Enhanced production of *Scenedesmus* spp. (green microalgae) using a new medium containing fermented swine wastewater

M.K. Kim a,*, J.W. Park a, C.S. Park b, S.J. Kim b, K.H. Jeune b, M.U. Chang b, J. Acreman c

a Korea Plankton Culture Collection for Industrialization (KPCCI), Marine Science Research Center, Yeungnam University, Gyongsan 712-749, Republic of Korea

b Department of Biology, Yeungnam University, Gyongsan 712-749, Republic of Korea

c University of Toronto Culture Collection of Algae and Cyanobacteria, Department of Botany, University of Toronto, Toronto, Canada M5S 3B2

Received 22 December 2005; received in revised form 29 August 2006; accepted 31 August 2006

Available online 1 November 2006

Abstract

Fermented swine urine (3%) (v/v) was added to a control medium (CT), named KEP I, and an aquatic microalgal culture (10% Bold’s Basal Medium) for growing mixed *Scenedesmus* species. During a two-month period, the KEP I medium effected, the delayed onset of the stationary phase of cell division in a batch culture. After 31 days, of culturing, the growth rate (3-fold), dry weight (2.6-fold) and amino acid levels (2.7-fold), and secondary metabolites including chlorophyll *a* (2.1-fold), astaxanthin (2.8-fold), lutein (2.7-fold) and *α* (greater than 30-fold) and *β*-carotene (greater than 5-fold) increased a greater degree in *Scenedesmus* grown in KEP I medium than in CT medium. Total lipids were much less in cells grown in KEP I than those grown in CT. An increased quantum yield of photosystem II of the aquatic microalgae. The KEP I medium should improve the cost efficiency of industrial mass batch cultures for CO2 sequestration, bioremediation, phytonutrients, agricultural fertilizers, and microalgal stock for the species preservation of aquaculture strains for use in young fish feed. It may also serve to attenuate negative environmental impact via the recycling of animal wastewater.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: *Scenedesmus*; Batch culture; Long-term culture; Fermented swine urine; Production; Pyrenoid

1. Introduction

A variety of aquatic microalgae, including the green alga *Scenedesmus*, have been studied for their possible efficacy as bioresources for applications as fish feed, human food, supplemental human nutrients and pharmaceutical products (Belay et al., 1993), and also for the bioremediation of polluted water (Chong et al., 2000). *Scenedesmus* is an ubiquitous organism, and frequently is a dominant microalga in freshwater lakes and rivers (Borowitzka and Borowitzka, 1998). The green algae are among the most common and taxonomically diverse of the chlorococcalean genera, within which over 200 species, and almost 1200 infraspecific taxa, have been identified (John et al., 2002).

Previous studies of *Scenedesmus* have focused principally on continuous or semicontinuous cultures, using culture media modified in a variety of ways (Voltolina et al., 1998; Adamsson, 2000). Biochemical and physiological changes in microalgal cells, such as the cells of *Scenedesmus*, can be effected by the culture media, growing conditions, and nutrient compositions, in addition to gene transformation technology (McLachlan, 1973; Nichols, 1973; Kim and Giraud, 1989; Kim and Smith, 2001). A variety of culture media, including Erdschreiber, Grund, ES, CHU-10, f/2 and ASP series media (McLachlan, 1973; Nichols, 1973), have been developed for the effective culturing of fresh and marine microalgae. These media, however, have considerable limits. They lack selected nutri-
Wastewater containing the biological wastes of animals, which harbors abundant inorganic nitrogen and phosphate, is one of the most significant causes of eutrophication in waterways. Microalgae, including *Scenedesmus*, have been utilized as a bioremediation agents in the removal of inorganic nutrients from polluted water to improve short-term quality (Martinez et al., 2000). This sort of wastewater has also been used as a microalgal culture medium (Olguin et al., 2001, 2003; An et al., 2003). These studies have focused primarily on the capacity to remove inorganic nutrients, particularly those from animal’s wastewater. We have become quite interested in a new technology involving fermented swine urine (Kawamoto, 1996). This technology produces beneficial organic matter (dissolved organic carbon) in wastewater subjected to fermentation and bioreaction via bacteria and the addition of soil humus and quartz sand, over 40 days of treatment. The majority of inorganic nutrients (N and P) in the wastewater are removed by this treatment.

The objective of this study was to determine whether a new medium, KEP I, to which treated swine urine had been added, could enhance the growth and quality of the microalgae, *Scenedesmus*, and alter the biochemical compositions of its cells. The yields of photosynthetic (Kaftan et al., 1999) and secondary metabolites (Kim et al., 1996; Burczyk et al., 1999; Orosa et al., 2000) were analyzed. The chloroplast ultrastructure was also investigated to verify the physiological biochemical changes occurring in microalgal cells grown in different media (Chang, 2001).

2. Methods

2.1. Culture conditions

The microalgae used in these experiments were obtained from the Korea Plankton Culture Collection for Industrialization (KPCCI) at Yeungnam University, Korea. Three species of *Scenedesmus* – *S. acutus* (P-F-6), *S. spinosus* (P-F-77), and *S. quadricauda* (P-F-70) – were mixed and grown in 1 L volumes in 2 L Erlenmeyer flasks. The inoculum for each strain was 5 ml, containing about 10^4 cells ml^-1. The strains were cultivated at 24 °C with a light:dark photoperiod of 14 h:10 h, with a light intensity of 115 μmol m^-2 s^-1, and were continuously stirred for aeration.

2.2. Developments of KEP I medium

Mixed *Scenedesmus* cultures were grown using dilute (10%) Bold’s Basal medium (BBM) (Nichols, 1973) containing the following components (mg L^-1) as the control medium: KH₂PO₄ (175), CaCl₂ 2H₂O (25), MgSO₄ 7H₂O (75), NaNO₃ (250), K₂HPO₄ (75), NaCl (25), Na₂EDTA (10), KOH (0.62), FeSO₄ 7H₂O (4.98), H₂SO₄ (0.001), H₃BO₃ (8.05) and 1 ml of trace metal (g L⁻¹) solution composed of H₂BO₃ (2.86), MnCl₂ 4H₂O (1.81), ZnSO₄ 7H₂O (0.22), NaMoO₄ 5H₂O (0.39), CuSO₄ 5H₂O (0.08), (CO(NO₃)₂ 6H₂O (0.05) in 1 L distilled water. The pH was adjusted to 6.8 with HCl and NaOH prior to autoclaving. The treated swine urine was obtained from a wastewater treatment pilot plant, located in a Yeung-Chun BMW (bacteria mineral water: the current designation for fermented swine urine) farm in Korea (Fig. 1; Table 1). The plant was constructed on the basis of a Japanese technique, which involves fermentation and bioreaction technologies for the pre-processing of swine urine through porous inorganic substrates (Kawamoto, 1996). Pellets containing soil humus and quartz sand at a pH of 2.88 were utilized in the culturing of the bacteria, which in turn effected the fermentation of the pre-processed wastewater to produce treated swine urine water, referred to as BMW (Nagasaki, 1998).

2.3. 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 (APHA, 1995). 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, Tekmar-Dohrmann, Cincinnati, Ohio, USA) after membrane (pore size: 0.45 μm; Millipore GF/C # HVLP04700, Cork, Ireland) filtration of the samples.

![Fig. 1. Pilot plant scheme for the swine urine fermentation process.](image-url)
Table 1

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

▲: Increase percent; ▼: decrease percent. Values are expressed as means ± s.d. (n = 3).

2.4. Growth and photosystem II activity of cells

Cell growth rates were evaluated via periodic counting using a haemocytometer, and growth kinetics were calculated using Guillard's formula (1973). The maximum quantum yields of photosystem II were measured with a pulse-amplitude-modulated fluorometer (Phyto-PAM Walz, Effeltrich, Germany). The total pigments were extracted in a solvent system consisting of acetone, methanol and water, at a ratio of 10:9:1 (v/v/v). Chlorophyll a and b were analyzed via the method of Kim and Smith (2001). For the measurement of total carotenoids, chlorophyll was removed from the extract via the addition of 5% KOH. The total lipids were extracted three times from the same samples cultured for 31 days, and subsequently hydrolyzed to effect the release of fatty acids, via the method of Kim et al. (1996). The fatty acids were then methylated by BF₃ methanol (Sigma–Aldrich #33356, St. Louis, MO, USA) and analyzed via GC (HP 6890 Series, Wilmington, NC, USA), using heptadecanoic acid (C₁₇:0) (Sigma–Aldrich #3500, St. Louis, MO, USA) as a standard. The individual fatty acid compositions were determined via the comparison of GC retention times with those of a standard mixture of fatty acid methyl esters (Sigma–Aldrich #189-19, St. Louis, Missouri, USA).

2.5. Analysis of fatty acids

2.6. Analysis of amino acids

Twenty milliliters of algal culture (5–10 × 10⁶ cells ml⁻¹) for each of the collected samples, after 31 days of cultivation, was freeze-dried, added to 2 ml of 6 N HCl, and briefly bubbled with nitrogen gas. The sample was subsequently 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 repeatedly with distilled water, and evaporated to dryness, in order to remove the HCl. It was subsequently then dissolved in 25 ml of buffer (0.2 M sodium citrate, pH 2.2), and the amino acid content was determined with an automatic analyzer (Biochrom 20, Pharmacia Biotech, Piscataway, New Jersey, USA).

2.7. Cell observations via transmission electron microscopy

After 3–4 washes with distilled water followed by 2 min of centrifugation at 15 rpm at ambient temperature, a Scenedesmus mixed culture was suspended in a solution of 2.5% glutaraldehyde in distilled water, and incubated for 90 min at 4 °C with gentle mixing every 10 min. The glutaraldehyde-treated cells were then centrifugated as before, followed by suspension in 0.1 M phosphate buffer, at a pH of 7.2. The cells were subsequently post-fixed for 90 min at 4 °C in 1% osmium tetroxide, to a final concentration of 2% (v/v), and buffered with 0.1 M phosphate. After the addition of melted agar at 60 °C, the sample was washed in the same phosphate buffer, then dehydrated by incubation in a graded series of 30%, 50%, 70%, 80%, 90%, and 100% ethanol (5 min each). The ethanol was then replaced by an embedding solution consisting of propylene oxide and epoxy resin (2:1, v/v). After 20 min of incubation, the dehydrated sample was replaced with fresh embedding solution, and incubated for an additional 20 min. The propylene oxide was removed via 4 h of vacuum pumping. The final samples in the embedding molds were then placed into a drying oven at 70 °C, and heated gradually to thoroughly polymerize the epoxy. After ultrathin sectioning, the samples were incubated for three successive 20-minute time periods in uranyl acetate for staining, incubated for 10 min in lead citrate for post-staining, and finally heated to 60 °C for drying. The stained and
dried samples were positioned on grids and visualized via TEM (Hitachi H-7600, Japan) (Chang, 2001).

2.8. Statistical analyses

Experiments for the determination of dry weight and photosynthetic rates, as well as the biochemical assays, were all repeated three times to ensure the clarity of the data. Standard deviations of values and error bars shown in the figures were determined using Microsoft Excel on a Compaq nc 4010 (Hewlett-Packard Co., L.P., USA). The number of replicates was 3, and the probability values were less than 0.05. Spearman rank correlation coefficient ($r_s$) was calculated by the formula:

$$r_s = 1 - \frac{6 \sum D_i^2}{n(n^2 - 1)}$$

where $n$ is the number of pairs and $D_i$ is the difference between ranks for each pair of numbers.

3. Results

3.1. Improvement of the water quality of the fermented swine urine

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 (Fig. 1). The agricultural and floricultural efficacy of the fermented swine urine has been repeatedly demonstrated since the initial 1996 studies on the subject in Japan (Kawamoto, 1996). 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.

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

3.2. Growth rates of Scenedesmus in KEP I medium

To determine the optimal concentration of fermented swine urine for maximal growth and quality of the cultured microalga, *Scenedesmus*, a series of 0.5, 2, 3, 5, 7, and 9 (v/v)% mixtures of fermented swine urine and 10% Bold’s Bases Medium were prepared as KEP I media. The growth rates of the cells and the correlation probabilities ($r^2$), listed by Spearman rank among cell numbers and culture time, are shown in Table 2. Twelve days after the start of the culture, growth rates were determined to be the highest (0.29) for CT, 10% BBM. When 3% treated swine urine was added to CT (10% BBM), higher maximal (0.46) growth rates at 51 days of culture were attained. The correlation probability ($r^2$) among the cell numbers was also found to be highest (0.98) in the culture with 3% fermented swine urine.

The algae were cultured in a medium containing 3% unfermented swine urine (WW: wastewater) to 10% BBM (CT), to conduct a comparison to cultures grown in the CT and KEP I media. This experiment clarified whether the physiological and biochemical changes occurring in the cells were attributed to the uptake of inorganic nutrients contained in the original swine urine without any fermentation process, or to organic bacterial metabolites produced in the swine urine during fermentation.

In Fig. 2, the *Scenedesmus* cells were cultured for up to 60 days in the CT controls, the KEP I fermented urine medium, and the WW medium. After 59 days, the cell density was determined to be higher in the KEP I medium ($21.2 \times 10^6$ cells ml$^{-1}$) than in the CT ($9.5 \times 10^6$ cells ml$^{-1}$) and WW ($13.3 \times 10^6$ cells ml$^{-1}$) media. In the CT and WW media, the cells entered stationary phase as early as...
the 31st day of culture. The *Scenedesmus* cells growing in the KEP I medium continued to divide after this threshold, and entered the stationary phase at approximately the 59th day of culturing.

The physio-biochemical activities of the *Scenedesmus* grown for 31 days in KEP I medium were compared to the activities of the cells grown in CT (Table 3). The growth rate of *Scenedesmus* was increased by over 3-fold in KEP I \((k = 0.12)\) as compared to the CT medium \((k = 0.04)\). The maximum PSII quantum yield of the cells in KEP I was found to be 0.7, whereas the PSII quantum yield of the cells in CT was 0.5. The total amino acid levels in the *Scenedesmus* cells grown in KEP I medium \((336 \text{ mg/g d wt})\) was found to be 2.7-fold higher than that in CT \((124 \text{ mg/g d wt})\). The quantity of essential amino acids in the *Scenedesmus* cells grown in the KEP I medium was higher \((119 \text{ mg/g d wt})\) than in the cells grown in the CT medium \((44 \text{ mg/g d wt})\). Individual amino acids were also found to be most abundant in the KEP I cultures (Fig. 3). Especially, the acidic amino acids of aspartic acid (Asp) and glutamic acid (Glu) in KEP I were about 3-fold more abundant than those in CT. The KEP I medium containing the acidified swine urine fermented by bacteria (Table 1) might stimulate to synthesize the acidic amino acids such as Asp and Glu of the *Scenedesmus* cells.

### 3.3. Physio-biochemical enhancement in KEP I medium

The Chl \(a\) \((3.6 \text{ mg/g d wt})\) concentration was determined to be the highest in the KEP I medium, whereas the chl \(b\) concentration was highest \((2.51 \text{ mg/g d wt})\) in CT (Table 3). The ratio of concentrations between chlorophyll \(a\) and \(b\) was 1.58 in the *Scenedesmus* cells grown in the KEP I medium, and was 0.67 in the CT medium. Chl \(a\) accumulated to a higher degree in the *Scenedesmus* culture grown in the KEP I medium than in the culture grown in CT. The ratios of concentrations among experimental factors in the *Scenedesmus* grown in the KEP I medium were considerably higher than those grown in CT. The total quantity of carotenoids in the *Scenedesmus* cells grown in the KEP I medium \((3.4 \text{ mg/g d wt})\) was 3.1-fold higher than was seen in the CT cultures \((1.1 \text{ mg/g d wt})\). *Scenedesmus* cells grown in the KEP I medium generated substantially greater amounts of \(\alpha\)-carotene \((0.3 \text{ mg/g d wt})\) and \(\beta\)-carotene \((0.05 \text{ mg/g d wt})\) than did the cultures in CT \((\text{less than }0.01 \text{ mg/g d wt})\). The amounts of astaxanthin and lutein detected in the *Scenedesmus* cells grown on the KEP I medium were also higher \((\text{ast: }1.12 \text{ mg/g d wt}; \text{lut: }1.88 \text{ mg/g d wt})\) than were seen in the CT cultures \((\text{ast: }0.4 \text{ mg/g d wt}; \text{lut: }0.7 \text{ mg/g d wt})\).

The total fatty acid content in the KEP I medium culture after 31 days of culturing \((9 \text{ mg/g d wt})\) was less than the total fatty acid content of the counterpart CT culture \((45.8 \text{ mg/g d wt})\) (Table 3). The quantities of individual fatty acids were lowest, among the tested media, in the KEP I cultures, as shown in Fig. 4. The high levels of

<table>
<thead>
<tr>
<th>Physio-biochemical characteristics</th>
<th>CT</th>
<th>KEP I</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry weight (mg L(^{-1}))</td>
<td>76.5 ± 1.2</td>
<td>197 ± 4</td>
<td>2.6</td>
</tr>
<tr>
<td>Growth rates (k/day)</td>
<td>0.04</td>
<td>0.12</td>
<td>3</td>
</tr>
<tr>
<td>Photosynthetic rates (Fv/Fm)</td>
<td>0.5 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Total amino acids (mg/g d wt)</td>
<td>124 ± 6</td>
<td>336 ± 8</td>
<td>2.7</td>
</tr>
<tr>
<td>Total chlorophylls (mg/g d wt)</td>
<td>4.2 ± 0.4</td>
<td>5.9 ± 1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Chl (a)</td>
<td>1.7 ± 0.1</td>
<td>3.6 ± 0.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Chl (b)</td>
<td>2.5 ± 0.3</td>
<td>2.3 ± 0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Total carotenoids (mg/g d wt)</td>
<td>1.1 ± 0.1</td>
<td>3.4 ± 0.2</td>
<td>3.1</td>
</tr>
<tr>
<td>(\alpha)-Carotene</td>
<td>0 ± 0.0</td>
<td>&gt;30</td>
<td></td>
</tr>
<tr>
<td>(\beta)-Carotene</td>
<td>0.05 ± 0.0</td>
<td>&gt; 5</td>
<td></td>
</tr>
<tr>
<td>Astaxanthin</td>
<td>0.4 ± 0.0</td>
<td>1.12 ± 0.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Lutein</td>
<td>0.7 ± 0.0</td>
<td>1.88 ± 0.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Fatty acids (mg/g d wt)</td>
<td>45.8 ± 3.1</td>
<td>9 ± 0.9</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Values are expressed as means ± s.d. \((n = 3)\).

\(^{\text{a}}\) Less than 0.01 mg/g d wt.

**Table 3**

Comparison of physio-biochemical activities of *Scenedesmus* cells cultured for 31 days in CT and KEP I media.
unsaturated fatty acids observed in the CT cultures may have induced an accumulation of neutral lipids and triglycerides in the cytosol, due to the fact that the Scenedesmus cells were compelled to tolerate a lack of nutrients over the long culture time (31 days) in the batch culture.

3.4. Pyrenoid formation in KEP I medium

The cultures utilized in the previous experiments were maintained for an additional 5 months (exactly, 156 days), to determine to what degree the cells in different media were able to tolerate long-term culture conditions in batch culture (Fig. 5). In the Scenedesmus cells grown in CT medium, lipid and starch droplets generally accumulated inside aging cells, primarily in the cytosol and stroma of the chloroplasts. The ultrastructure of the thylakoid membranes disappeared to a far greater degree in the CT cultures than in the KEP I cultures, according to the electron micrographs. However, the pyrenoids existing inside of the chloroplasts were distinctly formed in the KEP I medium cultures, and this was not accompanied by any chlorosis effects.

Fig. 4. Composition of fatty acids in Scenedesmus cultured in different media for 31 days.

Fig. 5. Electron micrographs of Scenedesmus. A (×8000) and B (×40,000) of Scenedesmus cultured for 5 months in CT. Lipid and starch droplets accumulated in the stroma of the chloroplasts, whereas the thylakoids disappeared; C (×8000) and D (×50,000) of Scenedesmus grown in KEP I medium for the same time period. The pyrenoids accumulating in the stroma of the chloroplasts were abundant, whereas few starch and lipid droplets were noted. L: lipid droplet; St: starch droplet; Th: thylakoid of chloroplasts.
4. Discussion

The higher biomass and the generally superior pigment and essential amino acid profiles of the *Scenedesmus* cells grown on KEP I media suggests a way to effectively process industrial and pharmaceutical sources of these nutrients for applications fish feed and human nutrient supplements.

In Fig. 2, the optimal growth period for the use of these microalgae at the commercial level might be approximately 40 days. The color of the cells grown on the CT medium changed completely, from green to yellow, via chlorosis, whereas the cells grown on the KEP I medium maintained their characteristic deep green color during long-term culture growth, for a minimum of 5 months. This phenomenon was attributed to the accumulation of both chl a and carotenoids in these cultures. Astaxanthin and lutein, similarly to β-carotene, have been proven to be very effective xanthophylls for use as chemopreventive agents (anticancer and antioxidants) (Kim and Lee, 1998; Dominguez-Bocanegra et al., 2004). Increases in the concentrations of these carotenoids ameliorates photoinhibition and photo-oxidation during photosynthesis, and protects this function under a variety of growth conditions (Kobayashi and Okada, 2000). The individual fatty acid levels in the microalgal cultures grown for 31 days on KEP I medium were found to be lower than those in the CT and WW media. In general, when cells are grown under environmental stress conditions, lipid and fatty acid concentrations tend to be higher than in cases in which they are grown under favorable conditions (Kim et al., 1996). Therefore, CT was shown to be less favorable for the growth of *Scenedesmus* cells than KEP I medium. In previous studies involving *Scenedesmus obliquus* (Borowitzka and Borowitzka, 1998), lipid levels were measured at 7% and 14% per dry weight. However, in this study, the lipid levels of the *Scenedesmus* cells grown in the KEP I medium were substantially lower (0.9%) than those grown in the CT medium (5%). Our previous study demonstrated that healthy microalgal cells grown under favorable growth conditions evidenced a high ratio of C16:0 and C18:1 (Kim et al., 1996). The ratio between the two fatty acids of the *Scenedesmus* cells in KEP I was also found to be higher (2.73) than that grown in the other media (CT: 1.84; WW: 1.28).

The finding that pyrenoids were formed in *Scenedesmus* cells cultured in KEP I medium was consistent with the results of previous studies (Suss et al., 1995; Rawat et al., 1996). Thylakoid membranes in the chloroplasts and on the inner surfaces of the pyrenoid membranes of *Chlamydomonas reinhardtii* are associated predominantly with the Calvin enzymes ribulose-1,5-bisphosphate carboxylase activase, nitrite reductase, ferredoxin-NADP⁺ reductase, and H⁺-ATP synthase (Suss et al., 1995). The amounts of rubisco within the pyrenoids can vary with varying growth conditions (Borkhensioius et al., 1998). In this study, the newly-developed KEP I medium, representing the most effective growth condition for *Scenedesmus*, stimulated the synthesis of pyrenoids, chl a, and carotenoids, thereby contributing to the maintenance of photosynthesis, despite the removal of inorganic nutrients (N, P, and C) from the cultures. The biochemical composition of the KEP I medium, including the minerals fundamentally required for cell growth and photosynthesis in a batch culture, will have to be analyzed and clarified in future research.

Despite the absorption of most inorganic nutrients (N, P, and C) from the batch culture medium by the microalgal cells within one week of inoculation (An et al., 2003), the cell density of the *Scenedesmus* samples grown in the KEP I medium continued to increase by 300% over the long-term culture period (minimum of 5 months), as compared to the density of the cultures grown on CT.

*Scenedesmus* cells grown in CT over the short-term (12 days), with sufficient N and P concentrations, yielded a substantially greater number of cells than were seen in the KEP I cultures. However, after the cells entered stationary phase (31 days), the cells grown in the CT and WW media began to starve, due to the limited quantities of inorganic nutrients, whereas the cells growing in the KEP I media continued to grow. The quantity of inorganic compounds in the fermented swine urine added to the KEP I media were far lower than those contained in the non-treated swine urine used for WW. However, the DOC of the treated swine urine was slightly increased (5.5%) compared to that of the non-treated swine urine. We surmise that this increased DOC value may be attributable to organic compounds contained in the humic substances, organic acids, vitamins, hormones or chelating agents, probably generated by the bacteria and soil humus via the fermentation of the swine urine. Despite the fact that the KEP I medium harbored less abundant inorganic nutrients than did the WW medium, a question exists with regard to whether or not the *Scenedesmus* grown in KEP I medium was exhibiting delayed cell divisions. Inhibition of growth and chlorosis are normally caused by a lack of the N and P necessary for photosynthesis (Schlee et al., 1985; Sayed, 1998; Shehawy and Kleiner, 2001; Wagner and Falkner, 2001). In the KEP I medium, the reduced quantities of N, P, and C in long-term batch cultures may stimulate the uptake of organic carbon in a photo-trophic-to-heterotrophic switch in the *Scenedesmus* cells, or the generation of pyrenoids associated with the synthesis of Calvin enzymes for CO₂ fixation. Fermented swine urine, which harbors a higher DOC content than untreated swine urine, may simultaneously stimulate the growth of cells within a constant carrying-capacity of culture, due to the low uptake-ratios of N/P resultant from interspecific competitions among three different *Scenedesmus* species.

Yang and Gao (2003) demonstrated that increased carbon concentration coupled with lowered pH values could affect the growth rate and photosynthesis of microalgae, and also demonstrated enhanced growth rates as the result of carbon enrichment combined with a limitation of N and P levels. In this study, *Scenedesmus* cells cultured in KEP I medium to which 3% treated swine urine was added, and in which the DOC content had been enriched, absorbed a
greater quantity of organic compounds in the presence of reduced N and P under long-term culture conditions (minimum of 5 months).

The growth rate of the *Scenedesmus* cells increased by 3-fold in the KEP I medium as compared to the cells grown on CT medium. This indicates that the use of KEP I medium may reduce the expenses normally associated with culture media, as well as the attendant time and labor costs. This medium may also generate substantially higher yields, without the need for medium additions, in long-term batch cultures. The observation of enhanced pigment and amino acid production in the *Scenedesmus* cells grown in KEP I medium may indicate that the medium bears great potential in several industrial applications, including fish feed, phyto-nutrients, and pharmaceutical compounds (Shon et al., 2004), including β-carotene, astaxanthin, and lutein (Kobayashi and Okada, 2000; Orosa et al., 2000).

5. Conclusions

In this study, the newly-developed KEP I medium was determined to be an optimal growing medium for *Scenedesmus* cells in a batch mass culture, maintained for a minimum of 5 months, without any culture medium addition. The KEP I culture, to which fermentation-treated swine urine has been added, should improve the cost efficiency of industrial mass batch cultures and microalgal stock for species preservation, and may also prove to ameliorate certain environmental damages, via the recycling of animal wastewater. The KEP I medium containing fermented swine urine harbors organic materials (organic acids, enzymes, and hormones) generated by bacteria during the fermentation process. These materials serve to accelerate the physiological and biochemical activities of the growing cells, and also effect a delay in the onset of stationary phase of the cell divisions of *Scenedesmus*, despite the shortage of inorganic nutrients (N, P, and C) within the medium. In future studies, we hope to further assess the suitability and preferable of the addition of treated swine urine to microalgal culture media.

Acknowledgements

This work was supported by Grant No. R04-2000-00048 from the Korea Science and Engineering Foundation. We greatly appreciate Prof. Jerry Brand, UTEX Algae Collection, College of Natural Science, University of Texas at Austin, USA, who corrected carefully our text and Mr. Jong-Soo Suk, owner of Yeung-Chun Bacteria Mineral Water farm, who offered kindly treated swine urine for this work.

References


