Mem. Fac. Fish. Kagoshima Univ.,
Vol. 59, pp. 17~25（2010）

Purification of an Algicidal Substance Produced by Marine *Pseudomonas* sp. C55a-2

Taizo Sakata, Takeshi Yoshikawa* and Satomi Nishitarumizu

Key words: algicidal substance, *Chaetoceros, Chattonella, Heterosigma, Pseudomonas*

Abstract

A marine bacterium, C55a-2 strain was selected among various algicidal bacteria isolated from coastal sea water of Kagoshima Bay, Japan, which killed diatom cells on a double layer agar plate. The algicidal strain was identified as *Pseudomonas* sp. tentatively on physiological and biochemical examinations.

Pseudomonas sp. C55a-2 was demonstrated to produce an algicidal substance extracellularly in marine nutrient medium Z-CII. An algicidal substance was purified from the culture supernatant of strain C55a-2 by a series of purification steps including ethyl acetate extraction, Sep-Pak treatment, Sephadex LH-20 column chromatography and high performance liquid chromatography (HPLC) with Mightysil RP-18GP column. The substance was shown to have algicidal activity against *Chaetoceros ceratosporum, Chattonella marina* and *Heterosigma akashiwo* strains by use of a microplate culture method.

Microorganisms possessing algal-killing activity have been divided into two groups, one of which produces extracellular algicidal substances and kills algal cells by action of the substances (indirect attack group), and the other kills them in direct contact with host algal cells (direct attack group).[1-5] Many strains belonging to genera *Pseudomonas* and *Pseudoalteromonas* spp. were isolated from aquatic environments as the former group bacteria, and various strains of genera *Cytophaga*[6,7] and *Saprospira*[8-10] were found to be the latter group bacteria. These strains exhibited algicidal activity on double-layer agar plates with diatom cells or in liquid cultures with microalgal cells in a microplate. However, it is still unclear what kinds of compounds have algicidal activity and what mechanisms are involved in direct attack against algal cells.

In this article, we reported that a substance algicidal to marine microalgae, produced by a marine algicidal bacterium was purified from the culture supernatant of the bacterium.

Materials and Methods

Bacteria strains

Marine algicidal bacterial strains were isolated by using microalgae double-layer agar plates from sea water in the coastal regions of Kagoshima Bay, Kagoshima, Japan in May and June, 2001 as shown in Fig. 1. The sea water samples were taken from the surface water of rocky foreshore including various macroalgae. The double-layer plates contained 20 ml of basal agar medium (Provasoli's enrichment sea water medium with 1.5% agar) and overlayered soft agar medium, which was composed of 0.1 ml of sea water sample, 1.0 ml of concentrated *Chaetoceros ceratosporum* C-16 cell suspension and 2.5 ml of Provasoli's enrichment sea water medium (PES) with 0.8% agar as described in previous papers.[9, 10] After 6 days incubation at 25°C, the bacteria which formed clear zone around their colonies on the double-layer agar plates were decided as algicidal bacteria. Algicidal bacterial strains were

鹿子島大学水産学部食品・資源利用学分野　(Department of Biochemistry and Technology of Marine Food and Resources, Faculty of Fisheries, Kagoshima University, 4-50-20 Shimoarata, Kagoshima 890-0056, Japan)
* Corresponding Author, Email: yoshi@fish.kagoshima-u.ac.jp

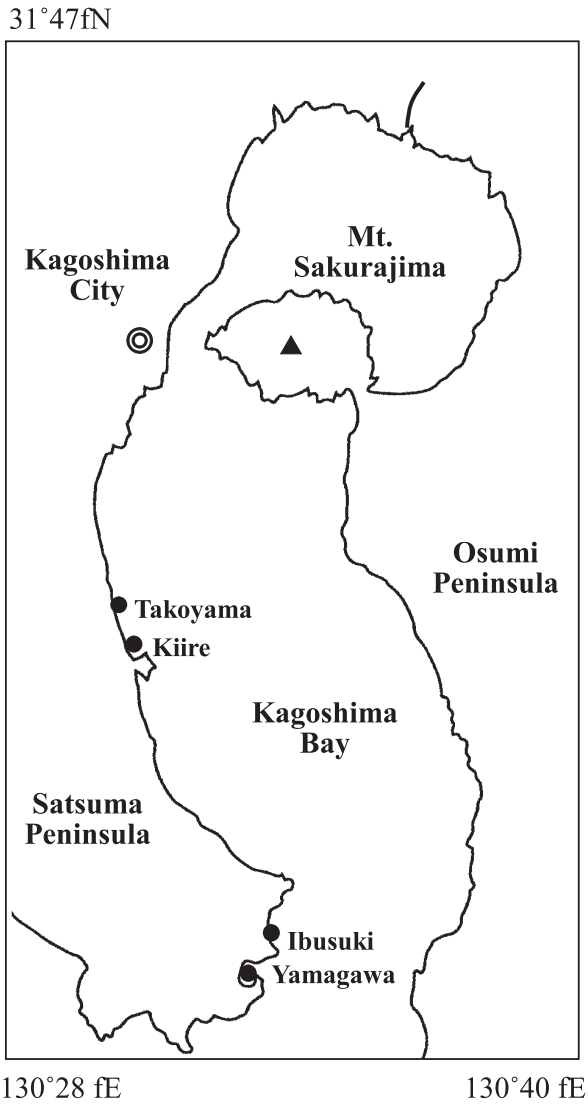

Fig. 1. Sampling sites for coastal sea water in Kagoshima Bay, Kagoshima, Japan.
● indicates sampling sites.

purified by the streaking plate method on nutrient-enriched sea water agar plates (Z-CII agar) as described in a previous paper. [11)]

Bacteriological characterization of algicidal bacteria

Bacteriological characterization was carried out on isolated algicidal strains according to the standard bacteriological methods. Colony and cell morphology was observed about colonies on Z-CII agar medium. Macromolecule hydrolysis was examined on Z-CII agar plates with macromolecule substances such as starch, casein, lecithin, tributyrin, or chitin. Fermentation of glucose and sucrose was examined in OF test medium with glucose or sucrose. Oxidase, catalase, indole production, Voges-Proskauer (VP) test and amino acid dehydrolation to lysine and arginine were examined based on the standard methods.

Purification of algicidal substance

Bacterial culture:

One algicidal strain C55a-2 was selected as a test strain and incubated shakingly in 250 ml of Z-CII liquid medium at 25°C for 5 days. The culture was centrifuged at 6,000 *g* for 20 min and the supernatant was recovered.

Ethyl acetate extraction:

The supernatant of the bacterial culture was concentrated to about half volume by using a rotary evaporator and then mixed with equal volume of ethyl acetate. After keeping the mixture for 60 min in a separation funnel, ethyl acetate layer was collected and the same procedure was repeated twice. Ethyl acetate extract obtained was evaporated and the remaining materials were dissolved in 10 ml of methanol. UV absorption spectrum of the substances in methanol solution was determined by using a spectrophotometer (U-2010, Hitachi, Japan).

Sep-Pak treatment:

The methanol solution of ethyl acetate extract was evaporated and dissolved in 1.0 ml of methanol and applied to a Sep-Pak cartridge (silica cartridges, Waters, USA). *n*-Hexane (10 ml) was passed through and then adsorbed materials on the Sep-Pak cartridge were eluted by 10 ml of methanol.

Sephadex LH-20 column chromatography:

Two ml of methanol solution was applied to Sephadex LH-20 column (2.5 x 30 cm, Amersham Biosciences, USA) and eluted by methanol:water (3:7) solution as mobile phase. The eluate was collected with a fraction collector (DC-1000, EYELA, Japan) as 3 ml of each fraction. UV absorbance of each fraction was determined at 242 nm by use of a spectrophotometer.

High performance liquid chromatography (HPLC):

Main peak fraction (Fraction 3) from Sephadex LH-20 column chromatography was evaporated and dissolved in 1.0 ml of methanol. The methanol solution (10-100 μl) was applied to HPLC column (Mightysil RP-18GP, 250 x 10.0 mm or 250 x 4.6 mm, Kanto Chemical, Japan) and eluted with 25% acetonitrile aqueous solution as mobile phase by using a liquid chromatography system (LC-10AT, Shimadzu, Japan). The eluate was monitored at 242 nm by use of UV-Vis detector (SPD-10A VP, Shimadzu, Japan).

Algicidal activity

Double-layer method:

An aliquot (15-20 μl) of each fraction (0.3-0.5 ml) was impregnated to a paper disk (0.7 cm in diameter) and it was placed on the surface of double-layer agar plate with *C. ceratosporum* cells. Algicidal activity was confirmed on the basis of clear zone around a paper disk after 6 days incubation under illumination (12 h light:12 h dark cycle) at 23°C.

Microplate culture method:

Each 10 μl of a series of ten-fold dilution of each test solution was added to a mixture of 90 μl of PES liquid medium and 10 μl of microalgal cell culture in each well of a microplate (96 wells). *C. ceratosporum* C-18, *Heterosigma akashiwo* NIES-6, and C*hattonella marina* NIES-121 strains were used as test microalgae strains. Algal growth in test culture wells was compared with that of control culture well (without test solution) after 6 days cultivation under illumination (12 h light:12 h dark) at 23°C.

Results

Characteristics of algicidal bacteria

Representative strains showing algicidal activity on double-layer agar plates were isolated from sea water samples in coastal regions of Kagoshima Bay. Bacteriological characterization of marine algicidal strains is shown in Table 1. Most strains possessed hydrolytic activities against starch and casein and some strains showed lipolytic activities. One strain, C25a-1 showed fermentative activity in fermentative-oxidative (OF) test medium with glucose or sucrose and was identified as genus *Vibrio*. On the other hand, most of algicidal strains were identified as genus *Pseudomonas* tentatively. A strain C55a-2 was selected as a target strain based on noticeable algicidal activity on double-layer agar plate.

Ethyl acetate extraction and Sep-Pak treatment

As shown in Fig. 2, ethyl acetate extract from the culture supernatant of C55a-2 strain indicated distinctive algicidal activity to *C. ceratosporum* on a double-layer agar plate. UV absorption spectrum of ethyl acetate extract solution (in methanol) did not exhibit significant absorption maxima in a range over 242 nm but that of Sep-Pak-adsorbed fraction in methanol solution possessed absorption maxima at 219 and 278 nm.

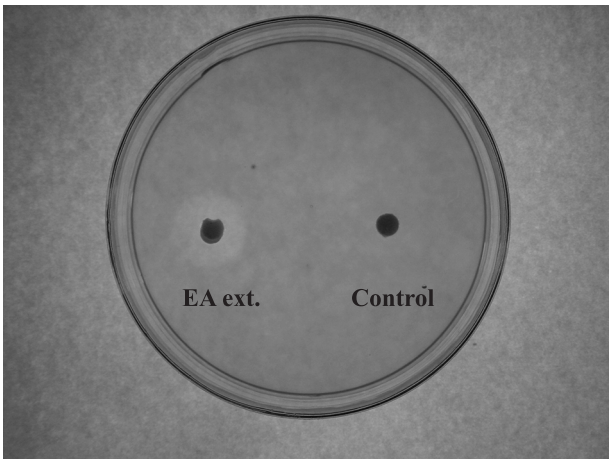

Fig. 2. Algicidal activity of ethyl acetate extract from the culture supernatant of *Pseudomonas* C55a-2 strain. 'EA ext.' indicates a paper disk impregnated with ethyl acetate extract in methanol and 'Control' corresponds to a paper disk impregnated with methanol and dried up.

Sephadex LH-20 column chromatography

An elution profile of adsorbed substances recovered from a Sep-Pak cartridge on Sephadex LH-20 column chromatography is illustrated in Fig. 3. Four major fractions were separated by monitoring UV absorbance of eluted solution at 242 nm. Among 4 fractions, Fraction 3 (Fr-3) presented absorption maxima at 243.5 and 291 nm and broad absorption between 400 and 500 nm giving pale yellow in color of the methanol solution as shown in Fig. 4. Fraction 3 only exhibited algicidal activity on a double-layer agar plate with *C. ceratosporum* cells as shown in Fig. 5.

HPLC on Mightysil RP-18GP column

High performance liquid chromatography of Fraction 3 from SephadexLH-20 column chromatography was carried out by using Mightysil RP-18GP column (250 x 4.6 mm). As shown in Fig. 6, four major fractions were detected by monitoring the eluate at 242 nm. Fraction III from HPLC on Mightysil RP-18GP (250 x 10.0 mm) was collected and its UV absorption spectrum in methanol was determined. As shown in Fig. 7, Fraction III possessed absorption maxima at 247 and 280.5 nm. The algicidal fractions dissolved in methanol were diluted ten-fold serially and their algicidal activities were determined in the microplate culture method. Fig. 8 exhibits the microplate containing *C. ceratosporum* cells incubated for 6 days. First ten-fold dilution of original fraction (0.5 ml of methanol solution) obtained from each purification step showed apparent algicidal activities against *C. ceratosporum*. After 6 days incubation of test cultures in the microplate, dis-

Table 1. Bacteriological characteristics of marine algicidal bacteria isolated from coastal sea waters in Kagoshima Bay.

Strains	Sampling site	Colony color	Motility	Cell form	Oxidase test	Catalase test	Glucose ferment.	Sucrose ferment.	Indole product.	VP test	Starch	Casein	Lecithin	Tributyrin	Tween 80	Chitin	Lys	Arg
C25a-1	K[*1]	WG[*2]	+[*3]	R[*4]	+	+	F[*5]	F	−	−	+	+	−	−	−	+	+	+
C31a-1	I	Y	−	R	+	+	−	−	−	−	+	−	−	−	−	−	−	−
C32a-1	Y	WG	+	LR	+	+	F	F	−	−	+	+	+	−	+	−	−	−
C32b-1	Y	WG	+	LR	+	+	F	F	−	−	+	+	+	−	+	−	−	−
C42a-1	K	Y	−	LR	+	+	−	−	−	−	+	+	−	−	−	+	−	−
C43a-1	K	Y	−	LR	+	+	−	−	−	−	+	+	−	−	−	+	−	−
C43a-3	K	Y	−	LR	+	+	−	−	−	−	+	+	−	−	−	+	−	−
C43a-4	K	Y	−	LR	+	+	−	−	−	−	+	+	−	−	−	−	−	−
C47a-2	K	Y	−	SR	+	+	−	−	−	−	+	+	−	−	−	+	−	−
C47b-1	K	R	+	R	+	+	O	O	+	−	+	+	+	+	+	+	−	−
C49a-1	K	Y	−	LR	+	+	−	−	−	−	+	+	−	−	−	+	−	−
C49a-2	K	Y	−	R	+	+	−	−	−	−	+	+	−	−	−	+	−	−
C54a-3	T	O	−	R	+	+	−	−	−	−	+	+	−	−	+	+	−	−
C55a-2	**T**	**WG**	**+**	**R**	**+**	**+**	**−**	**−**	**−**	**−**	**+**	**+**	**+**	**+**	**+**	**−**	**−**	**−**
C55a-3	T	WG	+	R	+	+	−	−	−	−	+	+	+	+	+	−	−	−
C55b-1	T	WG	+	R	+	+	O	O	−	−	+	+	+	+	+	+	−	−
C56a-1	T	WG	+	R	+	+	O	−	−	−	+	+	+	−	+	−	−	−
C56a-3	T	WG	+	R	+	+	O	−	−	−	+	+	+	−	+	−	−	−

[*1] Sampling site: I, Ibusuki; Y, Yamagawa; K, Kiire; T, Takoyama (district).

[*2] WG, whitish grey; Y, yellow; R, red; O, orange.

[*3] +, positive; −, negative.

[*4] R, rod; LR, long rod; SR, short rod.

[*5] F, fermentative; O, oxidative; −, negative.

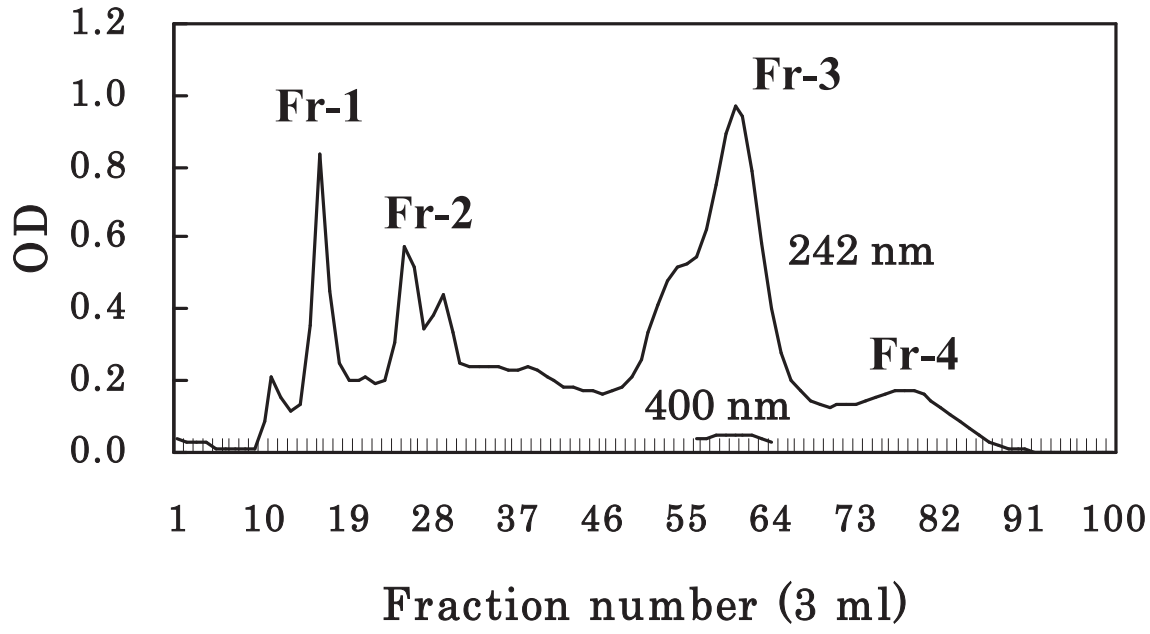

Fig. 3. Elution profile of Sep-Pak-adsorbed fraction on column chromatography of Sephadex LH-20 (2.5 x 30.0 cm column).

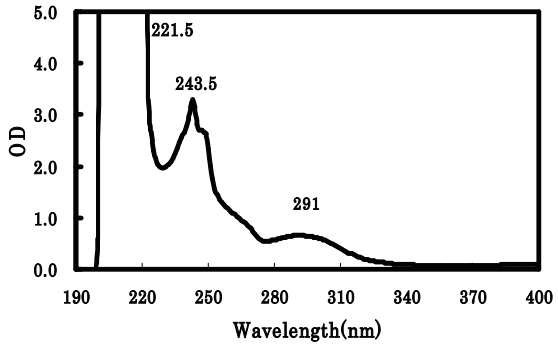

Fig. 4. UV absorption spectrum of Fraction 3 (Fr-3) obtained by using Sephadex LH-20 column chromatography in methanol.

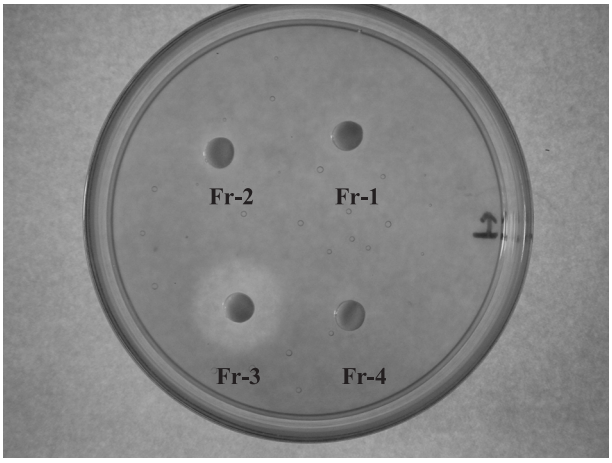

Fig. 5. Algicidal activity of each fraction from Sephadex LH-20 column chromatography on the double-layer agar plate with *C. ceratosporum*.

rupted or lyzed algal cells in the wells including microalgal cells and first ten-fold dilution of Fraction III were observed under a light microscope as shown in Fig. 9. In conclusion, it was found that Fraction III exhibits algicidal activities against three microalgal strains including *C. ceratosporum, H. akashiwo* and *C. marina* in the microplate culture method.

Discussion

Various algicidal microorganisms which kill or lyse microalgal cells have been isolated from aquatic environments. However, few algicidal substances have been purified and identified. For example, Mitsutani and Takesue[12] isolated and purified a protease from the culture medium of a cyanobacteria-lytic *Lysobacter* sp. strain LB-1. Lee et al.[13] reported that *Pseudoalteromonas* sp. A28 produced extracellular proteases exhibiting lytic activity toward marine algae. Lovejoy et al.[14]

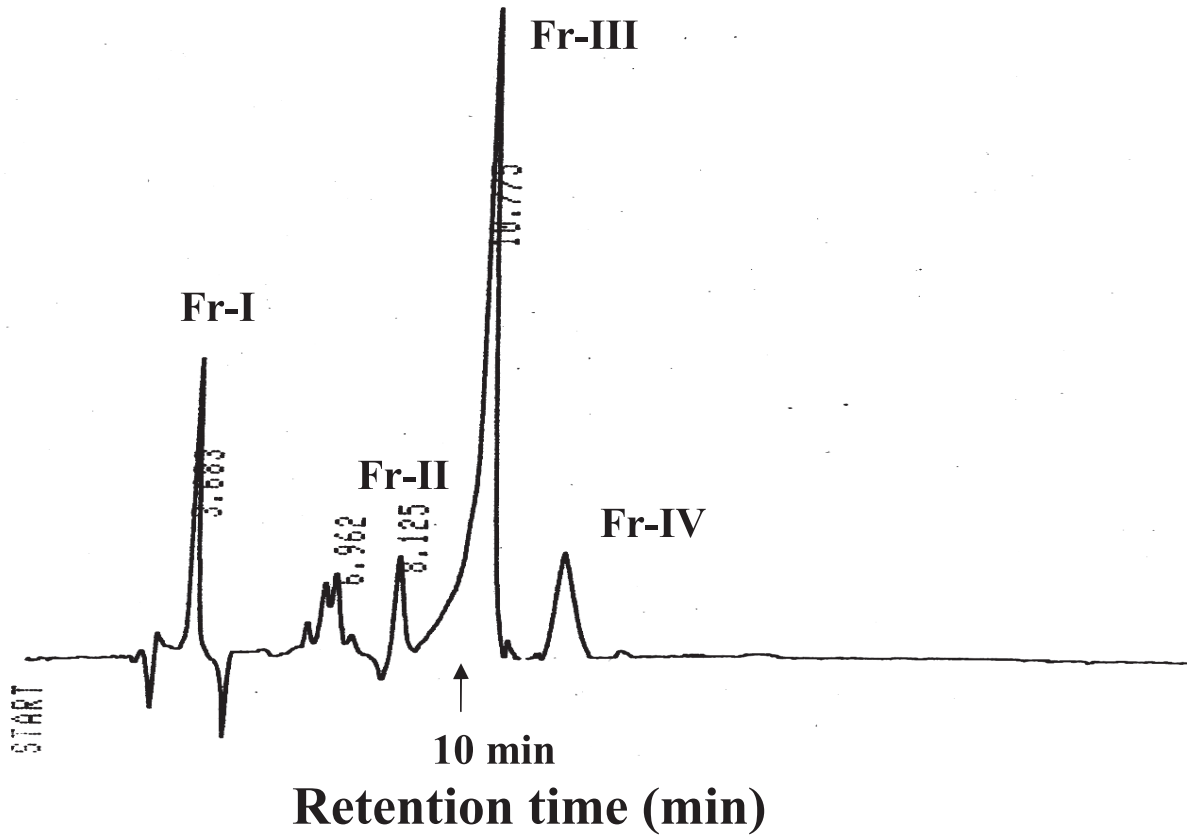

Fig. 6. Elution profile of Fraction 3 obtained from Sephadex LH-20 column chromatography on HPLC with Mightysil RP-18 GP (250 x 4.5 mm column). Vertical axis indicates relative abundance.

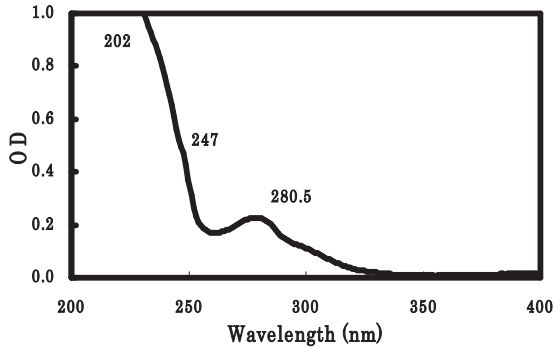

Fig. 7. UV absorption spectrum of Fraction III (Fr-III) obtained by using HPLC with Mightysil RP-18 GP in methanol.

demonstrated that marine *Pseudoalteromonas* sp. strain Y excreted heat-labile algicidal substance(s) which lyze cells of *Gymnodinium catenatum, Chattonella marina* and *Heterosigma akashiwo*. Recently, Long et al.[15] and Wigglesworlth-Cooksey et al.[16] demonstrated that synthetic 2-pentyl-4-quinolinol, an antibiotic from a marine *Alteromonas* sp. inhibited the growth of marine diatoms that usually grow as biofilms. del Castillo et al.[17] and Sakata et al. [18] also reported that a quinolinol derivative, 2-*n*-pentyl-4-quinolinol, isolated from the culture supernatant of marine *Pseudoalteomonas* sp. A1-J11 strain as an anti-*Vibrio* substance, showed algicidal activity against a diatom, *Chaetoceros ceratosporum* strain C-16 on a double-layer agar plate. So far various synthetic chemicals reported to have algicidal activity but few natural substances produced extracellularly by algicidal microorganisms have been identified.

In this study we isolated an algicidal substance from the culture supernatant of marine *Pseudomonas* sp. C55a-1 and demonstrated that it possessed algicidal activity against three microalgal strains including *C. ceratosporum, C. marina* and *H. akashiwo*. Further study is needed in order to identify chemical structure of the algicidal substance produced by this strain and clarify its algicidal mechanism.

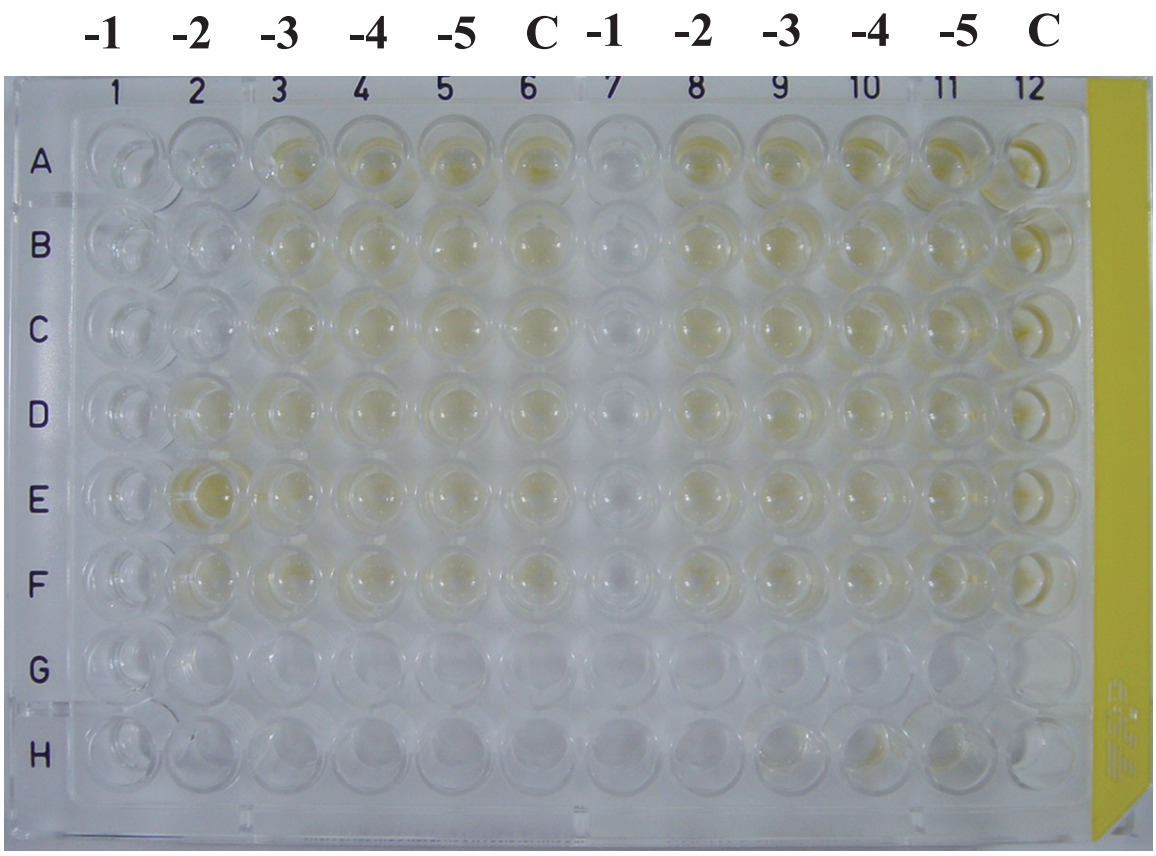

Fig. 8. Algicidal activity of each fraction from the culture of strain C55a-2 against *C. ceratosporum* in a microplate. Lines A, B, and C of the left half wells, ethyl acetate extracts; lines A, B and C of the the right half, Sep-Pak fraction; lines D, E and F of the left half, Fraction 3 of Sephadex LH-20 column chromatography; line D, E, and F of the right half, Fraction III from HPLC with Mightysil RP-18 GP. Figures -1, -2, -3, -4, and -5 indicate ten-fold serial dilution of the algicidal fractions and 'C' is the control cultures without algicidal fractions.

References

1) Stewart, W. D. P. and M. J. Daft (1976). Algal lysing agents of freshwater habitats. In "Microbiology in Agriculture, Fisheries and Food" (ed. by F. A. Skinner and J. G. Carr). Academic Press, New York, pp. 63-90.
2) Baker, K. H. and D. S. Herson (1978). Interactions between the diatom *Thallasiosira pseudonanna* and an associated pseudomonad in a mariculture system. *Appl. Environ. Microbiol*., **35**(4): 791-796.
3) Burnham, J. C., S. A. Collart, and B. W. Highison (1981). Entrapment and lysis of the cyanobacterium *Phormidium luridum* by aqueous colonies of *Myxococcus xanthus* PCO2. *Arch. Microbiol*., **129**: 285-294.
4) Cole, J. J. (1982). Interactions between bacteria and algae aquatic ecosystems. *Ann. Rev. Ecol. Syst*., **13**: 291-314.
5) Holmstrom, C. and S. Kjelleberg (1999). Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiol. Rev*., **30**: 285-293.
6) Stewart, J. R. and R. M. Brown Jr. (1969). *Cytophaga* that kills or lyses algae. *Science*, **164**: 1523-1524.
7) Imai, I., Y. Ishida, and Y. Hata (1993). Killing of marine phytoplankton by a gliding bacterium *Cytophaga* sp., isolated from the coastal sea of Japan. *Mar. Biol*., **116**: 527-532.
8) Sakata, T. (1990). Occurrence of marine *Saprospira* sp. possessing algicidal activity for diatoms. *Nippon Suisan Gakkaishi*, **56**(7): 1165.
9) Sakata, T., Y. Fujita, and H. Yasumoto (1991). Plaque formation by algicidal *Saprospira* sp. on a lawn of *Chaetoceros ceratosporum*. *Nippon Suisan Gakkaishi*, **57**(6): 1147-1152.
10) Furusawa, G., T. Yoshikawa, A. Yasuda, and T. Sakata (2003). Algicidal activity and gliding motility of *Saprospira* sp. SS98-5. *Can. J. Microbiol*., **49**(2): 92-100.
11) del Castillo, C. S., M. I. Wahid, T. Yoshikawa, and T. Sakata (2008). Isolation and inhibitory effect of anti-*Vibrio* substances from *Pseudoalteromonas* sp. A1-J11 isolated from the coastal sea water of Kagoshima Bay. *Fish. Sci*., **74**: 174-179.
12) Mitsutani, A. and K. Takesue (1993). Purification and properties of a protease from a cyanobacteria-lytic bacterium *Lysobacter* sp.

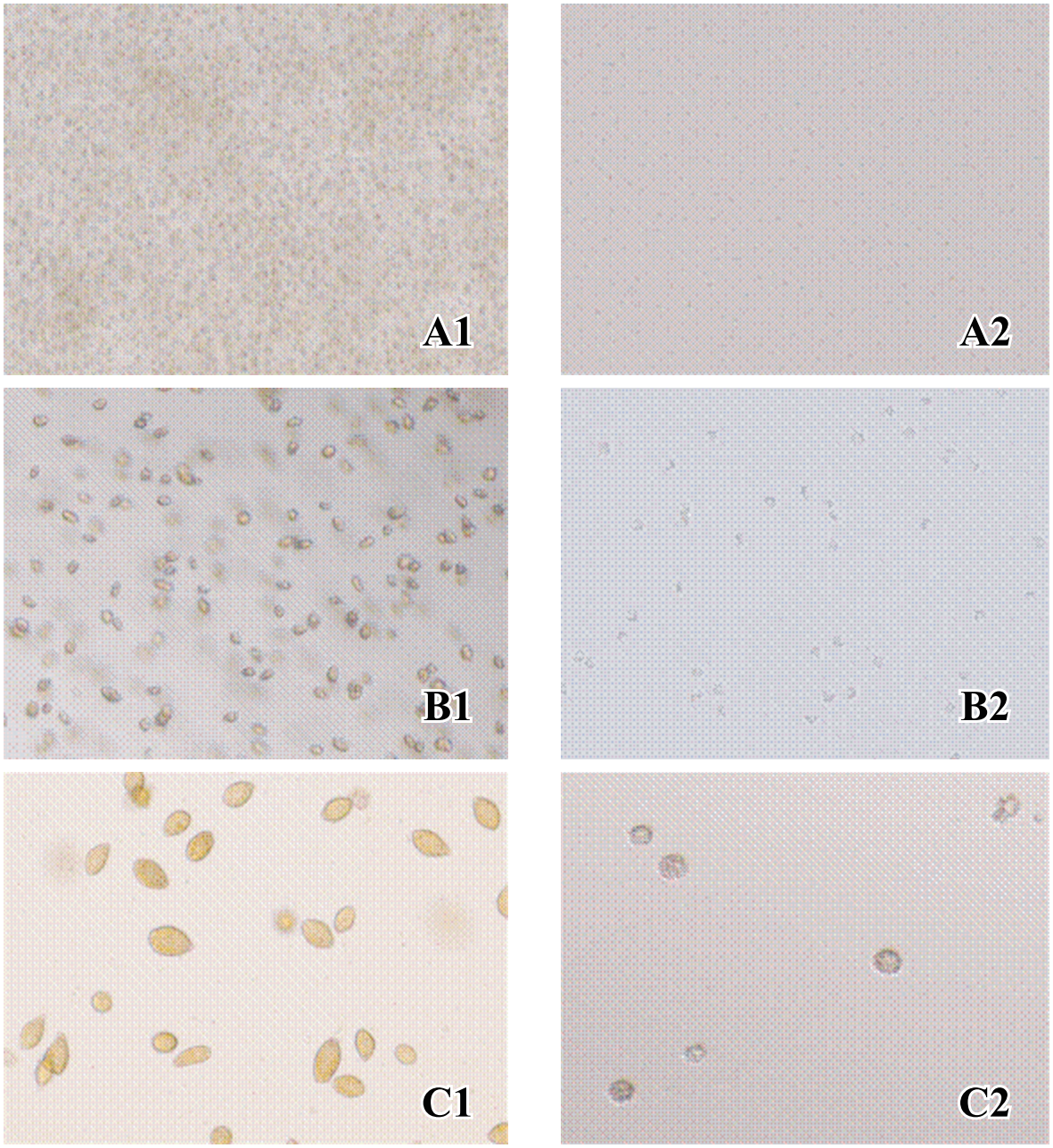

Fig. 9. Cell forms of three microalgal strains during microplate cultures with Fraction III from HPLC observed under a light microscope. A1 and A2, *C. ceratosporum* ;B1 and B2, *H. akashiwo*; C1 and C2, *C. marina*. A1, B1, and C1, without Fraction III; A2, B2, and C2, with Fraction III.

LB-1. *J. Shimonoseki Univ. Fish*., **41**(2): 65-75 (In Japanese).

13) Lee, S-O., J. Kato, N. Takiguchi, A. Kuroda, T. Ikeda, A. Mitsutani, and H. Ohtake (2000). Involvement of an extracellular protease in algicidal activity of the marine bacterium *Pseudoalteromonas* sp. strain A28. *Appl. Environ. Microbiol*., **66**: 4334-4339.

14) Lovejoy, C., J. P. Brown, and G. M. Hallegraeff (1998). Algicidal effects of a novel marine *Pseudoalteromonas* isolate (class proteobacteria, gamma subdivision) on harmful algal bloom species of the genera *Chattonella, Gymnodinium*, and *Heterosigma*. *Appl. Environ. Microbiol*., **64**: 2806-2813.

15) Long, R. A., A. Qureshi, D. J. Faulkner, and F. Azam (2003). 2-*n*-Pentyl-4-quinolinol produced by a marine *Alteromonas* sp. and its potential ecological and biogeochemical roles. *Appl. Environ. Microbiol*., **69**(1): 568-576.

16) Wigglesworlth-Cooksey, B., K. E. Cooksey, and R. Long (2007). Antibiotic from marine environment with antimicrobial fouling activity. *Environ. Toxicol*., **22**(3): 275-280.

17) del Castillo, C. S., T. Yoshikawa, M. Hashimoto, and T. Sakata (2008). Correlation between chemical structures and inhibitory activities of anti-bacterial substances from marine *Pseudoalteromonas* sp. A1-J11. *Fish Patholog*., **43**(2): 65-71.

18) Sakata, T., C. S. del Castillo, T. Yoshikawa, and M. Hashimoto (2008). Chemical structures of quinolinol compounds produced by marine *Pseudoalteromonas* sp. A1-J11. *Mem. Fac. Fish. Kagoshima Univ.*, **57**: 29-35.