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Isolation and algae-lysing characteristics of the algicidal bacterium B5

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Abstract

Water blooms have become a worldwide environmental problem. Recently, algicidal bacteria have attracted wide attention as possible agents for inhibiting algal water blooms. In this study, one strain of algicidal bacterium B5 was isolated from activated sludge. On the basis of analysis of its physiological characteristics and 16S rDNA gene sequence, it was identified as *Bacillus fusiformis*. Its algae-lysing characteristics on *Microcystis aeruginosa*, *Chlorella* and *Scenedesmus* were tested. The results showed that: (1) the algicidal bacterium B5 is a Gram-negative bacterium. The 16S rDNA nucleotide sequence homology of strain B5 with 2 strains of *B. fusiformis* reached 99.86%, so B5 was identified as *B. fusiformis*; (2) the algal-lysing effects of the algicidal bacterium B5 on *M. aeruginosa*, *Chlorella* and *Scenedesmus* were pronounced. The initial bacterial and algal cell densities strongly influence the removal rates of chlorophyll-*a*. The greater the initial bacterial cell density, the faster the degradation of chlorophyll-*a*. The greater the initial algal cell density, the slower the degradation of chlorophyll-*a*. When the bacterial cell density was 3.6×10^7 cells/ml, nearly 90% of chlorophyll-*a* was removed. When the chlorophyll-*a* concentration was less than 550 μg/L, about 70% was removed; (3) the strain B5 lysed algae by secreting metabolites and these metabolites could bear heat treatment.

Key words: algicidal bacteria; water blooms; algae-lysing characteristic; 16S rDNA; *Bacillus fusiformis*

Introduction

Water blooms have become a serious problem all over the world in recent years. These blooms cause significant decreases in dissolved oxygen, water transparency and recreational amenity (Kolmakov *et al*., 2002; Kang *et al*., 2005), negatively impact the drinking water supply and may cause expensive problems in water treatment plants such as filter clogging and reduced efficiency of coagulation and sedimentation. In particular, the blooms of *Microcystis aeruginosa* cause heavy damages almost every year in China and other countries. With the aim of eliminating water blooms, several approaches including physical, chemical and biological methods have been tried. But these approaches cannot resolve the problems completely and water blooms are still very serious. Therefore, research on economic and feasible approaches to algae removal has important theoretical and practical significance (Pei *et al*., 2005).

Previous studies have reported that bacteria are important algal lysing agents in many lakes (Caiola and Pellegrini, 1984; Yamamoto and Suzuki, 1977). Recent work is focusing on the identification of bacteria capable of inhibiting or degrading water blooms in marine and freshwater environments (Lovejoy and Bowman, 1998; Wu *et al*., 1998; Doucette *et al*., 1999; Imai *et al*., 2001; Manage *et al*., 2001). Bacteria which can control water blooms or red tides can be called algicidal bacteria. Algicidal bacteria, as an important fraction of biological species in hydrophytic ecosystems, play important roles in controlling water blooms and red tides and limiting algal biomass (Zhao and Liu, 1996; Lee *et al*., 2000; Imamura *et al*., 2001; Wu *et al*., 2002; Lovejoy and Bowman, 1998; Imai and Ishida, 1993; Imai *et al*., 1995; Imai and Kim, 1998; Mitsutani and Yamasaki, 2001). Some algicidal bacteria have algicidal effects and are involved in the termination and decomposition of algal blooms (Fukami *et al*., 1996). In this contribution, one strain of algicidal bacterium B5 was isolated from activated sludge and its algae-lysing characteristics on *M. aeruginosa*, *Chlorella* and *Scenedesmus* were studied. Several important parameters were obtained and the molecular sequence of the 16S rDNA was analyzed.

1 Materials and methods

1.1 Tested strain and culture medium

The tested strain, numbered B5, was isolated from activated sludge collected from a sewage treatment plant. The

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removal of total algae and chlorophyll-*a* by the activated sludge was favorable after cultivation with the tested algae for two months.

The bacterium B5 was cultured in LB medium (Shen *et al*., 2002).

1.2 Physiological identification

After Gram staining, the strain B5 was observed under a light microscope. The utilization of carbon sources by the strain B5 was tested on a 96-well microplate (Biolog, USA). According to the results of Gram staining, different test plates were chosen and the tested strain B5 was cultured in BUG+B agar culture medium. Then the B5 bacterial cell suspension was inoculated into the 96-well microplate. After it had been cultured for 24 h at 35°C, the color reaction was detected by the microbial identification system (Biolog, USA) and the data were analyzed by MicroLog4 software. The growth curve of the bacterium B5 was determined according to the reference (Shen *et al*., 2002).

1.3 PCR amplification of 16S rDNA and sequencing

Each isolate was grown on a 0.5% LB for 24–48 h. The cells were lysed by heating at 96°C for 10 min, immediately cooled on ice, centrifuged, and suspended in TE buffer with lysozyme (10 mg/ml). The total DNA was extracted using a DNA extraction kit according to the manufacturer's instructions (Genetech, China). PCR was performed in a total volume of 25 μl containing 100 ng DNA, 10 mmol dNTP, 10 pmol each of PCR the universal primers 27F (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTAC-GA-3′), 2.5 μl of PCR buffer and 4 U Taq polymerase with 30 thermal cycles of amplification (1 min for 94°C, 1 min for 55°C, 1 min for 72°C), followed by 10 min at 72°C. The PCR product was purified and cloned into pMD 18-T vector followed by sequencing which was performed by Shanghai Genetech Biotechnological Company. The obtained nucleotide sequences were edited using the software Seaview (Galtier *et al*., 1996).

1.4 Tested algae, culture medium and water samples

The algae tested were *M. aeruginosa*, *Chlorella* and *Scenedesmus*, which came from the Institute of Hydrobiology, Chinese Academy of Sciences. They were cultivated at 25°C and 3000 lx of illumination intensity after being activated.

M. aeruginosa medium (BG11 medium): $NaNO_3$ 150 mg, K_2HPO_4 4 mg, $MgSO_4{\cdot}7H_2O$ 7.5 mg, $CaCl_2{\cdot}2H_2O$ 3.6 mg, $Na_2SiO_3{\cdot}9H_2O$ 5.8 mg, citric acid 0.6 mg, Ferric ammonium citrate 0.6 mg, EDTA 0.1 mg, Na_2CO_3 2 mg, A5 solution + Co 0.1 ml, distilled water 99.9 ml (A5 solution: H_3BO_3 286 mg, $MnCl_2{\cdot}4H_2O$ 181 mg, $ZnSO_4{\cdot}7H_2O$ 22 mg, $CuSO_4{\cdot}5H_2O$ 7.9 mg, $Na_2MoO_4{\cdot}2H_2O$ 3.9 mg, distilled water 100 ml).

Chlorella and *Scenedesmus* medium (SE medium): $NaNO_3$ 25 mg, $CaCl_2$ 2.5 mg, $MgSO_4{\cdot}7H_2O$ 7.5 mg, K_2HPO_4 7.5 mg, KH_2PO_4 17.5 mg, NaCl 2.5 mg, soil extraction 4 ml, $FeCl_3{\cdot}6H_2O$ 0.5 mg, Fe-EDTA 0.1 ml, A5 solution 0.1 ml, distilled water 95.8 ml.

The water samples of cultured algae looked deep green and smelled terrible. Water temperature ranged 16–24°C. The pH was 7.0. The algal cell density was $(1–6)\times10^5$ cells/ml and the chlorophyll-*a* concentration was 700–1000 μg/L. The water samples were used after being diluted. The algal cell density was determined by a hemocytometer under a microscope. The chlorophyll-*a* of the water sample was extracted with 90% acetone and measured with a UV-2100 spectrophotometer at λ=630, 645, 663, 750 nm (Wei *et al*., 2002).

1.5 Degradation test of the bacterium B5 on algae

The algicidal effect of the bacterium B5 on algae was evaluated by determining the decrease in chlorophyll-*a* concentration of the water samples.

1.5.1 Influence of bacterial cell density on algicidal efficiency

The bacterium B5 was incubated in liquid LB medium at 30°C overnight when it reached logarithmic growth phase. The bacterial cell density was 3.6×10^8 cells/ml which was determined by the spread plate method (Adams, 1959). After being diluted with sterilized LB medium, 20 ml of B5 bacterial cell suspension, whose cell density was 0 (C1), 7.2×10^5 (C2), 7.2×10^6 (C3), 3.6×10^7 cells/ml (C4), respectively, confected the 4 water samples whose chlorophyll-*a* concentration were 412.3 μg/L. Then the 4 water samples were oscillated at 200 r/min. The chlorophyll-*a* concentration of the 4 water samples was measured every 24 h.

1.5.2 Influence of algal cell density on algicidal efficiency

Samples of 20 ml of 5.6×10^6 cells/ml bacterial culture were added to the 4 water samples containing algal cells at differing densities. The water samples were oscillated at 200 r/min. The chlorophyll-*a* concentration was measured every 24 h.

1.6 Mechanism of algal lysis

To test the algae-lysing mode of the bacterium B5, 50 ml of B5 bacterial cell suspension, 50 ml of heat-treated (121°C) B5 cell suspension and 50 ml of B5 cell suspension filtered with 0.22-μm cellulose acetate membrane were poured into three identical water samples. Their algicidal effects were testified after 24 h by measuring the removal of chlorophyll-*a*. Chlorophyll-*a* was extracted from the water samples with 90% acetone and measured on the UV-2100 spectrophotometer at λ=630, 645, 663, 750 nm.

2 Results and discussion

2.1 Physiological identification

The bacterium B5 was determined to be Gram-negative (Fig.1). It was a white and elliptical floccule in liquid LB. It can move and its cell size is (0.6–1.0)×4.0 μm. The results of its utilization of 95 carbon sources are shown in Table 1.

Table 1 Utilization of 95 carbon sources by the bacterium B5

Carbon source	Utilization	Carbon source	Utilization	Carbon source	Utilization
Water	0	Glycogen	V	Soil temperature of 80	–
l-Arabinose	–	Fructose	–	Gentiobiose	–
α-*d*-Lactose	–	*d*-Mannose alcohol	–	α-Methyl-*d*-galactosi	–
β-Methyl-*d*-glucopyranoside	–	*d*-Raffinose	–	Sedoheptulose	–
d-Tagatose	–	*l*-Malic acid	–	γ-Hydroxybutyrate	–
Lactamide	–	*d*-Xylose	–	*a*-Keto-gluconic acid	+
l-ALT	–	*l*-Asparagine	–	*l*-Serine	–
Adenosine	V	Floxuridine	V	*d*-Fructose-phosphate	–
Cyclodextrin	–	Inulin	–	N-acetyl-*d*-glucosamine	–
Arab sugar alcohol	–	*l*-Fucose	V	*d*-Gluconate	–
Lactulose	–	*d*-Mannose	–	β-Methyl-*d*-galactosi	–
α-Methyl-*d*-mannosidase	–	*l*-Rhamnose	–	*d*-Sorbitol	–
d-Fucose	–	Acetic acid	–	*p*-Hydroxy-phenylacetic acid	–
d-Methyl lactate	–	Methyl acetone	–	Succinic acid	–
d-Alanine	–	*l*-Glutamic acid	–	Putrescine	–
2′-Deoxyadenosine	V	5′-AMP	V	α-*d*-Glucose-phosphate	–
l-Histidine	+	Mannan	V	Mannosamine	V
Arbutin	–	*d*-Galactose	–	*l*-Ornithine	+
Maltose	–	*d*-Melezitose	–	β-Methyl-glucose	–
Paltinose	–	*d*-Ribose	V	Sugar water Su	–
Turanose	–	α-Hydroxyhutyrate	–	Succinic acid	+
l-Lactic acid	–	Monomethyl succinate	–	α-Ketogluconic acid	V
l-Alanine	–	Glycyl-*l*-glutamic acid	–	2,3-Butanediol	–
Inosine	+	5-Thymidine-phosphate	–	*d*-Glucose-6-phosphate	–
Dextrin	–	*l*-Proline	+	Amygdalin	–
Cellobiase	–	*d*-Galacturonic acid	–	*m*-Inositol	V
Maltotriose	–	*d*-Melibiose	–	α-Methyl-*d*-glucoside	–
d-Allulose	–	Salicylaldehyde	–	Saccharose	–
Xylitol	–	Alanine	–	α-Aminolevulinicaid	V
d-Malic acid	–	β-Hydroxybutyrate	V	N-acetyl-*l*-glutamic acid	V
l-Alanyl-glycin	–	*l*-Pyroglutamic acid	–	Glycerine	V
Thymidine	+	5′-Floxuridine-phosphate	V	*d*-*l*-α-Glycerophosphate	–

+: positive; –: negative; V: neutral.

The bacterium B5 could utilize such carbon sources as *l*-histidine, inosine, thymidine, *l*-proline, *a*-keto-gluconic acid, *l*-ornithine and succinic acid. It showed relatively weak use of 16 carbon sources including adenosine, 2′-deoxyadenosine, glycogen, floxuridine, etc. The other 72 carbon sources could not be used.

The growth curve of the bacterium B5 in liquid LB culture medium (Fig.2) showed that it entered logarithmic growth phase after 4 h of lag phase. Its stationary phase was from 16 to 28 h and then it entered decline phase.

2.2 Sequence analysis of 16S rDNA and molecular identification

The length of the PCR product of the bacterium B5 was 1.5 kb (Fig.3). The sequence obtained is available in GenBank under accession number AY822613. The 16S rDNA sequence homology of strain B5 with many strains of *Bacillus* all reached above 99.7%. In particular, the 16S rDNA sequence homologies of strain B5 with 2 strains of *Bacillus fusiformis* reached 99.86%. Together with the physiological identification, B5 was identified as *Bacillus fusiformis*.

2.3 Influence of bacterial cell densities on algicidal efficiency

The influence of bacterial cell density on algal lysis is shown in Fig.4. Results showed that the strain B5 had good algicidal efficiency and the removal rates of chlorophyll-*a*

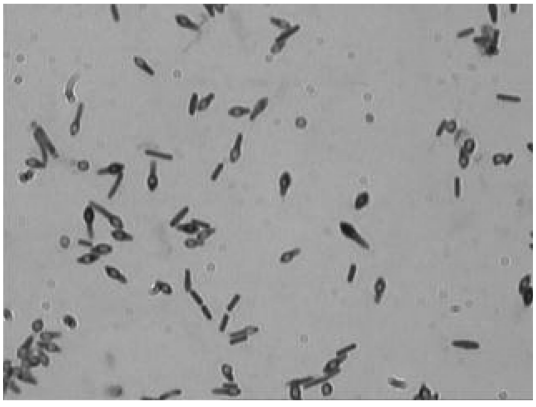

Fig. 1 Micrograph of Gram-stained bacterial strain B5.

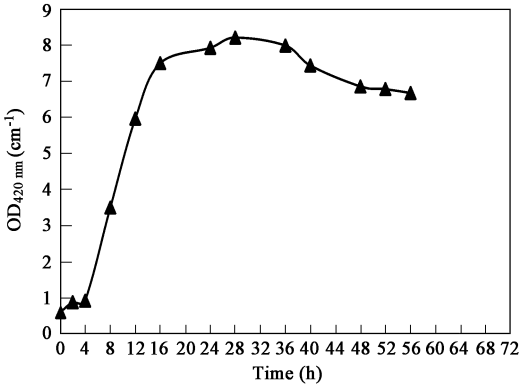

Fig. 2 Growth curve of the bacterium B5.

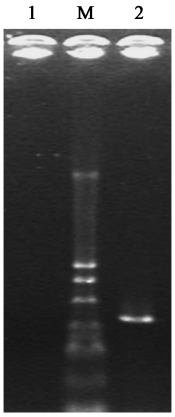

Fig. 3 Amplification of B5 16S rDNA. M is the marker and the molecular weights are 9, 4, 3, 2.1, 0.8, 0.6, 0.3 and 0.2 kb. (1) negative control; (2) strain B5.

increased as bacterial cell density increased. When the bacterial cell density was 7.2×10^6 cells/ml, over 70% of chlorophyll-*a* was removed. When the bacterial cell density was 3.6×10^7 cells/ml, nearly 90% of chlorophyll-*a* was removed. Initially, the amount of chlorophyll-*a* decreased very quickly. On the first day, the removal of chlorophyll-*a* of the four samples was 3.85%, 45.65%, 58.92% and 73.12%, respectively. With the experiment continued, the removal velocity slowed, which may be connected with the activity of the strain B5 (Zhou and Gao, 2000). The strain's growth curve showed that it reached stationary phase after 16 h. On the first day, the strain was in the logarithmic growth phase, and its metabolic velocity was fast, so the algicidal efficiency was high. When the strain entered lag phase and stationary phase, its activity decreased and death velocity was higher than growth velocity. Therefore, the removal rate of chlorophyll-*a* was reduced.

2.4 Influence of algal cell densities on algicidal efficiency

The influence of algal cell density on algal lysis is shown in Fig.5. Results showed that the reduction of chlorophyll-*a* was considerable and the removal rates decreased as algal cell densities increased. When the chlorophyll-*a* concentration began at more than 650 μg/L, about 60% was removed. When the chlorophyll-*a* concentration began at less than 550 μg/L, about 70% was removed. When the chlorophyll-*a* concentration began at less than 100 μg/L, as much as 80% was removed. The chlorophyll-*a* concentration in natural water blooms is often less than 100 μg/L, so the strain B5 could be of great practical significance in treating water blooms. The removal rate of chlorophyll-*a* was high on the first day then slowed down in a manner consistent with the decrease in bacterial growth as shown in Fig.2. This indicated that good removal of chlorophyll-*a* could be obtained during a short period. Therefore, the reaction cycle could be shortened to save total investment during practical water treatment.

2.5 Mechanism of algal lysis

As shown in Fig.6, separate B5 bacterial suspensions were given no treatment, or heat-treated, or filtered, and then mixed with water samples containing algae. Neither treatment had a great effect on the extent of algal lysis. Therefore algal lysis does not depend on the bacteria itself, but is affected by secreted metabolites. These metabolites can withstand high pressure and temperature.

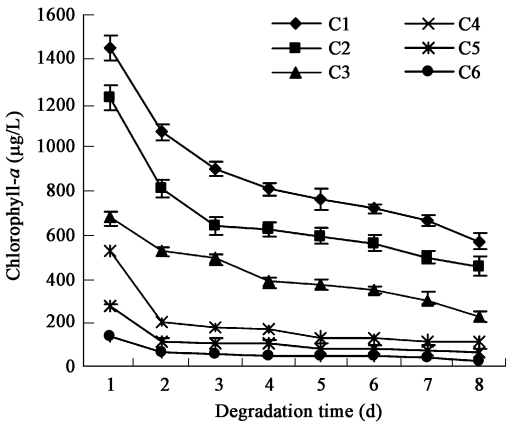

Fig. 5 Chlorophyll-*a* removal rate of B5 at different algal cell densities. Data are mean±SD from at least three independent assays. C represents different chlorophyll-*a* densities (μg/L) (C1: 1445; C2: 1219; C3: 683; C4: 524; C5: 274; C6: 134).

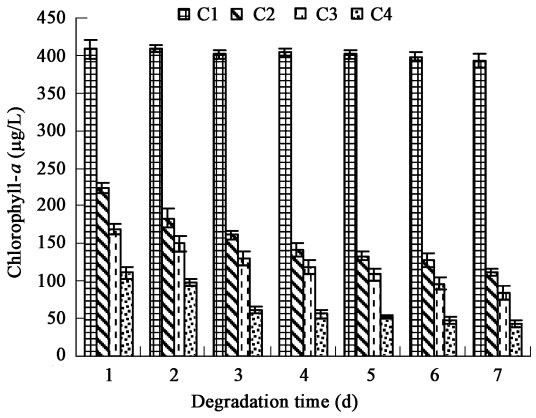

Fig. 4 Chlorophyll-*a* removal rate of B5 at different bacterial cell densities. C represents bacterial cell densities (cell/ml) (C1: 0; C2: 7.2×10^5; C3: 7.2×10^6; C4: 3.6×10^7); data are mean±SD from at least three independent assays.

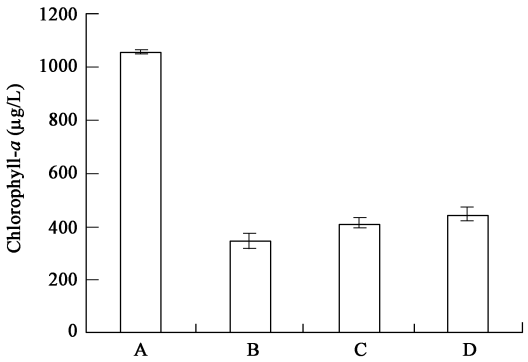

Fig. 6 Effects of different treatments of bacterial cultures on algal lysis. Data are mean±SD from at least three independent assays. (A) original water sample; (B) water sample plus B5 cell suspension; (C) water sample plus heat-treated (121°C) B5 cell suspension; (D) water sample plus B5 cell suspension filtered through 0.22-μm cellulose acetate membrane.

3 Conclusions

The algicidal bacterium B5 isolated from activated sludge had pronounced algicidal effects. Physiological identification showed that the bacterium B5 was Gram-negative and could utilize 7 carbon sources. On the basis of analysis of 16S rDNA sequence and physiological identification, the bacterium B5 was identified as *Bacillus fusiformis*. The tests of the degradation of chlorophyll-*a* in algae by the bacterium B5 indicated that the bacterial and algal cell densities had strong influences on the removal rates of chlorophyll-*a* in water samples. The results of the experiment to determine the mechanism of algal lysis showed that the bacterium B5 lysed algae and removed chlorophyll-*a* by secreting metabolites and these metabolites could bear heat treatment (121°C).

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