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Enzyme Profiles of Alga-Lytic Bacterial Strain AK-13 Related with Elimination of Cyanobacterium *Anabaena cylindrica*

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Abstracts - To investigate bacteria with alga-lytic activities against *Anabaena cylindrica* when water blooming occurs and to study enzyme profiles associated with alga-lytic activity, various bacterial strains were isolated from surface waters and sediments in eutrophic lakes or reservoirs in Korea. Among 178 isolates, only nine isolates exhibited lytic abilities against *A. cylindrica* on the agar plates, and then the isolate AK-13 was selected as the strongest in lysing the cyanobacterium *A. cylindrica*. The strain AK-13 was characterized and identified as *Sinorhizobium* sp. based on fatty acid methyl ether profiles and 16S rDNA sequence. According to the results of the enzyme assays, in the strain AK-13 of *Sinorhizobium* sp., alginase, amylase, proteinase (caseinase and gelatinase), carboxymethyl-cellulase (CMCase), laminarinase, and lipase was produced, namely CMCase, laminarinase and protease were highly active. None of glycosidase was produced. Therefore, enzyme systems of *Sinorhizobium* sp. AK-13 were very complex to degrade cell walls of *A. cylindrica*. The peptidoglycans of *A. cylindrica* may be hydrolyzed and metabolized to a range of easily utilizable monosaccharides or other low molecular weight organic substances by *Sinorhizobium* sp. AK-13.

Key words : alga-lytic bacteria, *Sinorhizobium* sp., hydrolytic-enzymes, *Anabaena cylindrica*

INTRODUCTION

Many cyanobacteria are ecologically important microorganism in view of their nitrogen-fixing ability (Fay 1992; Khan *et al.* 1994). They can, however, bring out severe trouble such as water blooms in many freshwater lochs and reservoir around the world due to mass development (Carmichael 1994; Reyssac and Pletikosic 1990). Water blooms by cyanobacteria, particularly in genera *Microcystis* and *Anabaena*, are widely distributed in nature freshwater ecosystems during summer, and have often resulted in a deterioration of water quality with adverse effects on lake ecology, livestock, human water supply, and recreational amenity. They produce the toxic substances (Carmichael *et al.* 1992; Harada 1996) and offensive odors (Slater and Blok 1983; Tsuchiya *et al.* 1992). Several approaches have been tried to control cyanobacterial blooms from lakes and reservoirs. The frequently used algicidal agents were copper sulfide (McGuire *et al.* 1984) and simazine, which block photosynthesis (Reyssac and Pletikosic 1990), but this is expensive and potentially damaging on the environment. An alternative approach is to reduce the nutrient required the algal growth, although it is not easy to control the amount of nutrient reaching lakes and reservoirs. None of these methods have been successful so far (Burnham *et al.* 1976, 1981). Therefore, biological con-

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trol was on the rise for a more suitable method to regulate cyanobacterial blooms and bacterial pathogens that inhibit growth of cyanobacteria have been isolated (Reyssac and Pletikosic 1990; Yamamoto and Suzuki 1990; Yamamoto *et al.* 1998). Only a few cases, however, has been elucidated the nature of the antagonistic mechanisms (Burnham *et al.* 1976, 1981). Moreover, the role of physiological adaptation and selection of alga-lytic bacterial species with respect for algal degradation are not easily investigated in natural systems due to its obvious interactions that are more complex exist in nature microbial communities (Gonzalez *et al.* 1996). With the aim of obtaining other microorganisms, which can be used for controlling cyanobacterial blooms, the authors isolated and identified bacterial strains with alga-lytic activities against *Anabaena cylindrica* and investigated enzyme profiles of alga-lytic bacteria. The activities of protease (gelatinase and caseinase), lipase, amylase, carboxymethyl-cellulase (CMCase), chitinase, agarose, fucodian hydrolase, laminarinase, alginase, β-glucosidase, α- and β-galactosidase, β-N-acetylglucosaminidase, β-xylosidase, and α-mannosidase were examined.

MATERIALS AND METHODS

1. Organism and culture conditions

Anabaena cylindrica NIES-19 that used as host for alga-lytic bacteria was kindly supported 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 *l* under continuous illumination of cool white fluorescent lamps giving an incident light intensity of 35~80 μmol photon 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 *l* (Cattenholz 1988). And Fe solution is plus $FeSO_4 \cdot 7H_2O$ 2.0 g and H_2SO_4 0.26 ml per 1 *l*.

2. Isolation of alga-lytic bacteria

Various surface waters and sediment cores were collected from Pal'tang reservoir and Sukchon Lake in Korea where eutrophic lakes in which cyanobacteria are dominant phytoplankton during summer season. Alga-lytic bacteria were isolated by the soft agar overlayer technique (Shilo 1970). Axgenic cultures of *A. cylindrica* NIES-19 were grown 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 μmol photon m^{-2} s^{-1}. Bacterial colonies that produced clear zones on lawns of *A. cylindrica* were picked, purified, and maintained on MDM agar plates. Pure cultures were stored at −80°C in MDM medium containing 0.1% (v/v) yeast extract supplemented with 20% (v/v) of glycerol.

3. Determination of biochemical and physiological characteristics of bacterial isolates

Purified bacterial isolates were precultured on nutrient agar (peptone 5.0 g, NaCl 5.0 g, yeast extract 2.0 g, beef extract 1.0 g, and agar 15.0 g per 1 *l*) for following tests. The biochemical and physiological characteristics were determined using the method of Gerhardt *et al.* (1994) and further investigated using carbon utilization, the optimum pH, and temperature for growth, a hemolysis test, and gram straining. Oxidase and catalase activity, gelatin liquefaction, arginine dehydrolase, ornithine decarboxylase, etc. were further examined. For investigation of DNA base composition, average guanine-plus-cytosine (G+C) content was determined by using the thermal denaturation method (De Lay and Van Muylem 1963) and was calculated by using the equation of Marmur and Doty (1962), as modified by De Lay (1970).

4. Analysis of fatty acid composition

Amount of fatty acids in whole-cell hydrolysates were

determined as methyl ester by temperature programmed gas chromatographic separation on a Hewlett Packard (Avondale, PA, USA) 5890 Series II with a flame ionisation detector and hydrogen as the carrier gas. To prepare the ethers, harvested and washed cells of approximately 10 mg dry mass were saponified with sodium hydroxide, methylated with acidic methanol, extracted in a mixture of n-hexane/methyl tetra-butyl ether and base-washed according to procedure described by MIDI Inc. (www.midi-inc.com) in technical notes supplied with their microbial identification system. The fatty acids were identified with the aid of standards, using derivatisation procedures and mass spectrometry as described by Härtig *et al.* (1999). The degree of saturation was defined as the ration of saturated fatty acids to total amount of fatty acids. For fatty acid analysis, cells were grown for 40 h on Columbia agar (Oxoid Ltd., Basingstoke, England) containing 5% (v/v) defibrinated horse blood. The isolates were grown at 28°C.

5. Test for antibiotic sensitivity

An antibiotic as chemical compounds are commonly released to freshwater ecosystems, so it is important to choose antibiotic resistant bacteria for use in wastewater treatment systems. The bacterial suspensions of isolate AK-13 precultured for 24 h were spread onto the surface of Antibiotic Medium II (Difco) agar plate with sterile swabs to create a bacteria lawn. The thirteen different kinds of antibiotic discs (Difco) were then placed on the bacterial lawn using sterile forceps. The plates with a bacterial lawn and antibiotic discs were incubated at 30°C for about 24~48 h to verify their antibiotic resistance. The antibiotics used for this study were bacitracin, carbenicillin, cephalothin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, lincomycin, penicillin G, rifampin, streptomycin, and tetracycline.

6. Identification of bacterial isolates with phylogenetic analysis

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′-AGAAAGGAGGTGATCCAGCC-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-13 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 *Sinorhizobium* 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.

7. Preparation of cell-free extracts

Cells of isolate AK-13 were harvested by centrifugation at 20,000 rpm and 4°C for 20 min and washed twice with 50 mM phosphate buffer with pH 7.2 and resuspended in the same buffer. The cells were homogenized by sonication (MSE 100 watt Ultrasonic Disintegrator, MSE, London, UK) and centrifuged at 20,000 rpm for 20 min under 4°C to remove cell debris. The supernatant was used as the crude enzyme solution. The cell-free extracts were kept at −20°C until used.

8. Enzyme assays

To determine the activities of chitinase, laminarinase (β-1,3-D-glucanse), alginase, agarase, carboxymethyl-cellulase (CMCase), a chitin, laminarin, alginate, agar, and CM-cellulose used as substrates were purchased from Sigma-Aldrich Ltd., USA. The most of enzyme activities were measured by colorimetric analysis of the reducing sugar content determined by the procedure of Somogyi (1952) and Nelson (1955). Enzymes and substrate blanks were also included. A unit of enzyme activities is defined as the amount of enzyme catalyzing the release of 1 μmol of correspondent substrate per 1 mg of protein. Protein concentration was measured by the method of Bradford (1976), with bovine serum albumin as the standard. Chitinase and alginase were determined by development of clear zones around the

colonies. Congo red was used for detection of *in vivo* CMCase activity as follows: AK-13 colonies on agar plates were lysed by chloroform vapor during 15 min. The plates were overlaid with 5 ml of 50 mM phosphate citric acid buffer, pH 5.2, at 50°C supplemented with agar (0.5% w/v) and CM-cellulose (1% w/v) at 50°C. After 12 h of incubation at 30°C, the plates were flooded with Congo red (1% w/v) for 20 min and washed 1 M NaCl. Active clones were surrounded by a yellow halo on a red background. One unit of cellulase activity corresponds to 1 μmol D-glucose equivalent released min^{-1}. Laminarinase activity was determined by measuring the amount of reducing sugar releasing from laminarin. A standard assay mixture (1 ml) was containing enzyme solution properly diluted, 4 mg of laminarin, and 50 mM potassium acetate buffer with pH 5.5. The reaction were run for 30 minutes at 30°C and stopped by boiling for 5 min and reducing-sugar content was determined by the procedure of Somogyi (1952) and Nelson (1955). A unit of laminarinase activity is defined as the amount of enzyme catalyzing the release of 1 μmol of glucose equivalent per minute. Glycosidase activities were measured with the *p*-nitrophenyl derivatives of relevant monosaccharides (Sigma-Aldrich Ltd., USA) viz., *p*-nitrophenyl-β-D-glucopyranoide, *p*-nitrophenyl-α-D-galatopyranoide, *p*-nitrophenyl-β-D-galatopyranoide, *p*-nitrophenyl-β-N-acetyl-D-glucosaminopyranoside, *p*-nitro- phenyl-β-N-acetyl-D-galctopyranoside, *p*-nitrophenyl-α-L-fucoside, *p*-nitrophenyl-α-D-manno-pyranoside, and umbelliferyl-β-D-xylopyranoside. Glycosidases activities were determined in 100 mM phosphate buffer with pH 7.0 at 28°C. A 0.05 ml of *p*-nitrophenyl glycoside in 50 mM phosphate buffer with pH 7.0 and 0.1 ml of cell-free extract were mixed thoroughly and 0.1 ml of 1 M Na_2CO_3 was added to stop the reaction. Specific activities of glycosidase were expressed as amount of enzyme that converted 1 μmol of *p*-nitrophenol per hour.

RESULTS

1. Isolation of alga-lytic bacteria against *Anabaena cylindrica*

A number of bacteria (78 strains from surface waters and 100 from sediments) were isolated from surface waters and sediments. Only nine isolates exhibited lytic abilities against *A. cylindrica* NIES-19 on the agar plates, and then isolate AK-13 was selected as the strongest in lysing the cyanobacterium *A. cylindrica* NIES-19. The characteristics of strain AK-13 are shown in Table 1.

Table 1. Morphological, physiological, and biochemical characteristics of isolate AK-13

Characteristics	AK-13
Cell shape	Rod
Cell diameter	0.5~1.0 μm
Gram staining	−
Optimum temperature	30°C
Growth on 42°C	+
Optimum pH	6~8
Motility	+
Catalase	+
Oxidase	+
Arginine	−
Ornithine	−
Lysine	−
Citrate utilization	+
Hemolysis	−
Production of	
Amylase	+
Caseinase	+
Gelatinase	+
Agarase	−
Carboxymethyl-cellulase (CMCase)	+
Lipase	+
Chitinase	+
Alginase	−
Laminarinase	+
Fucodian hydrolase	−
Carbohydrate utilization of	
D-Lactate	−
D-Mannose	+
Lactose	+
D-Arabinose	+
D-Galactose	+
Sorbitol	−
D-Fucos	−
D-Ribose	+
D-Xylose	+
Glycerol	−
Mannitol	+
Cellobiose	+
Trehalose	+
Sucrose	−
Coenzyme Q	Q-10
G+C content (mol %)	60.3%

2. Determination of characteristics of alga-lytic bacterial strain AK-13

Enrichment cultures were obtained from fresh water sediments with added alga-lytic bacteria to favor growth of hydrolytic enzyme-producing microorganisms. Only bacterial isolate AK-13 was clearly picked on the plates during the monitoring algal degradation. In strain AK-13, amylase, protease (caseinase and gelatinase), CMCase, lipase, and laminarinase (β-1, 3-glucanase) were detected. Ubiguinone Q-10 was listed coenzyme, and G+C content of the DNA was 60.3 mol%. Other phenotypic features are shown in Table 1. Thus, the strain AK-13 was found to be very similar to *Rhizobium* sp. based on its biochemical and physiological characteristics. The antibiotic sensitivity testing of the strain AK-13 was listed in Table 3. The strain AK-13 was resistance only to gentamycin, kanamycin, rifampin and streptomycin.

3. Analysis of FAME (fatty acid methyl ester) profiles

The FAME of strain AK-13 contained a total of 18 fatty acids (Table 2). Only the fatty acid 16 : 0 and the unresolved fatty acid mixture in Summed Features 7 were present in over 15% of total fatty acids. The main components were 23.18% for 16 : 0, 16.01% for 16 : 1 w7c, and 15.32% for summed Features 7. In addition, 19 : 0 CYCLO w8c was present in minor amounts 0.40%. According to the FAME profile characteristics, the bacterial strain AK-13 was tentatively identified strain *Sinorhizobium* sp.

4. Phylogenic analysis

The 16S rDNA sequence was analyzed to determine which species matched strain AK-13 with the highest homology among *Sinorhizobium* species cited in the GenBank. The phylogenetic tree constructed using the neighbor-joining method is shown in Fig 1. The 16S rDNA sequences of AK-13 were compared with 16S rDNA sequences of type strains of the genus *Sinorhizobium* and the representative of some other related genera, and exhibited 98.1% of the highest homology with the sequences of *Sinorhizobium* medicae A321^T.

Table 2. Fatty acid composition of isolate AK-13 grown on Columbia agar containing 5% (v/v) defibrinated horse blood at 30°C

Fatty acid	Fatty acid (%) of AK-13
12 : 0	5.30
Unsaturated	1.29
12 : 0 2OH	0
12 : 0 3OH	0
13 : 0	1.32
14 : 0	5.89
14 : 0 3OH	0
15 : 0	4.91
16 : 1 Alcohol	0
16 : 1 w9c[1)]	0
16 : 1 w7c[1)]	16.01
16 : 0	23.18
17 : 0	2.30
17 : 1	0.30
18 : 1 Alcohol	0
18 : 1 w9c	0
18 : 1 w9t	0
18 : 0	0
19 : 0 CYCLO w8c	0.40
Summed Feature 7[2)]	15.32

[1)] There is currently a trend away from use of "systematic" names and forward the use of omega positions of double bonds.
[2)] 18:1 w7c/w9t/w12t, 18:1 w12t/w9t/w7c, 18:1 w9c/w12t/w7c.

Table 3. Antibiotic sensitivity of bacterial strain AK-13

Antibiotic	AK-13
Bacitracin	S
Carbenicillin	S
Cephalothin	S
Chloramphenicol	S
Clindamycin	S
Erythromycin	S
Gentamycin	R
Kanamycin	R
Lincomycin	S
Penicillin G	S
Rifampin	R
Streptomycin	R
Tetracycline	S

S, Sensitivity; R, Resistance; I, Intermediate reaction

This phylogenetic relationship is consistent with the results of the phenotypic and chemotaxonomic evidence and allowed us to conclude that strain AK-13 of algae degradation bacterium was designated as *Sinorhizobium* sp. AK-13.

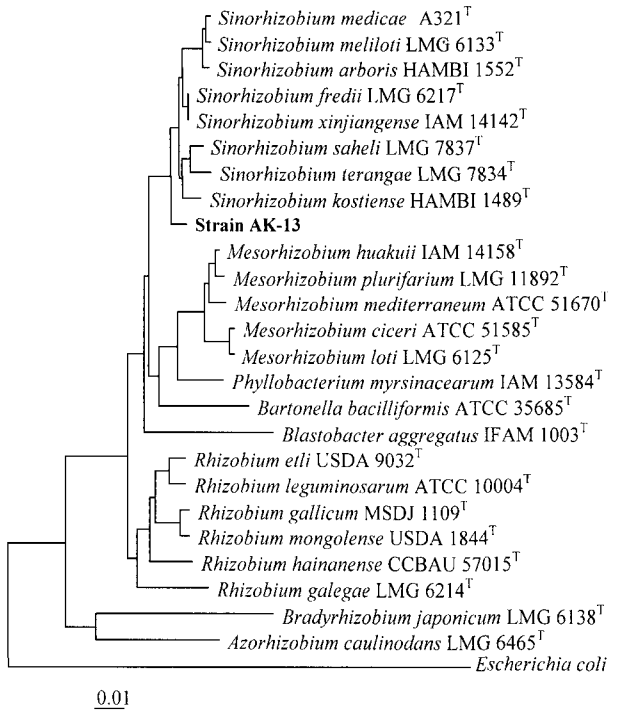

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the positions of strain AK-13, the type strains of *Sinorhizobium* species and the representatives of some other related taxa. The scale bar represents 0.01 substitutions per nucleotide position.

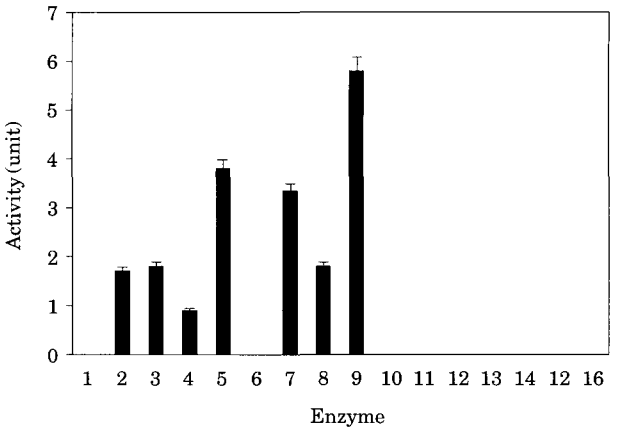

Fig. 2. Comparative enzyme activities and enzyme profile *Sinorhizobium* sp. AK-13. Values are given as an enzyme activity. One unit of gylcanase or glycosidase activity of enzyme that liberated 1 μmol from reducing ends of corresponding monosaccharides or 1 μmol from *p*-nitrophenol of corresponding substrates per 1 hour, respectively. 1, Agarase; 2, Alginase; 3, Amylase; 4, Chitinase; 5, CMCase; 6, Fucodian hydrolase; 7, Laminarinase; 8, Lipase; 9, Protease; 10, β-Glucosaminidase; 11, α-Galatosidase; 12, β-Galactosidase; 13, β-Glucosidase; 14, β-Xylosidase; 15, α-Fucosidase; 16, α-Mannosidase.

6. Enzyme profiles of *Sinorhizobium* sp. AK-13

In this study, we analyzed hydrolytic enzyme profile of *Sinorhizobium* sp. AK-13. The free-living bacterium, *Sinorhizobium* sp. AK-13, produced a protease, amylase, laminarinases (β-1, 3-glucanase) and CMCase that probably break down cell wells of *A. cylindrica* (Fig. 2). Fucodian hydrolase, β-galctosidase, β-glucosidase, β-glucosaminidase, and β-xylosidase that hydrolyzed mainly β-glycosidic bonds (Fig. 2) were not detected. And other glycosidase such as α-galctosidase, α-fucosidase and α-mannosidase, which degraded α-glycosidic bonds were not exhibited.

DISSCUSION

Monitoring of bacteria to control cyanobaceria causing water blooms, numerous samples were collected from lakes or reservoirs that have suffering from algal blooming by *Anabaena* spp. Our identification of only two algicidal strains from 178 isolates screened suggests that such activity may not be uncommon among freshwater bacteria. Daft and Stewart (1971) isolated only four strains of Myxobacteriales, which caused lyses of 40 strains of cyanobacteria. A recent report by Yamamoto *et al.* (1998), who isolated 83 actinomycete strains lethal to toxin-producing cyanobacterium *Microcystis aeruginosa*, provides further evidence for the compound of algicidal microorganism involved in lyses. The low densities of alga-lytic bacteria encountered in the environment may be due to insufficient inorganic fertility. In this work, two algicidal bacteria isolated from sediments in eutrophic lake are associated with a bloom population of the target algal species. The fatty acids compositions of alga-lytic bacteria are characteristic for Rhizobiaceae (Yokota *et al.* 1993; Graham *et al.* 1995; Jarvis *et al.* 1996). Eight common fatty acids, namely 14 : 0, 15 : 0, 16 : 1w7c, 16 : 0, 17 : 0, 17 : 1, C19 : 0 CYCLO w8c, and Summed Feature 7 that revealed in most of all *Rhizobium* species (Jarvis *et al.* 1996), are contained in the strain AK-13. However, 18 : 1 Alcohol, 18 : 1 w9c, 18 : 1 w9t, and 18 : 0 were not detected. It suggests that

AK-13 is distinguished with the genus *Rhizobium*. The strain AK-13 isolated from Pal'tang reservoir, however, which was designated *Sinorhizobium* sp. (Fig. 1) owing to 98.1% similarity of *Sinorhizobium medicae* A321^T based on its 16S rDNA analysis is shown high activities of protease, laminarinase and CMCase in Fig. 2. Any kinds of glycanase were not produced. Recently, two species of *Flexibacter* that lysed the cyanobacterium *Oscillatoria williamsii* produce lysozyme as one of cell inhibition compounds (Sallal 1994). Burnham *et al.* (1981) provided *Myxococcus xanthus*, which degraded the cyanobacterium *Phormidium luridum* var. *olivacea*, lyses the cells by the release of a lysozyme-like enzyme. Kim *et al.* (1997) reported *Moraxalla* sp. CK-1, which has been known to inhibit the growth of *Anabaena cylindrica*, produces aminodase, or endopeptidase. Carotenoids, peptidoglycan-associated proteins, and lipopolysaccharide (LPS), laminarinase and cellulose are one of the major constituents of cyanobacteria including *Microcystis* and *Anabaena* (Warren 1996). The peptidoglycan is to be covalently linked to a wall polysaccharide. In our results, highly active amylase and fucodian hydrolase are not active, and the highest activity of β-glucosidase and laminarinase also exhibited in to the strain studied herein. Laminarins, alginic acids, cellulose, and fucodians are the major constituents of algal cell walls up to 50~80% of defatted algal mass (Warren 1996). However, mainly laminarinase are often detected in microorganisms. This is most likely due to the important defense mechanisms by digesting fungal cell walls, etc., and releasing oligosaccharide that switch the production of antifungal compounds (Harmova and Fincher 2001), and their ability to hydrolyze the reserved β-1, 3-glucan. In Fig. 2, CMCase, laminarinase, lipase and protease in *Sinorhizobium* sp. AK-13, are showed high alga-lytic activities. Proteins such as lysozyme, protease, and lipase extracted from microorganisms caused cell lyses (Burnham 1976; Mitsutani *et al.* 1988; Yamamoto and Suzuki 1990). In conclusion, the results obtained in this study suggest that *Sinorhizobium* sp. AK-13 is able to metabolite peptidoglycans. The present work, hence, enhance our understanding of the functional interaction between algal cell structures and enzymes of alga-lytic bacteria during degradation of the cyanobacterium *A. cylindrica*. Identification and purification of the most active enzyme is now in progress. Elucidation of the mechanism of the selective alga-lytic enzyme against harmful blue-green algae is necessary to control water blooms. Nevertheless, algicidal bacterial strain AK-13 might be used as a biocontrol agent for management of cyanobacterial blooms in eutrophic lakes.

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