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Enhanced Production of *Phaeodactylum tricornutum* (Marine Diatoms) Cultured on a New Medium with Swine Wastewater Fermented by Soil Bacteria

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Abstract There have been a number of studies of methods for recycling animal wastewater to provide new bioresources. In the present work, a marine algal culture medium, designated KEP II, was prepared by adding swine waste (3% v/v) fermented by soil bacteria to a dilution of f/2 culture medium (CT). When *Phaeodactylum tricornutum* was grown in batch culture in KEP II, the cells lasted long at the exponential phase producing the specific growth rate and biomass; the production of total amino acids and secondary metabolites rose up to 5-fold. It also substantially enhanced the maximum quantum yield of photosystem (PS) II of *P. tricornutum*, greatly increased the level of thylakoid membranes containing PS, and stimulated the production of pyrenoids, including enzymes for CO_2 fixation in chloroplasts. KEP II should improve the cost efficiency of industrial mass batch cultures and the value of microalgae for long-term preservation of fresh aquaculture feed as well as production of anticancer and antioxidant agents. Specifically, a low-cost medium for growing the diatoms of aquaculture feed will be economically advantageous.

Key words: Enhanced production, batch culture, *Phaeodactylum tricornutum*, swine wastewater, fermentation

Phaeodactylum tricornutum, a marine benthic diatom [37], is cosmopolitan and one of the most important photosynthetic microalgae for ecophysiological research [30]. Culturing techniques for physiobiochemical enhancements [1, 33, 40–42] and bioremediation of the seawater ecosystem [22, 38] have been extensively studied. The biochemical activities of microalgae are dependant on the growth conditions and specific nutrient elements of their culture medium [18, 20].

Animal, urban, domestic, and human wastewaters having major eutrophic compounds containing nitrogen and phosphorus cause adverse effects on the water ecosystem. Microalgae like *Chlorella kessleri*, *C. vulgaris*, *Scenedesmus obliquus*, *S. bijugatus*, and *Selenastrum capricornutum* have been studied for eliminating the nutrients through purification facilities [4, 8, 23–25, 27, 39]. Recently, microalgae wastewater treatment plant models have been investigated [12, 31]. The biodegradation of organic solution [34] and heavy metals [7, 15, 20, 34] derived from mine wastewater stimulating the ecotoxicity to freshwater biota have been attempted by using microalgae.

Recently, microalgae have been siginificantly applied to industry as marine bioresources. However, they have general defects to be preserved for long-term and to be abundantly grown. For these reasons, it will be necessary to develop more efficient culture media for preserving microalgae for the long-term, lowering the culture cost and labor, and improving their cell densities and bioactivities such as antibiotic, anticancer, chemoprevention, and antioxidation activites [35].

Media, such as Erdschreiber, Grund, ES, CHU-10, f/2, and the ASP series [29] have been developed for effective culture of fresh and marine microalgae. This study was performed to investigate whether addition of secondary swine urine (SSU) fermented by soil bacteria [14, 28] to the culture medium could enhance and sustain the vitality of *P. tricornutum* under conditions that might permit batch mass culture at lower cost. For this purpose, we analyzed and compared the photosystems of photosynthesis and secondary metabolites of *P. tricornutum* grown in different media.

Materials and Methods

Culture Conditions

The *Phaeodactylum tricornutum* used in these experiments originated from the Korea Plankton Culture Collection for Industrialization (KPCCI). *P. tricornutum* (KPCCI P-M-

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23) was grown in 1-l volumes in 2-l Erlenmeyer flasks. They were cultivated for 44 days at 17°C with a light:dark photoperiod of 14 h:10 h, using a light intensity of 180± 5 μmol $m^{-2}s^{-1}$, and continuously shaken at 140 rpm. The growth density (k) was calculated by Guillard's method [13]. To observe the changes of cell organelles by transmission electron microscopy, *P. tricornutum* was continually cultivated for 14 months.

Dry Weight of Biomass

Microalgae of 50 ml were centrifuged at 5,000 rpm for 10 min, dried in an oven at 75°C for 24 h, kept in a desiccator for 24 h, and measured with a balance (Ohaus, U.S.A.).

Developments of KEP II Medium

Cultures of *P. tricornutum* were grown using f/2 as the control medium (CT) [13]. It contained the following components (g/l): $NaNO_3$ (0.075), $NaH_2PO_4 \cdot 2H_2O$ (0.006), EDTA Na_2 (4.36), $FeCl_3 \cdot 6H_2O$ (3.15), $CuSO_4 \cdot 5H_2O$ (0.01), $ZnSO_4 \cdot 7H_2O$ (0.02), $CoCl_2 \cdot 6H_2O$ (0.01), $MnCl_2 \cdot 4H_2O$ (0.18), $NaMoO_4 \cdot 2H_2O$ (0.006), cyanocobalamin (0.0005), thiamine HCl (0.1), and biotin (0.0005) per liter of filtered natural seawater. The pH was adjusted to 8.0 before autoclaving. The technique used to generate SSU, termed Bacterio-Mineral-Water (BMW), has been granted international patents involving fermentation and bioreaction technologies [14]. Pellets containing soil humus at a pH of 2.88 are used to culture bacteria such as actinomycetes and yeasts in swine urine, which in turn ferments the pre-processed wastewater to produce SSU [28]. Three percent SSU was added to a dilution of f/2 medium to make KEP II medium.

Analysis of Inorganic and Organic Compounds in Treated Swine Urine

The concentrations of total nitrogen (T-N) and total phosphorus (T-P) as well as the weight of the suspended solids (SS) were determined via Standard Methods [3] (Table 1). Total carbon (T-C) contents were measured using an Elemental Analyzer (EA1108, Fison, Italy), and the concentration of dissolved organic carbon (DOC) was analyzed using a TOC Analyzer (Phoenix 8000, TekmarDohrmann, Cincinnati, OH, U.S.A.) after membrane (pore size: 0.45 μm; Millipore GF/C # HVLP04700, Cork, Ireland) filtration of the samples.

Table 1. Suspended solids, pH, and inorganic and organic compounds of treated swine urine after the fermentation process.

Elements	Original swine urine	Treated swine urine	Variation value of percent (%)
pH	7.97±0.4	3.73±0.8	-
T-C (mg/l)	561.7±27	489.4±31	▼ 12.9±0.9
DOC (mg/l)	153.5±9	162±14	▲ 5.5±0.5
TN (mg/l)	662.4±39	86.4±11	▼ 87±8
TP (mg/l)	120±12	20.2±1	▼ 83.2±3
SS (mg/l)	440±42	32.8±3	▼ 92.6±6

▲, Increase percent; ▼, decrease percent. Values are expressed as means±SD (n=3).

Measurement of Growth and Photosystem II Activity

Cell density was determined in triplicate cultures by periodic counting with a hemocytometer, and growth rates were calculated using the formula of Guillard [13]. Maximum quantum yields of photosystem II (Fv/Fm) were measured using a pulse-amplitude-modulated fluorometer (Phyto-PAM Walz, Effeltrich) with dark-adapted samples for 5 min while being stirred. Total pigments were extracted in a solvent system consisting of acetone, methanol, and water, 10:9:1 (v/v/v). Chlorophylls *a* and *c* were analyzed by the method of Kim and Smith [20]. For measurements of total carotenoids, chlorophyll was removed from the extract by adding 5% KOH (w/v) to a methanol/acetone extract of pigments, and saponification was carried out overnight in the dark as described by Kim and Lee [19]. Samples (300 to 500 μl) were then streaked onto TLC plates (20×20 cm, 250 μm thickness, Merck 60 F-254), and the pigments separated in petroleum ether/benzene/ethanol (10:2:1, v/v/v) development solvent as described by Kim and Lee [19]. To determine the amounts of individual carotenes and xanthophylls, the samples were analyzed by HPLC, using an inertsil ODS-2 column (5 μm, 4.6×250 mm, GL Science, Tokyo). Total carotenoids, eluted with acetonitrile/methanol/dichloromethane (65/30/5, v/v/v) at a flow rate of 1 ml/min, were analyzed in triplicate using a photodiode array UV-VIS detector at 445 nm (SPD-M6A, Shimadzu, Kyoto).

Analysis of Fatty Acids

Total lipids were extracted in triplicate, and hydrolyzed to release fatty acids by the method of Kim *et al.* [17]. Fatty acids were methylated with BF_3 methanol (Sigma #33356) and analyzed by gas chromatography (GC) (HP 6890 Series) using heptadecanoic acid (C17:0) (Sigma #3500) as the standard. The amounts of individual fatty acids were determined by comparing GC retention times with those of a standard mixture of fatty acid methyl esters (Sigma #189-19).

Analysis of Amino Acids

A 20-ml sample of algal culture ($5-10\times10^6$ cells/ml) was freeze-dried, succeeded in 2 ml of 6 N HCl, and bubbled briefly with nitrogen gas. It was then oven-dried for 24 h at 110°C. The hydrolyzed sample was filtered through a membrane (pore size: 0.5 μm, Millipore FH) to remove particulates, rinsed repeatly with distilled water, and evaporated to dryness to remove HCl. It was then dissolved in 25 ml sodium loading buffer (0.2 M sodium citrate, pH=2.2), and the amino acid content was determined using an automatic analyzer (Biochrom 20, Pharmacia).

Transmission Electron Microscopy (TEM)

After washing 3–4 times with distilled water by gentle centrifugation at ambient temperature, a culture of *P. tricornutum* was suspended in a solution of 2.5% glutaraldehyde in distilled water and incubated at 4°C for 90 min with gentle mixing every 10 min. The glutaraldehyde-treated cells were washed by centrifugation as before, followed by suspension in 0.1 M phosphate buffer, pH 7.2. They were then post-fixed at 4°C for 90 min in 1% osmium tetroxide, to a final concentration of 2% (v/v), buffered with 0.1 M phosphate. After adding melted agar at 60°C, the sample was washed in the same phosphate buffer, and then dehydrated by incubating for 5 min each in an increasing ethanol series. The ethanol was replaced by an embedding solution consisting of propylene oxide and epoxy resin (2:1, v/v). After 20 min incubation, the embedding solution was replaced by fresh solution and incubation continued for an additional 20 min. The propylene oxide was removed by vacuum pumping for 4 h, and the final samples in embedding molds were placed in a drying oven and gradually heated to fully polymerize the epoxy. Ultrathin sections were cut and incubated for three successive 20-min periods in uranyl acetate to stain them, then for 10 min in lead citrate for post-staining, and finally heated to 60°C to dry them. The stained and dried samples were placed on grids and observed by TEM (Hitachi H-7600, Japan) [6].

RESULTS

Fermented swine urine refers to original swine urine from which the inorganic nutrients have been removed by 40 days of treatment in a fermentation apparatus (Table 1). The agricultural and floricultural efficacies of the fermented swine urine have been repeatedly demonstrated since the initial 1996 studies on the subject in Japan [14]. Our study represents the first demonstration that recycled animal wastewater could be used as a medium for microalgal cultures. It is the first to address the possible industrial potential of this substance as a recycling bioresource, as well as an agent for amelioration of negative environmental effects such as eutrophication.

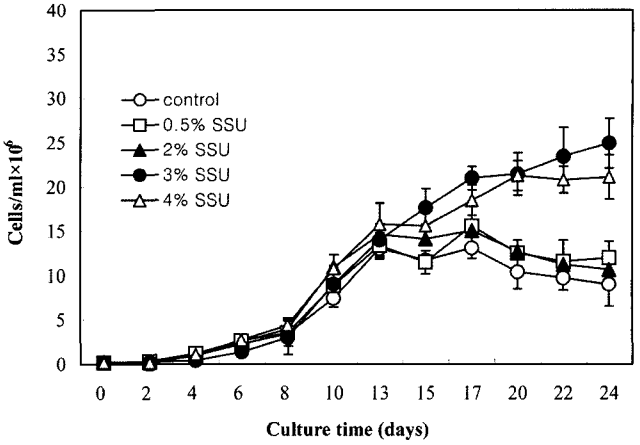

Fig 1. Growth curves of *Phaeodactylum tricornutum* in control medium (f/2) containing different concentrations of secondary swine urine. SSU: secondary swine urine. f/2 Medium (CT: ○); 0.5% SSU (□); 2% SSU (◇); 3% SSU (▲); 4% SSU (△).

The T-C, T-N, and T-P of the original swine urine, after 40 days of fermentation treatment, were reduced to 12.9% (T-C), 87% (T-N), and 83.2% (T-P) of the original values, and the pH was acidified, from an initial 7.97 to a final 3.73 (Table 1). The treated swine urine was acidified to a pH of 3.73. The inorganic nutrients in the fermented swine urine were removed from the original non-fermented swine urine, to a level of 87% of total T-N and 83.2% of T-P. The SS was reduced to 92.6%, whereas the DOC was

Table 2. Comparison of physiobiochemical activities of *Phaeodactylum tricornutum* cultured in CT and KEP II media on the stationary stage for 44 days. Cell numbers for inoculation were 0.41×10^6/ml in CT and 0.26×10^6/ml in KEP II, and final cell counts on the 44th day were 0.9×10^7/ml in CT and 1.2×10^7/ml in KEP II. Values represent means±SD (n=3).

Physiobiochemical elements	CT	KEP II	Ratios
Growth rates (*k*/day)*	0.33±0.1	0.71±0.3	2.2
Maximal PSII quantum yield (Fv/Fm)	0.36±0.03	0.78±0.04	2.2
ATP (μg/l)	$5.52\pm0.8\times10^{-14}$	$1.15\pm0.2\times10^{-13}$	2.1
Chlorophyll *a* (mg/g dry wt)	0.64±0.08	2.08±0.71	3.3
Chlorophyll *c* (c_1+c_2) (mg/g dry wt)	0.2±0.01	0.8±0.12	4
Beta-carotene (mg/g dry wt)	0.2±0.04	0.7±0.03	3.5
Total xanthophylls (mg/g dry wt)	54.61±5.11	263±49.23	4.8
Fucoxanthin (mg/g dry wt)	28.41±7.46	130.26±29.28	5.2
Diadinoxanthin (mg/g dry wt)	18.69±5.76	93.37±11.4	4.6
Diatoxanthin (mg/g dry wt)	7.51±1.89	39.38±8.55	5.0
Amino acids (mg/g dry wt)	75.6±23.1	98.1±19.4	1.3
Fatty acids (mg/g dry wt)	46.7±4.2	31.89±3.9	0.7

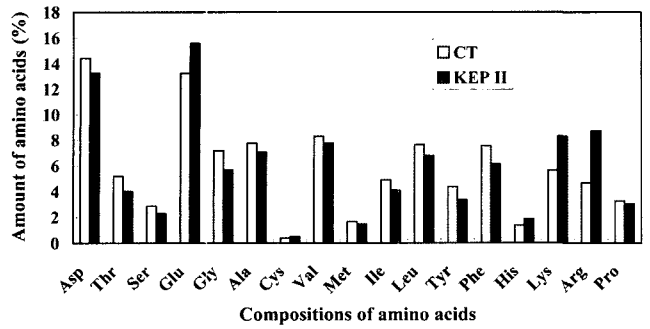

Fig. 2. Compositions of amino acids of *P. tricornutum* cultured in CT and KEP II media for 44 days.

increased (12.9%) above the levels observed in the original swine urine samples (Table 1).

To determine which concentration of secondary swine urine was most favorable for the growth of *P. tricornutum*, cultures were prepared in f/2 medium (CT), containing 0.5%, 2%, 3%, and 4% (v/v) SSU, in batch culture rather than continuous culture to reduce maintenance costs. The highest cell densities of *P. tricornutum* were obtained with 3%SSU (KEP II medium) at pH 6.6 (Fig. 1). The stationary phase in cells grown in CT as well as in 0.5% and 2% SSU was reached on the 13th day of culture, whereas cells in 3% SSU continued to grow until the 17th day. CT growth rates and maximal quantum yields of photosystem II (Fv/Fm) of *P. tricornutum* cultured in KEP II medium were higher than those in CT (Table 2). The dominant amino acids were aspartic acid (14% in CT and 13% in KEP II) and glutamic acid (13% in CT and 16% in KEP II). The amounts of glutamic acid, histidine, lysine, and arginine were higher in KEP II than in CT (Fig. 2). The dominant fatty acids were C16:0 (30%) and C16:1 (38%) in CT, whereas they were C16:0 (25%) and C20:5 (26%) in KEP II. The ratio

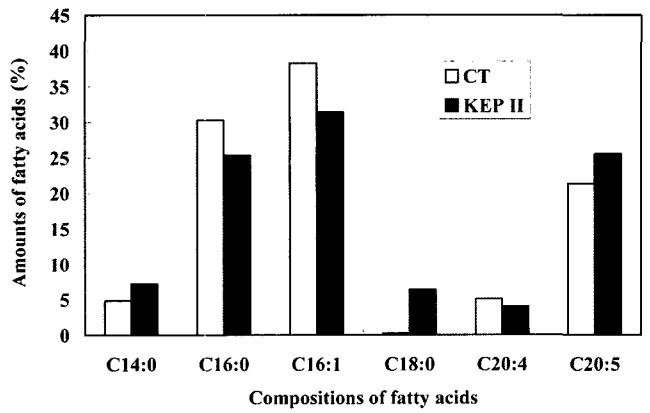

Fig. 3. Compositions of fatty acids of *P. tricornutum* cultured in CT and KEP II media for 44 days. Each amount of C16:1, C18:1, C18:2, C18:3, and C22:6 showed less than 0.1 mg/g dry wt (1% of total fatty acids).

of fatty acids between C16:0 and C18:1 of *P. tricornutum* was also higher (1.83) in KEP II than in CT (1.10) (Fig. 3).

Levels of ATP (adenosine triphosphate), chlorophyll *a*, chlorophyll *c*, β-carotene, total xanthophylls such as fucoxanthin, diadinoxanthin, and diatoxanthin, as well as of total amino acids of cultures in KEP II were up to 5-fold higher than those in CT, although the fatty acid content was lower (Table 2).

Batch culture of *P. tricornutum* grown for 14 months in control medium (CT) had the pigments bleached by chlorosis, cell wall damaged and separated from the cell membrane by plasmolysis (Fig. 4). The cell organelles were broken up. The thylakoid of the chloroplasts had also disappeared. On the contrary, *P. tricornutum* grown in KEP II medium continued to produce the chlorophylls *a*, *c*, β-carotene, diadinoxanthin, fucoxanthin, and violaxanthin participating in photosynthesis and secondary metabolism. Pyrenoids could be visable in the stroma chloroplasts, and thylakoid membranes were still present.

These results indicate that microalgae in KEP II could be useful as biosources of fish food and human nutrient supplements such as antioxidants, cancer chemopreventives, etc.

DISCUSSION

In general, xanthophylls like fucoxanthin, diadinoxanthin, and diatoxanthin are accumulated by microalgae under stress and nutrient deficiency over short culture periods [9, 10, 19, 29]. In KEP II, xanthophylls remained at a high level over long periods of culture. The increase of carotenoids, including xanthophylls, probably reduces photoinhibition of photosynthesis and protects photosynthesis under various growth conditions [21]. These xanthophylls have considerable value as chemopreventive (anticancer and antioxidizing) agents [9, 10]. The aspartic acid of *P. tricornutum* in CT and KEP II (13–14%) was higher than the previous studies (9.5%) [16]. Levels of individual fatty acid in the microalgae grown in KEP II medium for 44 days were lower than those in CT. However, the amount of polyunsaturated fatty acids increased more in KEP II (30%) than in CT (26%). When microalgae are grown under environmental stress, lipids and fatty acids typically accumulate to higher concentrations [11, 17]. Thus, CT is probably more stressful for *P. tricornutum* than KEP II. We previously showed that healthy cells of microalgae grown under favorable condition have a high ratio of C16:0 to C18:1 fatty acids [17, 18]. Thus, the physiological activities of *P. tricornutum* in KEP II having a higher ratio of C16:0 to C18:1 were superior to that in CT.

The facts that pyrenoids were higher in *P. tricornutum* grown in KEP II agrees with previous studies [32, 36] demonstrating that the pyrenoids in chloroplasts are associated

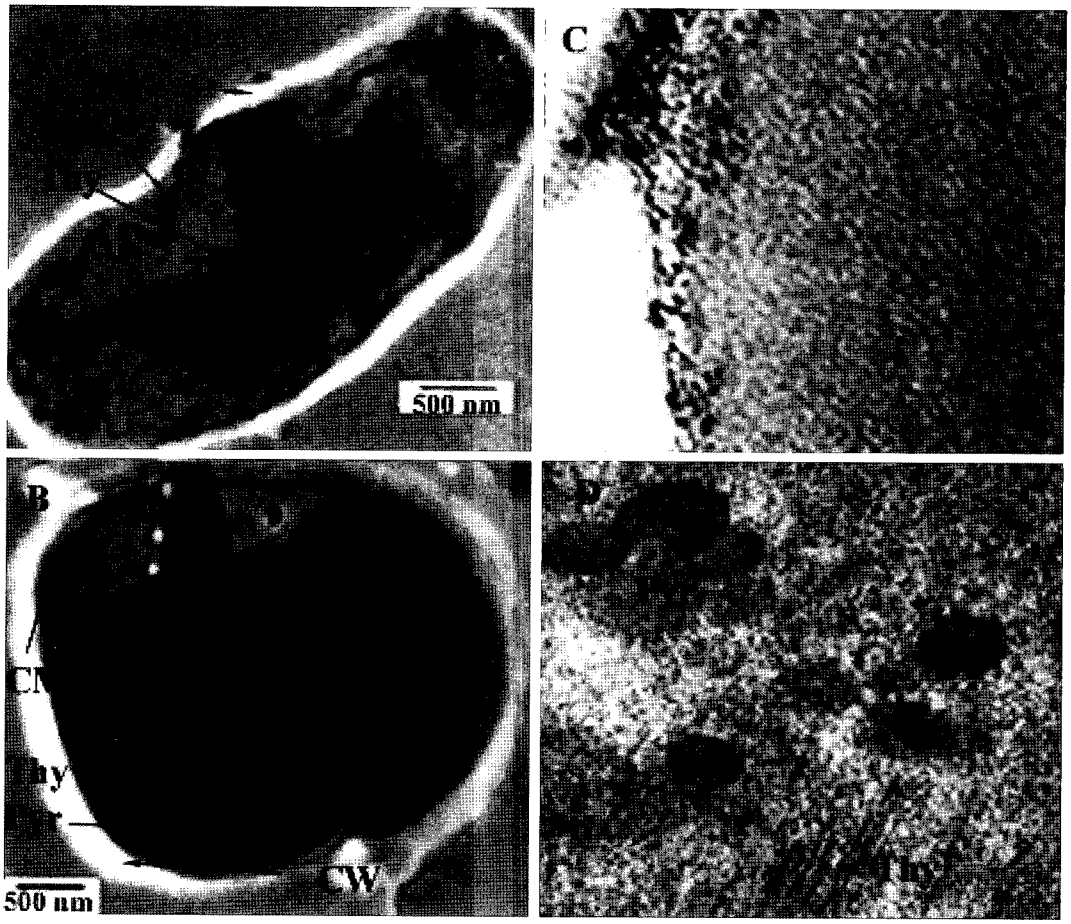

Fig. 4. Electron micrographs of *P. tricornutum*.
A (×5,000) and **C** (×20,000): *P. tricornutum* cultured in CT for 14 months. The cell wall was damaged and separated from the cell membrane by plasmolysis. Cell organelles were broken up. The thylakoid of the chloroplasts have also disappeared. **B** (×4,000) and **D** (×20,000): *P. tricornutum* grown in KEP II medium for the same time. Pyrenoids could be seen in the stroma chloroplasts, and thylakoid membranes were present. CW, cell wall; CM, cell membrane; Thy, thylakoid of chloroplast; Pyr, pyrenoid.

predominantly with the Calvin enzyme, ribulose-1,5-bisphosphate carboxylase activase, involved in CO_2 fixation in photosynthesis [5]. KEP II medium promotes the formation of the pyrenoids, chlorophyll *a*, chlorophyll *c*, and carotenoids in order to maintain photosynthetic activity in batch culture over long periods during which nutrients are largely depleted. The microalgae grown on KEP II medium will be able to be applied as bioresources for biotechnological industry (health food, cosmetics, feed etc.) [35].

The inorganic nitrogen and phosphorus of batch cultures were usually exhausted within one week of inoculations [2], yet the cell density of *P. tricornutum* in KEP II medium during long-term culture was 2.2 times of that in CT, and this increase was maintained for more than one year (Fig. 5). In batch culture, nutrients must be regularly supplied to the microalgae to provide for their basal metabolism. It is surprising that the vitality and high pigment content of *P. tricornutum* can be sustained for long periods of time in KEP II medium without supplying additional nutrients.

The composition of the SSU added to the medium for *P. tricornutum* was altered significantly by the process of swine urine treatment. Total carbon in the secondary swine urine was to 12.9% of that in the original swine urine, whereas total nitrogen was 87% and total phosphate 83.2% of that in the original urine. The pH of the original urine (7.97) was reduced to 3.73 after treatment. However, dissolved organic carbon (DOC) in the treated swine urine was slightly higher (by 5.5%) than in non-treated swine urine. The increase in DOC may have arisen from the fermentative production of organic compounds such as humic substances, organic acids, vitamins, hormones, or chelating agents by bacteria of soil humus. The chelating agent in treated wastewater might stimulate that the onset of stationary phase is delayed in KEP II, despite the lower level of inorganic nutrients. Inhibition of growth, and chlorosis, of microalgae is usually caused by starvation of

the N and P needed for photosynthesis and metabolism [38]. Even though there is the severe starvation for N and P during prolonged batch culture, KEP II medium may stimulate the uptake of dissolved organic carbon including the fermentative production of organic compounds such as humic substances, organic acids, vitamins, hormones, and chelating agents from the SSU or the production of pyrenoids. The new medium also maintains high vitality within a constant carrying capacity of the batch culture despite the low uptake ratios of N/P. Accordingly, KEP II can reduce the maintenance expenses of long-term culture and promote higher yields without needing additional nutrients. The increased production of pigments and amino acids in KEP II enhances the value of the *P. tricornutum* as fish food, health food supplement, and pharmaceutical [35].

KEP II should improve the cost efficiency of industrial mass batch culture and microalgal stock for long-term species preservation, and decrease the negative impact on the environment by recycling animal wastewater. The higher KEP II content of organic matter such as natural intermediate products produced by bacteria during fermentation may accelerate the biochemical activities of *P. tricornutum* and delay the onset of the stationary phase despite the shortage of inorganic nutrients in the batch culture. We propose, in future studies, to investigate how the small addition of treated swine urine favors the microalgal culture, by analyzing the dissolved organic carbon compounds in SSU with the aim of producing a culture medium of value for higher plants as well as microalgae.

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References

1. Acién Fernándaz, F. G., D. O. Hall, G. E. Cañizares, K. Krishna Rao, and E. G. Grima. 2003. Outdoor production of *Phaeodactylum tricornutum* biomass in a helical reactor. *J. Biotechnol.* **103:** 137–152.
2. An, J. Y., S. J. Sim, J. S. Lee, and B. W. Kim. 2003. Hydrocarbon production from secondarily treated piggery wastewater by the green alga *Botryococcus braunii*. *J. Appl. Phycol.* **15:** 185–191.
3. APHA. 1995. *Standard Methods for the Examination of Water and Wastewater.* 19th Ed. American Public Health Association, Washington, DC, U.S.A.
4. Bich, N. N., M. I. Yaziz, and N. A. K. Bakti. 1999. Combination of *Chlorella vulgaris* and *Eichhornia crassipes* for wastewater nitrogen removal. *Wat. Res.* **33:** 2357–2362.
5. Borkhsenious, O. N., C. B. Mason, and J. V. Moroney. 1998. The intracellular localization of ribulose-1,5-bisphosphate carboxylase/oxygenase in *Chlamydomonas reinhardtii*. *Plant Physiol.* **116:** 1585–1591.
6. Chang, M. U. 2001. *Plant Viruses in Korea.* Junghaeng-Sa Press, Suwon, Korea.
7. Charles, A. L., S. J. Markich, J. L. Stauber, and L. F. De Filippis. 2002. The effect of water hardness on the toxicity of uranium to a tropical freshwater alga (*Chlorella* sp.). *Aquatic Toxicol.* **60:** 61–73.
8. Craggs, R. J., P. J. Mcaulley, and V. J. Smith. 1997. Wastewater nutrient removal by marine microalgae grown on a corrugated raceway. *Wat. Res.* **31:** 1701–1707.
9. Fábregas, J., A. Dominguez, D. G. Álvarez, T. Lamela, and A. Otero. 1998. Induction of astaxanthin accumulation by nitrogen and magnesium deficiencies in *Haematococcus pluvialis*. *Biotechnol. Lett.* **20:** 623–626.
10. Fábregas, J., A. Dominguez, A. Maseda, and A. Otero. 2003. Interactions between irradiance and nutrient availabillity during astaxanthin accumulation and degradation in *Haematococcus pluvialis*. *Appl. Microbiol. Biotechnol.* **61:** 545–551.
11. Fidalgo, J. P., A. Cid, E. Torres, A. Sukenik, and C. Herrero. 1998. Effects of nitrogen source and growth phase on proximate biochemical composition, lipid classes and fatty acid profile of the marine microalga *Isochrysis galbana*. *Aquaculture* **166:** 105–116.
12. Grönlund, E., A. Klang, S. Falk, and J. Hanæus. 2004. Sustainability of wastewater treatment with microalgae in cold climate, evaluated with energy and socio-ecological principles. *Ecol. Eng.* **22:** 155–174.
13. Guillard, R. R. L. 1973. Division rates, pp. 289–311. *In* J. R. Stein (ed.), *Handbook of Phycological Methods - Culture Methods and Growth Measurements*. Cambridge University Press, Cambridge.
14. Kawamoto, S. 1996. *Experiment Results of BMW Techniques in the Farm of Kamegawa.* Ecopeace Press, Daegu.
15. Khoshmanesh, A., F. Lawson, and I. G. Prince. 1996. Cadmium uptake by unicellular green microalgae. *Chem. Eng. J.* **62:** 81–88.
16. Kim, S. K., H. C. Baek, H. G. Byun, O. K. Kang, and J. B. Kim. 2001. Biochemical composition and antioxidative activity of marine microalgae. *J. Korean Fish. Soc.* **34:** 260–267.
17. Kim, M. K., J. P. Dubacq, J. C. Thomas, and G. Giraud. 1996. Seasonal variation of triacylglycerols and fatty acids in *Fucus serratus*. *Phytochemistry* **43:** 49–55.
18. Kim, M. K. and G. Giraud. 1989. Characters of neutral lipids of *Detonula* sp. in culture. *Korean J. Phycol.* **4:** 55–61.
19. Kim, M. K. and H. W. Lee. 1998. Changes of β-carotene in fresh and dry thalli of *Undaria pinnatifida* and *Enteromorpha compressa* from Korea. *Algae* **13:** 151–155.
20. Kim, M. K. and R. E. H. Smith. 2001. Effect of ionic copper toxicity on the growth of green alga, *Selenastrum capricornutum*. *J. Microbiol. Biotechnol.* **11:** 211–216.

21. Kobayashi, M. and T. Okada. 2000. Protective role of astaxanthin against U.V.-B irradiation in the green alga *Haematococcus pluvialis*. *Biotechnol. Lett.* **22:** 177–181.
22. Kudo, I., M. Miyamoto, Y. Noiri, and Y. Maita. 2000. Combined effects of temperature and iron on the growth and physiology of the marine diatom *Phaeodactylum tricornutum*. *J. Phycol.* **36:** 1096–1102.
23. Lee, K. and C.-G. Lee. 2001. Effect of light/dark cycles on wastewater treatment by microalgae. *Biotechnol. Bioprocess Eng.* **6:** 194–199.
24. Lee, K. and C.-G. Lee. 2002. Nitrogen removal from wastewaters by microalgae without consuming organic carbon sources. *J. Microbiol. Biotechnol.* **12:** 979–985.
25. Martínez, M. E., S. Sánchez, J. M. Jiménez, F. E. Yousfi, and L. Muñoz. 2000. Nitrogen and phophorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. *Biores. Technol.* **73:** 263–272.
26. McLachlan, J. 1973. Growth media - marine, pp. 25–51. *In* J. R. Stein (ed.), *Handbook of Phycological Methods - Culture Methods and Growth Measurements*. Cambridge University Press, Cambridge.
27. Megharaj, M., H. W. Pearson, and K. Venkateswarlu. 1992. Removal of nitrogen and phosphorous by immobilized cells of *Chlorella vulgaris* and *Scenedesmus bijugatus* isolated from soil. *Enzyme Microb. Technol.* **14:** 656–658.
28. Nagasaki, H. 1998. *Bacteria Save the Earth - Challenge of BMW Technology*. Ecopeace Press, Daegu.
29. Nichols, H. W. 1973. Growth media - freshwater, pp. 7–24. *In* J. R. Stein (ed.), *Handbook of Phycological Methods - Culture Methods and Growth Measurements*. Cambridge University Press, Cambridge.
30. Oswald, W. J. 1988. *Micro-Algal Biotechnology*, pp. 305–328. Cambridge University Press, Cambridge.
31. Pehlivanoglu, E. and D. L. Sedlak. 2004. Bioavailability of wastewater-derived organic nitrogen to the alga *Selenastrum capricornutum*. *Wat. Res.* **38:** 3189–3196.
32. Rawat, M., M. C. Henk, L. L. Lavigne, and J. V. Morney. 1996. *Chlamydomonas reinhardtii* mutants without ribulose-1.5-bisphosphate carboxylase/oxygenase lack a detectable pyrenoid. *Planta* (Berl.) **198:** 263–270.
33. Reis, A. 1996. Eicosapentaenoic acid-rich biomass production by the microalga *Phaeodactylum tricornutum* in a continuous-flow reaction. *Biores. Technol.* **55:** 83–88.
34. Semple, K. T. 1997. Biodegradation of phenols by a eukaryotic alga. *Res. Microbiol.* **148:** 365–367.
35. Shon, Y. H., K. S. Nam, and M. K. Kim 2004. Cancer chemopreventive potential of *Scenedesmus* cultured in medium based on swine wastewater. *J. Microbiol. Biotechnol.* **14:** 158–161.
36. Suss, K. H., I. Prokhorenko, and K. Adler. 1995. *In situ* association of Calvin cycle enzymes, ribulose-1,5-bisphosphate carboxylase/oxygenase activase, feredoxin-$NADP^+$ reductase, and nitrate reductase with thylakoid and pyrenoid membranes of *Chlamydomonas reinhardtii* chloroplast as revealed by immunoelectron microscopy. *Plant Physiol.* **107:** 1387–1397.
37. Tomas, C. R. 1997. Marine diatoms, pp. 5–361. *In* G. R. Hasle and E. E. Syvertsen (eds.), *Identifying Marine Phytoplankton*. Academic Press, San Diego.
38. Torres, E., D. Cid, C. Herrero, and J. Abalde. 2000. Effect of cadmium on growth, ATP content, carbon fixation and ultrastructure in the marine diatom *Phaeodactylum tricornutum* bohlin. *Water Air Soil Pollut.* **117:** 1–14.
39. Tredici, M. R., M. C. Margheri, G. C. Zitelli, S. Biagiolini, and E. Capolino. 1992. Nitrogen and phosphorus reclamation from municipal wastewater through an artificial food-chain system. *Biores. Technol.* **42:** 247–253.
40. Lee, J. Y., T. S. Kwon, K. T. Baek, and J. W. Yang. 2005. Biological fixation of CO_2 by *Chlorella* sp. HA-1 in a semi-continuous and series reactor system. *J. Microbiol. Biotechnol.* **15:** 461–465.
41. Kim, J. P., C. D. Kang, S. J. Sim, M. S. Kim, T. H. Park, D. H. Lee, D. J. Kim, J. H. Kim, Y. K. Lee, and D. W. Pak. 2005. Cell age optimization for hydrogen production induced by sulfur deprivation using a green alga *Chlamydomonas reinhardtii* UTEX 90. *J. Microbiol. Biotechnol.* **15:** 131–135.
42. An, J. Y., S. J. Sim, B. W. Kim, and J. S. Lee. 2004. Improvement of hydrocarbon recovery by two-stage cell-recycle extraction in the cultivation of *Botrycoccus braunii*. **14:** 932–937.