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Characterization of a Novel Alga-Lytic Bacterium, *Acidovorax temperans* AK-05, Isolated from an Eutrophic Lake for Degradation of *Anabaena cylindrica*

Kim, Jeong-Dong and Myung-Soo Han*

(Department of Life Science, Hanyang University, Seoul 133-791, Korea)

부영양 호수에서 분리한 *Acidovorax temperans* AK-05의 *Anabaena cylindrica* 분해 특성.

김정동 · 한명수* (한양대학교 생명과학과)

부영양 호수로부터 살조 세균을 분리하고 동정한 결과 *Anabaena cylindrica* NIES-19를 유릴 탄소원으로 이용하는 double layer 방법으로 15종의 살조세균을 분리하였으며 높은 살조 활성을 나타내는 4종의 살조세균 AK-05, AK-07, AK-13 그리고 AK-28을 선별하여 살조 능력을 비교하였다. 이들 중에 AK-05가 가장 높은 살조 활성을 나타내었으며 이를 16S rDNA 염기서열을 분석한 결과 *Acidovorax temperans*와 99.5%의 유사성을 나타내어 *Acidovorax temperans* AK-05로 명명하였다. *A. temperans* AK-05의 배양 여액을 *A. cylindrica* NIES-19에 뚜렷한 살조 활성을 나타냈었으며, 이것의 살조 활성 능력을 분석한 결과 살조 활성에 관여하는 주요 물질은 non-protein이며 열에 안정적이었다. 이러한 살조 활성 능력은 알칼리 조건과 25～30°C에서 가장 높게 나타냈다. 따라서 이와 같은 특성은 일반적으로 알칼리 조건을 야기하는 Cyanobacteria에 의한 water blooms이 발생하는 호수에 적용하는데 매우 유리할 것으로 여겨진다.

Key words : alga-lytic bacteria, *Acidovorax temperans*, extracellular substances, algal blooms

INTRODUCTION

Cyanobacteria (blue-green algae) are common members of the phytoplankton in mesotrophic to hypereutrophic lakes, where it frequently dominates as a nuisance under stratified condition (Paerl, 1988). Massive accumulations of cyanobacteria are worldwide in lakes and reservoirs during the summer. Especially, they abundantly inhibiting in freshwater ecosystems frequently form blooms in mesotrophic and eutrophic lakes and ponds. *Anabaena* spp. and *Microcystis* spp. are well known as one of the most common bloomforming cyanobacteria, which cause many problems for scenery, anxiety about toxicity (Carmichael, 1992; Harada, 1996) and unpleasant odors (Kikuchi *et al*., 1973; Tsuchiya *et al*., 1992). Therefore, various microorganisms such as bacteria, actinomycetes, fungi, phages and amoebae have been treated in the termination and decomposition of cyanobacteria blooms (Mitsutani *et al*., 1987; Fukami *et al*., 1992; Yamamoto *et al*., 1998; Jang *et al*., 2003; Kim and Han, 2004).

In the present work, we isolated a bacterial strain AK-05 showing high lytic activity against a cyanobacterium, *Anabaena cylindrica*. Identification of the isolates based on 16S rDNA sequence information was conducted. Some specific characteristics of the isolate were investigated on a alga-lytic test. In addition, the effect of pH and temperature on the lytic activity was investigated to

* Corresponding author: Tel: +82-2-2290-0956, Fax: +82-2-2296-1741, E-mail: hanms@hanyang.ac.kr

evaluate the applicability of the isolate as a biological control agent for cyanobacterial blooms.

MATERIALS AND METHODS

1. Host alga and culture conditions

A. cylindrica NIES-19 that used as host for alga-lytic bacteria was kindly supplied by National Institute for Environmental Studies, Japan, and a clonal axenic culture was cultivated and maintained in MDM medium including KNO_2 10 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, K_2HPO_4 2.5 g, NaCl 1.0 g, A5 solution 1 mL, and Fe solution 1 mL per 1 liter under continuous illumination of cool white fluorescent lamps giving an incident light intensity of 35~80 μmE m^{-2} $s^{-1}$and at 25~28°C with agitation at 150 rpm on rotary shaker. A5 solution is composed with H_3BO_3 2.86 g, $MnSO_4 \cdot 7H_2O$ 2.5 g, $ZnSO_4 \cdot 7H_2O$ 0.222 g, $Na_2MoO_4 \cdot 2H_2O$ 0.021 g, $CuSO_4 \cdot 5H_2O$ 0.079 g per 1 liter (Cattenholz, 1988). In addition, Fe solution is plus $FeSO_4 \cdot 7H_2O$ 2.0 g and H_2SO_4 0.26 mL per 1 liter.

2. Isolation of alga-lytic bacteria

Various samples collected from Pal'tang reservoir and Sukchon Lake in Korea in which cyanobacteria are dominant phytoplankton during summer were used as the isolation source. Initial screening was conducted using skim milk agar plates containing 10 g of skim milk (Difco), 1 g of yeast extract (Difco) and 15 g of agar in 1 liter of distilled water. Then, the double-layer method (Shilo, 1970) using *A. cylindrica* NIES-19 as a sole nutrient was used for second screening method (Yamamoto, 1981). Axgenic cultures of *A. cylindrica* NIES-19 were grown in MDM medium for 1 week, and 1 mL of *A. cylindrica* NIES-19 cultures was mixed with 1 mL of filtered (200 μm filter) suspension of surface waters or sediment samples and molten MDM soft agar equilibrated to 50°C. The mixture was immediately poured onto a MDM soft agar plate. After the agar had solidified, the plates were incubated at 25~28°C with continuous illumination of cool white fluorescent lamps giving an incident light intensity of 35~80 μE m^{-2} s^{-1}. Bacterial colonies that produced clear zones on lawns of *A. cylindrica* were picked, purified, and maintained on PY medium slants containing 1.0% (w/v) of peptone, 0.1% (w/v) of yeast extract and 1.5% (w/v) of agar (pH adjusted to 7.0). Isolated bacteria were stored at −80°C in PY medium supplemented with 20% (v/v) of glycerol.

3. Identification of isolates

The chromosomal DNA was isolated using a method described elsewhere (Yoon *et al*., 1997). The amplification of the 16S rDNA was conducted using two primers according to Stackebrandt and Liesack (1993), 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AGAAAGGA-GGTGATCCAGCC-3'. A PCR was run for 35 cycles in a DNA thermal cycler, Genetic analyzer 377 (Perkin-Elmer, Boston, USA), employing the thermal profile according to Yoon *et al*. (1997). The 16S rDNA sequence of bacterial isolate AK-05 was aligned using CLUSTAL W software (Nigam *et al*., 2000). The evolutionary distance matrices were calculated with the DNADIST program within the PHYLIP package (Felsenstein, 1993). The sequence of representative species of the genus *Acidovorax* and related taxa were cited using the GenBank Database. The values of 16S rDNA similarity were calculated from the alignment, while the evolutionary distances were calculated using a Kimura two-parameter correction. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) based on the calculated distance matrix.

4. Extraction and measurement of cyanobacterial chlorophyll *a*

A solution of 90% methanol was used for extraction of chlorophyll *a* (chl-*a*) from cyanobacteria. Measurement of chlorophyll *a* was according to the method of Parson *et al*. (1984). Cyanobacteria filtrated by GF/C (Whatman, Uppsala, Sweden) were subjected to extraction with 90% acetone at 4°C for 24 h. After extraction, the solid suspension was removed by centrifugation. Then, the absorbance of extracts at 750, 647, 644 and 630 nm was measured using a spectrophotometer (model HP8453, Hewlett Packard, MI, USA). The concentration of chlorophyll *a* was calculated using following equation.

Chlorophyll *a* (μg L^{-1}) = Ca × (V_a V_b^{-1})

Ca : Enumerated wavelength value

V_a : amount of 90% aceton (mL)

V_b : amount of sample filtered (L)

5. Assay of alga-lytic activity

The alga-lytic activity of the isolates was tested as fallows. Supernatants of the bacterial isolates cultivated at 30°C and 120 rpm for 24 h in Erlenmeyer flask including PY medium were separated from the cells used for the assays and were suspended in sterile lake water avoid any influence of chemicals from the MDM medium during the assay. The lake water used in this study was collected from Pal'tang reservoir and sterilized by membrane filtration (0.2-μm pore size, Whatman, Uppsala, Sweden). The concentration of host cyanobacterial cells, 1×10^7 cells mL^{-1}, was added in the sterilized lake water and the supernatant of the bacterial isolates was mixed with lake water containing host cyanobacterial cells at a ratio of 1 : 1 (v/v). Alga-lytic activities were evaluated by the change in chlorophyll *a* concentration or in cyanobacterial cells.

6. Alga-lytic activity of supernatant under heat and proteinase treatment conditions

Several treatments were carried out on the bacterial supernatant to investigate the change of alga-lytic activity. For heat treatment, bacterial supernatant was deposed into a test tube and incubated 90°C in a water bath for 30 min. A 1 mL of bacterial supernatant was incubated at 55°C in a water bath for 3 after mixing with 5 μL of proteinase-K solution (Sigma).

7. Effect of various pHs and temperatures

The *Anabaena*-lytic activity of bacterial supernatant was investigated under different pH conditions between 4.5~11.0 generated using NaOH and HCl. To determine the influence of NaOH or HCl additions on cyanobacterial cells, both distilled water and fresh PY medium the pH of which was adjusted by NaOH or HCl were used as a control. And the effect of temperature in the *Anabaena*-lytic activity of the bacterial supernatant toward *A. cylindrica* was investigated at various temperatures, 5, 10, 15, 20, 25 and 30°C.

Table 1. Reduction ratios of chlorophyll *a* of *A. cylindrica* NIES-19 upon incubation with the bacterial supernatants of four isolates cultivated at 30°C for 48 h in PY medium.

Bacterial isolate	[a]Reduction ratios of Chl-*a* (%)
AK-05	45
AK-07	23
AK-13	26
AK-26	10

[a]Reduction ration = (decreased amount of Chl-*a* / the initial amount of Chl-*a*) × 100

RESULTS AND DISCUSSION

1. Isolation and identification of alga-lytic bacteria

Fifteen isolates showing clear zones on the host *A. cylindrica* NIES-19 over-layer agar were productively isolated. Among the isolates, only four-isolates lyse *A. cylindrica* NIES-19 when co-culturing was carried out. The alga-lytic activities of the supernatants of four isolates were shown in Table 1. Cyanobacterial chlorophyll *a* was reduced from 10 to 45% by the supernatants of four isolates after 48 h of incubation. The isolate AK-05 exhibited the highest activity. This strain was identified based on 16S rDNA sequence and showed 99.5% homology with *Acidovorax temperans* CCUG 11779^T (Fig. 1). To illuminate its lytic mechanism, the alga-lytic test by bacterial supernatant in the present or absence of *A. temperans* AK-05 cells was carried out. The cells of *A. cylindrica* NIES-19 were highly aggregated by the cells of AK-05, but no significant difference in the alga-lytic activity based on the chlorophyll *a* measurement was observed during 48 h of incubation. It indicates that the alga-lytic activity of *A. temperans* AK-05 was attributed to extracellular products and direct contact with bacteria cells was not responsible for the lyses of cyanobacterial cells.

2. Alga-lytic activity

After examination of the alga-lytic activity against *A. cylindrica*, the chlorophyll *a* was decreased to 42% of the initial chlorophyll; however, 99.8% of the cyanobacterial cells were complete destroyed (Table 2). This differences in the residual amounts of chlorophyll *a* and cyanobacterial cells demonstrated that alga-lytic substance degraded the cell or cells wall but not chlorophyll *a*. In addition, relationship between bacterial growth phase and alga-lytic activity was investigated. As shown in Fig. 1, the alga-lytic activity of the supernatant was minimal, although the growth of *A. temperans* AK-05 reached the sta-

Table 2. Reduction ratio of chlorophyll *a* and cell number of *A. cylindrica* NIES-19 after treatment of the supernatant of *A. temperans* AK-05.

Treatment	[a]Chlorophyll *a* (mg L^{-1})	[b]Cell number (cells mL^{-1})	[c]Reduction ratio of chlorophyll *a* (%)	[c]Reduction ratio of cell number (%)
Supernatant	1.05	2.0×10^5	58	99.8
PY medium	2.43	3.1×10^7	2.8	3.9

[a]The initial cells: 1.0×10^8, [b]The initial chlorophyll *a*: 2.5 mg L^{-1}, [c]Reduction ratio = (decreased amount/the initial amount) × 100

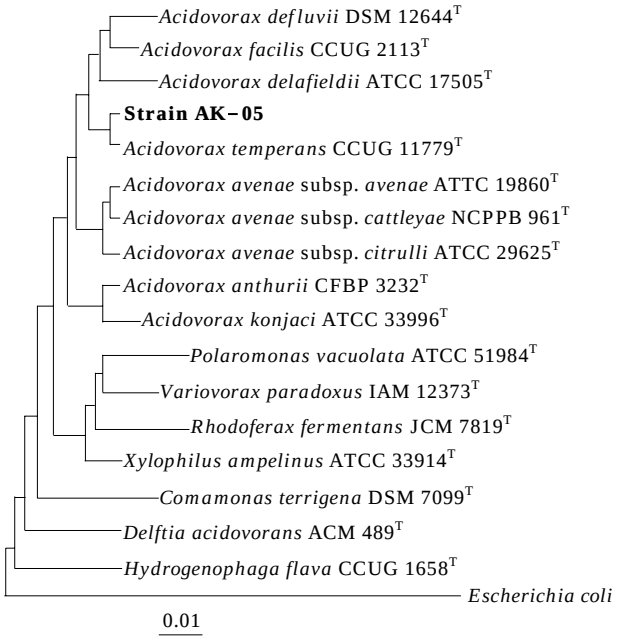

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the positions of strain AK-05, the type strains of *Acidovorax* species and the representatives of some other related taxa. Scale bar represents 0.01 substitutions per nucleotide position.

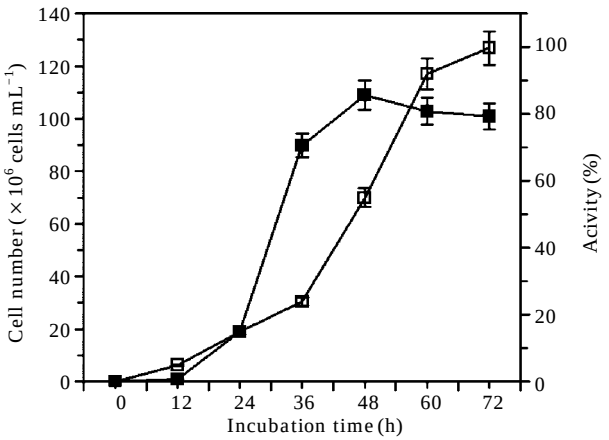

Fig. 2. Alga-lytic activity of the bacterial supernatant of *A. temperans* strain AK-05 related with bacterial growth phages.

tionary. In exponential growth phase, the alga-lytic activity gradually increased. The supernatant obtained from AK-05 cultivated for 72 h exhibited the highest activity. This explained that the alga-lytic substances were produced after stationary phase. The producing of antibiotics during the early stages is common in bacteria (Katz and Demain, 1977). These results suggested that alga-lytic substances produced from *A. temperans* AK-05 are possibly antibiotic analogues.

3. Alga-lytic activity under several conditions

The alga-lytic activity of the bacterial supernatant under several conditions was investigated. PY medium was used as a control to evaluate any influence of components in the culture medium. In Fig. 3, cyanobacterial cells was not decreased even after 96 h incubation with PY medium and it is suggesting that PY medium did not influence on cyanobacterial cells. On the other, the bacterial supernatant lyses 90% of the cyanobacteria cells over a period of 72 h. Therefore, it was obvious that some substance produced by *A. temperans* AK-05 in supernatant were responsible for lyses. To test the heat stability of the alga-lytic substance in the supernatant, alga-lytic activity was investigated after heat treatment at 90°C. As shown in Fig. 4, 75% of cells were degraded after 60 h of incubation. Although the alga-lytic activity was somewhat decreased by heat treatment, the activity of bacterial supernatant were still reminded in high. The result indicated that the major alga-lytic substances were heat stable. To determine whether the alga-lytic substances were proteins or not, proteinase-K was treated. 66% reduction of alga-lytic activity by bacterial supernatant pretreated with proteinase-K was observed. This result demonstrated that the major alga-lytic substances were not degraded by proteinase-K.

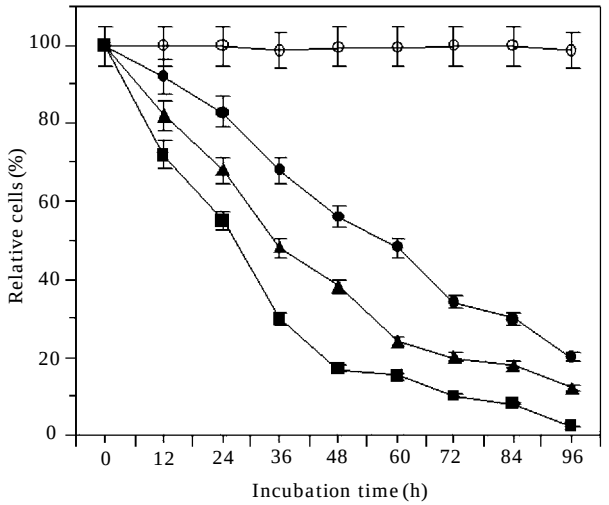

Fig. 3. Alga−lytic activity of the bacterial supernatant of *A. temperans* strain AK−05 against *A. cylindrica* NIES−19 under several conditions. Samples are (○), PY medium as a control; (■), bacterial supernatants without any treatment; (▲), bacterial supernatant pretreated with heat; (●), bacterial supernatant pretreated with proteinase−K.

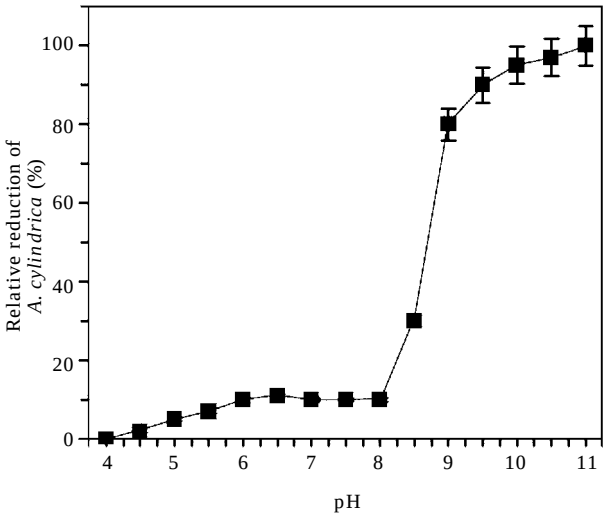

Fig. 4. Influence of pHs on alga−lytic activity of *A. temperans* strain AK−05 bacterial supernatant against *A. cylindrica* NIES−19.

Nevertheless, *A. temperans* AK−05 made clear zone on skim milk medium, it produced any protease. Thus, protease contributing alga−lytic activity is considered minor.

4. Effect of pH and temperature

Alga−lytic activities of the supernatant of bacterial culture investigated at different pH values were shown in Fig 4. *Anabaena*−cells were not ruptured in the acidic pHs, but, in the alkaline conditions, cyanobacterial cells were dramatically decreased. i.e., cyanobacterial cells were not degraded by the bacterial supernatant at pH 4, but 100% cell lyses were detected at alkaline condition, pH 11. Cyanobacterial blooms often alkalify the aquatic environments because some cyanobacteria including the genus *Microcystis* and *Anabaena* can use HCO_3 more efficiently than CO_2 (Matsuda *et al*., 1999). Accordingly, this alkaline pH−stable characteristic of alga−lytic substances produced by *A. temperans* AK−05 is advantage for application to control water bloom in aquatic environments.

Temperature is very important factor in the control of water blooms in eutrophic lakes (Tujimura *et al*., 2001). Therefore, the effect of temperature

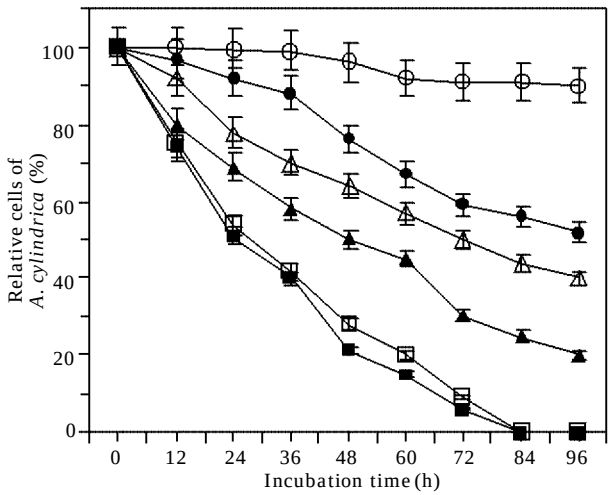

Fig. 5. Influence of temperature on alga−lytic activity of *A. temperans* strain AK−05 bacterial supernatant against *A. cylindrica* NIES−19. Symbols are bacterial supernatants treated at (○), 3°C; (●), 10°C; (△), 15°C; (▲), 20°C; (□), 25°C; (■) 30°C.

of alga−lytic activity to degrade *A. cylindrica* NIES−19 was examined at various temperatures (Fig. 5). At 25 and 30°C, the cells of *A. cylindrica* were almost lysed up to 100%. This is telling that the cells were ruined more competently as the temperature increases. At 4°C, the alga−lytic activity was not detected since the cyanobacterial cell membrane was considered to motionless at

low temperature (Goldman and Carpenter, 1994; Tujimura *et al*., 2001). In consequent, optimal culture conditions for growth of cyanobacteria such as high temperature and alkaline pH can make active transport of the alga-lytic substances to cyanobacterial cells and successive rapid lyses.

ABSTRACT

Isolation and identification of alga-lytic bacteria were carried out. Fifteen isolates of alga-lytic bacteria were screened by the double layer method using *A. cylindrica* NIES-19 as a sole nutrient and four isolates among them were compared with their alga-lytic activity. The isolate AK-05 exhibiting the highest alga-lytic activity was identified as *Acidovorax temperans* base on its 16S rDNA sequence. The culture supernatant of the isolate AK-05 was reliable for the alga-lytic. Alga-lytic activity assays of culture supernatant revealed that the major substances for alga-lytic activity were non-proteins and heat stable. The highest alga-lytic activity was practical under alkaline conditions and at 25~30°C. It is indicating an advantage for the application of water blooms by cyanobacteria in eutrophic lakes where the pH is generally in alkaline region.

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REFERENCES

Carmichael, W.W. 1992. A status report of planktonic cyanobacteria (blue-green algae and their toxins). #EPA/600/R-92/079. United States Environmental Protection Agency, Washington, DC, USA.

Castenholz, R.W. 1988. Culturing methods for cyanobacteria. *Methods Enzymol*. **167**: 8-92.

Felsenstein, J. 1993. PHYLIP: Phylogenetic Inference Package. Version 3.5. Seattle, University of Washington, Washington, USA.

Fukami, K., A. Yuzawa, T. Nishijima and Y. Hata. 1992. Isolation and properties of a bacterium inhibiting the growth of *Gymnodinium nagasakiense*. *Nippon Suisan Gakkaishi* **58**: 1073-1077.

Goldman, J.C. and E.J. Carpenter. 1974. A kinetic approach to effect of temperature on algal growth. *Limnol. Oceanogra*. **19**: 756-766.

Harada, K.-I. 1996. Chemistry and detection of microcystins. pp.103-148. *In* Toxic *Microcystis* (Watanabe, M.F, K.-I. Harada, W.W. Carmichael and H. Fujiki, eds). CRC Press

Jang, E.-H., J.-D. Kim and M.-S. Han. 2003. Isolation and characterization of alga-lytic bacterium HY0210-AK1 and its degradability of *Anabaena cylindrica*. *Korean J. Environ. Biol*. **21**: 194-201.

Katz, E. and A.L. Demain. 1977. The peptide antibiotics of *Bacillus*; chemistry biogenesis and possible functions. *Bacteriol. Rev*. **41**: 449-474.

Kikuchi, T., T. Miura, K. Harimaya, H. Yano, T. Arimoto and T. Masata. 1973. Odorous metabolites of blue-green alga *Schizothrix muelleria* Nägeli collected in the southern basin of Lake Biwa: Identification of geosmin. *Chem. Pharm. Bull*. **21**: 2342-2343.

Kim, J.-D. and M.-S. Han. 2004. Enzyme profiles of alga-lytic bacterial strain AK-13 associated with elimination of cyanobacterium *Anabaena cylindrica*. *Korean J. Environ. Biol*. **22**: 184-191.

Matsuda, Y., T.G. Williams and B. Colman. 1999. Quantification of the rate of CO_2 formation in the periplasmic space of microalgae during photosynthesis. A comparison of whole cell rate constant for CO_2 and HCO_3 uptake among three species of the green alga *Chlorella*. *Plant Devel. Environ*. **22**: 397-405.

Mitsutani, A., A. Uchida and Y. Ishida. 1987. Occurrence of blue-green algae and algal lytic bacteria in Lake of Biwa. *Bull. Jpn. Soc. Microbiol. Ecol*. **2**: 21-28.

Nigam, P., G. Armour, I.M. Banat, D. Singh and R. Marchant. 2000. Physical removal of textile dyes and solid-state fermentation of dye-adsorbed agricultural residues. *Bioresour. Technol*. **72**: 219-226.

Parson, R.T, Y. Matia and C.M. Lalli. 1984. A manual of chemical and biological methods for seawater analysis. 1st ed. Pergamon Press Ltd. Oxford.

Pearl, H.W. 1988. Growth and reproductive strategies of freshwater blue-green algae (cyanobacteria). *In*: Growth and Reproductive Strategies of Freshwater Phytoplankton pp 262-315. (Sandgreen, C.D., ed.). Cambridge University Press, Cambridge.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. **4**: 406-425.

Shilo, M. 1970. Lysis of blue-green algae by myxobacter. *J. Bacteriol*. **104**: 453-461.

Tsuchiya, Y., M.F. Watanabe and M. Watanabe. 1992. Volatile organic sulfur compounds associated with blue-green algae from inland waters of Japan. *Water Sci. Technol*. **25**: 123-130.

Tujimura, S., K. Ishikawa and H. Tsukada. 2001. Effect of temperature on growth of the cyanobacterium *Aphanizomenon flos–aquae* in Lake Biwa and Lake Yogo. *Phycol. Res.* **49**: 275–280.

Yamamoto, Y. 1981. Observation on the occurrence of microbial agents, which cause lysis of blue–green algae on Lake Kaumigaura. *Jpn. J. Limnol.* **42**: 20–27.

Yamamoto, Y., T. Huchiwa, Y. Hodoki, K. Hotta, H. Uchida and K.–I. Harada. 1998. Distribution and identification of actinomycetes lysing cyanobacteria in a eutrophic lake. *J. Appl. Phycol.* **10**: 391–397.

Yoon, J.–H., S.–T. Lee, S.–B. Kim, W.–Y. Kim, M. Goodfellow and Y.–H. Park. 1997. Restriction fragment length polymorphisms analysis of PCR–amplified 16S ribosomal DNA for rapid identification of *Saccharomonospora* strains. Int. *J. Syst. Bacteriol.* **47**: 111–114.

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