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Inhibition of *Microcystis aeruginosa* by the Extracellular Substances from an *Aeromonas* sp.

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Growth of *Microcystis aeruginosa* could be inhibited significantly within 24 h by the extracellular substances prepared from *Aeromonas* sp. strain FM. During the treatment, the concentration of extracellular soluble carbohydrates increased significantly in algal culture. Morphological and ultrastructural changes in *M. aeruginosa* cells, including breakage of the cell surface, secretion of mucilage, and intracellular disorganization of thylakoids, were observed. HPLC-MS analysis showed that the extracellular substances of *Aeromonas* sp. strain FM were a mixture of free amino acids, tripeptides, and clavulanate. Among these, the algae-lysis effects of lysine and clavulanate were confirmed.

Keywords: Algicidal bacteria, extracellular substances, *Microcystis aeruginosa*, *Aeromonas*

Cyanobacterial blooms severely impair water quality and pose operational problems to water treatment plants. Biological methods using algicidal bacteria have the potential to inhibit algal growth in freshwater and marine systems [5, 10–15, 17, 21, 23]. To date, most algicidal bacteria have been reported to exert effects via indirect attack, by synthesizing algicidal extracellular substances [4, 6, 14, 16, 18, 20, 25]. *Aeromonas* sp. strain FM was previously isolated from a reservoir as an algicidal bacterium [22]. In the present study, its extracellular substances associated with its algicidal effect were prepared, and the effect on *Microcystis aeruginosa* was investigated.

To prepare extracellular substances, strain FM was cultivated in LB medium for 36 h. The extracellular substances were extracted with butyl alcohol. The extracts were subsequently concentrated to dryness on a rotary evaporator, and then applied to a PAX-SPE column (150 mg/6 ml; Agela Technologies Co. Ltd., China) after dissolving in distilled water. The anticyanobacterial substances were eluted with formic acid–methanol solution (1:20 (v/v)), after which they were evaporated to dryness. Approximately 0.6 g of dry anticyanobacterial substance was obtained from 1,200 ml FM cultures. The collected extracellular substances were redissolved in sterilized water and subjected to algicidal test as described previously [26]. After 40 μg/ml extracellular substances were added to the *M. aeruginosa* cultures at the exponential phase (4.6×10^6 cells/ml), the color and dispersity of the algal culture changed significantly within 15 min. *M. aeruginosa* cells tended to aggregate and form a mass (Fig. 1). Simultaneously, *M. aeruginosa* cells were monitored by microscopic observation, and it was shown that most cells remained intact within 1 h. The cells were completely lysed after 24 h, whereas the biomass of *M. aeruginosa* increased in the untreated control (Fig. 1). Moreover, the effective concentration of the prepared extracellular substances against *M. aeruginosa* was 10 μg/ml.

During treatment with the prepared extracellular substances, the extracellular soluble carbohydrates in BG11 cultures of *M. aeruginosa* were monitored. The algal cells were discarded and the soluble carbohydrates in the BG11 medium were quantified spectrophotometrically by the phenol-sulfuric acid method using glucose as a standard [7]. Simultaneously, the BG11 cultures of *M. aeruginosa* without treatment were used as a control. As shown in Fig. 2, 1 h after the extracellular substances were added, the concentration of soluble carbohydrates increased from

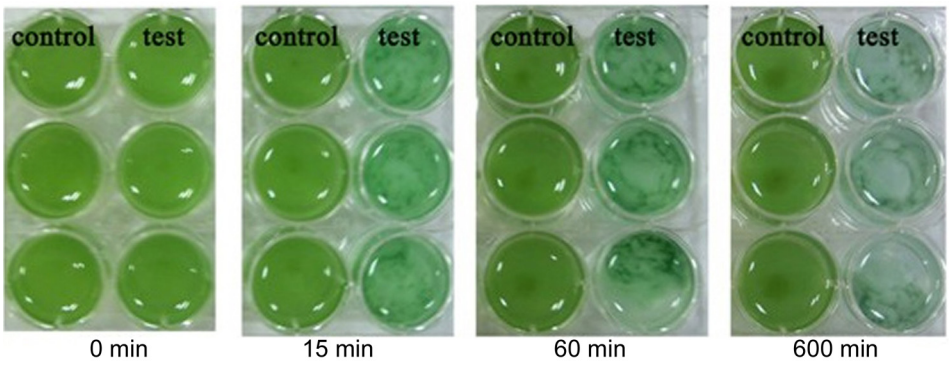

Fig. 1. Effects of extracellular substances prepared from *Aeromonas* sp. strain FM on *M. aeruginosa*.
Pairs of wells show *M. aeruginosa* cultured in BG11 with (right wells) and without (left wells) extracellular substances after treatment for 0, 15, 60, and 600 min.

10 to 76 μg/l, after which it slowly increased to 83 μg/l. It should be noted that the extracellular soluble carbohydrates concentration of the untreated control as well as that of the initially treated algal culture was relatively low (10 μg/l). These results indicate that the extracellular substances of strain FM stimulated *M. aeruginosa* to secrete extracellular soluble carbohydrates. *M. aeruginosa* is known to produce extracellular soluble carbohydrates with different compositions [8]. Similarly, an increase of carbohydrate contents was observed after *M. aeruginosa* FACHB-905 cell exposure to an algicidal *Streptomyces* sp. [19]. Moreover, it has been shown that the production of extracellular polysaccharide can serve as a protective response to adverse factors such as toxic metal ions [2], cyanobacterial toxin [9], and protozoan predation [29].

Additionally, cells of *M. aeruginosa* treated with the extracellular substances were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Samples were prepared according to Ozaki *et al.* [24] and Wang *et al.* [27]. As shown in Fig. 3, cells of *M. aeruginosa* changed morphologically and ultrastructurally during treatment with extracellular substances. Typical scanning electron micrographs, shown in Figs. 3A–3C, revealed changes in morphology. Gelatinous substances appeared on the cell surface after treatment with the extracellular substances for 1 h (Fig. 3B). The cell surface

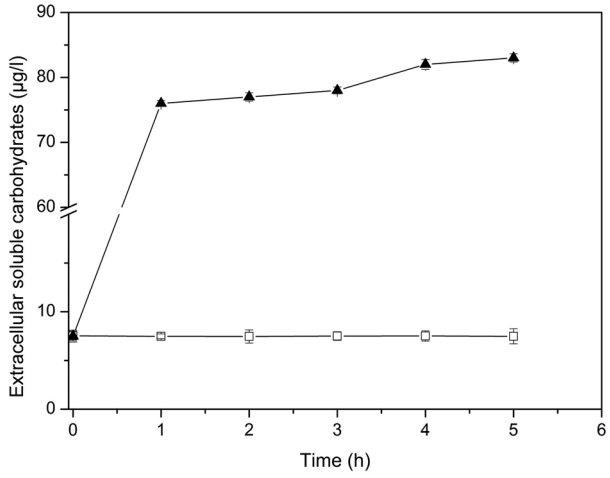

Fig. 2. Extracellular soluble carbohydrates in *M. aeruginosa* culture after the addition of extracellular substances from *Aeromonas* sp. strain FM (▲), in comparison with untreated *M. aeruginosa* control (□).
Data are the means ± standard deviation of three independent assays.

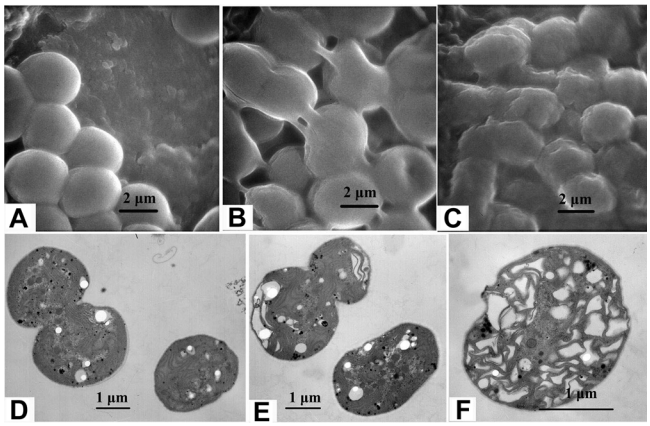

Fig. 3. SEM and TEM observations of *M. aeruginosa* during treatment with the extracellular substances from *Aeromonas* sp. strain FM.
Algal cell changes after treatment are shown in the SE micrographs A: 0 h, B: 1 h, and C: 24 h; and TE micrographs D: 0 h, E: 1 h, and F: 24 h.

was broken and changed from smooth to rough after 24 h (Fig. 3C). The morphological changes in *M. aeruginosa* cells indicated that extracellular polysaccharide was produced during the treatment, which was in accordance with the increase of extracellular soluble carbohydrates shown in Fig. 2. Moreover, the secretion of carbohydrates resulted in the assembly of algal cells, shown in Fig. 1. Transmission electron micrographs showed that the untreated cells appeared as normal prokaryotic cells (Fig. 3D). However, intracellular damage was observed 1 h after the addition of extracellular substances, and the thylakoids were disordered in some algal cells (Fig. 3E). After 24 h, distortion of the plasma membrane and disorganization of thylakoids were observed in most cells (Fig. 3F). The ultrastructural change of *M. aeruginosa* cells confirmed that the cells collapsed and growth was inhibited during the treatment. Ozaki *et al.* [24] used SEM to demonstrate types of morphological changes in the cyanobacterial cells corresponding to treatment with different anticyanobacterial agents, with terpenoids being found to cause cell stripping and basic amino acids to cause swelling followed by collapse. Similarly, allelochemicals produced by bacteria, such as indole and 3-oxo-a-ionone, could damage the thylakoid membranes of cyanobacteria and consequently decrease the effective quantum yields, eventually leading to the failure of photosynthesis [28].

To analyze the anticyanobacterial extracellular substances, high-performance liquid chromatography mass spectrometry (HPLC-MS) analysis was performed according to Wang *et al.* [26]. Free amino acids of the prepared extracellular substances were also analyzed using a high-speed automatic amino acid analyzer (Hitachi 835-50; Japan). The main components of the extracellular substances of *Aeromonas* sp. strain FM determined by HPLC-MS analysis included 16 amino acids (Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, Phe, Lys), tripeptides (Asp-Glu-Gln, Tyr-Lys-Ser, Pro-Phe-Glu, His-Trp-His) and clavulanate. The lysis effects of each compound on *M. aeruginosa* (10^6 cells/ml) were tested using the corresponding pure substance, and the algicidal activity was determined as described previously [26]. The results showed that the effective concentrations for significant inhibition against *M. aeruginosa* by lysine and clavulanate were 10 mg/l and 0.4 mg/l, respectively. However, Glu, His, and Arg showed significant inhibitory effects against *M. aeruginosa* at concentrations above 100 mg/l (Table 1). No significant inhibitory effects were detected for the other components. In addition, although clavulanate and lysine could significantly inhibit the growth of cyanobacteria, neither of them could induce an increase in the soluble carbohydrates concentration of the algal culture.

Table 1. Effective concentrations of algicidal extracellular substances components

Components	Minimum concentration (mg/l) for algicidal effect*
Clavulanate	0.4
Lysine	10
Glutamic acid	>100
Histidine	>100
Arginine	>100

*Algicidal activity ≥ 20%.

Algicidal bacteria strains produce different extracellular substances targeting specific ranges of algae. *Vibrio shiloi* biosynthesized an extracellular 12-residue proline-rich peptide to inhibit photosynthesis of coral symbiotic algae [1], and another strain of *Vibrio* sp. synthesized β-cyano-L-alanine against cyanobacteria [30]. Dissolved free amino acids were found to inhibit or strongly decrease the biomass and growth rates of diatoms, which may serve as a soluble factor to induce the secretion of extracellular carbohydrates [3]. Thus, the effects of extracellular substances against *M. aeruginosa* observed in the present study were likely the result of a combined activity of different substances. The function of each component and their correlation should be investigated in future studies.

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