African Journal of Biotechnology Vol. 11(2), pp. 460-465, 5 Januaary, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2303
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Inhibition of *Microcystis aeruginosa* and microcystin-LR with one algicidal bacterium isolated from a eutrophic lake

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Accepted 16 December, 2011

Toxic *Microcystis aeruginosa* blooms have been increased in freshwater body in China in recent years. The discovery of algicidal bacteria has brought renewed attention in the treatment of the harmful algae blooms (HABs). In this study, one strain of algicidal bacterium number SDK2 was selected from a eutrophic lake and its inhibition effects on *M. aeruginosa* and microcystin-LR released by *M. aeruginosa* was studied. The algicidal activity showed that the strain SDK2 had significant algicidal efficiency on *M. aeruginosa*. The removal rate of chlorophyll a increased when the bacterial cell density increased and it reached a maximum of 80%. It inhibited *M. aeruginosa* indirectly by releasing extracellular algicidal matter. It also had good degradation effects on microcystin-LR and the removal rate was 65%. According to the physiological and biochemical characteristics and the16S rDNA sequencing analysis of the strain SDK2, it was identified as *Ochrobactrum* sp.

Key words: Removal, *Microcystis aeruginosa*, microsystins, algicidal bacterium.

INTRODUCTION

Recent years, most of the lakes and reservoirs are in eutrophic conditions in China because of the discharge of industrial wastewater and domestic sewage. One of the consequences of eutrophication is the increase of the harmful algae biomass or named harmful algae blooms (HABs), which is harmful to people's health. People who contact with polluted water containing HABs may result in uncommon symptoms and strange ailments, while animals that drink this water may cause harsh diseases (Hunter, 1988). Some approaches such as physical, chemical and biological methods have been tried in order to eliminate HABs, but it still occurred every year. Long-term solutions like decreasing nutrients of the freshwater body will take years to achieve results (Hare et al., 2005). People have found that bacteria and virus may play important roles in the prevention and termination of HABs (Ren et al., 2010). Using antagonistic microorganisms is becoming an effective method in order to eliminate water blooms (Mu et al., 2009). Various algicidal bacteria have been reported to possess the ability to inhibit algal blooms (Xavier and Farooq, 2004; Pei et al., 2005; Mu et al., 2009; Wang et al., 2010; Su et al., 2011).

Microcystis aeruginosa is the commonest strain during harmful algae blooms. It can release extracellular toxin known as microcystins after the cell death, which is harmful to people and can cause liver cancer. The microcystins are the cyclic heptapeptide toxins and very difficult to remove from the water body (Diehnelt et al., 2006; Tsuji at al., 2006). It is reported that the problem of microcystin-contaminated water bodies is increasing due to the eutrophication of lakes and reservoirs (Hernandez et al., 2009). Seldom research has reported the degradation of microcystins by algicidal bacteria.

In this study, one algicidal bacterium numbered SDK2 was isolated from a eutrophic lake. The *algicidal* experiments showed that it could inhibit and stop the

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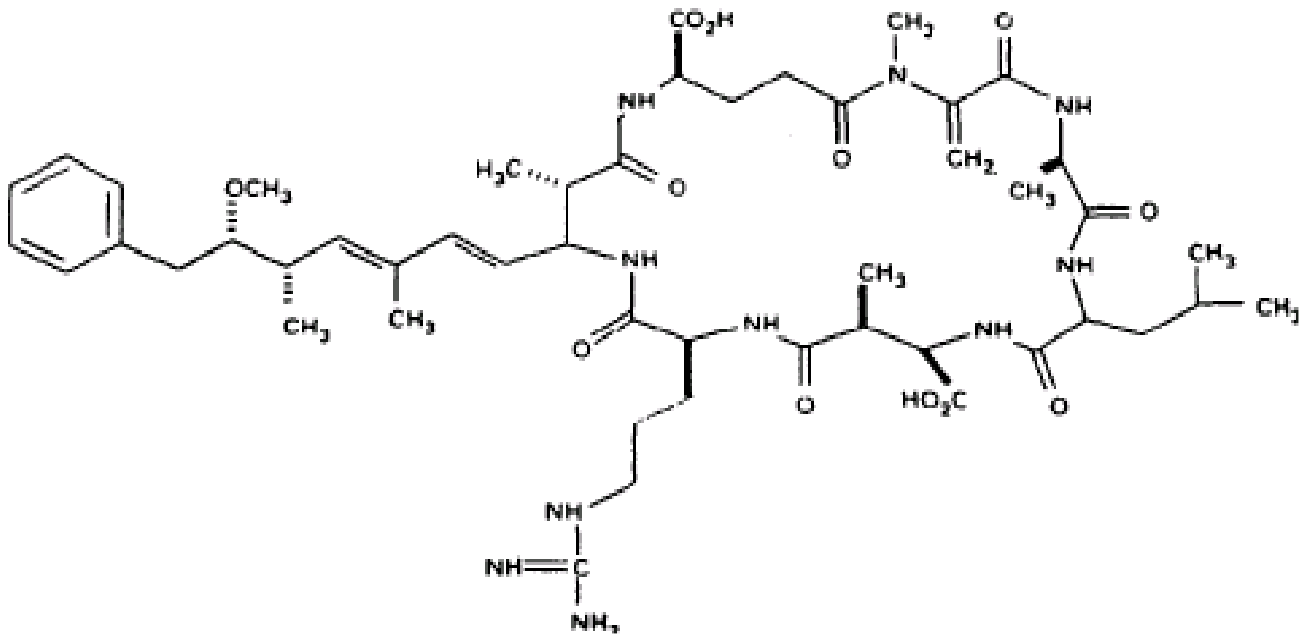

Figure 1. Structure of microcystin-LR.

growth of *Microcystis aeruginosa.* We also discovered that it was effective in degrading the microcystins-LR (Figure 1), one of the microcystins releasd by *Microcystis aeruginosa*, which was the commonest and most toxic among microcystins (Fu and Xu, 2006).

MATERIALS AND METHODS

Algal and bacterium culture

The tested algae *Microcystis aeruginosa* (No.905) was purchased from the Institute of Hydrobiology, Chinese Academy of Sciences. It was maintained at 25°C and on a 12:12 h light: dark cycle. The cultural medium was BG11 medium and it was sterilized before using. The *Microcystis aeruginosa* water samples looked deep green and smelled terrible. The tested water samples contained over 5.0×10^6 algal cells ml^{-1} and the chlorophyll a concentrations were 750 to 1100 μgL^{-1}.

The tested bacterium strain numbered SDK2 was isolated from a eutrophic lake of Shandong Province and it was maintained in LB agar medium (Shen et al., 2002).

Physiological identification

The physiological and biochemical characteristics of the strain SDK2 were identified according to Manual of Determinative Bacteriology (Dong and Cai, 2001).

PCR amplification of 16S rDNA and sequencing

The bacterial cells were lysed by heating at 100°C for 10 min, then immediately cooled on ice, centrifuged and suspended in TE buffer with Lysozyme (10 mg/ml). The total DNA was extracted with a DNA extraction kit. PCR was performed in a total volume of 50 µl containing 100 ng DNA, 10 mmol dNTP, 10 pmol each of the PCR universal primers 27F (5'-*GAG AGT TTG ATC CTG GCT CAG*-3') and 1512R (5'-*ACG GCT ACC TTG TTA CGA CT*-3') , 2 µl of PCR buffer and 2 µl Taq polymerase with 33 thermal cycles of amplification (1 min for 94°C, 1 min for 53°C, 2 min for 72°C, followed by 15 min at 72°C). The PCR product was purified and cloned into pMD 18-T vector followed by sequencing. The obtained nucleotide sequences were edited using the software clustalx 1.83 (Thompson et al., 1997).

Algicidal activity

The bacterium SDK2 was incubated on a shaker table at 200 rpm, 25°C. When it entered logarithmic phase after 14 h, the bacterial suspension was added into the water samples to test the removal effect of SDK2 on *Microcystis aeruginosa.* To testify the influence of bacterial cell density on algicidal efficiency, the bacterial suspension was diluted 5 times, 10 times and 50 times with pure water respectively and then added the water sample. Then the water samples were oscillated at 200 rpm. The chlorophyll a concentration of the 4 water samples was measured every 8 h. The chlorophyll a in the water sample was extracted with 90% acetone and measured on a UV-2100 spectrophotometer at λ = 630, 645, 663, 750 nm (Wei at al., 2002).

Mechanism of algicidal activity

To testify if the strain SDK2 inhibited *Microcystis aeruginosa* directly or indirectly and make sure the mechanism of algicidal activity, the SDK2 bacterial suspension was treated by heat (121°C), filtration (0.22-µm cellulose acetate membrane) and supersonic wave respectively and then added into the water samples. Their algicidal effects were testified after 24 h by measuring the removal rate of chlorophyll a.

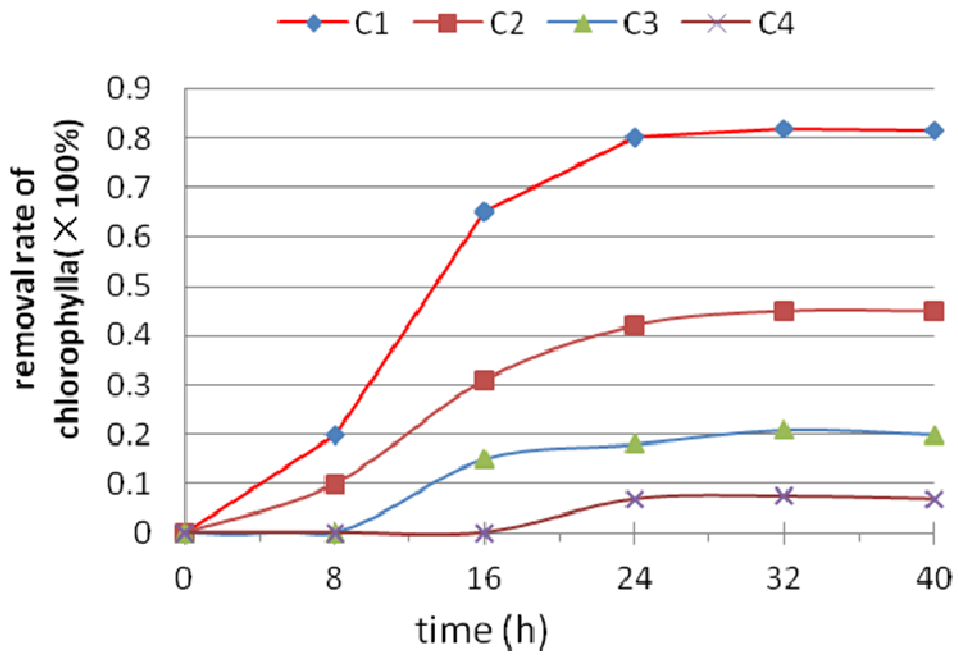

Figure 2. Removal rate of different densities of SDK2 on Microcystis aeruginosa (C1, C2, C3, C4 represented the bacterial suspension without dilution, diluted by 5 times, 10 times and 50 times respectively).

Detection of microcystins-LR

The microcystin-LR standard (Sigma Co., USA) was stored at -20℃ until required. Stock solutions were prepared by initial solubility in 1ml methanol.

The *Microcystis aeruginosa* water samples were freeze-thaw 3 cycles by liquid nitrogen to disrupt the algal cells and release microcystins. After disruption, the sample was centrifuged 8000 rpm and the supernatant was used. The microcystin-LR was detected by the HPLC (Agilent 1100) method. The water sample was carried out on a reverse SB-C18 column which was maintained at 40℃. Mobile phase was Milli-Q water (A) and methanol (B) both containing 0.1% TFA. Samples (10 µl) were separated using a gradient increasing from 15% to 60% B over 25 min at a flow rate of 0.5 ml/min followed by ramp up to 100% B and re-equilibration over the next 10 min. The microcystin-LR was quantified at 238 nm.

RESULTS

The bacterium SDK2 was determined to be Gram-negative. Its length of the PCR product was 1.5 kb. It was identified by PCR amplification of the 16S rDNA, BLAST analysis and comparison with sequences in the GenBank nucleotide database. The results show that the 16S rDNA sequence homology of strain SDK2 with one strain of *Ochrobactrum* sp. reached 99%. Together with the physiological identification, it was identified as *Ochrobactrum* sp..

The algicidal activity (Figure 2) showed that the strain SDK2 had significant algicidal efficiency on *Microcystis aeruginosa.* The color of the tested water sample turned to yellow from green rapidly after the algicidal bacterial suspension was added. The logarithmic-phase bacterial suspension could remove 80% chlorophylla of *Microcystis aeruginosa* 24 h later at 25℃ and 200 rpm. The removal rate of chlorophylla increased as bacterial cell density increased.

When the logarithmic-phase bacterial suspension was diluted, the removal rate of chlorophylla decreased clearly. When the bacterial suspension was diluted by 50 times, the removal effect was almost zero. The data also indicated that the cell density of SDK2 played an important role in inhibiting *Microcystis aeruginosa.*

Figure 3 shows that the algicidal efficiency of the strain SDK2 still existed after the bacterial suspension was treated by heat, filtration and supersonic wave and the algicidal effects did not change clearly, which illustrated that the strain SDK2 removed *Microcystis aeruginosa* not by the bacterium itself directly but indirectly inhibited the algae by releasing algicidal matter.

The result also indicated that the algicidal matter could take effect under high temperature, so they were not proteins or enzymes.

Figure 4 and 5 were the chromatograms of microcystin-LR detected by high performance liquid chromatography (HPLC), which illustrated that the strain SDK2 had good removal effect on *microcystin*-LR released by *Microcystis aeruginosa.* The microcystin-LR concentration of the control sample was 98.5 mgL^{-1} and it decreased to 34.2 mgL^{-1} four days later. The removal rate of microcystin-LR reached 65%.

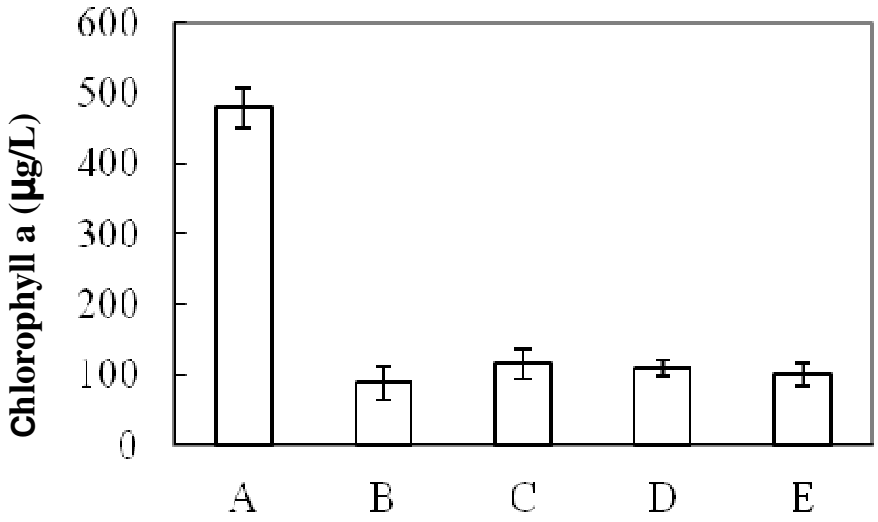

Figure 3. Algicidal efficiency of the strain SDK2 by different treatments after 24 h. A, the control water sample; B, water sample treated by bacterial suspension; C, water sample plus heat-treated (121°C) SDK2 bacterial suspension; D, SDK2 bacterial suspension filtered through 0.22-µm cellulose acetate membrane; E, water sample plus supersonic treated SDK2 bacterial suspension.

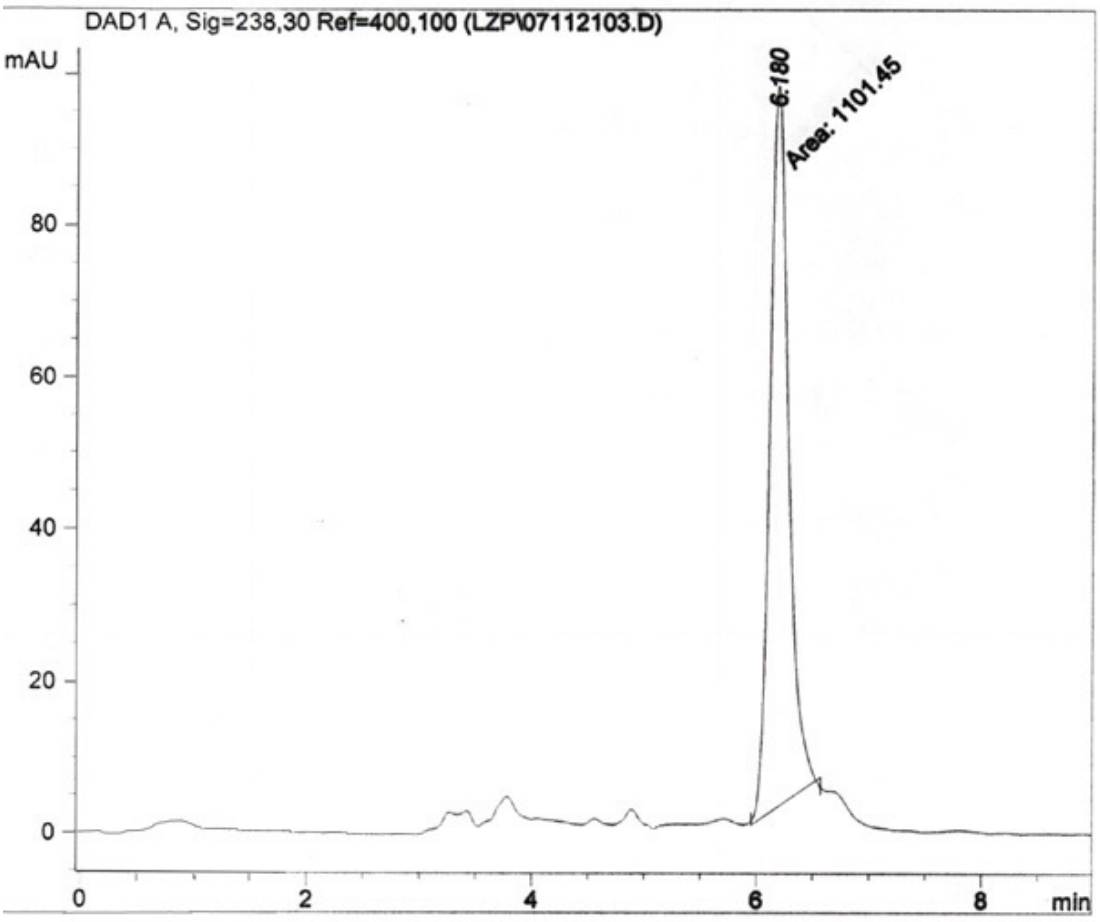

Figure 4. The chromatogram of microcystin-LR of the control sample.

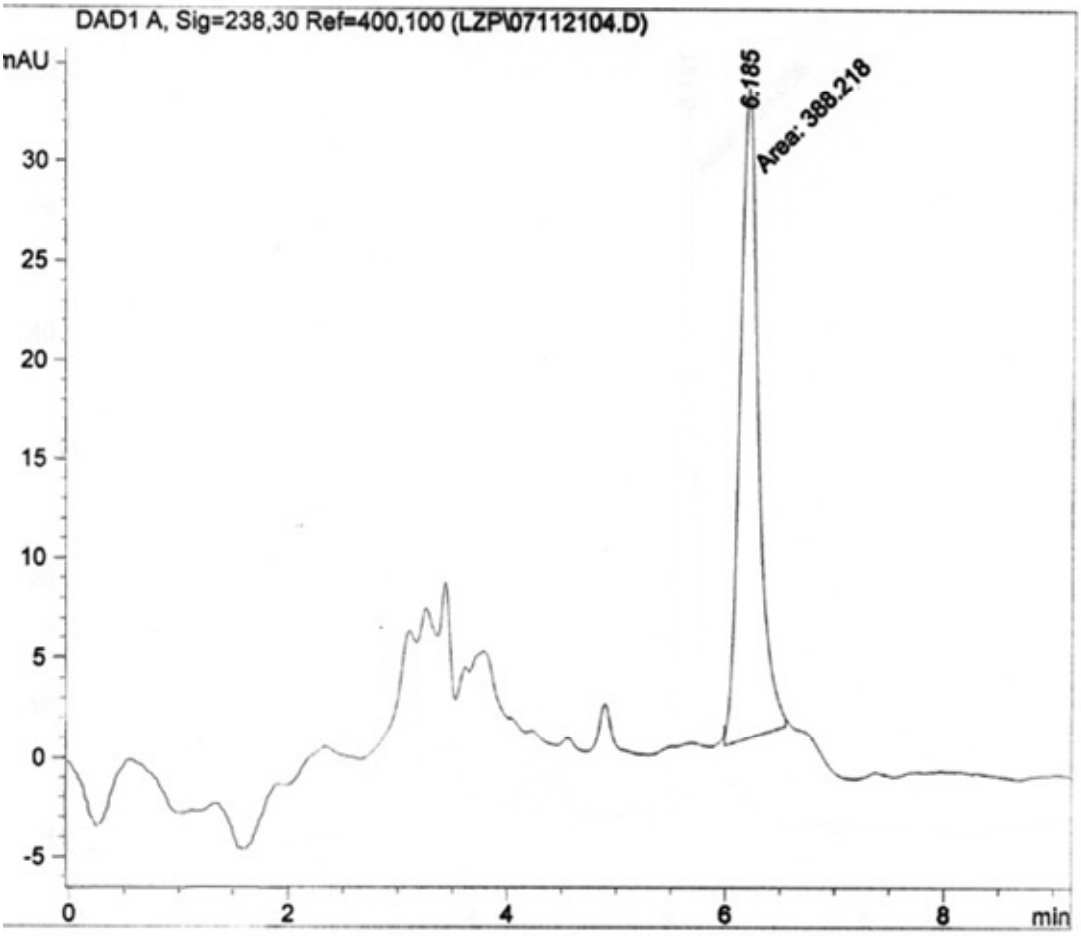

Figure 5. The chromatogram of microcystin-LR degraded by SDK2.

DISCUSSION

The characteristics of algicidal activity of the strain SDK2 illustrated that the algididal effects increased as the bacterial cell density increased. When the original bacterial suspension was diluted by 50 times, the algicidal activity nearly ended. Also, the algicidal activity was very active from 0 to 24 h, and the algicidal effects stopped increasing 24 h later. The reason was that the strain SDK2 was in a logarithmic phase during this period and its ability to reproduce and metabolism were in the highest point. Therefore, the removal of *Microcystis aeruginosa* was very clear. These results show that the removal rate of *Microcystis aeruginosa* could reach the maximum rapidly after the logarithmic-phase algicidal bacterium being added.

The results of algae-lysing mechanism revealed that the strain SDK2 inhibited the algae growth and removed chlorophylla not by itself but by releasing algicidal matter. The algicidal effects still existed after the bacterial cells were broken up by supersonic wave, which illustrated the extracellular materials were taking effect. The bacterial suspension treated by heat (121℃) was still efficient stated that the algicidal matters could endure high temperature and they were chemical compounds except protein and enzyme.

The microcystin-LR degradation experiment showed that there was a stagnation phase of 2 days and the removal rate was very little during the degradation period and the degradation effects were apparent after the stagnation phase. If the *algicidal* bacterium degraded microcystin-LR, it must break up the structure of microcystin-LR. Because the cyclic chain structure of microcystin-LR was very stable, it is very difficult for the algicidal bacterium to break it up. Therefore, the stagnation phase of 2 days was when the strain SDK2 was converting the toxic cycle structure of microcystin-LR into nontoxic linear structure. Yan (2002) reported that some enzymes took important effects during the degradation of microcystin-LR by bacteria. To testify the point, protease inhibitors were added into the bacterial suspension and the degradation experiment was duplicated. The final result was that the removal of microcystin-LR almost disappeared, which illustrated that

some protease released by the algicidal bacterium played key roles during the degradation of microcystin-LR.

ACKNOWLEDGEMENTS

We are grateful for the support of National Natural Science Foundation of China (No.30900047 and No.51078224) and State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology (No. ES201001).

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