content and lipid production rate of *C. reinhardtii* in the sewage effluent with glucose, fructose or sucrose were equal to or lower than those in the sewage effluent without supplementing organic carbon (Table 5).

**DISCUSSION**

In this study, we compared the growth and lipid production of *C. reinhardtii* in C medium under photoautotrophic and mixotrophic conditions, supplemented with one of the four commercial compounds (glucose, fructose, sucrose, and acetate) or two organic wastes (molasses and CSL). Biomass production of *C. reinhardtii* in C medium supplemented with the organic carbon was significantly enhanced compared to that in C medium under photoautotrophic conditions (Fig. 1 and Table 4). *C. reinhardtii* also utilized the tested organic carbon under mixotrophic conditions (Fig. 1). Acetate is well recognized as the suitable carbon source for *C. reinhardtii* growth under heterotrophic and mixotrophic conditions. Supplementing volatile fatty acids or glucose in a synthetic medium could also enhance growth of *C. reinhardtii*. In this study, the commercial organic compounds as well as two organic wastes were utilized by *C. reinhardtii* and supported its biomass production. The molasses and CSL were a mixture of sugars, proteins, lipids, nutrients (nitrogen and phosphorus) and various minerals (Table 2). Our findings showed that these mixed components were suitable for *C. reinhardtii* growth. Furthermore, molasses enhanced biomass production of *C. reinhardtii* more powerfully than CSL and to the same level as acetate (Fig. 1 and Table 4). Overall, our results clearly demonstrated that the two organic wastes, in particular molasses, were an effective carbon substrate for enhancing biomass production of *C. reinhardtii* under mixotrophic conditions.

We further compared the growth and lipid production of *C. reinhardtii* in C medium and sewage effluent collected from the final settling tank. The biomass and lipid production rates of *C. reinhardtii* in the sewage effluent were higher than those in C medium without supplementing organic carbon (Tables 4 and 5). The nitrogen, phosphate, and minerals, which had lower concentrations in the sewage effluent compared to C medium (Table 3) were sufficient for *C. reinhardtii* growth and lipid synthesis. Although TOC was present in the sewage effluent, the utilization of the TOC by *C. reinhardtii* was not clearly observed. Our results clearly indicate that the sewage effluent can be replaced with the synthetic

| Cultivation mode | Organic carbon source | TOC removal rate (mg/L/d) | TOC removal efficiency (%) | Biomass concentration (mg/L) | Biomass productivity (mg/L/d) | Lipid content (%) | Lipid productivity (mg/L/d) |
|------------------|-----------------------|---------------------------|---------------------------|-----------------------------|-----------------------------|------------------|-----------------------------|
| Mixotrophic cultivation (Sewage effluent) | Glucose | 5 ± 1 | 9 ± 2 | 367 ± 14 | 45 ± 3 (1.1)* | 26.6 ± 3.3 | 12 ± 2 (1.1) |
|                  | Fructose | 7 ± 1 | 11 ± 1 | 362 ± 6 | 44 ± 1 (1.1) | 25.1 ± 0.9 | 11 ± 0 (1.0) |
|                  | Sucrose | 6 ± 1 | 10 ± 1 | 418 ± 8* | 52 ± 1* (1.2) | 17.9 ± 7.0 | 9 ± 3 (0.8) |
| Mixotrophic cultivation (Sewage effluent supplemented with 1 g/L organic compound) | Acetate | 12 ± 2 | 27 ± 5 | 547 ± 33* | 74 ± 4* (1.8) | 41.9 ± 2.9* | 31 ± 0* (2.8) |
|                  | Molasses | 18 ± 1 | 50 ± 3 | 544 ± 35* | 73 ± 5* (1.8) | 37.1 ± 5.0* | 27 ± 2* (2.4) |
|                  | CSL | 4 ± 1 | 12 ± 4 | 396 ± 14* | 52 ± 2* (1.3) | 33.5 ± 3.3* | 17 ± 1* (1.6) |

Values are mean ± SD (n = 3).

* shows the significant difference compared with photoautotrophic condition (P<0.05).

* The number in a parenthesis indicates the ratio of each value to mixotrophic cultivation (sewage effluent without supplementing organic carbon) experimental value.
medium for *C. reinhardtii* and reduce the cost of its culture. Furthermore, supplementing the organic compounds into the sewage effluent increased biomass production of *C. reinhardtii*. Supplementing acetate or molasses enhanced biomass production of *C. reinhardtii* more powerfully, compared to other organic compounds. Acetate and acetyl-CoA are the major intermediate metabolites in the lipid synthesis pathway\(^\text{26}\), and assimilation of acetate can enhance lipid accumulation in *C. reinhardtii*\(^\text{27,28}\). Similar to the findings in the cited studies, in this study, supplementing acetate in a C medium and sewage effluent significantly increased lipid content and lipid production rate of *C. reinhardtii* (Figs. 1 and 2, Tables 4 and 5). In contrast, excepting the fructose into C medium, supplementing glucose, fructose, or sucrose into C medium and sewage effluent significantly decreased lipid content in *C. reinhardtii*, although biomass of *C. reinhardtii* increased under these conditions. Glucose and glucose-6-phosphate are precursors for starch in *C. reinhardtii*\(^\text{26,29}\). Although starch content in *C. reinhardtii* was not determined in this study, carbon flux in *C. reinhardtii* might be changed from lipid synthesis to starch synthesis under mixotrophic conditions supplemented with the sugars. Supplementing fructose into C medium increased lipid content (Fig. 1 and Table 4). However, this reason could not be revealed in this study. On the other hand, interestingly, supplementing the two organic wastes, in particular molasses, into C medium and sewage effluent increased the lipid content in *C. reinhardtii*. Molasses and CSL used in this study consisted of various organic compounds such as sugars, proteins, and lipids (Table 2). Other studies reported that molasses contained diverse amino acids and organic acids including acetate\(^\text{30,31}\), and CSL contained various amino acids, polypeptides, fatty acids, and organic acids\(^\text{32}\). Although the contribution of each component in the molasses and CSL to increase lipid accumulation in *C. reinhardtii* was not determined, these mixed organic compounds were found to be suitable for lipid synthesis and accumulation in *C. reinhardtii*. In particular molasses, acetate might be the most effective component for enhanced lipid accumulation in *C. reinhardtii*.

Enhanced biomass and lipid productivities of microalgae by addition of commercial and waste organic carbon sources into microalgal culture have been demonstrated in many previous studies\(^\text{7-10}\). On the other hand, wastewater has been recognized as a cost-effective culture medium for microalgal lipid production. Biomass productivity at range between 6 to 2,000 mg/L/d and lipid productivity at range between 0.54 to 505 mg/L/d of microalgae cultured in various wastewater conditions have been reported\(^\text{15}\). This is the first study for the comprehensive evaluation of biomass and lipid production by *C. reinhardtii* under mixotrophic conditions, using organic wastes or commercial compounds as organic carbon source and wastewater or synthetic C medium as a culture medium. Our results clearly demonstrated the enhanced biomass and lipid production by *C. reinhardtii* in the sewage effluent supplemented with molasses under mixotrophic conditions. Biomass (73 mg/L/d) and lipid (27 mg/L/d) productivities of *C. reinhardtii* cultured in sewage effluent supplemented with 1 g/L molasses were 1.8 and 2.4 times higher than those in sewage effluent alone, respectively. Thus, the mixotrophic cultivation of microalgae including *C. reinhardtii* using sewage effluent and organic waste can be a promising strategy for overcoming the bottlenecks (low lipid productivity and high cultivation cost) of microalgal biofuel production. This strategy can be coupled reasonably with wastewater treatment plant and can transform a wastewater treatment plant into the base of resource recycling and sustainable biofuel production. For the practical application, exploring the suitable organic wastes and wastewater for microalgal lipid production, and optimization of the mixotrophic conditions such as amending organic waste concentrations and using various combinations of organic wastes should be explored in future studies.

**CONCLUSIONS**

We compared production of biomass and lipid content by *C. reinhardtii* under
mixotrophic cultivation using C medium/ sewage effluent and supplementing these with different organic carbons. Mixotrophic cultivation using sewage effluent supplemented with molasses proved the enhanced biomass and lipid productivity of C. reinhardtii. The biomass and lipid productivity was significantly higher in the above conditions than under photoautotrophic cultivation using a synthetic C medium and mixotrophic cultivation using a C medium supplemented with glucose, fructose, or sucrose, and at the same level as under mixotrophic conditions using acetate. These results indicate that the mixotrophic cultivation using sewage effluent and molasses can be a promising strategy for a cost-effective, sustainable and highly efficient biofuel production by C. reinhardtii.

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