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Inhibition of *Microcystis aeruginosa* using *Brevundimonas* sp. AA06 immobilized in polyvinyl alcohol-sodium alginate beads

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ABSTRACT

Application of algicidal bacteria is a potential bio-technique to inhibit harmful algal growth and remove algal blooms. At present, algicidal bacteria are mainly used in the form of freely suspended cells. In this study, the bacterium *Brevundimonas* sp. AA06 against *Microcystis aeruginosa* was isolated from soil and the algicidal effects of immobilized strain AA06 were investigated. The spherically immobilized beads (ϕ = 3.0~4.0 mm) were prepared by polyvinyl alcohol (PVA) and sodium alginate (SA). Scanning electron microscopy (SEM) images of polyvinyl alcohol-sodium alginate (PVA-SA) beads showed that there were a large number of pores inside and that the bacterial cells adhered to the skeleton. Cyanobacterial dose-relationship tests showed that the PVA-SA beads exhibited weaker algicidal effects than freely suspended cells. At an initial *M. aeruginosa* cell density of 2.0 × 10^9 cells/L, PVA-SA beads inhibited the growth of *M. aeruginosa* for up to 60 days, which was much longer than freely suspended cells (34 days). When *M. aeruginosa* was exposed to PVA-SA beads at $EC_{70,96h}$ (concentrations for 70% of maximal algicidal effect after 96 h), the total microcystin-LR (MC-LR) concentrations (intra and extra cellular) remained stable at 40.0±5 μg/L, with intracellular MC-LR concentrations declining.

Keywords: Algicidal bacteria; *Brevundimonas* sp.; Immobilization; *Microcystis aeruginosa*

1. Introduction

Harmful algal blooms (HABs) have become a worldwide aquatic environmental problem due to increasing water eutrophication and climate change [1]. They destroy the stability of the ecosystem and threaten human health [2], particularly those blooms caused by toxic algal species [3]. The most frequently reported toxic bloom-forming organism in freshwater is *M. aeruginosa*, which is capable of producing cyclic peptide hepatotoxins referred to as microcystins [4]. It is well known that microcystins can bioaccumulate in aquatic animals and can be transferred along the food web to high trophic levels, including human beings [5]. In order to inhibit HABs, reducing external nutrient inputs to aquatic ecosystem is the ultimate strategy. Meanwhile, it is also necessary to treat or remove harmful algae when they overgrow. To date, many measures have been implemented, including physical (e.g. clays or flocculants) and chemical (e.g. copper sulfate or sodium hypochlorite)

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methods [6]. However, biological methods receive more attention due to their eco-friendliness and efficiency [7].

Biological methods of HAB-control include utilization of inhibitory phytoplankton, protozoans, and microorganisms [8]. Among microorganisms, including virus [9], protozoa [10], and bacteria [11], bacteria have complex relationships with the algae that cause HABs. Some bacteria can supply cobalamin for algal growth [12], and, in turn, they get nutrients from algae. In contrast, other bacteria can compete with algae for nutrients [13], and even produce secondary metabolites with a diverse range of algicidal activities that can inhibit or kill algae cells [14]. In consequence, algicidal bacteria from the environment have been isolated and identified [15], such as *Acinetobacter* sp. J25 [16], *Pseudomonas asplenii* [17], and *Raoultella* sp. R11 [18]. At present, algicidal bacteria are mainly used in the form of free suspended cells. However, freely suspended cells are easily washed away or eaten by other organisms in water.

Cell immobilization is a beneficial measure that can overcome the problems posed by free suspended cells [19]. It can efficiently avoid loss of bacterial cells and improve the utilization rate to produce a high cell density. It also provides bacteria with a stable growth environment by mitigating adverse factors [20]. Among the cell immobilization techniques, the embedded immobilization technique is a new and rapidly-developing method in the biological engineering field [21,22]. It is characterized by direct coating with immobilization carrier, thus the operation is relatively simple and has little effect on microbial activity [23]. When using the embedded immobilization technique, the gel matrices are very important to immobilization. Polyvinyl alcohol (PVA) is able to form strong gels in boric acid solution [24], resulting in a polymer matrix system that has good strength and flexibility. This method not only solves the difficulty of bead-forming, but also decreases the loss of microbial activity during preparation.

Indeed, micro-organism immobilization techniques have been extensively applied in industrial processes for production of alcohols (e.g. ethanol, butanol, isopropanol) [25], bioremediation of toxic chemicals (e.g. phenol, 4-chlorobenzoate, pyridine, 2,4-dichlorophenoxyacetic acid, naphthalene) [26] and wastewater treatment [27]. Thus, immobilization of algicidal bacteria can be an attractive alternative for freely suspended cells. Moreover, many previous studies have only studied the short-term algal inhibition effects,the microalgal inhibition time designed is usually about 2, 6, or 15 d. However, the duration of algal bloom is about two months in natural water body. To date, few studies have focused on the immobilization of algicidal bacteria to inhibit harmful algal blooms during long-term algicidal procedure, even though this technique might have potential to continuously inhibit algal blooms for a long time.

In the present study, a new strain of *Brevundimonas diminuta* AA06, which exhibited algicidal activity against *Microcystis aeruginosa*, was isolated and identified. In order to further improve the application of the strain AA06, thereby promoting sustainable inhibitory activity against *M. aeruginosa*, polyvinyl alcohol-sodium alginate (PVA-SA) beads were prepared. The algicidal effects of PVA-SA beads and freely suspended cells were investigated in long-term experiments. Meanwhile, the morphological variation of PVA-SA beads was measured during the algicidal treatment using scanning electron microscopy (SEM). The release of microcystin-LR (MC-LR) was also studied.

2. Materials and methods

2.1. Chemicals and materials

Polyvinyl alcohol (normal degree of polymerization = 1750, molecular weight 75,000–80,000) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Sodium alginate (SA) was obtained from Xiya Chemical Industry Co., Ltd. (Shandong, China). Beef extract and peptone (biochemical reagent grade) were purchased from Aoboxing Bio-tech Co., Ltd. (Beijing, China). $CaCl_2$, H_3BO_3, sodium citrate, $NH_4H_2PO_4$, $MgSO_4$, and K_2HPO_4 were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Methanol, acetonitrile and trifluoroacetic acid (TFA) were HPLC grade and were purchased from Xiya Chemical Industry Co., Ltd. (Shandong, China). The MC-LR standard solution was prepared by dissolving solid MC-LR (Express Technology Co., Ltd., Beijing, China). All reagents were of analytical reagent grade and used without further purification, except for beef extract and peptone.

2.2. Algal culture

M. aeruginosa (FACHB-905) was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). Algal cells were cultured in sterilized 500 mL glass flasks containing 250 mL BG-11 media prepared with sterilized distilled water. The flasks were incubated at 30±0.5°C under illumination (28 μmol/m²/s) with a 14-h light/10-h dark cycle.

2.3. Isolation and identification of algicidal bacteria

Soil samples were collected from a garden (40°0′17″N, 116°20′27″E) at the Beijing Forestry University and immediately transferred into sterile bottles. Using the screening approach with slight modifications [28], strain AA06, which showed strong algicidal activity against *M. aeruginosa* 905, was isolated from the samples and selected for further study. The purified isolate was obtained after being re-streaked three times onto beef extract peptone agar plates.

Bacterial chromosomal DNA was extracted. The 16S rDNA was amplified by PCR using primers 27F and 1492R [29]. The PCR products were sequenced on an ABI-Prism 3730 automated sequencer (PE Applied Biosystems, USA). The 16S rDNA sequence of strain AA06 was aligned with 7 sequences of related organisms that were retrieved from the GenBank database using the BLAST algorithm. Sequence alignment was conducted with Clustal X software [30], and the neighbor-joining phylogenetic tree was constructed using Bioedit and MEGA 7.0 [31].

The strain AA06 was cultured for 96 h at 37°C in modified medium (sodium citrate, $NH_4H_2PO_4$, $MgSO_4$, and K_2HPO_4 were added to distilled water at a final concentration of 0.5, 1.0, 0.2, and 0.5 g/L, respectively). During the following experiments, bacterial cultures were diluted with sterilized water to an optical density at 600 nm (OD_{600}) of 1.00.

2.4. Preparation of algicidal PVA-SA beads

The preparation method for the immobilized *Brevundimonas* sp. AA06 was shown in Fig. 1. For immobilization of strain AA06, 10.0 g PVA was dissolved in 100 mL distilled water at 80°C. Then, 0.5 g SA was added and stirred until the solution became homogenous. The PVA-SA solution was sterilized at 121°C for 30 min and cooled to room temperature. The bacterial culture (OD_{600} = 1.00) was mixed thoroughly with the PVA-SA solution at 1:2 ratio (v/v). The bacterial mixture was aliquoted into a syringe, dropped through a needle into the saturated H_3BO_3 solution containing 20.0 g/L $CaCl_2$, and immersed for 2 h to form spherical PVA-SA beads with a diameter of 3.0–4.0 mm. The formed PVA-SA beads were kept in the solution at 4°C for 24 h to complete the gelation process, and then washed several times with distilled water.

2.5. Characterization of PVA-SA beads

The porous structure and surface morphology of the PVA-SA beads were characterized using SEM (SU-8010, Hitachi, Japan) at various magnifications. The PVA-SA beads were rinsed with 0.1 mol/L phosphate buffer three times, and then fixed with 2.5% glutaraldehyde solution for 12 h at 4°C. Subsequently, the samples were washed and dehydrated in a graded series of ethanol solutions (30%, 50%, 70%, 80%, 90% and 100%). The dewatered samples were dried by the critical point method and prepared for SEM observation.

2.6. Dose-response relationship

The tests were conducted using the 96-h growth inhibition bioassay. Algae samples were exposed to different serial concentration gradients (e.g. 6.68×10^5, 1.04×10^6, 1.51×10^6, 2.17×10^6, 3.03×10^6, 4.82×10^6, 7.08×10^6, 1.38×10^7, 2.20×10^7, 3.63×10^7, 7.15×10^7, 1.35×10^8, 2.09×10^8, 4.92×10^8, 6.68×10^8 CFU/mL) of PVA-SA beads and freely suspended cells. The algae samples without any PVA-SA beads or freely suspended cells were used as the control group. Each treatment was performed in triplicate, with 250 mL algae cultures in 500 mL flasks. The initial algal cell density of each treatment was 2.0×10^9 cells/L. The cultures were incubated under the same conditions described above. Algal cell density was measured daily by counting cell numbers with the microscope (BX51, Olympus, Japan) and a hemocytometer. The 96-h median effective concentrations (EC_{20}, EC_{50}, EC_{70}, and EC_{90}) were calculated by the probit unit method.

2.7. Algicidal inhibition experiments

To compare the difference between PVA-SA beads and freely suspended cells in algal growth during long-term algicidal procedure, the exposure of *M. aeruginosa* was operated using a sequencing batch mode. The effective concentrations (EC_{20}, EC_{50}, EC_{70}, EC_{90}) of PVA-SA beads and freely suspended cells were added to 500 mL flasks containing 250 mL algae cultures (~2.0×10^9 cells/L). When the algal cell density was less than 1.0×10^8 cells/L during algicidal treatment, the concentrated fresh *M. aeruginosa* (~5.0×10^{10} cells/L) were added to the algae-bacteria coculture, resulting in a final cell density of ~2.0×10^9 cells/L. There were no dosing of PVA-SA beads or freely suspended cells in the following 60 d of algicidal treatment. Algal cultures without addition were used as control samples. The cell density of samples was measured daily and the inhibitory rate (*IR*%) was calculated using Eq. (1):

$$IR(\%) = \frac{C_{control} - C_{treatment}}{C_{control}} \times 100 \quad (1)$$

where $C_{control}$ and $C_{treatment}$ are the cell density of the control samples and treated samples on the corresponding days, respectively [32]. All experiments were conducted in triplicate. Results are reported as the mean ± S.D.

2.8. MC-LR variation in the algicidal procedure

PVA-SA beads and freely suspended cells were added into a 1.2 L algae cultures (2.0×10^9 cells/L) at EC_{70}. Sam-

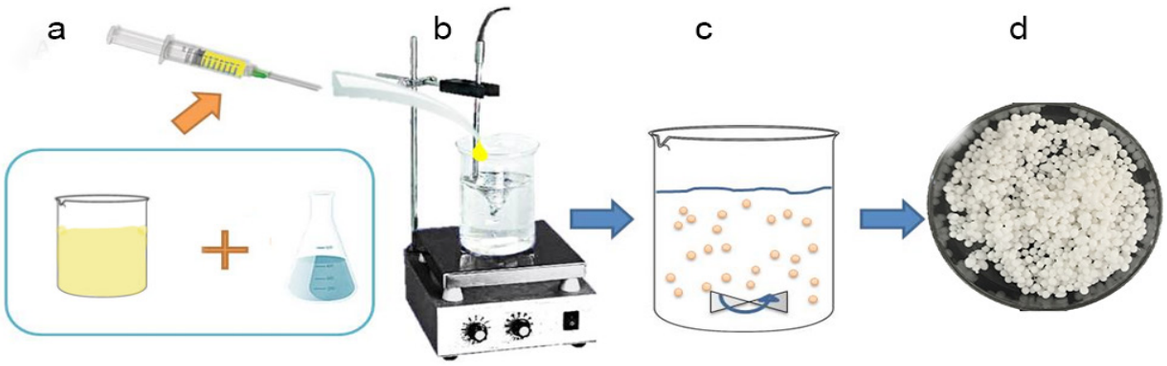

Fig. 1. Schematic preparation of PVA-SA beads. (a) The bacterial culture was mixed thoroughly with the PVA-SA solution at 1:2 ratio (v/v). (b) The beads were obtained by dropping the mixture into the saturated H_3BO_3 solution containing 20.0 g/L $CaCl_2$ using a syringe with a blunt ended needle at a mixture speed of 500 rpm. (c) They were kept for 2 h in the saturated H_3BO_3 solution containing 20 g/L $CaCl_2$ at this mixture speed, and then were kept at 4°C for 24 h. (d) Beads were then collected, rinsed for several times with distilled water.

ples were incubated under the same conditions described above. Aliquots (100 mL) of the suspensions were sampled on days 0, 2, 4, 8, 16, 24, and 30. Intracellular and extracellular MC-LR were extracted according to the methods of Liang [33]. The MC-LR was measured by HPLC (Agilent 1200 series, Agilent Technologies, Waldbronn, Germany) with the Hyper ODS-2 C18 4.6×250 mm column at a monitoring wave length of 238 nm. Mobile phase A consisted of MilliQ water containing 0.05% (v/v) trifluoroacetic acid (TFA) and mobile phase B was acetonitrile. The flow rate was 1 mL/min.

3. Results and discussion

3.1. Characterization and identification of strain AA06

A total of 75 bacterial strains were isolated from the garden soil samples. Strain AA06 exhibited the strongest algicidal activity against *M. aeruginosa*, and strain AA06 was deposited in the China General Microbiological Culture Collection Center (CGMCC), with accession number of CGMCC11571. The bacterial cells were Gram-negative, catalase positive and could produce NH_3. Strain AA06 could grow at temperatures ranging from 15 to 40°C, with an optimal temperature of 30–37°C. Phylogenetic analysis of strain AA06, based on the 16S rRNA gene sequence (1318 nt), indicated that strain AA06 belonged to the genus *Brevundimonas diminuta* (Fig. 2), sharing the highest similarity of 100%.

3.2. Dose-response relationship

The algal inhibition effects of PVA-SA beads and freely suspended cells were investigated. *M. aeruginosa* exhibited a typical dose response in cell density inhibition with increasing concentrations of freely suspended cells and PVA-SA beads (Fig. 3). According to the estimation from the dose-response curve, the EC_{50} value of freely suspended cells against *M. aeruginosa* (2.0×10^9 cells/L) was 1.37×10^7 CFU/mL. Among the currently isolated algicidal bacteria, the EC_{50} value against *M. aeruginosa* (5.0–8.2×10^9 cells/L) ranged from 3.0×10^5 CFU/mL to 2.0×10^7 CFU/mL [34]. The EC_{50} value of freely suspended cells showed slightly weaker algicidal activity compared with other reported species, namely *Aeromonas* sp. strain GLY-2107 [35], and *Stenotrophomonas* F6 [36]. Because on average one PVA-SA bead embedded freely suspended cells at ~2.4×10^4 CFU, the EC_{50} value of the PVA-SA beads was equivalent to 2.18×10^7 CFU/mL, which exhibited weaker algicidal effects than freely suspended cells. According to the dose response relationship, different effective concentrations (EC_{20}, EC_{50}, EC_{70}, and EC_{90}) of PVA-SA beads were selected for long-term algicidal assays, namely 7.06×10^6, 2.18×10^7, 4.67×10^7 and 2.39×10^8 CFU/mL, respectively. In order to compare this with freely suspended cells, the EC_{20}, EC_{50}, EC_{70}, and EC_{90} of freely suspended cells were also investigated, namely 4.11×10^6, 1.37×10^7, 2.87×10^7, and 1.12×10^8 CFU/mL, respectively.

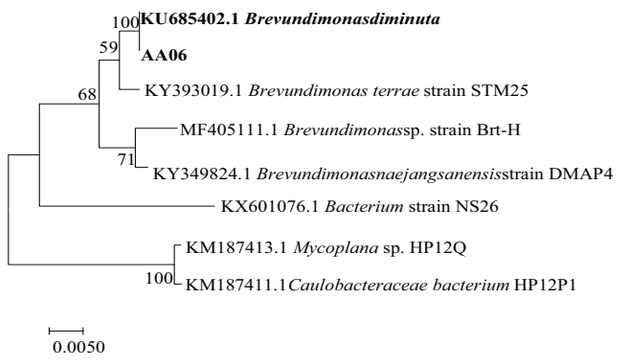

Fig. 2. Neighbor-joining phylogenetic tree of *Brevundimonas diminuta* strain AA06 and other related species on the basis of 16S rDNA sequences. Bootstrap values (percentages of 1,000 replications) were shown at the branch points. Scale bar = 0.0050 substitutions per nucleotide position (evolutionary distance).

3.3. Morphological variation of AA06 beads

The morphological characteristics of strain AA06 and PVA-SA beads were shown in Fig. 4. Colonies of strain AA06 on agar media had entire edges and were yellow, shiny, and compact (Fig. 4a). The fresh prepared PVA-SA beads were creamy white, smooth, spherical, and homogenous, with diameters ranging from 3.0 to 4.0 mm (Fig. 4b). The specific gravity of wet beads was approximately 1.03 g/cm^3; thus, they sank to the bottom of water. After washing and drying, the beads became rough and smaller.

Morphological examination via SEM revealed that strain AA06 was a rod-shaped bacterium without flagellum, at a width of 2.0–5.0 μm and a length of 5.0–15.0 μm (Fig. 4c). The SEM image depicted in Fig. 4d shows that the beads had a rough and relatively dense surface, which could ensure that the hole was large enough to maintain normal bacterial physiology and metabolism while avoiding large pores to reduce bacterial leakage. The freshly prepared beads possessed a large number of pores observed inside filament structures (Figs. 4e, f),

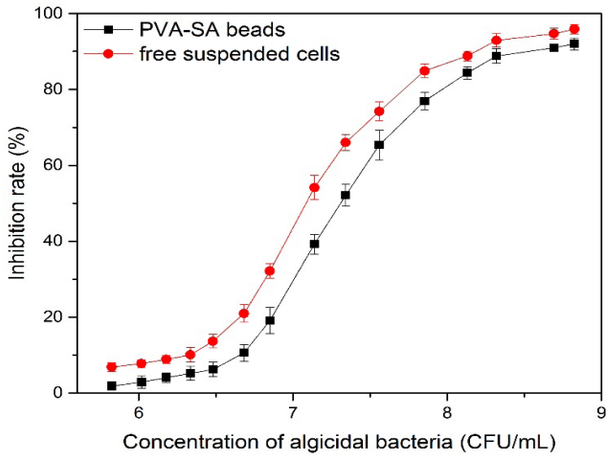

Fig. 3. Algicidal effects of freely suspended cells and PVA-SA beads on the growth of *M. aeruginosa* with the initial cell density of 2.0×10^9 cells/L. *M. aeruginosa* was incubated at 30 ± 0.5°C, 28 μmol/m^2/s, and a 14-h light/10-h dark cycle for 96 h.

providing a convenient channel for matter transmission. In addition, AA06 cells was attached to the pore walls (Fig. 4f). Attachment of algicidal bacteria to the filamentous substrate could make the AA06 cells grow better after immobilization in the beads. The large interspace of the carrier material structure allowed for good growth of strains and maintained a high degree of cell viability, meaning that AA06 cells could be successfully embedded in the gel carrier. On the other hand, the reticular structure had a huge specific surface area to provide more reaction sites, which promoted high efficiency between algae cells and algicidal substance.

SEM images of PVA-SA beads inhibiting algae cells at EC_{90} were used to detect the morphology variation during the algicidal procedure. To the naked eye, PVA-SA beads became larger after 15 d, but still remained spherical. SEM images of the beads immersed in the algae suspension for 15 d indicated that the surface of the beads was coarse and uneven (Fig. 4f). PVA-SA beads had a stable microstructure in intersecting surface (Fig. 4h), and some AA06 cells remained attached to the pore walls (Fig. 4i). Although the PVA-SA beads became softer, looser, and larger (ϕ = 4.0~5.0 mm) after 30 d, they also kept regular spheres. Meanwhile, the inside of PVA-SA beads melted and fused together,

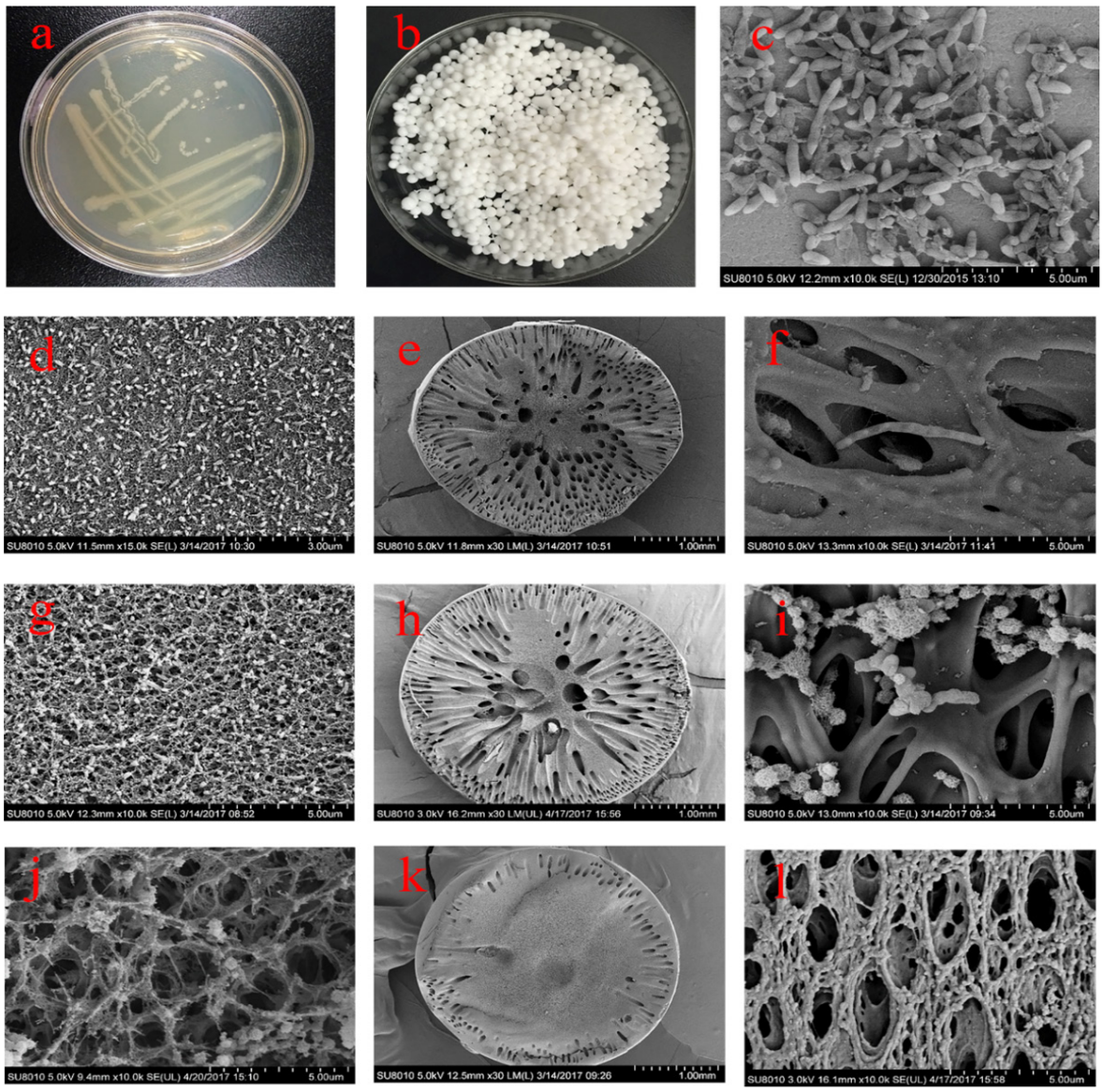

Fig. 4. Scanning electron microscopy (SEM) of the *Brevundimonas diminuta* AA06 and PVA-SA beads: (a) The strain AA06 colonies on agar medium, (b) Freshly prepared PVA-SA beads, (c) SEM images of strain AA06. (d), (e), and (f) were the SEM images of the surface, inside, and the attaching AA06 of freshly prepared PVA-SA beads. (g), (h), (i) were the surface, inside, and the attaching AA06 of PVA-SA beads immersed in algae culture for 15 d.(j), (k), (l) were the surface, inside, and the attaching AA06 of PVA-SA beads immersed in algae culture for 30 d.

resulting in the pore structure disappearing (Fig. 4k). The fiber skeleton of PVA-SA beads appeared more fragile and tenuous after 30 d. It was obvious that the pore structure of AA06 beads became damaged and broken during following long-term application. So the algicidal bacteria inside PVA-SA beads would lose the protection of this structure. The destruction of pore structure would affect algal inhibition effects of PVA-SA beads.

3.4. Analysis of algicidal activity

Exposure of *M. aeruginosa* to PVA-SA beads or freely suspended cells was operated in sequencing batch mode. One batch was also referred to as an algae lysing cycle in this study. Concentrated fresh *M. aeruginosa* was added to the algae-bacteria suspension at intervals, when the algal cell density was less than 1.0×10^8 cells/L. The freely suspended cells could inhibit algal cells for four cycles at EC_{90}, in which the lysing cycles were sustained for increasing time periods of 4, 7, 8, and 15 d (Fig. 5). It was obvious that the lysing cycles increased in length, with the algae cells beginning to grow normally during the fifth batch. However, when the *M. aeruginosa* was exposed to PVA-SA beads at EC_{90}, five lysing cycles were achieved, which were sustained for 4, 7, 14, 15, and 20 d. Although the fifth cycle was sustained for a long period, it still effectively inhibited the growth of *M. aeruginosa.*

Furthermore, freely suspended cells could inhibit algal cells for only two cycles at EC_{70}, sustaining 5 and 8 d. However, when *M. aeruginosa* was exposed to PVA-SA beads at EC_{70}, the PVA-SA beads could suppress algal cells for three cycles, sustaining 5, 15, and 17 d. Moreover, at the dosage of EC_{50}, freely suspended cells could not inhibit algal cells, while PVA-SA beads could suppress algae cells for one cycle. *M. aeruginosa* exposed to freely suspended cells at EC_{20} were similar to the control samples and grew normally, while the *M. aeruginosa* exposed to PVA-SA beads at EC_{20} showed a significant decrease from day 0 to day 8, then began to grow normally thereafter.

The results above showed that the PVA-SA beads exerted better sustainable inhibitory activity against *M. aeruginosa* than freely suspended cells in long-term experiments. The freely suspended cells inhibited algae cells for 34 d, while the PVA-SA beads resulted in much longer inhibition (approximately 60 d) at EC_{90}. Kang et al. reported that immobilization in/on a large pore-size carrier markedly increased the algicidal activity of SK09 against *S. hantzschii*, even at low water temperatures [37]. Greater packing and viable bacterial density of the immobilized technique in/on porous carriers resulted in a more protective environment that improved the efficacy of the procedure [38]. In particular, immobilization could increase bacterial productivity by providing a stable environment for the cells compared with the freely suspended cells. Thus, immobilization technique potentially enhanced the algicidal activity with a view towards applying algicidal bacteria in the natural environment. A previous study established that the algal-lysing bacteria induced its algicidal effects via indirect attack by secretion of algicidal compounds into the surrounding water rather than by the cell-to-cell contact lysis mechanism [39]. The results in this study indicated that strain AA06 could carry out normal physiological and biochemical activities in PVA-SA carrier, as well as secrete extracellular algicidal compounds.

It is an apparent seasonal phenomenon that cyanobacteria reemerge after winter dormancy in eutrophic water systems, with the cell density largely increasing from April to October [40]. For example, the cyanobacteria abundance of Lake Taihu peaked in October 2012 ($>1.0\times10^9$ cells/L) [41]. The duration of cyanobacteria blooms usually continues for several months. In order to control the cyanobacterial bloom, the algicidal bacteria need to be implemented frequently. If the algicidal bacteria possess longer lysing abilities, the dosage amount and numbers will be dramatically reduced. PVA-SA beads were prepared from natural compounds, including the PVA and SA, using a PVA-SA bead forming technique. The interaction between the freely suspended cells and the PVA-SA beads was suggested as complex formation rather than a simple physical adsorption. PVA and SA could be degraded by animals and specific bacteria, but overall they were not easily degraded by microorganisms in water [42]. Therefore, microbial activity

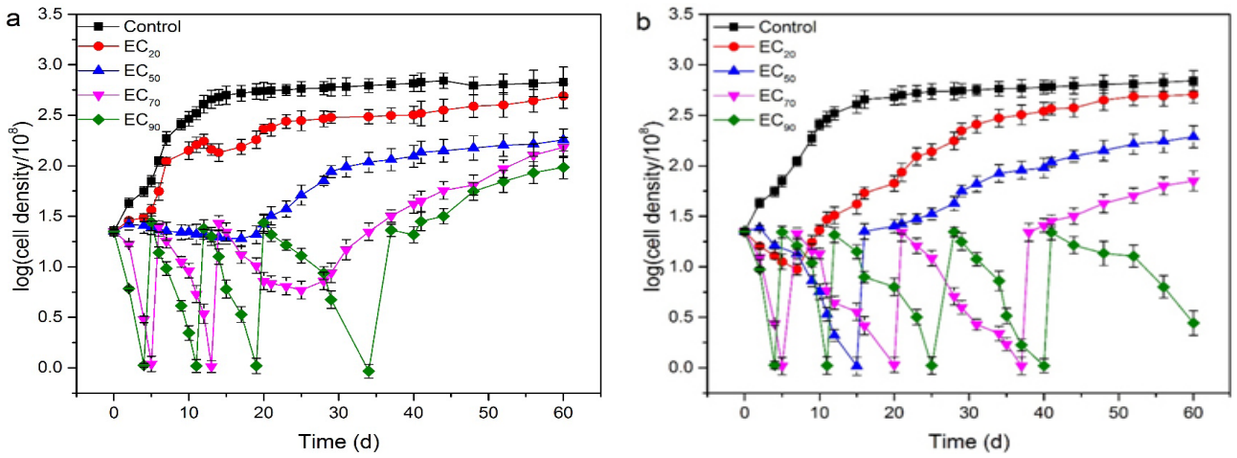

Fig. 5. Variations of cyanobacterial cell density under different dosing amount (Control, EC_{20}, EC_{50}, EC_{70}, EC_{90},). (a) Algae culture exposed to freely suspended cells; (b) Algae culture exposed to PVA-SA beads.

in water had insignificant effects on the life expectancy and release properties of the PVA-SA beads. Although freely suspended cells showed excellent inhibition of cyanobacterial growth in the short term (<34 d), the PVA-SA beads resulted in better algicidal effects in the long-term experiment. With a view towards applying strain AA06 in the natural environment, an immobilization technique could be used to support the bacterium and potentially enhance its algicidal activity. Therefore, application of PVA-SA beads in the season prior to the emergence of cyanobacteria was a promising technique to prevent cyanobacterial blooms.

3.5. MC-LR variation in the algicidal procedure

More than 90 microcystin isoforms have been detected at present [43]. Microcystins are chemically represented by the general structure cyclo(-D-Ala-L-X-D-erythrob-methylasparticacid-L-Y-Adda-D-Glu-Nmethyl-dehydroalanine) [44]. The main structural variations are observed in the L-amino-acid residues, designated "X" and "Y", with over 100 variants. Combinations of the two variable L-amino acids, X and Y, are used in the nomenclature of the toxins. The XY variable amino acids for MC-LR, MC-RR, and MC-YR are leucine (L), arginine (R), and tyrosine (Y) [45]. Among these variants, microcystin-leucine arginine (MC-LR) is the most abundant and the most toxic variant of microcystin [46]. Therefore, the variation of intracellular and extracellular MC-LR concentrations was investigated in the lysing of *M. aeruginosa*.

The PVA-SA beads and freely suspended cells were dosed at EC_{70} in a 1.2 L algal cultures with an initial cell density of 2.0×10^9 cells/L. The algal cell density in the control group increased throughout the entire cultivation period. Conversely, the algal cell density decreased gradually in the treated samples. The inhibition rates of PVA-SA beads (66.24%) and freely suspended cells (68.39%) had nearly no significant difference after 96 h (Fig. 6a).

The intracellular MC-LR concentrations in the control samples gradually increased from 22.4 μg/L to 380.3 μg/L after 30 d of incubation (Fig. 6b). The extracellular MC-LR concentrations remained nearly unchanged at 30.0 μg/L during the first 4 d. However, the extracellular MC-LR concentrations also increased gradually in the following cultivation, from approximately 30.0 μg/L to 210.0 μg/L after 30 d. In contrast to the control samples, the MC-LR produced and released from *M. aeruginosa* were very similar under the treatment of freely suspended cells and PVA-SA beads. The intracellular MC-LR concentrations of algae samples exposed to PVA-SA beads reached 22.5 μg/L on day zero (just after inoculation) and significantly decreased to 7.27 μg/L on day 24. Furthermore, there was no intracellular MC-LR detected on day 30 in the PVA-SA beads treatment groups. The extracellular MC-LR concentrations exposed to PVA-SA6 beads decreased slightly within the first 8 d. However, it significantly increased to 53.3 μg/L after 30 d, as a result of *M. aeruginosa* cytolysis. The inhibition of *M. aeruginosa* resulted in total MC-LR concentrations (intra plus extra cellular) significantly below the controls after 48 h. However, the extracellular concentrations still existed in the algae-bacteria suspension, indicating that strain AA06 couldnot effectively assimilate and degrade MC-LR.

Any damaging effects on algal cells in the treatment processes will contribute to the release of toxin into the water. Therefore,the release of microcystin-LR into solution is an important parameter in assessing cell damage. Previous studies showed that some types of amino acids, polyphenols, and phosphorus could increase the synthesis of microcystins in *M. aeruginosa* [47]. Without any external interference, the extracellular concentration of algal toxins was negligible during the early stages of algal cell culture, and remained at a relatively low level prior to the stable growth stage. However, following the death of algal cells, there was a large release of microcystins. Since inoculation doses of EC_{70} resulted in inhibition rate of over 99% within 24 d, this could be expected to suppress the release of MC-LR, along with the growth and metabolism of *M. aeruginosa*. The low concentration of MC-LR detected in cultures treated with these inoculation doses agreed with this expectation. The observed total decrease of MC-LR in the treatment group could be the result of reduction in algal cell numbers and/or microcystin synthesis ability. The number of *M. aeruginosa* exposed to PVA-SA beads and freely suspended cells obviously decreased (Fig. 6a). This led to

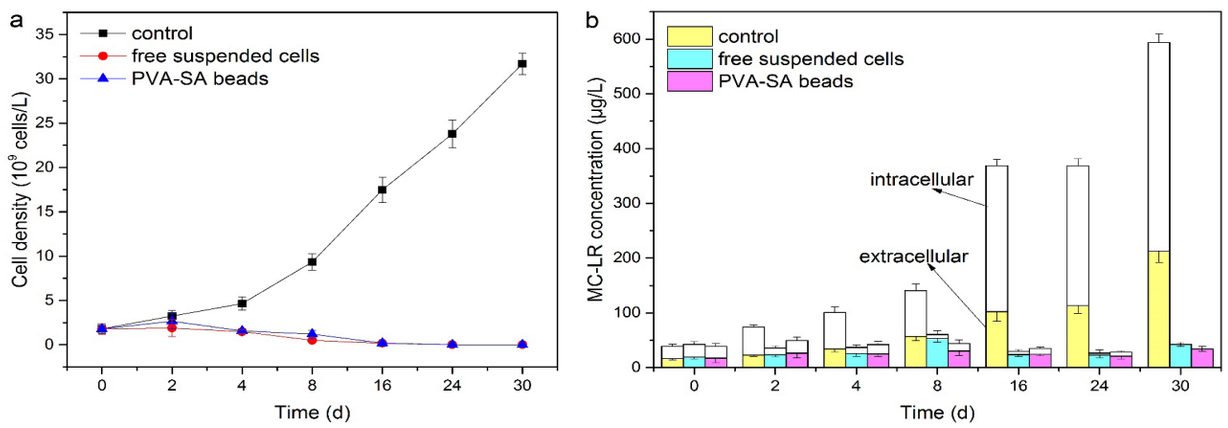

Fig. 6. The growth curve of *M. aeruginosa* treated with freely suspended cells and PVA-SA beads (a) and (b) the variations of intracellular and extracellular MC-LR concentrations.

a much lower total MC-LR concentrations in the treatment group than in the control group because algal cells were propagating with microcystins synthesis. Another possible reason was related to bacterial degradation of MC-LR. Extracellular MC-LR was detectable on day 30, probably due to the low degradation efficiency of strain AA06. One concern in using algicides to treat cyano-HABs was that increased the production and release of cyanotoxins, which could subsequently increase toxin levels and aggravate. As had been observed, PVA-SA beads and freely suspended cells caused similar variations in intracellular, extracellular, and total MC-LR concentrations of *M. aeruginosa* FACHB-905 cultures, without stimulating cells to produce and release toxins.

4. Conclusions

Embedding freely suspended cells inside PVA-SA beads was a feasible option in terms of controlling HABs. The dose-response relationship showed that the PVA-SA beads had an $EC_{50,96h}$ value of 2.18×10^7 CFU/mL after 96 h incubation with *M. aeruginosa*. The PVA-SA beads showed better sustainable inhibitory activity against *M. aeruginosa* than freely suspended cells in long-term experiment. The PVA-SA beads were still spherical after immersing in algae cultures for 30 d. The PVA-SA beads had less influence on MC-LR production by *M. aeruginosa*, and did not promote the synthesis of MC-LR in the cells. Based on the simple preparation process and the inhibitory efficiency, PVA-SA beads are a promising and environmentally friendly anti-cyanobacteria product. The results presented herein strongly suggest that immobilization of free algicidal bacteria will be an attractive alternative in the extensive application of eutrophic water systems, including ponds, lakes, and reservoirs.

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