Evaluation of Growth Characteristics of *Euglena gracilis* for Microalgal Biomass Production Using Wastewater

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**ABSTRACT**

Recently, fossil fuel depletion and global warming have become serious problems in the world. In order to solve these problems, renewable energy has attracted much attention. Here, a microalga, *Euglena gracilis* (*E.gracilis*), was focused on as a renewable biomass energy source. In our idea, *E. gracilis* biomass produced using nutrients in wastewater is mixed with sewage sludge, and anaerobically digested for methane recovery. It is considered that microorganisms and suspended solids in wastewater may have a negative impact against the growth of *E. gracilis*. Accordingly, their effect on the growth of *E. gracilis* was discussed in this research. Three types of culture media, supernatant fluid of wastewater, suspended solids-free wastewater, and suspended solids and microorganisms-free wastewater, were used for the cultivation test. The culturing conditions were a temperature of 25°C and photosynthetically active radiation of 98.2 μmol/(m\(^2\)·s). As a result, *E. gracilis* was able to increase in wastewater, though both microorganisms and suspended solids gave a negative influence. Since the negative effect of suspended solids was stronger than that of microorganisms, an introduction of a pre-removal process of suspended solids would be favored for the microalgal biomass production in wastewater.

**Keywords:** biomass production, euglenid, growth rate, phytoplankton, wastewater

**INTRODUCTION**

Recently, people live a very convenient life owing to the remarkable development of science and technologies. After the Industrial Revolution, on the other hand, natural ecosystem has collapsed due to global warming, air pollution by emission of acidic substances like sulfur oxides and nitrogen oxides, and other phenomena in the world. In addition, depletion of fossil fuels is a serious problem (Bentley, 2002). Currently, renewable energies being characterized as carbon neutral, attract much attention in order to solve these problems. For example, sustainable energy like solar power, wind power, geothermal power, and biomass energy have been developed to substitute for fossil fuel in Vietnam since the early 2000 (Nguyen *et al.*, 2013). Japanese government is also carrying out Biomass Nippon Strategy since 2002 (Kuzuhara, 2005). However, the production cost of biomass energy in Canada was about 50 US$/MWh, whereas that of fossil energy was only about 30 US$/MWh (Kumar *et al.*, 2003). In Belgium it was reported that biomass production cost 78% higher than fossil fuel production (Brammer *et al.*, 2006). Therefore, it is necessary to improve a cost-performance ratio of biomass energy for its penetration into our society.

In this study, we paid attention to microalgae as a biomass energy source. They can grow rapidly without soil (Widjaja *et al.*, 2009) and incept carbon dioxide during their growth. Therefore, they can contribute to solve the global warming problem (Avagyan,
Although a usage of food crops like corn as a biomass energy source may arouse a food crisis, microalgal biomass is unlikely to cause such a problem. Furthermore, microalgae can be harvested in shorter cultivation period than other crops (Schenk et al., 2008). Thus, the microalgal biomass is a potential substrate for sustainable biomass energy production (Harun et al., 2010).

Everyday people are producing a huge amount of municipal wastewater, which abundantly contains nitrogen and phosphorus. Benemann and Oswald (1996) reported that the usage of wastewater as a nitrogen and phosphorus source was estimated to cut down the microalgal biomass production cost by about 10 – 20%. Thus, microalgal biomass production in wastewater is a promising process for economical biomass production. However, there are few reports on microalgal biomass production in wastewater. Bhatnagar et al. (2010) successfully cultivated Chlorella minutissima (C. minutissima) using a mixture of wastewater and synthetic medium, but the cultivation in 100% wastewater deteriorated the growth of C. minutissima. Accordingly, cultivation of microalgae in wastewater is a challenging but important subject for the economical microalgal biomass production. In this research, we focused on Euglena sp. as a microalga for biomass production, because Mahapatra et al. (2013) reported that Euglena sp. was useful to wastewater treatment and might be produced in municipal wastewater.

The methane production from sewage sludge is one of the biomass energy processes. The addition of microalgal biomass produced in wastewater into a methane fermenter using sewage sludge is one of the potential alternatives to increase the methane production yield. It is considered as an alternative energy to fossil fuel (Chisti, 2007). Microalgal cultures in wastewater have also been discussed for wastewater treatment (Noüe et al., 1992). However, wastewater contains various microorganisms and suspended solids (SS), which may affect the growth of microalgae. Accordingly, we discussed the effect of microorganisms and SS on the growth of microalgae in wastewater to elucidate the feasibility of microalgal production using wastewater in this study.

MATERIALS AND METHODS

Microalga

An axenic and clonal strain of Euglena gracilis (E. gracilis) stocked in the National Institute for Environmental Studies, Japan (NIES-49, Kawachi et al., 2013) was used in this research. Although euglenid is classified as both animal (Häder and Liu, 1990) and plant (Chang et al., 1981), the classification of Euglenophyceae in Euglenozoa was adopted in this research according to the classification by the National Institute for Environmental Studies. This species is an oval-shaped single cell flagellate (Ascoli et al., 1978) with a high growth rate (Mahapatra et al., 2013) and can grow under both autotrophic and heterotrophic conditions because it is a mixotrophic alga (Takeyama et al., 1997). It is considered to be potentially useful for biomass energy, because of the production ability of wax esters combined with fatty alcohols and fatty acids (Inui et al., 1982).
Cultivation of *E. gracilis* in synthetic media

The HUT medium based synthetic media were used in this research. The normal HUT medium (Kawachi *et al*., 2013) was used for the evaluation of light and temperature effect. The compositions of the modified HUT media for the evaluation of nitrogen limitation and of phosphorus limitation are summarized in Table 1 and 2, respectively. Although vitamin B$_{12}$ and thiamine HCl contain nitrogen and phosphorus, the final concentrations derived from these chemicals were only 67 µgN/L and 0.012 µgP/L. Accordingly, the effects of nutrients from these chemicals were negligible. In the cultivation test, 100 mL of each medium was poured into a 200 mL Erlenmeyer flask with a loosely closed screw cap and sterilized for 20 minutes at 120°C in an autoclave (ES-315, Tomy Seiko, Tokyo, Japan). Then, 0.1 – 0.2 mL of pre-cultured *E. gracilis* was inoculated into each flask and cultured in a biotron (LH-55-RDS, Nippon Medical & Chemical Instruments, Osaka, Japan) for 13 days. The cultivation condition for nutrient limitation was a temperature of 25°C and a photosynthetically active radiation (PAR) of 98.2 µmol/(m$^2$·s). However, the temperature and PAR were changed from 10 to 35°C for the evaluation of temperature effect and from 58.4 to 178 µmol/(m$^2$·s) for the evaluation of light effect. The triplicated culture was used for all tests.

### Table 1 - Composition of the modified HUT medium for nitrogen limitation.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Nitrogen concentration [mgN/L]</th>
<th>Starvation medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>20 mg</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>0 mg</td>
<td></td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>0 mg</td>
<td>19.08 mg</td>
</tr>
<tr>
<td>MgSO$_4$ • 7H$_2$O</td>
<td>19.08 mg</td>
<td>38.17 mg</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>25 mg</td>
<td>76.36 mg</td>
</tr>
<tr>
<td>Potassium citrate</td>
<td>40 mg</td>
<td>152.7 mg</td>
</tr>
<tr>
<td>Polypeptone</td>
<td>0 mg</td>
<td>0 mg</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0 mg</td>
<td></td>
</tr>
<tr>
<td>Vitamin B$_{12}$</td>
<td>0.5 µg</td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.4 mg</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>1,000 mL</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2 - Composition of the modified HUT medium for phosphorus limitation.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Phosphorus concentration [mgP/L]</th>
<th>Starvation medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0 mg</td>
<td>4.394 mg</td>
</tr>
<tr>
<td>KCl</td>
<td>24.07 mg</td>
<td>13.18 mg</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>24.07 mg</td>
<td>21.97 mg</td>
</tr>
<tr>
<td>MgSO$_4$ • 7H$_2$O</td>
<td>21.97 mg</td>
<td>43.94 mg</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>43.94 mg</td>
<td>0 mg</td>
</tr>
<tr>
<td>Potassium citrate</td>
<td>400 mg</td>
<td></td>
</tr>
<tr>
<td>Polypeptone</td>
<td>288.8 mg</td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>40 mg</td>
<td></td>
</tr>
<tr>
<td>Vitamin B$_{12}$</td>
<td>40 mg</td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.5 µg</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.4 mg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,000 mL</td>
<td></td>
</tr>
</tbody>
</table>
Cultivation of *E. gracilis* in wastewater

Wastewater drained from a restaurant in Ryukoku University was used in this research. As *E. gracilis* was reported to use ammonia nitrogen as nitrogen source rather than nitrate nitrogen (Cramer and Myers, 1952), we assumed that a cultivation tank for *E. gracilis* was installed just after a primary sedimentation tank. Therefore, two hours of sedimentation was applied to the wastewater and supernatant fluid (called “raw wastewater” hereafter) was used for cultivation. To omit the effect of large and abiotic SS, raw wastewater was filtered through a glass fiber filter with 2.7 μm particle retention (Whatman GF/D, GE Healthcare, Tokyo, Japan). This filtrate was referred to as large SS-free wastewater hereafter. The large SS-free wastewater was further filtered through a filter paper with particle retention of 0.45 μm (HAWP04700, Merck Millipore, Billerica, MA, USA) to remove the microorganisms. This filtrate was referred to as microorganism-free wastewater hereafter. The chemical oxygen demand (CODC), total nitrogen (TN), and total phosphorus (TP) concentrations of each wastewater analyzed in pursuance of APHA-AWWA-WEF (2012) were respectively 485 mg/L, 72.9 mgN/L and 3.67 mgP/L for raw wastewater; 368 mg/L, 70.8 mgN/L and 3.32 mgP/L for large SS-free wastewater; and 281 mg/L, 62.5 mgN/L and 3.16 mgP/L for microorganism-free wastewater. The 100 mL portion of each wastewater and 0.3 – 0.4 mL of pre-cultured *E. gracilis* were placed into a 200 mL Erlenmeyer flask with a loosely closed screw cap and cultured in a biotron (LH-55-RDS, Nippon Medical & Chemical Instruments, Osaka, Japan) for 20 days at a temperature of 25°C and a PAR of 98.2 μmol/(m²·s). The triplicated culture was used for all cultivations.

Evaluation of specific growth rate

Cultivation was continued until the cell density reached the maximum. The medium was sampled from each Erlenmeyer flask every 2 – 3 days and the cell density of *E. gracilis* was counted using a plankton counting chamber (MPC-200, Matsunami, Osaka, Japan) under an upright microscope (CX41, Olympus, Tokyo, Japan) after fixation by the addition of glutaraldehyde solution at a final concentration of 53.2 g/L. The cell density increases exponentially in the logarithmic growth phase. Accordingly, the growth curve was expressed by equation (1) (Suzuki *et al*., 2013),

\[ \ln X_t = \ln X_{t0} + \mu t \]  

where \( \mu \) is the specific growth rate [1/day], \( X_t \) is the cell density at day \( t \) [cells/mL], and \( X_{t0} \) is the cell density at the beginning of the logarithmic growth phase [cells/mL]. The specific growth rate was determined by the regression analysis based on equation (1) using the data in the logarithmic growth phase.

RESULTS AND DISCUSSION

Temperature effect

Figure 1 shows the specific growth rate and the maximum cell density observed in the HUT medium. The specific growth rate and the maximum cell density reached the maximum level at water temperature ranging from 25 – 30°C and 20 – 25°C, respectively. These results mostly accorded with the suitable temperature of 20 – 35°C reported by Buetow (1962), though 35°C was not favorable for the growth of *E. gracilis* in this study. Judging from the experimental results, the optimal temperature for *E.
E. gracilis biomass production was thought to be 25°C, at which the suitable temperature ranges for the specific growth rate and for the maximum cell density were overlapped. Under the optimal temperature of 25°C, the specific growth rate observed was 1.19 1/day. As the specific growth rates of *E. gracilis* were reported to be in the range of 0.43 – 1.08 1/day (Cramer and Myers, 1952; Maly, 1975; Ogbonna *et al.*, 1998), the strain used in this study had a slightly higher growth rate than the other strains previously reported.

Nakayama *et al.* (2007) reported that the temperature of municipal wastewater in Tokyo ranged from 16 to 28°C in influent and from 14 to 29°C in effluent. Accordingly, *E. gracilis* has a potential to grow in the municipal wastewater, though biomass productivity is reduced by half in winter.

**Nutrient concentration effect**

The specific growth rate and the maximum cell density observed under nitrogen limitation and phosphorus limitation are shown in Figs. 2 and 3, respectively. When *E. gracilis* was incubated in the nutrient-free medium, an increase in the cell density was observed during the first 4 days of incubation. The initial growth in *E. gracilis* would be caused by the carryover of nutrients with the pre-cultured *E. gracilis* inoculated. Accordingly, the specific growth rate was evaluated by the data after 4 days of incubation. The observed specific growth rates and maximum cell densities in Figs. 2 and 3 were relatively smaller than those at 25°C shown in Fig. 1. The original HUT medium contains 600 mg/L of polypeptone and 400 mg/L of yeast extract, which involved nitrogen at a concentration of 56 mgN/L and 20 mgN/L, and phosphorus at a concentration of 4.4 mgP/L and 2.9 mgP/L, respectively. Therefore, both polypeptone and yeast extract were removed in nutrient limitation tests. However, polypeptone and yeast extract also involve minerals and trace chemicals. Accordingly, the relatively small growth rate and small maximum cell density shown in Figs. 2 and 3 might be caused by the lack of some minerals and/or trace chemicals.

Fig. 1 - Dependency of specific growth rate (a) and maximum cell density (b) on temperature. The error bar represents the unbiased standard deviation of triplicated culture.
Fig. 2 - Dependency of specific growth rate (a) and maximum cell density (b) on nitrogen concentration. The error bar represents the unbiased standard deviation of triplicated culture.

Fig. 3 - Dependency of specific growth rate (a) and maximum cell density (b) on phosphorus concentration. The error bar represents the unbiased standard deviation of triplicated culture.

The specific growth rate under nitrogen limitation gradually increased with the rise in nitrogen concentration and reached the maximum at 20 mgN/L (Fig. 2(a)), but the difference in the specific growth rate in the range of 5 – 40 mgN/L was not so large. The maximum cell density showed similar dependency on nitrogen concentration with the specific growth rate (Fig. 2(b)), though the significant difference between ≤ 10 mgN/L and ≥ 20 mgN/L was observed. Thus, 20 mgN/L or higher concentration was thought to be suitable for the growth of *E. gracilis*.

The specific growth rate under phosphorus limitation jumped up at 1 mgP/L and reached a plateau at a higher concentration (Fig. 3(a)). The dependency of the maximum cell density on phosphorus concentration was unclear, but was similar to the specific growth rate (Fig. 3(b)). Thus, 1 mgP/L or higher concentration was thought to be suitable for the growth of *E. gracilis*.
Japan Sewage Works Association (2006) reported that the total nitrogen (TN) and total phosphorus (TP) concentrations in influent to 8 municipal sewage treatment plants in Japan were in the range of 23 – 36 mgN/L and 2.0 – 5.2 mgP/L, respectively. The suitable concentration range observed in this study covered the concentration range in municipal wastewater. Consequently, *E. gracilis* was evaluated to be applicable to biomass production in municipal wastewater from the viewpoint of nutrient concentration.

**Light effect**

Figure 4 shows the specific growth rate and the maximum cell density observed in the HUT medium. The specific growth rate and maximum cell density observed were almost constant in the PAR range tested. However, solar radiation is more than 1000 \( \mu \text{mol}/(\text{m}^2\cdot\text{s}) \) of PAR, even though it is cloudy (Keller *et al.*, 2001), and contains ultraviolet (UV) radiation. Actually, inhibition of the growth of *E. gracilis* by UV radiation was observed (Ekelund, 1993). Therefore, when *E. gracilis* is cultured under the sunlight, its growth may be inhibited by the UV radiation. Accordingly, it may be required to cut off the UV radiation in sunlight with an optical filter or to deepen the depth of a culture tank for providing an evacuation site.

**Growth characteristics in the wastewater**

Figure 5 summarizes the specific growth rate and the maximum cell density in three wastewater-based medium, raw wastewater, large SS-free wastewater, and microorganism-free wastewater. The specific growth rates in large SS-free wastewater and microorganism-free wastewater were significantly higher than that in raw wastewater. However, the specific growth rates observed were much smaller than that in the synthetic wastewater. The maximum cell density in microorganism-free wastewater was the largest of the three, but the density was also much less than that in the synthetic wastewater. Similar result of *C. minutssima* was reported by Bhatnagar *et al.* (2010), though the reason for the growth deterioration in 100% wastewater was not identified. Nevertheless, our results indicate that some factors except temperature, light, nitrogen, and phosphorus affect the growth of *E. gracilis* in wastewater.

The comparison of results in raw wastewater and in large SS-free wastewater revealed that large SS had a negative effect on both specific growth rate and maximum cell density. Since aggregates of *E. gracilis* with SS were found under microscopic observation in this study, the aggregation effect of SS would inhibit the growth of *E. gracilis*. 
Fig. 4 - Dependency of specific growth rate (a) and maximum cell density (b) on PAR. The error bar represents the unbiased standard deviation of triplicated culture.

Fig. 5 - Specific growth rate (a) and maximum cell density (b) in three types of wastewater-based medium. The error bar represents the unbiased standard deviation of triplicated culture.
The microorganisms in wastewater had a negative effect on the maximum cell density, but no significant influence on the specific growth rate. Two possible reasons for the effect of microorganisms are as follows: an attack against *E. gracilis* by microorganisms and a competition of substrate uptake. If the former occurs, a decrease in the specific growth rate is expected. However, no difference between the specific growth rates with and without microorganisms was observed as shown in Fig. 5(a). On the other hand, if the latter occurs, a depletion of limiting substrates limits the final biomass, namely maximum cell density. However, less influence on specific growth rate is expected in comparison with the maximum cell density, because the depletion of substrate shortens the period of logarithmic growth phase, but does not change the slope of the semi-logarithmic plot of cell density during the logarithmic growth phase. Therefore, Fig. 5 confirms that the latter reason was valid. Thus, it was inferred that some competition of substrate uptake between *E. gracilis* and microorganisms in wastewater limited the maximum cell density. It was unable to identify the limiting substrate at present but it was strongly suggested that trace chemicals limited the growth of *E. gracilis* as shown in the cultivation tests without polypeptone and yeast extract. Consequently, the pre-removal of SS will be recommended for *E. gracilis* biomass production in wastewater.

**CONCLUSIONS**

The growth characteristics of *E. gracilis* were evaluated by cultivation in synthetic and wastewater-based media to elucidate the feasibility of its biomass production using wastewater. The obtained results are summarized as follows:

1. The suitable temperature for the growth of *E. gracilis* was in the range of 20 – 30°C, and the optimal temperature was 25°C. As the temperature of municipal wastewater in Tokyo was ranging from 16 to 28°C, *E. gracilis* has a potential to grow in the municipal wastewater, though biomass productivity is reduced by half in winter.

2. The specific growth rate and the maximum cell density of *E. gracilis* reached the maximum at over 20 mgN/L as ammonia and 1 mgP/L as phosphate. As these concentration values were within a typical range in municipal wastewater, therefore, *E. gracilis* was thought to be applicable to biomass production in municipal wastewater from the viewpoint of nutrient concentration.

3. The SS in the wastewater had a negative effect on the growth of *E. gracilis*. Since aggregates of *E. gracilis* with SS were found under microscopic observation, the aggregation effect of SS would inhibit the growth of *E. gracilis*.

4. The microorganisms in the wastewater affected the maximum cell density, but did not have any significant influence on the specific growth rate. It was inferred that a competition of substrate uptake between *E. gracilis* and microorganisms in wastewater limited the maximum cell density.

5. The wastewater was available for the biomass production of *E. gracilis* if a pretreatment of wastewater for the removal of SS was introduced. However, the growth rate of *E. gracilis* in the wastewater was smaller than in the synthetic
medium, probably due to insufficient amount of substrate like minerals and/or trace chemicals. Accordingly, the identification of limiting substrates and the development of culture and biomass recovery system will be required for the application of E. gracilis to biomass production in wastewater.

REFERENCES
wastewater treatment and improvement of water environment. Japan Sewage Works Association, Tokyo, Japan.