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Biomass Production Potential of *Chlorella vulgaris* Under Different CO_2 Concentrations and Light Intensities

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ABSTRACT

The increasing demand of the crops (soybean and corn) for biofuel production has increased the focus of the animal nutritionists to look for alternative feeds, which are economic and environmental friendly. To identify microalgae as suitable candidate as an alternative feed, growth response of *Chlorella vulgaris* was studied under varying concentrations of carbon dioxide (0.07, 1.4, 3.0 and 5.0%) and photon densities (39.19, 72.97, 105.41, 116.22, 135.14, 175.68 μmol/m^2/s) by employing a photo-bioreactor. Swine wastewater was also investigated as nutritional source to economize the biomass production. Results showed that the higher biomass production was found to be at 3.0% CO_2 compared to other CO_2 concentrations. However, no difference in biomass production was found at 105.41 μmol/m^2/s and above photon densities with 12 h of photoperiodicity. It was observed that *C. vulgaris* could easily grow in 200 times diluted swine wastewater and growth was found to be similar with that of artificial medium. Provided the conducive conditions for optimal growth, it has also the potentiality of depleting ammonia nitrogen (NH_4-N) and orthophosphate (PO_4^{3-}-P) completely from the wastewater after 3~4 days of cultivation. Thus, growing *C. vulgaris* would not only solve the problem of animal feed, but also help in biological CO_2 mitigation and wastewater treatment.
(**Key words :** Swine wastewater, Biomass, Microalgae, Growth rate)

INTRODUCTION

The research activities world over has been moving towards the attainment of sustainable rural development. This change in approach arises from the threat to fragile environments due to population pressure and urbanization. The world fossil oil reserves will be exhausted soon due to staggering rates of consumption; therefore, to avert the imminent oil crisis, crops are used worldwide for the biofuel production. In this situation, for sustainable growth in animal production, new approaches are needed to identify alternative feed resources which are economic, sociological, ecological and, environmental friendly.

Traditionally, efforts have been directed to increase ruminant productivity by improving the quality and quantity of fibrous crop residues and also by-products of grain processing (Preston and Murgueitio, 1993 Khatun et al., 1994). The idea of feeding algae for animal production is unique, in the sense that they can use abundant solar energy and high ambient temperature for their growth (Chinnasamy et al., 2009). Among various microalgae, *Chlorella* seems to be a potential candidate for feed and food. Besides the high levels of protein (50~70%), lipids (2~22%) and carbohydrates (8~26%), it contains appreciable amounts of valuable vitamins and minerals. The protein of *Chlorella* contains most of the essential amino acids and, in suspension; living algae liberate simple sugars, alcohols, polysaccharide, glycolic acids, phenolic substances, hydrocarbon and aromatic compounds. *Chlorella* also has various efficacies such as heavy metal removal, degradation of toxic materials, control of arteriosclerosis, immunoprotective effects, anticancer activity and growth stimulating activity of intestinal bacteria (Kang et al., 2004).

Moreover, to address the present problem of global warming, use of microalgae that convert CO_2 from a point source into biomass has also attracted environmentalist worldwide. Microalgae use CO_2 efficiently because of their rapid growth (multiply 4~6 times overnight), perform 10~50 times more efficient photosynthesis than plants, require less energy, labor and can be readily incorporated into engineered

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systems, such as photo-bioreactors (Lee and Lee, 2003 Carvalho et al., 2006). The CO_2 fixation rate is related directly to light utilization efficiency and to cell density of microalgae. Therefore, biomass measurements or growth rate evaluations are critical in assessing the potential of a microalgae culture system for directly removing CO_2 (Cheng et al., 2006 Jin et al., 2006). Effects of CO_2 concentration and light intensity on growth of microalgae culture have been evaluated in several studies (Keffer and Kleinheinz, 2002 Chae et al., 2006; de Morais and Costa, 2007; Chinnasamy et al., 2009). However, these effects remain to be largely understood. Hence, to secure the mass production of microalgae for feed, present study was framed in order to assess the effect of different concentration of CO_2 and light intensities on growth of *C. vulgaris* as a first step.

Swine wastewater contains large amount of NH_4-N and PO_4^{3-}-P. Therefore, discharge of these nutrients to the environment is undesirable because it could accelerate eutrophication. Further, problem gets intensified as certain forms of nitrogen are toxic and may lead to disease if drinking water gets contaminated with these compounds. Thus, using nutrients-rich swine wastewater for algal cultivation may be novel if it enhances high cell density, and lowers the problem of eutrophication (Travieso et al., 2006). The choice of microalgae to be used in wastewater treatment is determined by their robustness against wastewater and by their efficiency to grow in and to take up nutrients from wastewater (Olguin 2003). Therefore, in the present work *C. vulgaris* was evaluated for its growth and nutrition depletion potential from swine wastewater under optimized growth parameters.

MATERIALS AND METHODS

1. Medium and cultivation conditions

In the present study, indoor culture of *C. vulgaris* was taken. M4N (artificial) medium of following composition (mg/L): {KNO_3 (5000); $MgSO_4 \cdot 7H_2O$ (2500); K_2HPO_4 (1250); NaFeEDTA (14); H_3BO_4 (2.86); $MnSO_4 \cdot 7H_2O$ (2.5); $ZnSO_4 \cdot 7H_2O$ (0.222); $CuSO_4$. $5H_20$ (0.079) and Na_2MoO_4 (0.021)} (Sung et al., 1999) was employed for growth. Cells were activated by inoculating a loop of a slant culture into 100 mL of the medium and were precultured under continuous illumination at 25±1ºC for a week. Cells were maintained at 4ºC by repeated sub culturing after one month interval.

2. Growth response of *C. vulgaris* to varied CO_2 concentration and luminous intensities

To optimize the CO_2 concentration and light intensity for maximum biomass production, growth response of the *C. vulgaris* in terms of biomass (g/L) under varying concentration of CO_2 (0.07, 1.4, 3.0 and 5.0%) and light intensities (39.19, 72.97, 105.41, 116.22, 135.14, 175.68 μmol/m^2/s) was investigated employing vertical tubular type photo-bioreactor (Fig. 1).

The photo-bioreactor (54 cm length, 8 cm diameter) with 2.4 L of total capacity and 2.0 L of working capacity was used throughout the experiment. The airstream with different CO_2 concentrations was adjusted by flow meters and sterilized by passing through the membrane filter (0.22 μ). External illumination of different light intensities was adjusted by mounting more fluorescent lamps (FPL36EX-D) of 36W, on both sides of the reactor and intensity was measured using a pocket luxmeter (ANA-F9). Culture in the photo-bioreactor was aerated continuously at the rate of 0.5 L/min. For conducting the entire experiment, we had six wooden chambers with fixed light intensity of 39.19, 72.97, 105.41, 116.22, 135.14, and 175.68 μmol/m^2/s. Each chamber contains two photo-bioreactors and CO_2 of required concentration (0.07, 1.4, 3.0 and 5.0%) was supplied into each photo-bioreactor.

The M4N medium containing balanced ratio of nitrogen and phosphorous was sterilized at 121ºC for 15 minutes and aseptically poured in the photo-bioreactor. Exponential phase

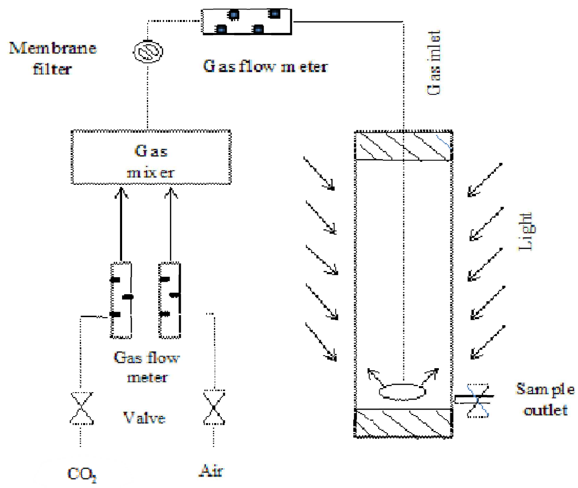

Fig. 1. Schematic of the photo-bioreactor experiment to study the effect of CO_2 concentration and light intensities on *C. vulgaris* culture.

of *C. vulgaris* (1.0%) was used as seed culture and allowed to grow at 25±1ºC, with a photo-periodicity of 12 h. The whole experiment was conducted for a period of 11 days in duplicate.

For each experiment (different CO_2 concentrations and light intensities) sample was taken at an interval of 24 h and subjected to absorbance at 660 nm and biomass measurement (g/L) employing standard protocols. For biomass measurement, supernatant from samples was removed by centrifugation (FLETA-5000, Hanil, USA) at 3000×g for 10 min and the micro algal pellet was collected. Biomass was measured after drying the microalgal pellet at 105ºC for 16 h (Takagi et al., 2006).

3. Biomass production of *C. vulgaris* in swine wastewater

To investigate the possibility of swine wastewater as a growth medium for *C. vulgaris*, 200 times diluted swine wastewater was taken in the photo-bioreactor and 1.0% of microalga was used as seed culture. The initial composition of swine wastewater collected from a local farm has 50~60 mg/L PO_4^{3-}-P and 1700-2400 mg/L NH_4-N. Light of different intensities (39.19, 72.97, 105.41, 116.22, 135.14, 175.68 μmol/m^2/s), were supplied with 3.0% CO_2 and the experiment was conducted at 25±1ºC with a photo-periodicity of 12 h for eight days in duplicate. Each sample was observed for biomass measurement as discussed in section 2. Similar experiment was conducted in artificial (M4N) medium providing the same cultural conditions and incubation time as described above.

4. Analysis of NH_4-N and PO_4^{3-}-P content

Samples collected from photo-bioreactor were centrifuged at 3000×g for 10 min and the supernatant was then used for chemical analysis. PO_4^{3-}-P and NH_4-N were analyzed with the auto water analyzer (Quick Chem 8500, LACHAT).

RESULTS AND DISCUSSION

1. Effect of varied CO_2 concentration on *C. vulgaris* biomass production at different luminous intensities

To investigate the effect of CO_2 concentration on biomass (g/L) production, *C. vulgaris in* photo-bioreactor was incubated for 11 days at 25±1ºC under different light intensities. Interestingly, the alga showed sharp increase in biomass production with increasing CO_2 and light intensities. At 5.0% CO_2, biomass production (Fig. 2a) was found to be maximum (1.83 g/L) at 175.68 μmol/m^2/s light intensity, whereas at lower light intensities (39.19 and 72.97 μmol/m^2/s), biomass was found to be low (0.81 and 1.22 g/L), respectively. However, at 105.41, 116.22 and 135.14 μmol/m^2/s light intensity, no difference in biomass production (1.57, 1.68 and 1.61) was found after 11 days of incubation.

At 3.0% CO_2 concentration, biomass production (Fig. 2b) after 11 days of cultivation was 0.86, 1.13, 1.81, 1.89, 1.93 and 1.80 g/L at photon densities of 39.19, 72.97, 105.41, 116.22, 135.14 and 175.68 μmol/m^2/s, respectively, showing that the biomass production of *C. vulgaris* at 3.0 is higher than that of 5.0% CO_2.

From figure (2c and 2d), it can be clearly observed that lowering the CO_2 concentration to 1.4 and 0.07%, further reduces the biomass production to 1.62 and 1.37 g/L respectively, even at 175.68 μmol/m^2/s of photon density. Hence, comparing the biomass production results at different CO_2 concentrations clearly indicates that at 3.0% CO_2 level, *C. vulgaris* could grow optimally and produce maximum biomass. Similar result was shown by Chiu et al. (2008) stating that *Chlorella* sp. growth increased rapidly at 2.0% CO_2 rather than 5.0% CO_2 concentration. However, for CO_2 mitigation, the response of algae to the elevated levels of CO_2 was observed by many researchers. Chinnasamy et al. (2009) stated that *C. vulgaris* ARC 1 could produce biomass 60 and 20 times more at 6% CO_2 rather than ambient (0.036%) CO_2. In addition, they found that at 16% CO_2 the biomass was comparable to those at ambient CO_2 but further increase in CO_2 level decreased biomass. Jeong et al. (2003) showed that *C. vulgaris* could grow optimally at 30% CO_2. *Chlorella* sp. KR-1 also showed good growth rate in air containing up to 30% CO_2 (Sung et al., 1999) and Kodama et al. (1993) reported that *Chlorella littorale* perform better at 20% CO_2 and another unicellular marine alga could grow rapidly at 60% CO_2 level. Wide variation showed by *Chlorella* in response to elevated CO_2 concentration suggests the possibility for the isolation of such naturally occurring algal forms that may scavenge CO_2 efficiently for biological CO_2 mitigation.

For mass cultivation of microalga besides using CO_2, considering light intensity is also important. It has been reported that growth gets inhibited if light is not optimal;

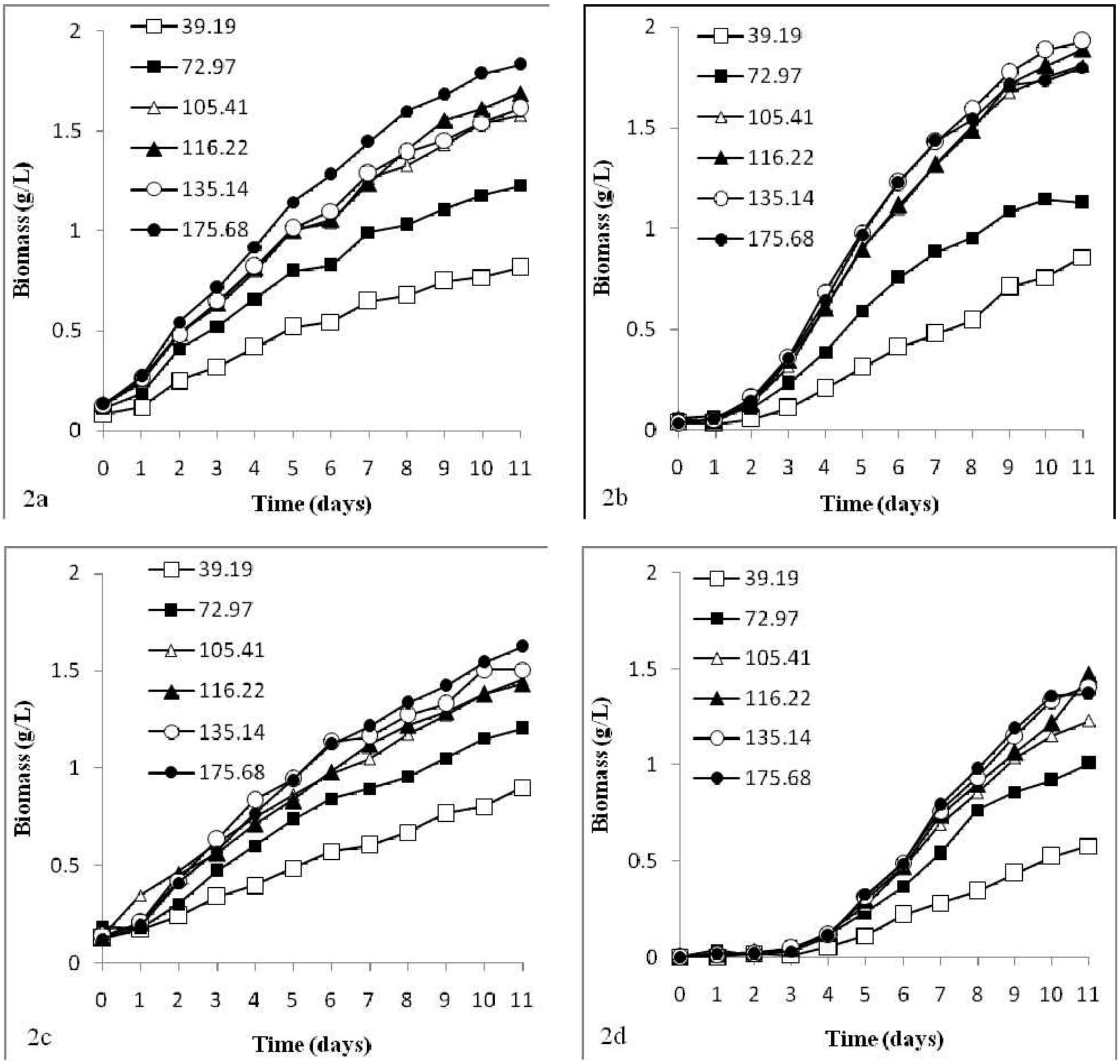

Fig. 2. Biomass production by *Chlorella vulgaris* at different CO_2 concentrations and light intensities.
(a) 5.0% CO_2 (b) 3.0% CO_2 (c) 1.4% CO_2 and (d) 0.07% CO_2

however at higher light intensities photo oxidative death of algae may occur (Phang, 1992). .Since, it is important to understand the mass culture conditions for *C. vulgaris*, growth rate were observed with the following range of intensities (39.19, 72.97, 105.41, 116.22, 135.14, 175.68 μmol/m^2/s). From the figure 3a-3d it is clearly observed that growth rate at all the CO_2 concentrations depending on photon densities increase proportionally with increased light intensities and R^2 value is more than 0.97 at 0.07, 1.4 and 5.0% CO_2 and 0.89 at 3.0% CO_2, however at all CO_2 concentrations, plateau is unknown near to 105.41μmol/m^2/s of photon density. Hence, the above results suggest that *C. vulgaris* can optimally grow at a brightness level of 105.41 μmol/m^2/s. Average growth rate at 105.41μmol/m^2/s of photon density were 0.14, 0.20, 0.13 and 0.12 g/L/d at 5, 3, 1.4 and 0.07% CO_2 level, respectively, showing much higher average growth rate at 3% CO_2 level. Average growth rate (g/L/d) was measured by using the formula:

$dC/dt = [C_t - C_0]/t$ (where; C = mass; C_0 = initial mass; t = time; C_t = final mass)

Sung et al. (1999) reported that Chlorella KR-1 maintained exponential growth phase at all the light intensities (40, 120, 310 and 470 μmol/m^2/s) and maximum specific growth was 0.13/h at 10% CO_2. This strain dependence of growth based on CO_2 fixation and light intensity may be due to physiological conditions of microalga, such as potential of growth, ability of CO_2 metabolism and adaptability to photon densities.

2. Biomass production of *C. vulgaris* in swine wastewater as medium

Swine wastewater treatment and disposal is one of the most important environmental problems in most of the countries. Swine wastewater is characterized by a high concentration of organic matter and a good balance of carbon and nutrients, which may fulfill the requirement of a microalgae culture (Kayombo et al., 2003 Travieso et al., 2006). In the present experiment, swine wastewater was diluted 200 times and growth was observed at 3.0% CO_2 level with different light intensities (39.19, 72.97, 105.41, 116.22, 135.14, 175.68 μmol/m^2/s). Wastewater is diluted 200 times to avoid any substrate based micro algal inhibition

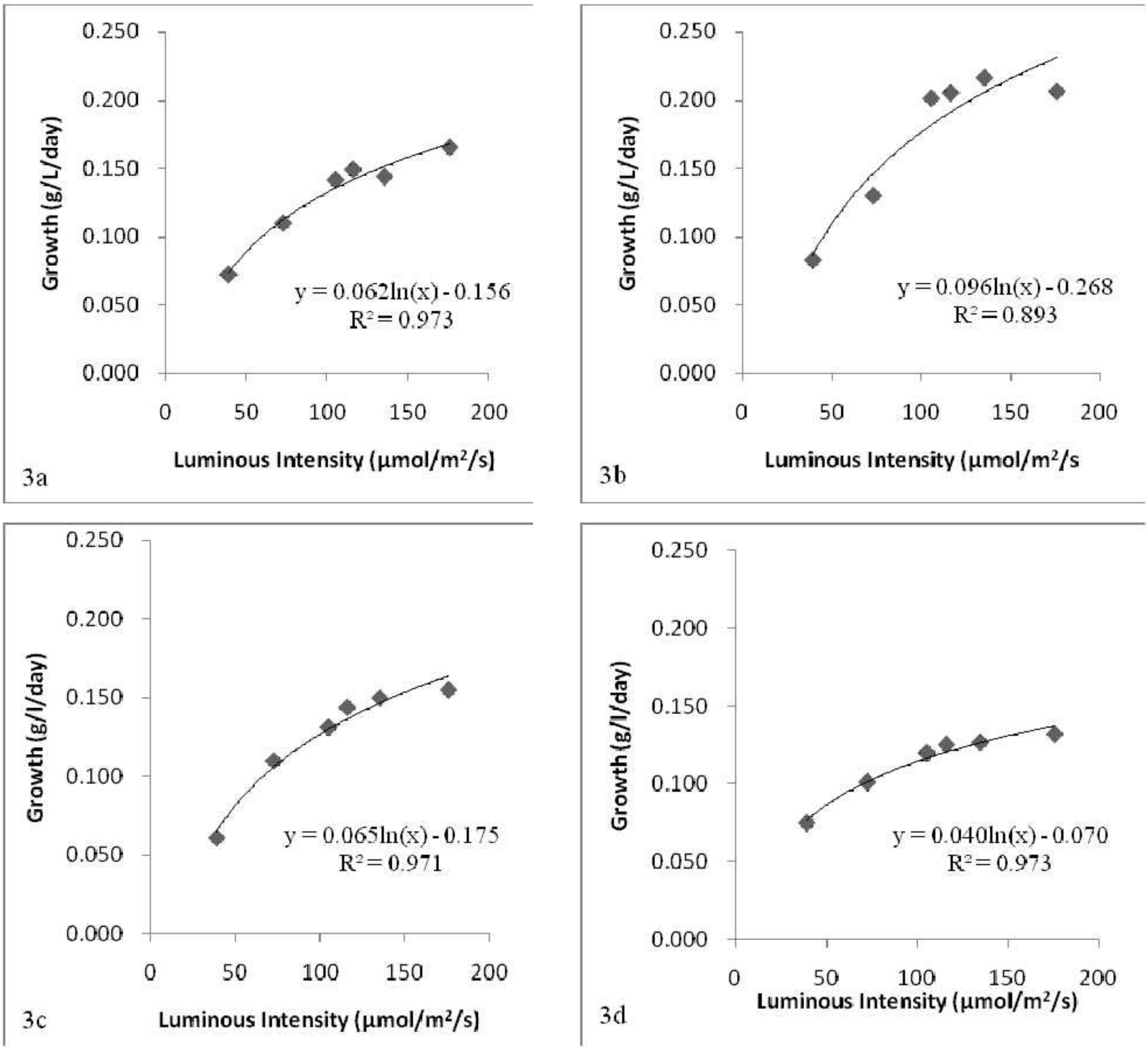

Fig. 3. Trend and predictability of biomass of *C. vulgaris* at different concentrations of CO_2 and light intensities. (a) Growth at different light intensities at 5.0% CO_2 (b) Growth at different light intensities at 3.0% CO_2 (c) Growth at different light intensities at 1.4% CO_2 (d) Growth at different light intensities at 0.07% CO_2.

(Travieso et al., 2006). Growth of *C. vulgaris* was also compared with growth in artificial medium under same conditions.

From the figure 4a-4f, it was observed that alga could grow in swine wastewater. At low light intensities (39.19 and 72.97 μmol/m^2/s), growth in terms of biomass (g/L) was nearly 1.10 and 1.58 g/L, respectively. However, at higher light intensities growth was similar initially for 1~2 days in both the media, but found to be slightly higher in swine wastewater compared to that of artificial medium after 7~8 days cultivation. After 8 days of growth in swine wastewater, it reached the plateau and was similar to artificial medium. This steadiness in growth might be due to nutrient's depletion and high growth rate as compared to that of artificial medium. With the increasing intensities (105.41, 116.22, 135.14, 175.68 μmol/m^2/s), the maximum biomass in diluted swine wastewater was 1.90, 1.86, 1.84, 1.85 g/L respectively, and is quiet higher than that of artificial medium (1.75, 1.80, 1.74 and 1.73 g/L). This observation might indicate that swine wastewater can be used as medium for the algal mass culture using 105.41 μmol/m^2/s of light intensity and 3.0% of CO_2. This growth of microalga in wastewater will help to produce maximum biomass in cost effective way.

Besides growth in swine wastewater, *C. vulgaris* was also investigated for its NH_4-N and PO_4^{3-}-P removal efficiency. Fig. 5a and 5b shows that NH_4-N (initial concentration; 13~14 mg/L) and PO_4^{3-}-P (initial concentration; 3.8~4.0 mg/L) were completely removed from the swine wastewater after 3~4 days incubation at higher light intensities (105.41, 116.22, 135.14, 175.68 μmol/m^2/s) (Fig. 4a and 4b). Hence, from the above results it can be concluded that NH_4-N and PO_4^{3-}-P might be taken up by *C. vulgaris* for biomass production. Shi et al. (2007) concluded that phosphate, ammonium and nitrate are taken up metabolically by the two microalgae and incorporated into their biomass and Travieso et al. (1996) stated that increasing the light intensity can lead to a higher microalgal activity and increased removal of nutrients from wastewater. According to Casadevall et al. (1985), green alga absorbs PO_4^{3-}-P and nitrogen from the

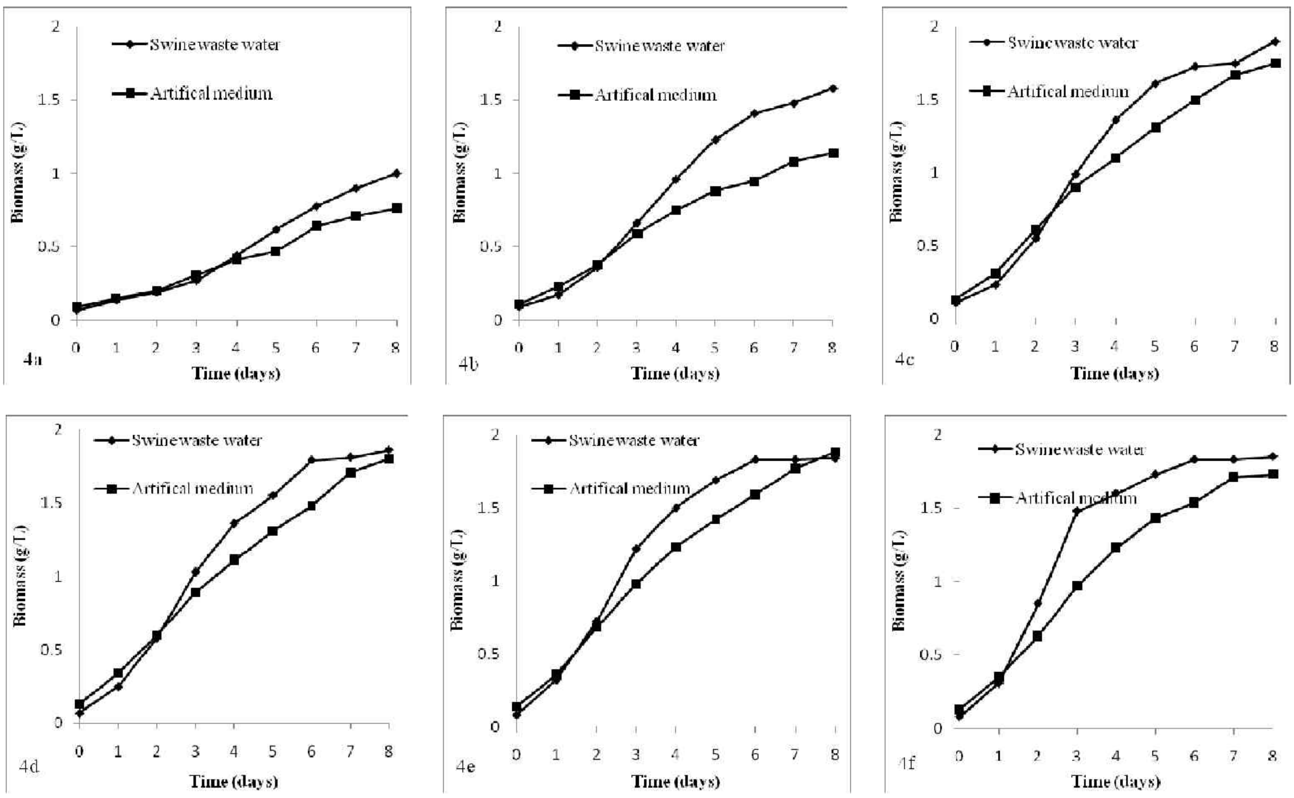

Fig. 4. Growth of *C. vulgaris* in artificial medium and swine wastewater at different light intensities and 3% CO_2 concentration.
(a) growth at 39.19 μmol/m^2/s (b) growth at 72.97 μmol/m^2/s (c) growth at 105.41 μmol/m^2/s
(d) growth at 116.22 μmol/m^2/s (e) growth at 135.14 μmol/m^2/s (f) growth at 175.68 μmol/m^2/s

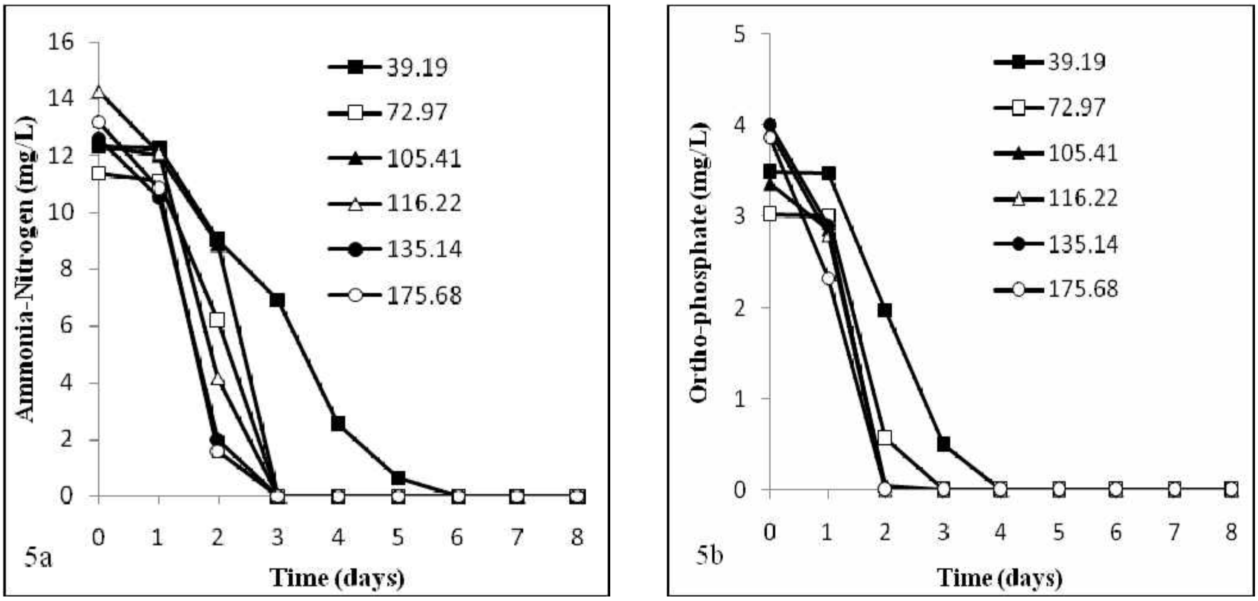

Fig. 5. Nutrient depleting ability of *C. vulgaris* from swine wastewater at 3.0% CO_2 and 105 μmol/m^2/s light intensity. (a) depletion of NH_4-N and (b) depletion of PO_4^{3-}-P.

medium efficiently and stores it in the form of polyphosphates and other intracellular granules. Therefore, after 3~4 days, if nutrients get depleted in the medium, alga doesn't get starved and continue to grow using these nutrient rich intracellular granules. Thus, our results are in corroboration with the above research.

The depletion of NH_4-N from the swine wastewater within 3~5 days depending on photon densities at 3.0% CO_2 by *C. vulgaris* was comparable to studies conducted by Martinez et al. (2000), who described elimination of NH_4^+ (between 79% and 100%) after about 8 days, and Gonzalez et al. (1997), who reported ammonium removal efficiencies of 90% from agro-industrial wastewater after 9 days. Eliminating ammonium from wastewater using microalgae does not generate secondary pollution by generation of NH_3 and the micro algal biomass can be harvested and used as a slow-release fertilizer or soil conditioner (de la Noue et al., 1992 Mallick, 2002; Mulbry et al., 2005 Shi et al., 2007).

According to regulations, the total phosphorus and nitrogen concentrations discharged from public animal wastewater treatment plants in Korea should be less than 8 mg/L total phosphorus and 60 mg/L total nitrogen. On observing the Fig. 5a and 5b, it could be suggested that these requirements could be met by exposing the algae to the effluent of treatment process for further removal of residual nutrients. However, actual residence time, of course, depends both on the initial concentration of total nitrogen and phosphorus in the wastewater and on the amount of algal biomass employed.

CONCLUSIONS

In this experiment, *C. vulgaris* culture conditions were optimized for maximum biomass production at different CO_2 concentrations and light intensities. Based on the results of this study, *C. vulgaris* showed maximum biomass production at 3.0% CO_2 and 105.41 μmol/m^2/s of photon density. At these conditions, the average growth rate of *C. vulgaris* was approximately 0.20 g/L/d. Further, to economize the biomass production cost and solve the issue of wastewater treatment, alga was allowed to grow in swine wastewater under the optimized culture parameters. The alga not only showed good growth utilizing diluted swine wastewater as source of nutrients but also completely depleted NH_4-N and PO_4^{3-}-P from wastewater. Hence, from the present investigation it can be concluded that *C. vulgaris* could grow economically and might be employed as an alternative feed with respect to the increasing demand of crops for biofuel production.

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