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Effect of carbon dioxide injection on photosynthetic wastewater treatment using microalgae *Chlorella vulgaris* and *Euglena gracilis*

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ABSTRACT

Photosynthetic wastewater treatment of secondary animal wastewater was investigated using two microalgae (*Chlorella vulgaris* and *Euglena gracilis*) under both control and CO_2-enriched conditions. The addition of CO_2 to the microalgae culture contributed to foster nutrient uptake for *C. vulgaris*, but *E. gracilis* culture was not affected much. *E. gracilis* culture under CO_2 injected condition showed the highest cell density and *C. vulgaris* showed the highest specific growth rate in the CO_2 enrichment experiment. *C. vulgaris* cells were 15-times smaller than those of *E. gracilis*. The noticeably distinguishable sizes affected the separation processes. *E. gracilis* possessed better filterability and dewaterability. The CO_2 injection greatly enhanced biomass protein production in both algae, but reduced lipid and carbohydrate fraction. By adding CO_2, lipid profile of *C. vulgaris* has changed greatly, as opposed to *E. gracilis* that has remained pretty much the same way.

Keywords: Carbon dioxide; *C. vulgaris*; *E. gracilis*; Gompertz model; Microalgae; Photosynthetic wastewater treatment

1. Introduction

Livestock wastewater is considered one of the most polluting wastewater because it contains a high concentration of nitrogen and phosphorus that causes eutrophication when discharged without proper treatment. Livestock waste is often subjected to integrated bio-treatment system including anaerobic digestion and advanced treatment process. However, it is difficult to meet the strict discharge standards, even in advanced systems. Since the 1950s, algal treatment has been applied along with a variety of processes to remove residual nutrients from wastewater [1]. Microalgae contain high nitrogen and phosphorus contents, approximately 10 and 1%, respectively, on a dry weight basis, which is several times greater than that of higher plants [2].

Microalgae and cyanobacteria can offer a low cost process and have been utilized as bioremediature agents to remove wastewater nutrients [3,4]. The use of microalgae has also attracted attention because the microalgae have the ability to provide greenhouse gas saving as it utilized large amount of CO_2 during the

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cultivation [5]. The application of CO_2 to microalgae cultivation, using wastewater as the culture medium, has provided active versatility to wastewater treatment, including the production of valuable protein and the mitigation of carbon. Microalgae is another valuable unicellular microalgae in the production of various proteins involved in photosynthetic reactions, including the biosynthesis of several substrates and metabolites, such as lipids, carotenoids, chlorophyll, alginate, and antioxidant vitamins [6]. Therefore, it can act as a substantial dietary supplement in food and feeds [7] as well as is currently considered the most promising sources for biofuel production based on its composition [8,9].

In particular, *Chlorella* spp. has broadly used in the wastewater treatment because they can be easily cultivated under different mixotrophic conditions [10]. *C. vulgaris* has been shown to actively assimilate nutrients during practical cultivation. Meanwhile, *E. gracilis* has been successfully employed on exposure to extreme pH conditions, though nutrient conditions affect *E. gracilis* motility [11]. *E. gracilis* has also been functionally applied due to its tolerance towards a diversity of high heavy metal concentrations [12,13]. However, due to complications in the harvesting and separation of microalgae, various investigations have focused on developing improved separation systems and a cost-effective approach for microalgae removal has been studied. The overall objectives of this study were to evaluate the performance of two microalgae (*C. vulgaris* and *E. gracilis*) grown in secondary swine wastewater and the impact of CO_2 enrichment on microalgae cells in terms of the growth kinetics, nutrient uptake, and biomass composition.

2. Materials and methods

2.1. Microalgae and wastewater

The unicellular microalgae used in this study were *C. vulgaris* (UTEX 265) and *E. gracilis* (SAG 1224). Before application to the wastewater medium, the microalgae were cultured in sterilized Bold Basal Media (BBM) for *C. vulgaris* and AF-6 media for *E. gracilis*. All inocula were subcultured by transferring 10% (v/v) of inoculate at 20-day intervals. *C. vulgaris* and *E. gracilis* were grown in 250 mL Erlenmeyer flasks at 20–25˚C on a 75 rpm rotary shaker. Initially loaded inocula were adjusted to 5×10^5 cell/mL for *C. vulgaris*, and 2.5×10^4 cell/mL for *E. gracilis*.

The secondary swine wastewater was collected from the aeration tank of an animal wastewater treatment plant in Hongseong, South Korea. The wastewater constituents varied with sampling time, and the stock wastewater was stored in the refrigerator at 4˚C. The color of the wastewater was pale brown and quite clear. The characteristics of the secondary swine wastewater were determined (Table 1). The chemical oxygen demand (COD), suspended solids (SS), total nitrogen (TN), and total phosphorus (TP) were higher than the Korea effluent standard. In this experiment, the secondary swine wastewater was diluted four times as the culture medium in order to neutralize and reduce the nutrient load used, but no pH adjustment was performed. The pH in the test cultures ranged from 8 to 10. The swine wastewater was controlled at 20–25˚C before applying to microalgae treatment.

2.2. Apparatus and culture condition

The apparatus consisted of a rectangular reactor with a working volume of 2.5 L and equipped with 13 watt warm white fluorescent tubes (Guangzhou Wanjiang Technology Co.) with controlled temperature (22–25˚C) and an illumination cycle of 16 h L: 8 h D (35–55 μmE/m^2/s). Air was bubbled through a diffuser to the culture at a continuous rate of 0.5 mL/min. The CO_2 injection system was equipped with a CO_2 generator set (CO_2 tank, regulator, flow meter, and diffuser). CO_2 was added to air adjusted with 3% (v/v) CO_2, and injected for 12 h a day, followed by a 4 h period with no CO_2 input during the light illuminated period.

2.3. Experimental and statistical analysis

Microalgae cells were observed under a microscope (Olympus BX51) at 20X magnification using a Superior Marienfeld 0.1 mm depth haemocytometer. Protein was analyzed using a standard Bovine Serum Albumin curve according to the Bradford method [14]. Carbohydrate was measured by the anthrone method

Table 1
Wastewater characteristics

Parameter	Unit	Value	Effluent standard*
pH		8.29–8.51	6.5–8.5
Turbidity	NTU	8.69–10.84	–
SS	mg/L	687–700	10
COD	mg/L	227.69–242.81	40
TP	mg/L	20.36–26.40	2
TN	mg/L	108.0–145.0	20
NO^{3-}–N	mg/L	61–74	–
NH_3–N	mg/L	1.69–1.90	–

*Source: Ministry of Environment, Republic of Korea.

and lipid was conducted by an infrared method modified from *Standard Methods* [15]. The fatty acid methyl esters (FAMEs) analyses were basically the same as described in previous study [16]. The FAMEs were analyzed using an HP 5890 gas chromatograph (Hewlett Packard, Rolling Meadows, IL) equipped with an HP Ultra 2 capillary column (cross-linked 5% phenyl methyl silicone, 25 m by 0.2 mm by 0.33 μm film thickness) and a flame ionization detector. Principal component analysis (PCA) was conducted to compare the lipid profiles of the two microalgae grown under different CO_2 conditions. COD, SS, pH, nitrogen, and phosphorus were measured according to *Standard Methods* [15]. Specific resistance to filtration (SRF) performed using a capillary suction time apparatus (Triton Type 319, Triton Electronics Ltd, England), and sludge volume index (SVI) and time to filtration (TTF) were analyzed according *to Standard Methods* [15]. Results were analyzed by graph fit, using a statistical program (MS Excel) and SigmaPlot 11 for growth model derivation.

3. Results and discussion

3.1. Microalgae growth

E. gracilis with CO_2 resulted in the highest biomass density and reached the maximum value at day seven. *C. vulgaris* also showed better growth with CO_2, although lag time was longer than *E. gracilis* (Fig. 1). Using methane content measurement and Gompertz equation [17], biochemical methane potential was calculated. The microalgae growth kinetics was expressed in terms of the Modified Gompertz model shown in Eq. (1).

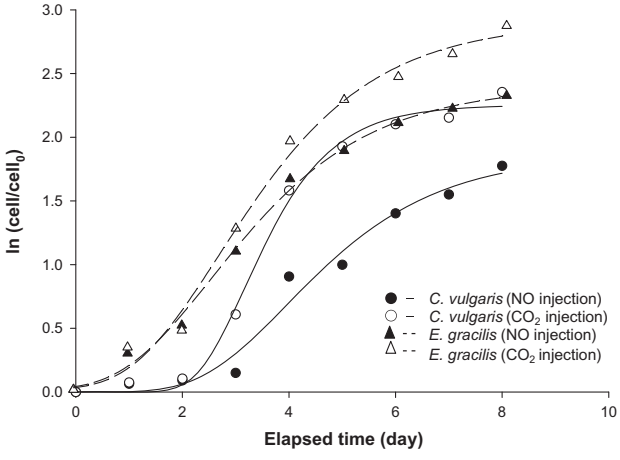

Fig. 1. The modified Gompertz model for the growth of microalgae (The model is shown as solid lines).

$$y = A \cdot \exp\left\{-\exp\left[\frac{\mu_m \cdot e}{A}(\lambda - t) + 1\right]\right\} \quad (1)$$

where y is the relative biomass concentration (ln(cell counts at time t/cell counts at initial time, t_0), A the relative maximum biomass concentration, μ_m the specific growth rate, λ the lag time, and t the time period.

As shown in Fig. 1, all experimental treatments were well-fitted with the model. A gradually increasing microalgae growth rate corresponded with CO_2 input, because microalgae converted CO_2 into biomass [18]. It is also probably due to pH reduction effecting the microalgae photosynthesis and metabolism. The addition of CO_2 to the *E. gracilis* culture showed the highest growth population (ln(cell/cell$_0$)). However, the highest growth rate occurring for *C. vulgaris* was investigated. CO_2 is renowned as a limiting source for algal cell metabolic activity, such as photosynthesis. Indeed, from retrospective studies, it has been known for a century that high CO_2 concentrations (beyond normal atmospheric level) improve algal metabolism and photosynthesis [19]. Peak and Peak [20] studied CO_2 fixation by *E. gracilis,* even in darkness, and found that CO_2 uptake by *E. gracilis* involved the carboxyl group in the cell catalytic metabolism. For *C. vulgaris*, the CO_2 addition increase cell population and it correspond to the previous report that describes raising concentration of CO_2 enhance cell density [21]. They have found that the maximum growth rate occurred with CO_2 levels in air up to 30%. Although the specific growth rate of *Euglena* cells has been found to be related to the light intensity [22], other influential factors, such as oxygen resource and light, were not considered in this experiment.

The mathematical parameters in the modified Gompertz model were derived from experimental data using the SigmaPlot11 statistical program. The mathematical parameters were calculated and are shown in Table 2. CO_2 was conducive to the cells in terms of cell concentration and specific growth rate, μ_m. The specific growth rate, μ_m, for *C. vulgaris* was increased twice by the addition of CO_2. This demonstrated that CO_2 was used as a carbon source for microalgae photosynthesis and respiration. CO_2 injection was more effective to *C. vulgaris* than *E. gracilis* in enhancement of biomass density and specific growth rate.

3.2. Nutrient removal

Nutrient removal was observed in terms of reductions in nitrate and phosphate. Fig. 2 shows the percentage nutrient uptake by microalgae between with

Table 2
The biokinetic parameters from microalgae treatments

Treatment	Maximum cell concentration ($\times 10^5$ cell/mL)	Lag time (λ)	Specific growth rate, μ_m (1/day)
C. vulgaris (No injection)	18.633	2.3568	0.426447
C. vulgaris (CO_2 injection)	22.537	2.2761	0.913598
E. gracilis (No injection)	0.24104	0.9078	0.537645
E. gracilis (CO_2 injection)	0.29215	2.085	0.665610

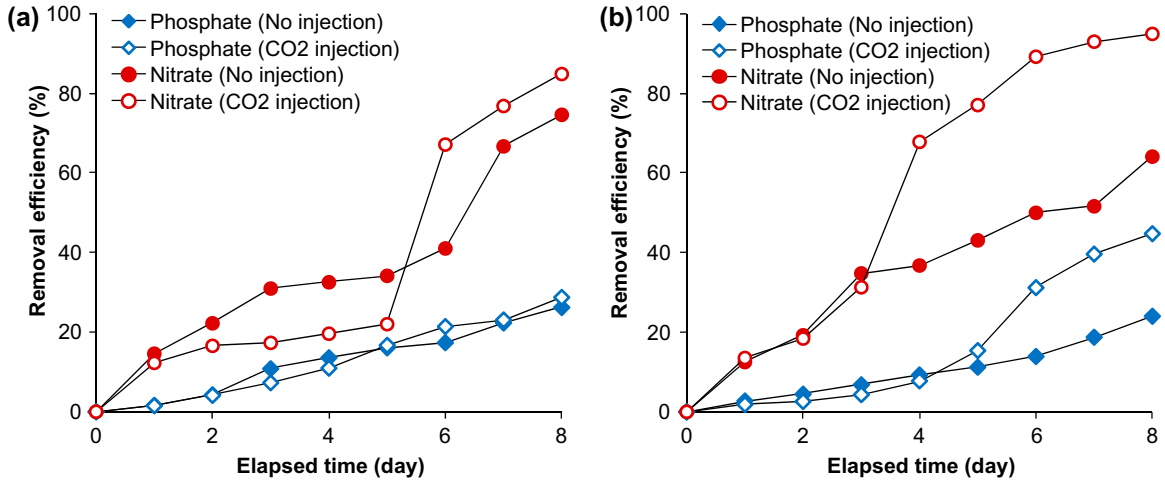

Fig. 2. Nutrient removal by microalgae: (a) *C. vulgaris* and (b) *E. gracilis*. All experiments were illuminated for 16 h with 35 μmE/m^2/s fluorescent lamps.

and without CO_2 injection. Compared with *C. vulgaris*, *E. gracilis* showed relatively fast removal of nitrogen. It was probably because, in *E. gracilis*, additional nitrate reductase could be derived from the ubiquinone pool [23]. Phosphate removal caused by the interaction with the nitrogen in wastewater was ascertained [24]. The phosphorus concentration was still high probably due to nitrogen being a limiting factor in the medium and; therefore, was abundantly employed [24]. They have also reported that less efficient phosphorus removal in *C. vulgaris* compared with other microalgae.

The injection of CO_2 to the wastewater reinforced the both nutrients removal in both microalgae cultures. Carbon and nitrogen are relatively requisite elements for algal growth and maintenance. Due to the use of gaseous CO_2 from air as a carbon source, resilient algae still required a nitrogen source in the culture medium. This feature will cause indiscriminate uptake of nitrogen from wastewaters without using organic carbon sources. *C. vulgaris*, a mixotroph, was capable in removing nitrate and phosphate from the wastewater, both with and without the addition of CO_2 [10]. However, *E. gracillis* played an ambiguous role in the assimilation of both nutrients under low CO_2 conditions as shown in Fig. 2(b).

3.3. Microalgae morphology and separation properties

The size and shape of both microalgae were enumerated prior to characterization of microalgae separation properties. The *C. vulgaris* and *E. gracilis* cells were magnified using a microscope, equipped with an image manager, to investigate the microalgae morphological characteristics. As shown in Fig. 3, *E. gracilis* cells were cylindrical, with a tapered tip compared to the rounded-shape of *C. vulgaris*, single cell green microalgae. The chloroplasts were apparently well-developed both in *C. vulgaris* and *E. gracilis*. *E. gracilis* was more than 15 times larger than *C. vulgaris*. The different sizes could affect separation and harvesting. The captured images clarified that the diminutive cells of *C. vulgaris* stressed harvesting and involved a high separation cost compared to *E. gracilis*.

The parameters on microalgae separation were investigated. The separation characteristics of *C. vulgaris* and *E. gracilis* were assessed in terms of

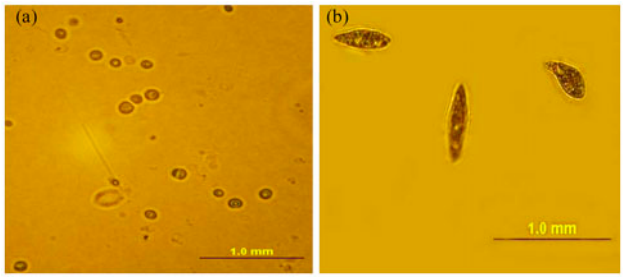

Fig. 3. The morphology of microalgae (a) *C. vulgaris*, (b) *E. gracilis*.

size, shape, SRF, SVI, and TTF. The separation parameters of *C. vulgaris* and *E. gracilis* were summarized as shown in Table 3. These characteristics mostly influenced the metabolism of cells and flocculation. The difference in sedimentation was clearly indicated by measuring the settled sludge volume after 30 min of settling, showing the tendency of biological solids in the sludge to become concentrated and thickened during the sedimentation or thickening process. The results show that *E. gracilis* possessed poorer settleability but better filterability, indicating smaller SVI and higher TTF values, than *C. vulgaris*. The SVI value of *E. gracilis* was twice as high as that of *C. vulgaris*.

SRF has been indicated as a filterability criterion for estimation of the fouling efficiency. Usually, TTF investigation is simpler than that of SRF, and has been used as a preliminary measurement to show the dewaterability trend prior to SRF analysis [25]. SRF tests for *C. vulgaris* indicated a poor filterability and significantly higher SRF compared with *E. gracilis*. This means *E. gracilis* are more effective than *C. vulgaris* in terms of dewaterability. Meanwhile, the low SRF induce optimal dewatering performance, filterability, and low running cost for a dewatering facility. The bio-flocculation of the biomass of *E. gracilis* has been observed during separation tests, and this could explain the enhanced filterability to microalagal biomass. Therefore, it could be assumed that comparative resistance of *E. gracilis* would allow more efficient dewaterability than that of *C. vulgaris*.

3.4. Biomass composition

Micoralgae also produce potentially valuable biomass which can be used as animal feed additives, a slow-release fertilizer, and biodiesel feedstocks [6,26]. The protein of algal biomass was a significant attraction as a refined protein source due to both the features of high protein content and reasonable quality of digestible protein [27]. The extraction of biomass protein from microalgae grown in wastewater for animal feed and dietary supplements is also attractive. Chojnacka [27] dentified carboxyl, phosphoryl and amino groups on the surface of *C. vulgaris*. In this study the production of protein from *C. vulgaris* and *E. gracilis* was investigated for potential use and the effect of the injection of CO_2 on microalgae protein composition was also studied. The protein yields of both microalgae were determined, and are displayed in Fig. 4. The protein composition of *C. vulgaris* was higher than that of *E. gracilis* under the same conditions. It was found that moderate pH, accompanied by CO_2 injection, favored protein production by *C. vulgaris*; whereas, *E. gracilis* preferred acidic conditions [28]. Chae et al. [22] showed that *E. gracilis* produced about 0.4 g of new biomass of each gram of CO_2 used in a continuous culture.

The algal cultivation also provides the biofuel because algae contain complex long-chain sugars (polysaccharides) in their cell walls [5]. These biofuels made from algal biomass are being issued as the most suitable alternative energy in current global and economical scenario. The biomass lipid produced from both microalgae showed similar gradually decreasing trends during the accumulating period. The addition of CO_2 to the reactors apparently increased the amount of cells but lipid content was reduced. Lipids profiles were analyzed using PCA (Fig. 5). PCA clearly separated the two microalgae strains grown without CO_2. *C. vulgaris* growth on wastewater with CO_2

Table 3
The microalgae cell characteristics

Microalgae	Size (μm^3)	TTF (s)	SVI (mL/g)	SRF ($\times 10^{13}$ m/kg)
C. vulgaris (No injection)	268.4 ± 89.2	238.9 ± 10.4	31.2 ± 3.6	8.3 ± 1.6
C. vulgaris (CO_2 injection)	375.9 ± 149.7	192.2 ± 9.2	37.0 ± 4.9	6.7 ± 1.3
E. gracilis (No injection)	4067.0 ± 249.8	30.5 ± 1.4	69.12 ± 10.9	1.7 ± 0.3
E. gracilis (CO_2 injection)	5940.1 ± 780.2	48.6 ± 2.2	86.4 ± 11.5	2.5 ± 0.7

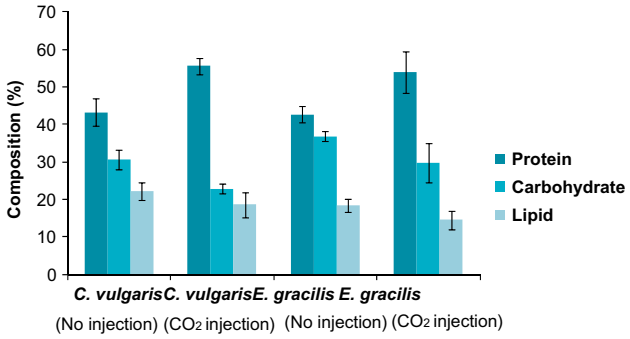

Fig. 4. Biomass composition produced from microalgae.

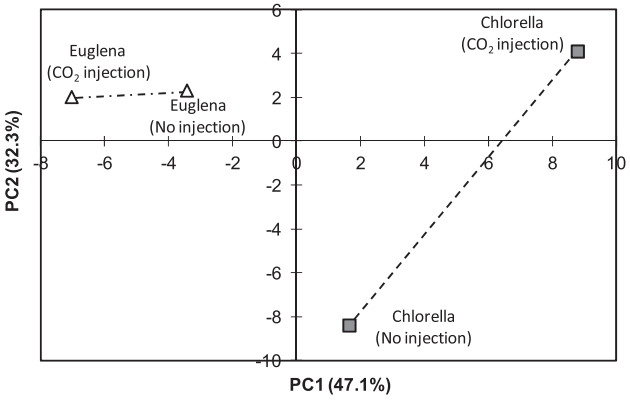

Fig. 5. Biomass lipid produced from microalgae.

addition resulted in different groupings but *E. gracilis* did not changed by CO_2 addition.

4. Conclusions

This study investigated the ability of two microalgae; *C. vulgaris* and *E. gracilis* to nitrogen and phosphorous removal, separation properties, and biomass composition from diluted secondary swine wastewater. These two microalgae were compared both with and without the injection of CO_2. From results, it can be concluded that the two microalgae have their own comparative advantages and constraints. The addition of CO_2 to microalgae cultivation impacted on the microalgae growth and resulted in enhanced nutrient removal and increased biomass protein yield for both species. *E. gracilis* prevailed in terms of rapid growth rate and easier filterability, but *C. vulgaris* provided high potential efficiency for nutrient removal without CO_2 injection. The injection of CO_2 to wastewater under favorable culture conditions lead to the increased relative content of protein, but fraction of lipid and carbohydrates decreased. Lipid profiles in *C. vulgaris* cultivation have been significantly changed by addition of CO_2, but *E. gracilis* was not directly affected.

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