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Algicidal Activity of Substance Purified from Marine Bacteria Metabolites against *Cochlodinium polykrikoides*

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Marine bacteria producing algicidal substance against *Cochlodinium polykrikoides* was screened and isolated from seawater. Metabolite of marine bacteria *Micrococcus* sp. LG-5 showed strong algicidal activity against *C. polykrikoides*. *C. polykrikoides* was inhibited above 90% at 5% solution of the metabolite within 24 hrs. Algicidal substance from the metabolite was extracted with ethyl acetate, and then purified by PTLC and reversed-phase HPLC. Algicidal activity of purified compound against *C. polykrikoides* was above 90% at 3.7, 11.0 and 33.0 μg/mL concentration after 12, 9 and 3 hrs, respectively. Ninety percent inhibition of other red tides, *Gymnodinium sanguineum* and *Gyrodinium impudicum* was observed when treated with 3.7 μg/mL of purified compound within a period of 12 hrs. The microscopic view of red tides treated with purified compound showed the deformations such as cell node cuts and swelling of cells.

Key words: Marine bacteria, Algicidal activity, *Cochlodinium polykrikoides*, Red tide

Introduction

Cochlodinium polykrikoides (*C. polykrikoides*) is one of major red tide species, which is a problematic microalgae due to its fast growth rate in embayments of Southern coast of Korea. These species have caused severe damage to aquaculture industry by killing fish and shellfish. The other, red tide species in Southern coast of Korea are *Ceratium fusus, Eutreptiella gymnastica, Gymnodinium sanguineum, Heterosigma akashiwo, Heterocapsa triquetra* and *Prorocentrum minimum* (Kim et al., 2000a). The damage caused by red tides to the fisheries industry in 1999 was three hundred million Korean Won (Kim et al., 2000a). In order to reduce the damage to fisheries industry by the red tides, many physiological and ecological studies have been conducted (Yanagi et al., 1995; Uchida et al., 1999; Jeong et al., 2000; Kim et al., 2000b).

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Harmful red tides were classified into two large categories (Nakanishi et al., 1996). One of these entails the phenomenon that certain kinds of phytoplankton grow to such a density that seawater is discolored and damage is caused to cultured fish and shellfish. In the other, fish and shellfish poisoning may occur as a result of eating toxic phytoplankton and accumulating toxic substances (Nakanishi et al., 1996). The most cultured fish suffer from damage to their gills and thus their oxygen intake is seriously restricted.

C. polykrikoides species were contained ichthyotoxic substances such as neurotoxic, hemolytic and hemagglutinating in intracellular of them (Onoue et al., 1989). However, Lee (1996) reported that the ichthyotoxic substances were not contained in *C. polykrikoides*. Therefore, the fish and shellfish were generally elucidated due to oxygen depletion and smothering of fish by massive dinoflagellate mucus production (Lee, 1996; Cho et al., 1999; Kim et al., 2000c).

Recently, destruction of red tides such as *Gymnodinium mikimotoi* (Yoshinaga et al., 1995), *Heterosigma akashiwo* (Kim et al., 1998), *Heterocapsa circularisquama* (Imai et al., 1998) and *C. polykrikoides* (Jeong et al., 2000) by marine algicidal bacteria was identified. These studies revealed that the marine bacteria have an algicidal activity against the red tides. But these studies were not elucidated algicidal substances contained in marine bacteria metabolites.

In this study, we could elucidate the algicidal substances derived from metabolite of the algicidal marine bacteria, which was screened from seawater.

Materials and Methods

Isolation of *C. polykrikoides*

The strain of *C. polykirkoides* used in this experiments was isolated from seawater samples collected from Masan bay on September 1996 (Jeong et al., 2000). Clonal culture of the strain was obtained by repeated washings using capillary pipettes (Droop, 1967). Bacteria-free cultures of the strain were obtained by several subcultures using enriched seawater medium containing an antibiotic mixture (final concentration of antibiotics in the medium: 100 μg/mL ampicillin, 10 μg/mL streptomycin sulfate, 10 μg/mL chloramphenicol, 10 μg/mL penicillin G, 50 μg/mL neomycin, 50 μg/mL gentamicin, 10 μg/mL kanamycin, 1.5 μg/mL nystatin) and keeping for 12 hrs. In previous study (Jeong et al., 2000), culture of the *C. polykirkoides* was maintained at 20℃, pH 8.2, 34‰ NaCl concentration, and 140 μEm photon $m^{-2}s^{-1}$ on f/2-Si broth (Guillard and Ryther, 1962).

Isolation and screening of algicidal bacteria from seawater

In our previous study (Jeong et al., 2000), we separated 12 species of marine bacteria having algicidal activity against *C. polykirkoides* from seawater of Masan bay. *Micrococcus* sp. LG-5 had the highest algicidal activity against *C. polykirkoides*. The isolated strain was kept as a frozen stock with 10% (v/v) glycerol. *Micrococcus* sp. LG-5 was cultured in 1-liter Erlenmeyer flask containing 300 mL PPES-II liquid broth (total 3 L) (Taga, 1968) at 25℃, pH 7.0, 34‰ NaCl for 18 hrs.

Measurement of algicidal activity

The algicidal activity of sample against *C. polykrikoides* was measured by using a 24-well plate. The bioassay plates containing *C. polykrikoides* (1.2×10^4 cells/mL) were incubated with known concentrations of the test samples at 20℃, pH 8.2, 34‰ NaCl, and 140 μEm photon $m^{-2}s^{-1}$ for 3 hrs. Then, the viable individuals swimming in each well were counted under the microscope (TMS, Nikon Co., Tokyo, Japan).

The algicidal activity was calculated as follow: Algicidal activity (%) = {1 − (viable individuals of *C. polykirkoides* after the treatment/initial individuals of *C. polykirkoides*)} × 100. The indices of algicidal activity were divided into four classes according to the dead individuals as above 90% (+++), 90~70% (++), 70~50% (+), and below 50% (-).

Purification of algicidal substance from the metabolite

The culture broth (3 L) was centrifuged (8,000×g, 10 min) at 4℃ and then supernatant was concentrated by using an evaporator (EYELA, Tokyo, Japan), the resulting aqueous solutions were extracted with an equal volume of ethyl acetate twice. The extract was dried over N_2 gas and concentrated by evaporator.

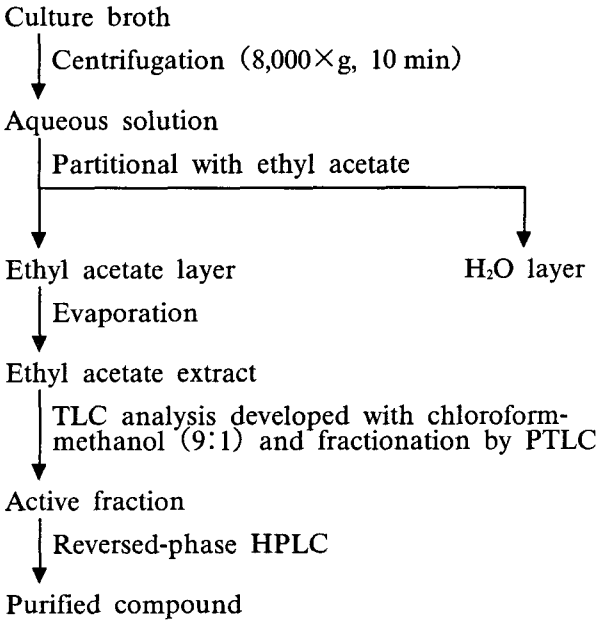

Fig. 1. Purification procedure of algicidal compound against *C. polykrikoides* from culture filtrate of *Micrococcus* sp. LG-5.

The procedure of purification and isolation of algicidal substances against *C. polykrikoides* is shown in Fig. 1. Purification of the algicidal substances against *C. polykrikoides* was done using thin layer chromatography (TLC), preparative TLC (PTLC) and reversed-phase HPLC (Shimadzu SCA-8A System Controller, Tokyo, Japan). TLC analysis of ethyl acetate extract was performed with TLC aluminium plate (0.25 mm silica gel 60 F_{254}, Merck Co., Darmstadt, Germany) developed with chloroform-methanol (9:1). Spots on TLC was detected by 1% $Ce(SO_4)_2$/10% H_2SO_4 and UV questing. The ethyl acetate extract was fractionated by PTLC glass plate (0.5 mm silica gel 60 F_{254}, Merck Co., Darmstadt, Germany). After the development in chloroform-methanol (9:1), the PTLC plate was divided into six sections from top to bottom (fraction 1 to 6), which were scraped off the plates. Six fractions were eluted from the silica with chloroform-methanol (1:1), and the eluate was recovered by centrifugation (850×g, 15 min). After evaporation in vacuum at 40℃, the fractionates were dissolved in methanol, and a bioassay was carried out to test the algicidal activity against *C. polykirkoides* using a 24-well plate.

The active fraction obtained from the PTLC was further purified by HPLC using ODS column (Develosil ODS-HG-5, ϕ4.6×250 mm, Nomura Chemical Co., LTD., Tokyo, Japan). The column was developed at a flow rate of 1 mL/min by a linear gradient (70% methanol, 40 min). The active fractions were collected and concentrated by using an evaporator, then further purified by step-wise gradient with methanol in the same column.

Results and Discussion

Effect of organic solvents against *C. polykrikoides*

Algicidal extracts first dissolved in organic solvents, and then evaporated the solvents or diluted with water for bioassay. Therefore, the effect of five organic solvents (acetone, ethanol, methanol, ethyl acetate and acetonitrile) on the culture of *C. polykrikoides* was examined. In our results (Table 1), ethyl acetate and acetonitrile were shown the algicidal activity against *C. polykrikoides* at above 6% (v/v) concentration. Acetone and methanol were found to be the most suitable solvent, and ethanol was also usable at the concentration of less than 7% (v/v). It should be noted that acetone damages the plastic assay plates. If necessary, the test samples were dissolved, in methanol or ethanol, then either evaporated the solvent in assay wells or diluted with water.

Table 1. Effect of organic solvents on algicidal activity against *C. polykrikoides*

Organic solvents	Concentration (%)									
	1	2	3	4	5	6	7	8	9	10
Acetone	−	−	−	−	−	−	−	−	−	−
Ethanol	−	−	−	−	−	−	−	+	++	++
Methanol	−	−	−	−	−	−	−	−	−	+
Ethyl acetate	−	−	−	−	−	+	++	++	+++	+++
Acetonitrile	−	−	−	−	−	+	+	++	+++	+++

The algicidal activity against 8,000~12,000 cells/mL of *C. polykrikoides* was measured after incubation for 48 hrs. All data were expressed bellow; +++, strong inhibition (>90% death rate); ++, inhibition (70~90% death rate); +, weak inhibition (50~70% death rate); −, no death.

Purification and algicidal activity of algicidal compound

Marine bacteria producing algicidal substances against *C. polykrikoides* were screened and isolated from seawater. Algicidal substances were screened from 110 strains of marine bacteria, which were isolated from seawater, and investigated to their activity against *C. polykrikoides*. Marine bacteria, *Micrococcus* sp. LG-5 showed strong algicidal activity. *C. polykrikoides* treated with 1, 5 and 10% (v/v) concentration of culture broth were inhibited above 90% at 36, 24 and 12 hrs, respectively (Table 2).

Table 2. The algicidal activities of the culture filtrate of *Micrococcus* sp. LG-5 against *C. polykrikoides*

Inoculation (%)	Algicidal activity			
	Incubation time (hrs)			
	12	24	36	48
1	+	++	+++	+++
2	++	+++	+++	+++
10	+++	+++	+++	+++

The algicidal activity against 8,000~12,000 cell2/mL of *C. polykrikoides* was measured after incubation for each time. All data were expressed bellow; +++, strong inhibition (>90% death rate); ++, inhibition (70~90% death rate); +, weak inhibition (50~70% death rate).

Fukami et al. (1992) reported that *Gymnodinium nagasakinese* was not inhibited at 25% solution of metabolite obtained from *Flavobacterium* sp. 5N-3, but was strong inhibited at 50% solution of the metabolite. Algicidal bacteria producing algicidal substances to outside of cell were *Acinetobacter, Alcaligenes, Alteromonas, Flavobacterium* and *Pseudomonas* (Mitsutani et al., 1992; Dakhama et al., 1993; Yoshinaga et al., 1995).

After the cultured broth was centrifuged, the supernatant concentrated by using a vacuum evaporator up to 1,000 mL. The ethyl acetate extracts from the supernatant were dried over N_2 gas and concentrated in vacuum, to yield was 198 mg of ethyl acetate extract. The algicidal activity of 167 μg/mL of the ethyl acetate extract showed above 90% at 9 hrs, and 500 μg/mL of the extract showed above 90% at 3 hrs (Table 3).

Table 3. The algicidal activities of the ethyl acetate extract isolated from culture filtrate of *Micrococcus* sp. LG-5 against *C. polykrikoides*

Inoculation (μg/mL)	Algicidal activity: Incubation time (hrs)			
	3	6	9	12
500	+++	+++	+++	+++
167	++	++	+++	+++
56	+/−	+/−	+/−	+

The algicidal activity against 8,000~12,000 cells/mL of *C. polykrikoides* was measured after incubation for each time. All data were expressed bellow; +++, strong inhibition (>90% death rate); ++, inhibition (70~90% death rate); +, weak inhibition (50~70% death rate); +/−, below 50%.

The extract was fractionated with PTLC glass plates in chloroform-methanol (9:1). The PTLC fractions were divided into six sections from bottom to top, which were scraped off from the plates. Fraction having Rf value of 0.40 showed the highest algicidal activity at the concentration of 167 μg/mL, and the yield of that fraction was 9.2 mg (Table 4). Algicidal activity of fraction having Rf value of 0.40 was tested with 6, 19 and 56 μg/mL concentrations. Strong inhibition (>90%) was observed at the test concentration of 19 μg/mL after 12 hrs, and inhibition (70~90%) was after 6 hrs. It took only 3 hrs for inhibition (70~90%) and 6 hrs for strong inhibition (>90%) by using 56 μg/mL of test concentration. Test concentration of 6 μg/mL was not even enough for weak inhibition (50%>) after 12 hrs (Table 5).

Table 4. The algicidal activities of the fractions isolated by preparative thin-layer chromatography (PTLC) against *C. polykrikoides*

Inoculation (μg/mL)	Algicidal activity: Rf values of PTLC fractions					
	0.06	0.25	0.40	0.46	0.52	0.58
167	+/−	+/−	+++	+/−	+	+

The algicidal activity against 8,000~12,000 cells/mL of *C. polykrikoides* was measured after incubation for 12 hrs. All data were expressed bellow; +++, strong inhibition (>90% death rate); ++, inhibition (70~90% death rate); +, weak inhibition (50~70% death rate); +/−, below 50%.

Table 5. The algicidal activities of PTLC active fraction (R_f value 0.40) against *C. polykrikoides*

Inoculation (μg/mL)	Algicidal activity: Incubation time (hrs)			
	3	6	9	12
56	++	+++	+++	+++
19	+	++	++	+++
6	+/−	+/−	+/−	+/−

The algicidal activity against 8,000~12,000 cells/mL of *C. polykrikoides* was measured after incubation for each time. All data were expressed bellow; +++, strong inhibition (>90% death rate); ++, inhibition (70~90% death rate); +, weak inhibition (50~70% death rate); +/−, below 50%.

The active fraction (Rf value 0.40) obtained using PTLC was further fractionated by HPLC using a pre-packed ODS column. The HPLC chromatogram of active fraction is shown in Fig. 2. The algicidal activity of the compound resulted after 10 minutes of retention time was tested with known concentrations (Table 6). The minimum inhibitory concentration (MIC) value against *C. polykrikoides* was 3.7 μg/mL.

In addition, above 90% inhibition of other red tide species, *Gymnodinium sanguineum* and *Gyrodinium impudicum* was observed when treated with 3.7 μg/mL within a period of 12 hrs (Table 7). The microscopic view of red tides treated with algicidal compound showed the morphological deformations such as cell node cuts and swelling of cells (Fig. 3).

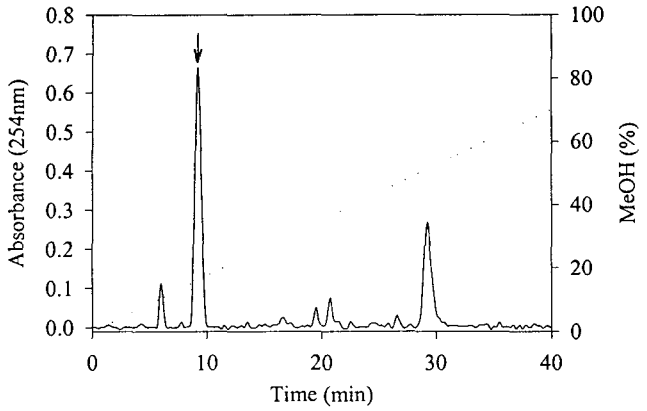

Fig. 2. HPLC chromatogram of active fraction (Rf value 0.4) isolated by PTLC. The sample was eluted with a linear gradient of 70% methanol in water. The flow rate was 1 mL/min.

Table 6. The algicidal activities of HPLC fraction against *C. polykrikoides*

Inoculation (μg/mL)	Algicidal activity			
	Incubation time (hrs)			
	3	6	9	12
33	+++	+++	+++	+++
11	+	++	+++	+++
3.7	+	+	++	+++

The algicidal activity against 8,000~12,000 cells/mL of *C. polykrikoides* was measured after incubation for each time. All data were expressed bellow; +++, strong inhibition (>90% death rate); ++, inhibition (70~90% death rate); +, weak inhibition (50~70% death rate).

In previous study, Kim et al. (2000c) reported that red sea bream and flounder in seawater containing *C. polykrikoides* of 8,000 cells/mL exhibited mortalities of 100% and 30% within 24 hrs, respectively.

Table 7. Algicidal effect of substance purified from culture filtrate of *Micrococcus* sp. LG-5 on the growth of six species of red tide-causing microalgae

Species	Algicidal activity
Dinophyceae	
Cochlodinium polykrikoldes	+++
Gymnodinium sanguineum	+++
Gyrodinium impudicum	+++
Prorocentrum minimum	+/−
Scrippsiella trochoidea	+/−
Chlorophyceae	
Chlamydomonas sp.	+/−

The algicidal activity against 8,000~12,000 cells/mL of *C. polykrikoides* was measured after incubation of 3.7 μg/mL for 12 hrs. All data were expressed bellow; +++, strong inhibition (>90% death rate); ++, inhibition (70~90% death rate); +, weak inhibition (50~70% death rate); +/−, below 50%.

The reason for the death of fish by *C. polykrikoides* is one of the direct causes of suffocation of fish, in spite of the sufficient oxygen in water (Lee, 1996). This was elucidated due to abnormal secretion of gill mucus and the inactivation of gill transport-related enzymes such as carbonic anhydrase and Na^+/K^+-ATPase by the *C. polykrikoides* (Kim et al., 2000b). The previous results (Lee, 1996; Kim et al., 2000b) indicated that only killing *C. polykrikoides* could not satisfactorily solve the problem of suffocation of fish. Therefore, the prevention of *C. polykrikoides* by various algicidal compounds is the most important approach by inhibiting its initial growth.

The algicidal compound purified from metabolite

(A) (B)

Fig. 3. Photomicrographs showing shapes of *C. polykrikoides* treated with algicidal compound (B) and the control (A).

of *Micrococcus* sp. LG-5 may proved to be a valuable as algicidal agent against initial growth of *C. polykrikoides*. Further researches are required to find out the chemical structure of this algicidal compound.

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