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Antialgal Effect of a Novel Polysaccharolytic *Sinorhizobium kostiense* AFK-13 on *Anabaena flos-aquae* Causing Water Bloom

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Abstract Isolation and identification of algal lytic bacteria were carried out. Nine strains of algal lytic bacteria were isolated by the double-layer method using *Anabaena flos-aquae* as a sole nutrient. The isolate, AFK-13, showing the highest algal lytic activity was identified as *Sinorhizobium kostiense* based on the 16S rDNA sequence. The algal lytic experiments of the culture supernatants of AFK-13 demonstrated that the bacterial cell growth reached a maximum at 36-h culture, but the supernatant of 72-h culture exhibited the highest activity. Components among the extracellular products in the crude enzyme of the supernatant from *S. kostiense* AFK-13 culture were responsible for degradation of cell walls of *Anabaena flos-aquae*. Algal lytic assay tests of the culture supernatants suggest that the main substances for algal lytic activity could be proteinaceous. The activity of glucosidase was observed highly by polysaccharolytic analysis using the crude enzyme from *S. kostiense* AFK-13, whereas activities of galactosidase, mannosidase, rhamnosidase, and arabinosidase were also detected in low levels. The molecular weights (MW) of α- and β-glucosidases were estimated to be approximately 50–100 kDa by the ultrafiltration method.

Key words: Algal lytic bacteria, *Sinorhizobium kostiense*, extracellular products, polysaccharolytic enzyme, *Anabaena flos-aquae*

Various are ecologically important microorganisms in view of their nitrogen-fixing ability [25]. They can, however, cause severe problems such as water blooms that are widespread in many freshwater lochs, lakes, and reservoirs around the world, especially during the summer owing to mass development [7]. Water blooms by cyanobacteria belonging to genera *Microcystis* and *Anabaena* are widely distributed in freshwater ecosystems and cause many problems for scenery, anxiety about toxicity [22], and unpleasant odors [45]. With the purpose to manage water blooms, several approaches have been tried using antialgal compounds such as copper sulfide [32] and simazine, which block photosynthesis [38], but this is expensive and potentially damaging on the environment. An alternative approach is to reduce the nutrient required for the algal growth, although it is not easy to control the amount of the nutrient reaching lakes and reservoirs. Therefore, none of these methods have been successful so far [5]. The other approaches using antagonistic microorganisms, such as bacteria, actinomycetes, fungi, phages, and amoebae, have been tried [33, 36, 47]. Among these, antagonistic bacteria are useful for potential bioagents to regulate algal blooms, and bacterial pathogens that inhibit growth of algae have been isolated [10, 24]. Only a few reports, however, have elucidated the nature of the antagonistic mechanisms [5, 6] because of its obvious interactions being are more complex in microbial communities [21, 26]. Moreover, there are only a few reports about the identification of antialgal compounds [27, 49]. In the present study, to obtain microorganisms that have the potential to be useful agents for algal blooms control, we isolated and identified a bacterial strain with antialgal activity against *Anabaena flos-aquae* from surface water of eutrophic lakes in order to search, purify, and determine for enzymes showing distinct algal lytic activity against the cyanobacterium.

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MATERIALS AND METHODS

Algal Culture

Anabaena flos-aquae NIES-75 used as the host for algalytic bacteria was kindly supplied by the National Institute for Environmental Studies, Japan. A colonial axenic culture was cultivated and maintained in BG-11 medium including $NaNO_3$ 1.5 g, K_2HPO_4 0.04 g, $MgSO_4 \cdot 7H_2O$ 0.075 g, $CaCl_2 \cdot 2H_2O$ 0.036 g, EDTA-disodium 0.001 g, Na_2CO_3 0.02 g, citric acid 0.006 g, ferric ammonium citrate 0.006 g,

and micronutrient 1 ml, per liter [39], under continuous illumination of cool white fluorescent lamps giving an incident light intensity of 35 μE/m^2/s and at 25±0.2°C with agitation of 150 rpm in a rotary shake incubator. The composition of the micronutrients was H_3BO_3 2.86 g, $MnCl_2 \cdot 4H_2O$ 1.81 g, $ZnSO_4 \cdot 7H_2O$ 0.222 g, $Na_2MoO_4 \cdot 2H_2O$ 0.39 g, $CuSO_4 \cdot 5H_2O$ 0.079 g, $Co(NO_3)_2 \cdot 6H_2O$ 0.0494 g, per liter [8]. The biomass was determined by measurement of chlorophyll-*a* by *in vivo* fluorometry (Turner Designs Model 10-AU, Sunnyvale, CA, U.S.A.).

Screening of Antialgal Bacteria

Algal lytic bacteria were isolated by the double-layer algal-lawn method [42] from sediment and surface water samples collected from Paldang, Seokchon, and Daecheong Reservoirs, where cyanobacteria blooms had often occurred in Korea. Cultures of *A. flos-aquae* were grown in BG-11 medium for 7 days and harvested by centrifugation at 3,000 ×*g* for 20 min. The culture pellet was mixed with molten BG-11 soft agar medium and poured onto a BG-11 agar plate (87 mm in diameter). After the cyanobacteria lawn was cultivated for 2–3 days under the *A. flos-aquae* culture conditions mentioned above, 200 μl of samples wat spread on the cyanobacteria lawn. After 5–10 days of incubation, the antialgal bacteria forming clear zones of inhibition on the lawn were isolated by serial streaking onto the nutrient agar (NA) plates to purify a monocolony. The isolates were axenically maintained in the dark on NA plates and kept at −76°C in NB medium containing 20% glycerol.

Measurement of Algal Growth

Growth characteristics were studied in terms of cell counts as determined with a hemocytometer, and the chlorophyll-*a* (chl-*a*) content according to the method of the APHA [1]. Chlorophyll-*a* of cyanobacteria was extracted by a solution of 90% methanol and measured according to the method of Parsons and Strickland [37]. Cyanobacteria were collected by filtration with GF/C (Whatmann, Uppsala, Sweden) and treated with 90% methanol at 60°C for 10 min to extract the chlorophyll-*a*. After extraction, the pellet was removed by centrifugation. Then, the absorbance of extracts at 664, 645, and 630 nm was measured using a spectrophotometer (Model HP8453B, Hewlett Packard, Waldron, Germany). Chlorophyll-*a* concentration was calculated using the formula

$$\text{Chlorophyll-}a\ (\text{mg/l}) = 11.64A_{664} - 2.16A_{645} - 0.10A_{630},$$

where A_{664}, A_{664}, and A_{630} are the optical densities at the respective wavelengths [1].

Extraction of EPS

A. flos-aquae EPS were extracted according to the method of Colombo *et al.* [15]. Cyanobacterial cells from a batch culture of 10 l were removed from the culture medium at the end of the exponential growth phase (15 days old) by tangential filtration in hollow fiber cartridges with 0.65 μm pore size membranes (A/G Technology Corporation, Needham, MA, U.S.A.). The medium, which contained the released EPS, was further concentrated (ca. 1,000 ml) by tangential filtration in hollow fiber cartridges with a 30-kDa pore size and washed (diafiltration) three times with three volumes of deionized water to eliminate low molecular weight compounds. The concentrated medium thus obtained was then freeze-dried to obtain the EPS and kept at −12°C until use.

Monosaccharide Composition of EPS

The monosaccharide composition of *A. flos-aquae* EPS was characterized by gas chromatography (GC) analysis of *O*-trimethylsilyl derivatives of methyl glycosides as described in Colombo *et al.* [15], and the protein content was determined according to the method of Bradford [4].

Algal Lytic Activity Test of Bacterial Strains on *A. flos-aquae*

To test the algal lytic activity of all isolates and/or select the most effective bacterium on *A. flos-aquae*, a liquid-culture test was carried out. The antialgal bacteria grown at the above condition were inoculated into test tubes (50 ml in capacity), which included 25 ml of *A. flos-aquae*, and cultured for 5 days. To measure the algal lytic activity of isolates, the concentration of chlorophyll-*a* was measured by *in vivo* fluorometry (Tuner Designs, Sunnyvale, CA, U.S.A.). Among 68 isolates tested, AFK-13 showed the best algal lytic activity on *A. flos-aquae* in this test. All tests had three replications.

Identification of Bacterial Strain AFK-13

The chromosomal DNA was isolated using a method described elsewhere [48]. The amplification of the 16S rDNA was conducted using two primers according to Stackebrandt and Liesack [44], 5'-GAGTTTGATCCTGG-CTCAG-3' and 5'-AGAAAGGAGGTGATCCAGCC AG-AAAGGAGGTGATCCAGCC-3'. A PCR was run for 35 cycles in a DNA thermal cycler, Genetic analyzer 377 (Perkin-Elmer, Boston, U.S.A.), employing the thermal profile according to Yoon *et al.* [48]. The 16S rDNA sequence of bacterial isolate AK-13 was aligned using CLUSTAL W software [35]. The evolutionary distance matrices were calculated with the DNADIST program within the PHYLIP package [19]. The sequence of representative species of the genus *Sinorhizobium* and related taxa were cited using the GenBank database. The values of 16S rDNA similarity were calculated from the alignment, and the evolutionary distances were calculated using a Kimura two-parameter correction. A phylogenetic tree was constructed using the neighbor-joining method [41] based on the calculated distance matrix.

Perception of Supernatants and Algal Lytic Activity of Enzymes

The supernatants from the culture of bacterial strain AFK-13 were separated from the cell biomass to be used as a cell-free extract. We also prepared the supernatant from the bacterial culture cocultivated in BG-11 medium with the cyanobacterium *A. flos-aquae*. To obtain the mixed-culture filtrate, the bacterial culture was centrifuged at 15,000 ×*g* for 20 min at 4°C and the supernatant was sterilized by syringe-filtering through a 0.2-μm pore membrane. The cell-free extracts were not obtained from the mixed-culture filtrate because of the coculture of the cyanobacterium.

The protein content of bacteria supernatants was quantified according to the Bradford method [4] using bovine serum albumin (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) as the protein standard. *A. flos-aquae* cells were treated with the bacterial supernatants (20% v/v) in 6-well plates (Falcon, U.S.A.), and the cells were counted with a hemocytometer after 24 h. A unit of enzyme was defined as the amount that reduced 10^4 algal cells after 24 h.

Treatments of Supernatants Under Specific Conditions

The following treatments were carried out on the bacterial supernatants prior to use in the antialgal activity test. (i) For heat treatment, the supernatant was placed into a test tube with a silicon stopper and autoclaved at 121°C for 20 min. To determine whether the algal lytic substances were protein-like, (ii) the bacterial cultures were centrifuged at 15,000 ×*g* for 20 min and the supernatants were filtrated using YM-10 membrane filter (exclusion size, 1 kDa MW cutoff; Millipore Corp, Billerica, MA, U.S.A.). (iii) Proteinase-K was added to the supernatants filtrated at (ii). One ml of the supernatants was incubated at 55°C in a water bath for 3 h after mixing with 5 μl of proteinase-K solution (Sigma). Then, the supernatants treated with proteinase-K were added to the algal cultures in the exponential growth phase to investigate the algal lytic activity.

Preparation of Cell Wall Powder from *Anabaena flos-aquae* (*Anabaena* Powder)

Anabaena flos-aquae was cultured at 25°C for 5 days using BG-11 broth as the seed culture. An aliquot of 10 ml of this culture was then inoculated into 10 l BG-11 medium in 20 pieces of 2-l Erlenmeyer flask and cultivated for 15 days under continuous illumination of cool white fluorescent lamps giving an incident light intensity of 35 μE/m^2/s and at 25± 0.2°C with agitation of 150 rpm in a rotary shake incubator.

The cell-wall powder from *A. flos-aquae* was prepared according to the modified method of Chet *et al.* [9]. The cultures of *A. flos-aquae* were centrifuged at 15,000 ×*g* for 20 min, and the precipitated cells were then washed with 100 ml of sterilized distilled water. The cells were homogenized with a blender at 10,000 rpm fro 5 min and sonicated at 30 W for 10 min (250/450 SONIFIER, Branson Co., Danbury, CT, U.S.A.). The sonicated sample was centrifuged at 8,000 ×*g* for 10 min to separate the coarse particles, and the supernatant with fine particles was further centrifuged at 15,000 ×*g* for 20 min. The precipitate containing the fine particles was subsequently washed repeatedly with 100 ml of methanol and sterilized distilled water until no proteins were detected in the waste solution by protein assay (BCA Protein Assay Kit, Pierce, Rockford, IL, U.S.A.). Finally, the freeze-dried cell walls were used as *Anabaena* powder.

Preparation of the Crude Enzymes of AFK-13

The *Anabaena flos-aquae*-degrading bacteria AFK-13 was inoculated in 200 ml of nutrient broth containing *Anabaena* power and cultivated at 25°C for 3 days with agitation at 150 rpm. The culture was centrifuged at 15,000 ×*g* for 20 min at 4°C. Solid ammonium sulfate was added to the supernatant to give 75% saturation and the mixture allowed to settle overnight at 4°C. Centrifugation at 15,000 ×*g* for 20 min at 4°C was carried out to collect the precipitates, which were dissolved in pure water. Then, the solutions were dialyzed and used as bacterial crude enzymes. Inactivated enzyme was prepared by boiling for 10 min at 100°C and used as controls.

Enzyme Assays

To determine the activities of glycosyl hydrolase, substrates such as β-arabinopyranoside (arabinose), β-mannopyranoside (mannose), β-galactoside (galactose), α-rhamnopyranoside (rhamnose), α-glucoside (glucose), and β-glucoside (glucose) based on the monosaccharide composition of *Anabaena*-EPS [15] were purchased from Sigma-Aldrich Co., U.S.A. Most of the enzyme activities of the samples were determined by 4-methylumbelliferone fluorescence (MUF) according to the method of Chróst and Krambeck [11]. Substrate saturation curves were obtained to establish the best MUF-substrate concentration. Autoclaved medium was used as a blank. The samples obtained from the experimental vessels were incubated for 30 min at 20°C, after which the reactions were stopped by the addition of 100 μl of 5 M NaOH, a procedure also employed to produce the maximum MUF fluorescence. The fluorescence was measured in a spectrofluorometer (Buckinghamshire, U.K.) at 450 nm. A MUF-calibration curve was prepared so that the measurements of relative fluorescence could be transformed into nmol/l/min.

Indirect Molecular Weights Analysis of the Polysaccharolytic Enzymes of AFK-13

To estimate the molecular weights of the glycosyl hydrolases present in the algal lytic bacterial crude enzymes, the polysaccharolytic activities of the crude enzymes were determined against each substrate after ultra filtration with 5 kDa, 30 kDa, 50 kDa, and 100 kDa molecular cutof membranes (Ultrafilter-MC, Milipore, Bedford, MA, U.S.A.).

Moreover, the remaining activities on the ultrafilters were also examined after washing with pure water.

RESULTS AND DISCUSSION

Isolation and Identification of Algal Lytic Bacterium AFK-13

Several attempts to screen useful indigenous algal lytic bacteria were carried out. To screen useful indigenous algal lytic bacteria, bacterial isolation was carried out by using double-layered plates that included the cyanobacteria *A. flos-aquae* NIES-75 as a sole nutrient. Nine strains exhibiting clear zones on the host cyanobacterium on overlay agar were successfully isolated. Among the strains, five bacteria could lyse *A. flos-aquae* when coculture was performed. The antialgal bacterium, AFK-13, isolated from a mud soil sample taken in Paldang Reservoir, showed the highest activities from liquid-culture tests. The optimal growing conditions for this bacterium were pH 7 and 30°C and it showed typical characteristics of Rhizobiaceae (data not shown). This identification was confirmed by comparing the 16S rDNA sequence of AFK-13 with the sequences of various type strains of *Sinorhizobium*. The 6S rDNA sequences of AFK-13 were most similar to that of *Sinorhizobium kostiense* HAMBI 1489^T (99.9%), prompting us to identify our isolate as *S. kostiense* AFK-13 (Fig. 1). In the phylogenetic tree, AFK-13 was the closest to *Sinorhizobium kostiense* HAMBI 1489^T and part of a robust monophyletic cluster with *S. terangae* LMG 7834^T, *S. saheli* LMG 7837^T, *S. xinjiangense* IAM 14142^T, and *S. fredii* LMG 6217^T. The level of sequence similarity of AFK-13 in the monophyletic cluster was greater than 98% (Fig. 1). This is the first time that *S. kostiense* has been reported as an algal lytic bacterium against *A. flos-aquae*.

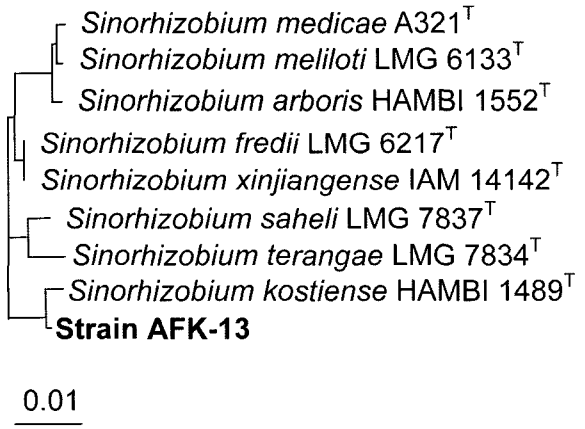

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the positions of the isolate AFK-13, the type strains of *Sinorhizobium* species, and the representatives of some other related taxa.
The scale bar represents 0.01 substitutions per nucleotide position.

Table 1. Reduction ratios of chlorophyll-*a* of *A. flos-aquae* by inoculation of five different algal lytic bacterial strains isolated from Paldang Reservoir.

Bacterial strain	Reduction ratio of chlorophyll-*a* (%)[a]
AFK-02	12.1±1.2
AFK-07	33.6±2.0
AFK-11	23.8±0.9
AFK-13	49.4±0.4
AFK-21	30.4±3.1

[a]Reduction ratio of chlorophyll-*a* (%)=$(1-Tt/Ct)\times100$, where T (treatment) and C (control) are the chlorophyll-*a* concentration of *A. flos-aquae* cell densities with and without algal lytic bacteria, respectively, and t is the inoculation time (t=3 days). Data are the mean±S.D. from at least three independent assays.

Algal lytic activities of bacteria culture supernatants were also tested. The results are shown in Table 1. The cyanobacteria chlorophyll-*a* was reduced from 10% to

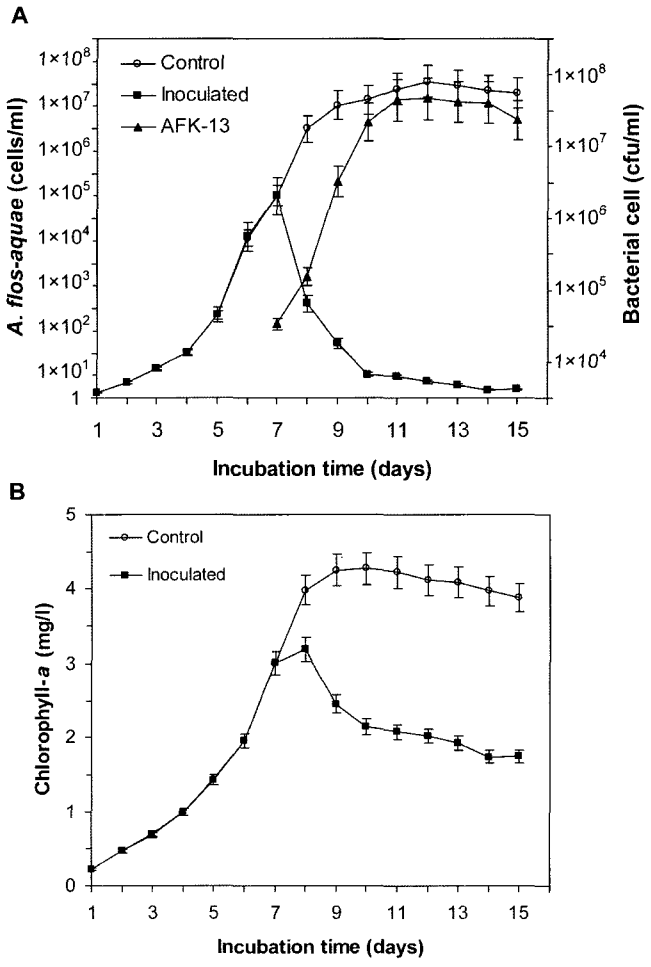

Fig. 2. Reduction ratio of cell concentration and the algal lytic bacterial cell growth (**A**) and chlorophyll-*a* (**B**) of *A. flos-aquae* upon inoculation with the algal lytic bacterium AFK-13.
Symbols are (○), control; (■), inoculated with AFK-13; and (▲), the cell growth of AFK-13.

50% in 3 days by the supernatants of the five algal lytic bacteria. The highest activity was observed in the supernatant of strain AFK-13. To clarify the algal lytic mechanism, the algal lytic activity test of *S. kostiense* AFK-13 was carried out. A high concentration of cyanobacteria cells were aggregated with *S. kostiense* AFK-13 cells, so that the cyanobacterial cells deceased drastically. No significant difference of the chlorophyll-*a* content was found over a period of 3 days. Namely, the cyanobacterial cells were completely lysed with only 5.03% remaining (Fig 2A); however, 50% of the chlorophyll-*a* still remained (Fig. 2B) after the algal lytic reaction with *A. flos-aquae* for 3 days. This suggested that *S. kostiense* AFK-13 produced extracellular substances to strongly associate with algal lytic activity, and direct contact with bacterial cells was not participated in the lysis of cyanobacteria. On the other hand, our microscopic observations (Fig. 3) revealed that the bacterium *S. kostiense* relies on cell-to-cell contact to induce cyanobacteria aggregation, and releases extracellular products for subsequently lysing most of the *A. flos-aquae* cells within 12 h. The algicidal mechanisms of bacteria generally proceed in one of three ways [16, 43]: (i) direct attack [23], (ii) release of extracellular compounds [30], or (iii) bacterial entrapment, which may involve lysis through release of lysozyme-like enzymes on the bacterial surface [5]. Here, we found that cells of *A. flos-aquae* were rapidly lysed by releasing the extracellular compound of bacterium AFK-13. Therefore, the difference of the reduction ratios of chlorophyll-*a* and cyanobacterial cells by treatment of AFK-13 indicated that the cyanobacterial cell or cell wall was markedly ruptured but chlorophyll-*a* did not degrade to the same level of cyanobacterial cells lysis by the extracellular substances released from the algal lytic bacterium. The result suggests that the algal lytic activity of bacterial supernatants could not be evaluated by the chlorophyll-*a* reduction ratio using the above equation [1]. Accordingly, in the subsequent experiment, the cyanobacterial cell count after algal lytic test was used to evaluate the algal lytic activity.

Algal Lytic Activity of Bacterial Supernatants

To investigate the algal lytic activity of bacterial supernatants, the supernatants collected at different growth phages against exponential phase *A. flos-aquae* were assayed. Aliquots of AFK-13 were incubated in 100-ml flasks containing 50 ml of NB, harvested every 6 h by centrifugation at 18,000 $\times g$ for 20 min, and the supernatants then used for algal lytic test. As shown in Fig. 4, although the bacterial cell growth reached a maximum at 36-h culture, the algal lytic activity of the supernatant showed low efficiency of the algal lytic activity and gradually increased following the exponential growth phase. The supernatant of 72-h culture exhibited the highest activity. This supposes that the extracellular products were released little by little at the lag phase and reached mainly after the stationary phase [29, 46].

The algal lytic activity of the bacterial supernatants put under multi conditions is shown in Fig. 5. Fresh NB medium was used as a control for each test to verify any influence of component in the culture medium on algal lytic activity. Any significant decrease of cyanobacteria cell number in the control during the growth period (Fig. 5A) indicated that fresh NB medium had no influence on the cells of *A. flos-aquae*, whereas the bacterial supernatants

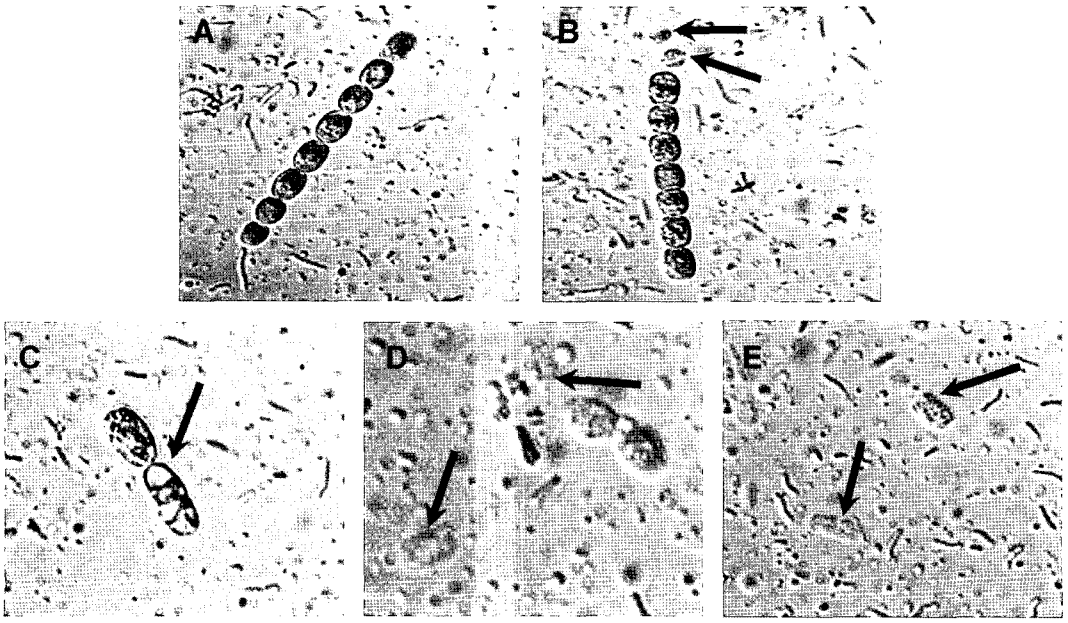

Fig. 3. Morphological destruction of *Anabaena flos-aquae* after inoculation of AFK-13 cells (approximately 1×10^4 cfu/ml).
A. Control (0 min); **B.** After 30 min; **C.** After 60 min; **D.** After 90 min; **E.** After 120 min.

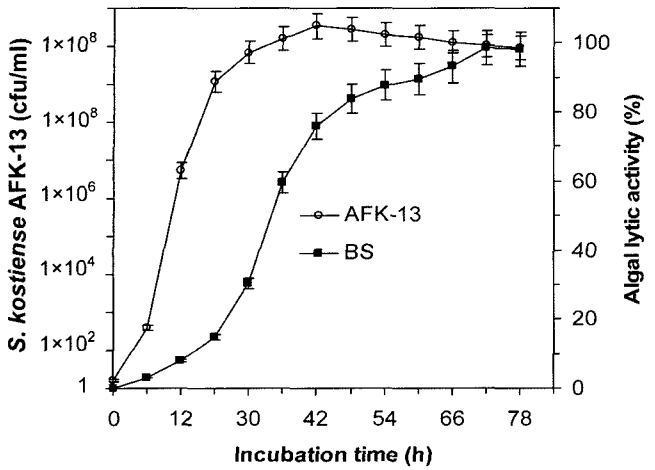

Fig. 4. Relationship between growth phase and algal lytic activity of the bacterial supernatants of *S. kostiense* AFK-13. Algal lytic activity was defined as the ratio of lysed cells in 24 h to the initial cell number (approximately 1.0×10^8 cells/ml).
Symbols are the cell growth (cfu/ml) of AFK-13 (○) and the algal lytic activity of the bacterial supernatants (■).

rapidly reduced over 90% of the cyanobacterial cells in 60 h of incubation (Fig. 5A). Thus, the extracellular substances released from the bacterium AFK-13 in the bacterial supernatants had a close relation to degrading the cells of *A. flos-aquae*. The heat stability of the extracellular substances in the bacterial supernatants was examined after autoclaving. Fig. 5B revealed that only 10% of the algal cells were mitigated for 84 h after incubation. Therefore, the algal lytic activity of the bacterial supernatants was clearly deactivated after heat treatment, although the algal lytic ability still remained. Thus, the extracellular substances in the bacterial supernatants were heat unstable and may be protein-like substances.

To clarify whether the extracellular substances associated with the algal lytic activity were protein-like or not, treatment with proteinase-K was carried out. As shown in Fig. 5C, only 20% of the algal lytic activity still remained compared with the bacterial supernatants only. This result implied that the major algal lytic substances were denatured by proteinase-K, indicating that the extracellular substances in the bacterial supernatants could be proteins. The algal lytic effect of the bacterial supernatants after proteinase-K treatment was similar to that of the heat treatment. Therefore, the contribution of proteins to algal lytic activity was considered to be major.

To estimate the molecular weight of algal lytic substances, the bacterial supernatants of AFK-13 were dialyzed using

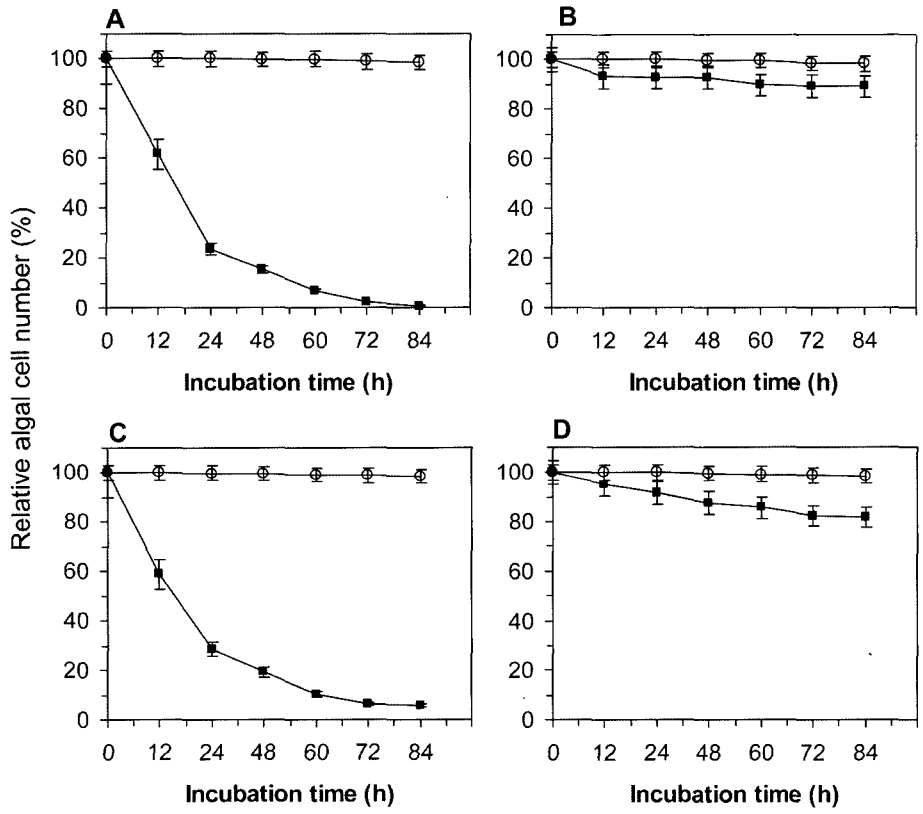

Fig. 5. Algal lytic effect on *A. flos-aquae* of the bacterial supernatants obtained from *S. kostiense* AFK-13 treated under several conditions.
A. The bacterial supernatants without any treatment; **B**. The bacterial supernatants treated with heat (121°C, 20 min); **C**. The bacterial supernatants filtrated using YM-10 membrane filter; **D**. The bacterial supernatants treated with proteinase-K.

a membrane filter having a molecular weight cutoff of 1 kDa. The algal lytic substances of the dialyzed bacterial supernatants was tested on *A. flos-aquae*. The result is shown in Fig. 5D. The dialyzed bacterial supernatants quickly degraded algal cells as soon as it was inoculated in the algal cell cultures. Since the dialyzed bacterial supernatants degraded 85% of the *A. flos-aquae* cells in 48 h after inoculation, the dialyzed bacterial supernatant showed closely the same algal lytic efficiency as the bacterial supernatant without any treatment.

Table 2. Algal lytic activity in the crude enzyme preparations of AFK-13 against *A. flos-aquae*.

Strain	Culture supernatant		Crude enzyme		Yield (%)
	Volume (ml)	Total activity (units)[a]	Volume (ml)	Total activity (units)	
AFK-13	200	413	1	358	86.7

[a]One unit of algal lytic activity was defined as the activity decreased by 0.01 of optical density at 660 nm per min.

Recovery of Algal Lytic Activity in the Crude Enzyme Preparation of AFK-13

The total activity in each 20-ml bacterial culture supernatant of AFK-13 and the activity of the crude enzyme are shown in Table 2. The yield of the algal lytic activity of AFK-13 was 86.7%, which considered that most of the enzymes associated with algal lytic effects on *A. flos-aquae* were recovered still after the salting-out procedure with ammonium sulfate.

Polysaccharolytic Activities of the Crude Enzyme

The monomeric composition of the *A. flos-aquae* EPS analyzed by GC was 33.2% of glucose, 21.6% of mannose, 19.9% of rhamnose, 6.8% of xylose, 5.6% of glucuronic acid, 5.2% of fucose, 2.8% of galactose, 1.4% of galacturonic acid, and 1.1% of arabinose. The EPS also contained 11.9% of protein. The composition of *A. flos-aquae* EPS was very similar to that of *A. spiroides* EPS based on these data [13, 15].

Polysaccharolytic activities of the crude enzymes were investigated using β-arabinopyranoside (arabinose), β-mannopyranoside (mannose), β-galactoside (galactose), α-rhamnopyranoside (rhamnose), α-glucoside (glucose), and β-glucoside (glucose) as substrates, assuming that these polysaccharides might constitute the cell walls of *Anabaena* [15]. The results are summarized in Table 3. The crude enzyme ruined the monosaccharides linkages mentioned above. This may be because the glycosides are very stable compounds and the chemical hydrolysis of substrates is improbable except under highly acidic conditions. The crude enzyme exhibited high polysaccharolytic activity against α-glucoside, β-glucoside, and β-galactoside, but it degraded a little of β-arabinopyranoside, β-mannopyranoside, and α-rhamnopyranoside. These results suggest that glucosidases, or β-galactosidase, or both might act as key enzymes in the degradation of *Anabaena* cell walls by the action of the crude enzyme present in the bacterial strain AFK-13. Furthermore, the high enzyme activity was exclusively due to glucosidase (refer Table 4), which might be justified by the marked concentration of both glucose in the cell walls of *A. flos-aquae* and may also be attributed to the polymer structure exposing the other monosaccharides to the subsequent hydrolases. Glucosidases (α and β) are the most studied among all glycosidases that are produced in aquatic environments, water and sediments, by bacteria and fungi and have been reported in several types of aquatic environments [12, 13, 34]. Most of such enzymes as galactosidase, mannosidase, rhamnosidase, and arabinosidase are also produced in pure cultures of fungi or bacteria [18, 20, 28, 31] and in aquatic environments [11, 34].

Molecular Weight of Polysaccharolytic Enzymes

The putative molecular weights of the glycosyl hydrolases were determined indirectly using ultrafilters (Table 4). The molecular weights of glucosidases from AFK-13 were estimated as approximately 50–100 kDa. Glucosidase splits α- or β-linked polysaccharides and the end product of glucosidase activity (α- or β-D-glucose) is energetically more important for the algal lytic bacterial growth [40]. Furthermore, β-glucosidase catalyzes the hydrolysis of compounds containing β-glucosidic linkage, and acts by splitting off the terminal β-D-glucose residue and releasing β-D-glucose as the reaction product [2, 3, 14]. Because of this activity, β-glucosidase plays an important role in the final step of cell wall degradation of *A. flos-aquae*. Thus, it

Table 3. Polysaccharolytic activity of algal lytic substances in the crude enzyme produced from AFK-13.

	Polysaccharolytic activity (unit/mg)[a]					
	Substrates					
Crude enzyme	β-Arabinopyranoside (Arabinosidase)	β-Mannopyranoside (Mannosidase)	β-Galactoside (Galactosidase)	α-Rhamnopyranoside (Rhamnosidase)	α-Glucoside (Glucosidase)	β-Glucoside (Glucosidase)
AFK-13	1.09±0.2	2.12±0.2	4.22±0.4	1.78±0.2	6.34±0.4	12.9±0.2

[a]Enzymes and substrate blanks were also included. A unit of enzyme activity is defined as the amount of enzyme catalyzing the release of one μmol of correspondent substrate per 1 mg of protein. Data are the mean±SD of triplicate experiments.

Table 4. Polysaccharolytic activity in the crude enzymes produced from AFK-13 and cutoff by ultrafiltration techniques.

	AFK-13	
Samples	β-Glucoside	α-Glucoside
Crude enzyme[a]	14.30±0.2	6.43±0.3
<5 kDa	0	0
>5 kDa	13.21±0.2	5.58±0.5
<30 kDa	0	0
>30 kDa	13.50±0.8	5.44±0.3
<50 kDa	0	0
>50 kDa	13.32±0.1	5.93±0.3
<100 kDa	13.52±1.1	6.16±0.2
>100 kDa	0	0

[a]The ultrafilters were used to cut off the components of crude enzymes with the molecular weights of 5 kDa, 30 kDa, 50 kDa, and 100 kDa. Data are the mean±SD of triplicate experiments.

might be possible that the hydrolyzing activity against β-glucosidic linkage of β-D-glucose is necessary for the efficient degradation of *A. flos-aquae* cell wall as well as α- glucosidic linkage.

To confirm our hypothesis of the effect of β-glucosidic linkage hydrolyzing activity against glucose on the degradation of *Anabaena* cell walls, further biochemical and molecular biological studies will be required to determine the kinetics, substrate specificity, and construction of the catalytic domain by using purified or recombinant glycosyl hydrolases [17]. Furthermore, this work enhances our understanding of the relationship between algal cell wall structures and algicidal substances during degradation of the cyanobacterium *A. flos-aquae*, and may provide a new strategy for controlling harmful blooms of this common freshwater cyanobacterium.

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