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A freshwater bacterial strain, *Shewanella* sp. Lzh-2, isolated from Lake Taihu and its two algicidal active substances, hexahydropyrrolo[1,2-a]pyrazine-1,4-dione and 2, 3-indolinedione

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Abstract Cyanobacterial blooms have become a serious problem in Lake Taihu during the last 20 years, and *Microcystis aeruginosa* and *Synechococcus* sp. are the two dominant species in cyanobacterial blooms of Lake Taihu. A freshwater bacterial strain, *Shewanella* sp. Lzh-2, with strong algicidal properties against harmful cyanobacteria was isolated from Lake Taihu. Two substances with algicidal activity secreted extracellularly by *Shewanella* sp. Lzh-2, S-2A and S-2B, were purified from the bacterial culture of strain Lzh-2 using ethyl acetate extraction, column chromatography, and high performance liquid chromatography (HPLC) in turn. The substances S-2A and S-2B were identified as hexahydropyrrolo[1,2-a]pyrazine-1,4-dione and 2, 3-indolinedione (isatin), respectively, based on liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and hydrogen-nuclear magnetic resonance (H-NMR) analyses, making this the first report of their algicidal activity toward cyanobacteria. S-2A (hexahydropyrrolo[1,2-a]pyrazine-1,4-dione) had no algicidal effects against *Synechococcus* sp. BN60, but had a high level of algicidal activity against *M. aeruginosa* 9110. The LD50 value of S-2A against *M. aeruginosa* 9110 was 5.7 μg/ml. S-2B (2, 3-indolinedione) showed a potent algicidal effect against both *M. aeruginosa* 9110 and *Synechococcus* sp. BN60, and the LD50 value of S-2B against *M. aeruginosa* 9110 and *Synechococcus* sp. BN60 was 12.5 and 34.2 μg/ml, respectively. Obvious morphological changes in *M. aeruginosa* 9110 and *Synechococcus* sp. BN60 were observed after they were exposed to S-2A (or S-2B) for 24 h. Approximately, the algicidal activity, the concentration of S-2A and S-2B, and the cell density of Lzh-2 were positively related to each other during the cocultivation process. Overall, these findings increase our knowledge about algicidal substances secreted by algicidal bacteria and indicate that strain Lzh-2 and its two algicidal substances have the potential for use as a bio-agent in controlling cyanobacterial blooms in Lake Taihu.

Keywords Algicidal bacteria · Cyanobacterial bloom · *Microcystis aeruginosa* · *Synechococcus* sp. · 2, 3-indolinedione (isatin) · Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione

Introduction

Lake Taihu, the third largest freshwater lake in China, is a typical shallow freshwater lake located in eastern China (surface area 2,338 km^2, mean depth 1.9 m) (Wu et al. 2007) and a vital economic source and natural treasure for 30 million people living in the basin. Due to agricultural intensification and industrial pollution in the lake basin, the lake's water quality is decreasing and has been in a eutrophic state since the 1980s. As a result, cyanobacterial blooms have become a serious problem in Lake Taihu during the last 20 years (Qin et al. 2010; Rinta-Kanto

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et al. 2005; Tian et al. 2009; Ye et al. 2011) and caused a severe drinking water problem in the city of Wuxi in 2007 (Qin et al. 2010). It is reported that *Microcystis* and *Synechococcus* are the two most dominant species in the cyanobacterial blooms of Lake Taihu (Ye et al. 2011).

Pollution control is the essence for the governance of cyanobacterial blooms, but other methods also make sense. Physical and chemical techniques have been used to manage the blooms, such as application of yellow loess (Lee et al. 2009), UV irradiation (Sakai et al. 2007), copper sulfate (Haughey et al. 2000), and hydrogen peroxide (Nowack et al. 2011; Paerl et al. 2011). However, chemical methods are usually not environment-friendly due to their side effects to aquatic ecosystems, and physical methods are usually too costly to apply. Up to date, there isn't an effective solution about cyanobacterial blooms. On the other hand, biological control agents such as algicidal bacteria (Kang et al. 2005; Mayali et al. 2008; Nakashima et al. 2006b; Su et al. 2007; Tian et al. 2012; Zheng et al. 2012), viruses (Cai et al. 2011), and protozoa (Sigee et al. 1999) are getting more and more attention. As the increase of algicidal bacteria usually coincides with the ebb of a bloom, it is supposed that algicidal bacteria could cause the decline of cyanobacterial or algal blooms (Jeong et al. 2005; Mayali and Azam 2004).

To date, lots of algicidal bacterial strains that kill cyanobacterial or microalgal cells have been isolated from aquatic environments (Hare et al. 2005; Jeong et al. 2000; Lovejoy et al. 1998; Su et al. 2007; Tian et al. 2012; Wang et al. 2005), while only a few algicidal substances secreted by these bacteria have been purified and identified due to the difficulty of purification. Among these identified algicidal substances, most of them (rhamnolipid biosurfactants (Wang et al. 2005), L-amino acid oxidase (Chen et al. 2011), bacillamide (Jeong et al. 2003), prodigiosin (Jeong et al. 2005; Kwon et al. 2010; Nakashima et al. 2006a; Nakashima et al. 2006b), isatin (Taizo et al. 2011), and others (Zheng et al. 2012) were extracted from marine bacterial strains; only one substance (argimicin A) was extracted from a freshwater bacterial strain (Imamura et al. 2000). Some studies have also been conducted to investigate algicidal bacteria isolated from Lake Taihu (SI Table. S1), including strain *Exiguobacterium* A27 (Tian et al. 2012), strain *Pseudomonas aeruginosa* R219 (Ren et al. 2010), and strain *Aeromonas* sp. LTH-1 (Yang et al. 2013), but so far, no algicidal substances secreted by bacteria from Lake Taihu have been identified.

In this study, the algicidal bacterium, *Shewanella* sp. Lzh-2, was isolated from lake Taihu, and two algicidal substances (hexahydropyrrolo[1,2-a]pyrazine-1,4-dione and 2, 3-indolinedione) secreted by Lzh-2 were purified and identified.

Materials and methods

Cyanobacterial and algal cultures

Microcystis aeruginosa 9110, *Synechococcus* sp. BN60, *Oscillatoria* sp. BN35, *Chlorophyta* sp. B1, and *Chlamydomonas* sp. BS3 were isolated from Lake Taihu, while *M. aeruginosa* PCC7806, *Microcystis viridis* FACHB-979, and *Chroococcus* sp. FACHB-191 were purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences, China (FACHB). All cyanobacterial and algal strains were cultivated in BG11 medium at 25 °C under 40 μmol photons/(m^2·sec) and a 12 h:12 h (light: dark) cycle (Tian et al. 2012). Cell densities of *M. aeruginosa* 9110 and *M. aeruginosa* PCC7806 were quantified using a hemocytometer under a light microscope (magnification ×400), while chlorophyll-*a* concentrations of *Synechococcus* sp. BN60, *M. viridis* FACHB-979, *Chroococcus* sp. FACHB-191, *Oscillatoria* sp. BN35, *Chlorophyta* sp. B1, and *Chlamydomonas* sp. BS3 were determined spectrophotometrically by the acetone method (Welschmeyer 1994) as their equivalent cell densities.

Cyanobacterial-lawn test

Cyanobacterial-lawn was used to preliminarily screen the algicidal metabolites from bacteria. To develop the cyanobacterial-lawn, 10-mL axenic cultures of *M. aeruginosa* 9110 were grown in 90-mL BG11 medium and cultivated for 1 week; after which, cells in 40-mL cultures were harvested by centrifugation at 4,000×*g* for 20 min. The harvested cells was mixed with 20 mL of molten BG11 soft agar (1.0 % agar, equilibrated to 53 °C), and the mixture was immediately poured onto a BG11 agar plate (1.5 % agar) and then incubated for 2 days to build the cyanobacterial lawn under 40 μmol photons/(m^2·sec) and a 12 h:12 h (light: dark) cycle at 25 °C. A paper disc which was soaked by bacterial metabolite solution was placed on the middle of the cyanobacterial-lawn plate; after which, the lawn was maintained at 25 °C under 40 μmol photons/(m^2·s) and a 12 h:12 h (light: dark) cycle for 2 days. The algicidal effect of the bacterial metabolite was evaluated based on whether there was a visible clear inhibition zone around the paper disc on the cyanobacterial lawn or not.

Isolation and identification of algicidal bacteria

The sampling site was located at the Taihu Ecosystem Research Station (31° 24′ N, 120° 13′ E) in Meiliang Bay, which is the most hypertrophic part in Lake Taihu. Water samples in cyanobacterial blooms were collected using a Ruttner Standard Water Sampler, immediately transferred into sterile bottles, and transported to the laboratory in a mini-icebox in

October, 2010. After that, 10-mL water sample was inoculated into 90-mL *M. aeruginosa* 9110 cultures in exponential growth phase, and the mixture was cultured at 25 °C under 40 μmol photons/($m^2 \cdot s$) and a 12 h:12 h (light: dark) cycle. A negative control was tested using sterile water inoculation instead of the water sample inoculation. When the cell density of *M. aeruginosa* 9110 declined to below 10 % of the negative control, 1-mL aliquots of the cultures were serially diluted with sterile water and spread onto beef extract peptone medium (composed of 10 g peptone, 5 g NaCl, 3 g beef extract per L) agar plates; after which, the agar plates were incubated for 24 h at 28 °C. Individual colonies of distinct morphology were streaked onto new beef extract peptone medium agar plates for further purification (Yamamoto and Suzuki 1990), and individual purified colonies were cryopreserved at −70 °C in beef extract peptone medium containing 20 % glycerol.

In experiments conducted to test the algicidal activities of the isolated bacterial strains, each isolated bacterial strain was inoculated into a test tube containing 10 ml of beef extract peptone medium and cultivated at 28 °C for 24 h with a shaking speed of 200 rpm; after which, the bacterial culture was then inoculated into a test flask containing 90 mL of exponential-phase cultures of *M. aeruginosa* 9110 (1.0×10^7 cells/ml) and cultivated at 25 °C under 40 μmol photons/($m^2 \cdot sec$) and a 12 h:12 h (light: dark) cycle. A negative control was tested using sterile beef extract peptone medium inoculation instead of the bacterial culture inoculation (Imamura et al. 2001). The algicidal activity (A, %) of tested bacterial strain was evaluated by the following equation:

$$A = \left(1 - \frac{D_{t-\text{treatment}}}{D_{t-\text{control}}}\right) \times 100$$

where, $D_{t\text{-treatment}}$ (cells/mL) and $D_{t\text{-control}}$ (cells/mL) are the cell densities of *M. aeruginosa* 9110 in the test and control, respectively, and t (day) is the cultivation time.

Strain Lzh-2 had the strongest algicidal activity among the isolated bacterial strains and was therefore selected for further study. Cell densities of strain Lzh-2 in cultures were determined by the colony-forming unit (CFU) method performed on beef extract peptone medium agar plates (Yamamoto and Suzuki 1990).

For identification of the algicidal bacteria strain Lzh-2, pure culture for algicidal bacterial isolate was grown on beef extract peptone medium for 24 h at 28 °C and then subjected to taxonomic analysis using conventional physiological and biochemical tests (XiuZhu and Cai 2001). Bacterial chromosomal DNA was extracted (Cai et al. 2011); after which, the 16S rDNA was amplified by PCR using primers 27F (5′-AGAGTT TGATCATGGCTCAG-3′) and 1492R (5′-GGTTACCTTG TTACFACTT-3′) (Wu et al. 2007). The PCR products were then sequenced on an ABI-Prism 3730 automated sequencer (PE Applied Biosystems, USA). Identification of algicidal bacteria strain Lzh-2 was accomplished by analyzing its 16S rRNA gene sequence (GenBank accession number HQ896842) using RDP classifier software (version 2.2) and comparing it with sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/blast).

Purification of algicidal substances secreted by strain Lzh-2

Bacterial culture and ethyl acetate extraction

Strain Lzh-2 was incubated in beef extract peptone liquid medium and cultivated with a shaking speed of 200 rpm at 28 °C for 48 h. The bacterial culture was mixed with an equal volume of ethyl acetate. After keeping the mixture in a separation funnel for 24 h, the ethyl acetate layer was collected and evaporated; after which, the remaining materials were dissolved in the water and filtered through a 0.22-μm membrane filter to get filtrate.

Column chromatography

The filtrate was subjected to column chromatography (commercial silica gel, Qingdao Haiyang Chemical Group Co., China, 200–300 mesh; 1×50 cm) at a flow rate of 1 mL/min and monitored at 254 nm. Each fraction of the effluent of column chromatography was collected and evaporated; after which, it was dissolved in 0.2 ml of water. The algicidal effect of each fraction was evaluated based on the cyanobacterial-lawn test.

Semi-preparative high performance liquid chromatography (HPLC)

The algicidal fraction from the silica gel column was applied to a semi-preparative HPLC column (Supersil™ C18-EP, 5 μm, 10.0×250 mm, Dikma, China, flow rate 4 mL/min) and eluted with 30 % methanol aqueous solution as mobile phase on a liquid chromatography system (1260 Infinity, Agilent, USA). The eluate was monitored at 210 nm using an UV-Vis detector (G1314F, Agilent, USA). The collected fractions of eluate were evaporated and then dissolved in 0.2 ml of water, respectively. The algicidal effect of each fraction was evaluated based on the cyanobacterial-lawn test. Fractions A and B from the eluate of semi-preparative HPLC were verified to have algicidal effect.

Further-purification HPLC

The fractions A and B recovered from the semi-preparative HPLC were further purified by a reversed-phase HPLC column (Supersil™ C18-EP, 5 μm, 4.6 mm×250 mm, Dikma, China; flow rate1 mL/min; UV detection at 210 nm) and eluted with 100 % ultrapure water and 20 % methanol

aqueous solution as mobile phase on a liquid chromatography system (1260 Infinity, Agilent, USA), respectively. The purified two substances S-2A and S-2B were verified to have algicidal effect based on the cyanobacterial lawn test.

Identification of the algicidal substances S-2A and S-2B

Liquid chromatography-mass spectrometry (LC-MS) analysis

Algicidal substances S-2A and S-2B were dissolved in 1 ml of methyl alcohol for LC/MS analysis, respectively. Ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-TOF MS) were recorded on LC/MS spectrometers (Agilent Technologies HPLC 1290-MS 6230, USA). The separation was performed on a ZORBAX Extend-C18 column (1.8 μm, 2.1×50 mm) using an eluent composed of 5 % methanol aqueous solution. The injection volume was 2 μL, and the flow rate was 0.2 mL/min. The total effluent from the detector was transferred directly to the hybrid IT/TOF mass spectrometer without splitting. The mass spectrometer was equipped with an electro-spray ionization source and operated in positive mode for S-2A and negative mode for S-2B. Mass spectrometric analyses were carried out on a full-scan MS over a mass range of m/z 100–2000 using data-dependent MS/MS acquisition of the suspected precursor ions. Data acquisition and processing were carried out using the LC/MS Qualitative Analysis B.04.00 software supplied with the instrument. Any mass numbers corresponding to particular elemental compositions were also calculated by the formula predictor (Liu et al. 2011).

Gas chromatography-mass spectrometry (GC-MS) analysis

Algicidal substances S-2A and S-2B were dissolved in 1 ml of methyl alcohol and subjected to GC/MS analysis. Gas mass spectra (EI-MS) were recorded on GC/MS spectrometers with HP-5 ms columns (0.25 μm, 0.25 μm×30 m) (Agilent Technologies 6850/5975C, USA, for EI-MS). Helium was used as the carrier gas, and the flow rate was maintained at 1 ml/min. Column temperatures were increased from 100 °C to 300 °C at 10 °C/min. The mass spectra of S-2A and S-2B were compared with those in the NIST/EPA/NIH Mass Spec. Library (Version 2.0), respectively.

Hydrogen-nuclear magnetic resonance (H-NMR) analysis

Algicidal substances S-2A and S-2B were dissolved in D_2O and chloroform-d3, respectively, and applied to an NMR spectrometer (400 MHz, Avance III, Bruker, Switzerland). Chemical shifts were expressed as *d* values (PPM) with trimethylsilyl (TMS) as the internal standard.

LD50 of S-2A and S-2B against *M. aeruginosa* 9110 and *Synechococcus* sp. BN60

Different amount of S-2A (or S-2B) were added to 10 mL exponential-phase cultures of *M. aeruginosa* 9110 or *Synechococcus* sp. BN60, respectively. The serial concentrations of S-2A or S-2B in the cultures ranged from 5 to 100 μg ml^{-1}. After 24 h of incubation, the cell densities of *M. aeruginosa* 9110 and the chlorophyll-*a* (chl-*a*) concentrations (equivalent cell densities) of *Synechococcus* sp. BN60 in the serial cultures were measured. The viability of cyanobacteria was calculated by the following equation: $V(\%)=D_{t\text{-treatment}}/D_{t\text{-control}}\times 100$, where V indicates viability, $D_{t\text{-treatment}}$ and $D_{t\text{-control}}$ are viable cells of *M. aeruginosa* 9110 (or the chlorophyll-*a* concentrations of *Synechococcus* sp. BN60) with and without S-2A (or S-2B), respectively. The LD50 of S-2A (or S-2B) is the concentration of S-2A (or S-2B) that causes the death of 50 % cyanobacterial cells in the culture after 24 h incubation. The LD50 value of S-2A and S-2B was calculated from the dose-response curve.

Observation of morphological change in *M. aeruginosa* 9110 and *Synechococcus* sp. BN60

S-2A (or S-2B) was added to the cell suspensions of *M. aeruginosa* 9110 and *Synechococcus* sp. BN60 at an initial concentration of 10 μg ml^{-1}, respectively, and the suspensions were incubated at 25 °C for 24 h under the growth conditions described above. After incubation, the *M. aeruginosa* 9110 and *Synechococcus* sp. BN60 cells were observed at a magnification of ×1000 using an Eclipse 50i Microscope (Nikon) (Nakashima et al. 2006a).

Quantification of S-2A and S-2B by LC-MS

Ten milliliters of the bacterial culture of strain Lzh-2 was inoculated into test flasks containing 90-mL *M. aeruginosa* 9110 cultures or *Synechococcus* sp. BN60 cultures, respectively. In the meantime, 10-ml sterile beef extract peptone medium was inoculated into 90-mL *M. aeruginosa* 9110 cultures or *Synechococcus* sp. BN60 cultures, respectively, as a control. The mixtures were cultured at 25 °C under 40 μmol photons/(m^2·s) and a 12 h:12 h (light: dark) cycle. From the 1st day to the 6th day, cell densities of strain Lzh-2 in the cocultures were determined by the CFU method performed on beef extract peptone medium agar plates (Yamamoto and Suzuki 1990), and the cell densities of *M. aeruginosa* 9110 and the chl-*a* concentrations of *Synechococcus* sp. BN60 were measured as described above; after which, aliquot portion of the cocultures were mixed with an equal volume of ethyl acetate to conduct the quantification of S-2A and S-2B. After keeping the mixture in a separation funnel for 24 h, the ethyl acetate layer was collected and

evaporated; after which, the remaining materials were dissolved in 1-mL water and filtered through a 0.22-μm membrane filter to get filtrate. The filtrates and standard solutions of S-2A and S-2B were subjected to LC-MS analysis in positive mode, respectively. MS data was acquired and processed using the LC-MS Qualitative Analysis B.04.00 software supplied with the instrument. Using this program, mass chromatograms corresponding to S-2A and S-2B were extracted and integrated from the total ion chromatogram. S-2A and S-2B's concentrations in the filtrate were determined by comparing the peak areas of S-2A (or S-2B) with those of the standards (Armando et al. 2012). Ethyl acetate extraction recoveries were evaluated at three levels, which were 1, 10, and 100 μg ml^{-1} for S-2A (or S-2B), respectively. Recoveries were determined by comparing the peak areas of S-2A (or S-2B) before and after extraction (Ran Ran et al. 2013). The concentrations of S-2A (or S-2B) in the cocultures were calculated according to S-2A (or S-2B)'s concentrations in the filtrates, the volume of cocultures to be extracted, and the extraction recoveries.

Results

Isolation and identification of algicidal bacteria

Fifty-six bacterial strains were isolated from the surface water samples collected from Meiliang Bay of Lake Taihu in October 2010. Among these strains, eight strains exhibited algicidal activity toward *M. aeruginosa* 9110, and strain Lzh-2 appeared to exert the strongest algicidal activity.

Strain Lzh-2' colony was round, slightly convex and ivory-white, measuring 1.5–2 mm in diameter for 24-h incubation (SI Fig. S1). The biochemical profiles of strain Lzh-2 were shown in SI Table S2. The isolate was gram-negative, motile, and oxidase and catalase positive. It could produce H_2S, but not indole. Strain Lzh-2 could grow at temperatures ranging from 20 to 40 °C. The isolate Lzh-2 demonstrated similar phenotypic characteristics to *Shewanella* sp. Comparison of the 16S rRNA gene of strain Lzh-2 with those available in the GenBank database (http://www.ncbi.nlm.nih.gov/blast) indicated that it was most closely related to *Shewanella* sp. S4 (99 % homology, accession number FJ589031). The 16S rRNA gene sequence of strain Lzh-2 has been deposited in the GenBank database under accession number HQ896842, and the strain was deposited in the China General Microbiological Collection Center (CGMCC) under accession number CGMCC-6549.

Algicidal activity of strain Lzh-2

The algicidal activities (t=6 days) of strain Lzh-2 against the dominant cyanobacterial species of Lake Taihu and other cyanobacterial or algal species are shown in Table 1. Strain Lzh-2 exhibited algicidal activity toward all four tested cyanobacterial species isolated from Meiliang Bay of Lake Taihu, especially toward *M. aeruginosa* 9110 (algicidal activity=92.3 %, t=6 days) and *Synechococcus* sp. BN60 (algicidal activity=84.9 %, t=6 days), which were previously reported to be the two most dominant microorganisms in the cyanobacterial blooms of Lake Taihu (Ye et al. 2011). The bacterial strain also showed algicidal activity against one eukaryotic algal strain from Lake Taihu, *Chlamydomonas* sp. BS3 (algicidal activity=93.1 %, t=6 days).

The algicidal process of strain Lzh-2 against *M. aeruginosa* 9110 and *Synechococcus* sp. BN60 were shown in SI Fig. S2a and SI Fig. S2b, respectively. The strain Lzh-2 (in the presence of 1×10^8 cells/ml (initial concentration)) did not significantly inhibit the growth of *M. aeruginosa* 9110 and *Synechococcus* sp. BN60 in the first 2 days, but after that cell density of *M. aeruginosa* 9110 and the chl-*a* concentrations of *Synechococcus* sp. BN60 were obviously decreased compared to the control (without Lzh-2). Most of the *M. aeruginosa* 9110 (>92 %) and *Synechococcus* sp. BN60 (>84 %) died within 6 days.

Extraction and purification of algicidal substances

After ethyl acetate extraction and silica gel column chromatography, there was found one fraction with algicidal effect in the effluent of the chromatographic column. Semi-preparative HPLC of the algicidal fraction from the column chromatography was shown in SI Fig. S3. Fraction A (retention time values=4.5–5.6 min) and B (retention time values=14–15.2 min) in the semi-preparative HPLC (SI Fig. S3) were found to have algicidal effect (SI Fig. S4). Fractions A and B

Table 1 Algicidal activity of strain Lzh-2 against several cyanobacterial and algal strains

Strain	Algicidal activity (A, %)
Microcystis aeruginosa 9110[a]	92.3±6.8
Synechococcus sp. BN60[a]	84.9±3.9
Chlorophyta sp. B1[a]	97.9±6.2
Oscillatoria sp. BN35[a]	97.2±10.5
Chlamydomonas sp. BS3[a]	93.1±7.3
Chroococcus sp. FACHB-191	29.5±9.7
Microcystis viridis FACHB-979	86.3±8.5
Microcystis aeruginosa PCC7806	84.9±3.8

[a] Isolated from Meiliang Bay of Lake Taihu; the algicidal activity of strain Lzh-2 against these organisms was calculated using the following equation: $A\ (\%)=(1-D_{t\text{-treatemt}}/D_{t\text{-control}})\times100$, where A is the algicidal activity, $D_{t\text{-treatment}}$ (cells/mL) and $D_{t\text{-control}}$ (cells/mL) are the concentrations of cyanobacteria (or algae) in cultures with and without inoculation of strain Lzh-2, respectively, and t is the cocultivation time (here, t=6 days). Data are the means±SD from at least three independent assays

were then further purified by further-purification HPLC, respectively. S-2A (retention time value=15–17 min, SI Fig. S5) in the further-purification HPLC was found to have algicidal effect (SI Fig. S6), and S-2B (retention time value=24.5–26.5 min, SI Fig. S7) in the further-purification HPLC was also found to have algicidal effect (SI Fig. S8).

Identification of the algicidal substances S-2A and S-2B

The electro-spray ionization (ESI) mass spectrum of S-2A displayed a molecular ion at 155.0824 m/z $(M+H)^+$, and the molecular formula was determined to be $C_7H_{10}N_2O_2$ based on the UPLC-TOF MS data (SI Fig. S9). The EI mass spectrum of S-2A indicated that ions at 154, 111, 83, and 70 corresponded to molecule (M), M-C_3H_7, M-C_4NH_9, and M-$C_3NO_2H_2$ ions, respectively (SI Fig. S10a). The EI mass spectrum of S-2A was similar to that of hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (MW 154, molecular formula: $C_7H_{10}N_2O_2$) in the GC/MS library (SI Fig. S10b), with a similarity index (SI)>850. The NMR data for S-2A were as follows: ^{1}H NMR (400 MHz, D_2O) δ 4.22 (s, 1H), 4.04 (d, J=2.7 Hz, 1H), 3.77 (d, J=17.3 Hz, 1H), 3.44 (dd, J=8.7, 4.8 Hz, 2H), 2.23 (s, 1H), 1.96 (s, 1H), 1.84 (d, J=5.6 Hz, 2H) (SI Fig. S11). According to the results of LC-MS, GC-MS, and H-NMR, S-2A is hexahydropyrrolo [1,2-a]pyrazine-1,4-dione (Fig. 1).

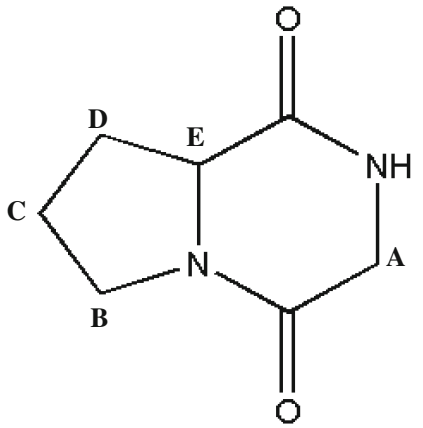
The ESI mass spectrum of S-2B displayed a molecular ion at 146.0251 m/z $(M-H)^-$ (SI Fig. S12), and the molecular formula was determined to be $C_8H_5NO_2$ upon UPLC-TOF MS analysis. The EI mass spectrum of S-2B indicated that ions at 147, 119, and 92 correspond to molecule (M), M-CO, and M-2CO, respectively (SI Fig. S13a). The EI mass spectrum of S-2B was similar to that of 2, 3-indolinedione (isatin, MW 147, molecular formula: $C_8H_5NO_2$) in the GC/MS library (SI Fig. S13b), with a similarity index>850. The NMR data for S-2B were as follows: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (s, 1H), 7.76 (d, J=7.5 Hz, 1H), 7.70 (s, 1H), 7.26 (t, J=7.6 Hz, 1H), 7.05 (d, J=7.9 Hz, 1H) (SI Fig. S14). According to the results of LC-MS, GC-MS, and H-NMR, S-2B is 2, 3-indolinedione (Fig. 2).

LD50 of S-2A and S-2B against *Microcystis aeruginosa* 9110 and *Synechococcus* sp. BN60

As shown in Fig. 3a, S-2A had no algicidal effects on *Synechococcus* sp. BN60, but had potent algicidal effects on *M. aeruginosa* 9110. The LD50 value of S-2A against *M. aeruginosa* 9110 was calculated to be approximately 5.7 μg/ml from the dose-response curve. As shown in Fig. 3b, S-2B had potent algicidal effects on both *M. aeruginosa* 9110 and *Synechococcus* sp. BN60, with LD50 values of 12.5 μg/ml and 34.2 μg/ml, respectively.

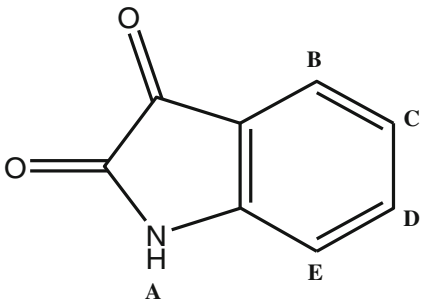
Morphological change in *Microcystis aeruginosa* 9110 and *Synechococcus* sp. BN60

Normal *M. aeruginosa* 9110 cell is shown in Fig. 4a; after it was exposed to S-2A for 24 h, its intracellular structure broke down and cell wall integrity lost (Fig. 4c). Exposure to S-2B for 24 h could cause abnormality of *M. aeruginosa* 9110 cell division (Fig. 4d). For comparison, normal *M. aeruginosa* 9110 cell division is shown in Fig. 4b.



Assignation	Sift (ppm)
A	4.04/3.77
B	3.44
C	1.84/1.96
D	2.23/1.96
E	4.22

Fig. 1 Molecular structure of S-2A (hexahydropyrrolo[1,2-a]pyrazine-1,4-dione) and its chemical shifts of ^{1}H signals



Assignation	Sift (ppm)
A	8.11
B	7.76
C	7.70
D	7.26
E	7.05

Fig. 2 Molecular structure of S-2B (2,3-indolinedione) and its chemical shifts of ^{1}H signals

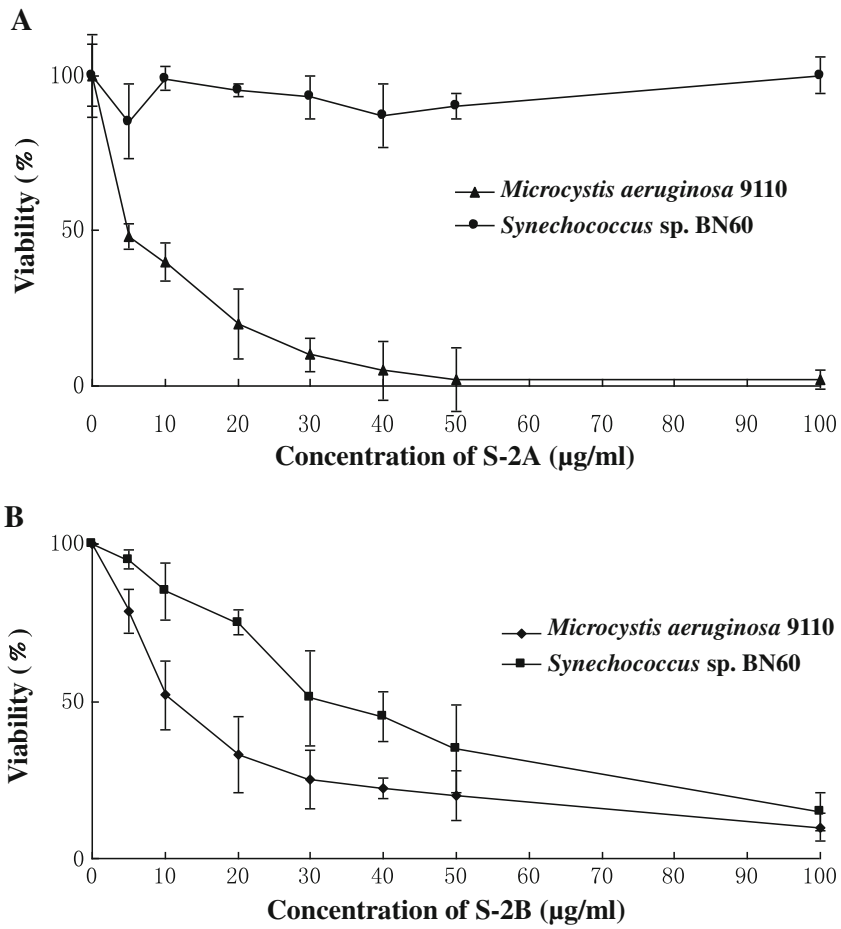


Fig. 3 LD50 of S-2A against *Microcystis aeruginosa* 9110 (*filled triangle*) and *Synechococcus* sp. BN60 (*filled circle*) (**a**) and LD50 of S-2B against *Microcystis aeruginosa* 9110 (*filled diamond*) and *Synechococcus* sp. BN60 (*filled square*) (**b**). Different amount of S-2A (or S-2B) were added to 10-mL exponential-phase cultures of *Microcystis aeruginosa* 9110 or *Synechococcus* sp. BN60, respectively. The serial concentrations of S-2A or S-2B in the cultures ranged from 5 to 100 μg ml^{-1}. After incubation for 24 h, the viability of cyanobacteria was calculated by the following equation: $V\ (\%)=D_{t\text{-treatemt}}/D_{t\text{-control}}\times 100$, where V indicates viability, $D_{t\text{-treatment}}$ and $D_{t\text{-control}}$ are viable cells of *Microcystis aeruginosa* 9110 (or the chlorophyll-*a* concentrations of *Synechococcus* sp. BN60) with and without S-2A (or S-2B), respectively. The LD50 value of S-2A and S-2B was calculated from the dose-response curve. Experiments were performed in triplicate. *Error bars* represent the SD

Compared to normal *Synechococcus* sp. BN60 cell (Fig. 4e), *Synechococcus* sp. BN60 had no morphological changes after exposure to S-2A (Fig. 4f), but after it was exposed to S-2B for 24 h, its cell shape was changed from round to irregular (Fig. 4g).

Dynamics of concentration of S-2A and S-2B, cell density of Lzh-2, and biomass of cyanobacteria in the cocultures

The cell densities of strain Lzh-2, cell densities of *M. aeruginosa* 9110, and the concentrations of S-2A and S-2B in the cocultures of *M. aeruginosa* 9110-strain Lzh-2 were shown in Fig. 5a, b. At the end of the first day, strain Lzh-2' density was up to 5.3×10^8 cells/ml, while S-2A's concentrations were detected (0.13 μg/ml) and S-2B was not detected, resulting in a very low algicidal activity. With the strain Lzh-2' density going up to 1.5×10^9 cells/ml at the end of the second day, S-2B was detected (0.64 μg/ml) and S-2A's concentrations kept rising to 0.56 μg/ml, while the cell density of *M. aeruginosa* 9110 was obviously decreased compared to the control (without Lzh-2 inoculation). From the end of the second day to the end of the sixth day, the cell density of Lzh-2 kept increasing and the S-2A and S-2B's concentrations as well as the algicidal activity were positively related to the cell density increase. At the end of the sixth day, most of the *M. aeruginosa* 9110 (algicidal activity>94 %) died, while the cell density of strain Lzh-2 was up to 1.4×10^{10} cells/ml and the concentrations of S-2A and S-2B reached to 2.53 and 4.46 μg/ml, respectively.

As shown in Fig. 6a, b, at the end of the first day, strain Lzh-2' density was 2.6×10^8 cells/ml, S-2A's concentrations was detected (0.21 μg/ml) (S-2A had no algicidal effects against *Synechococcus* sp. BN60), and S-2B was not detected in the cocultures of *Synechococcus* sp. BN60-strain Lzh-2,

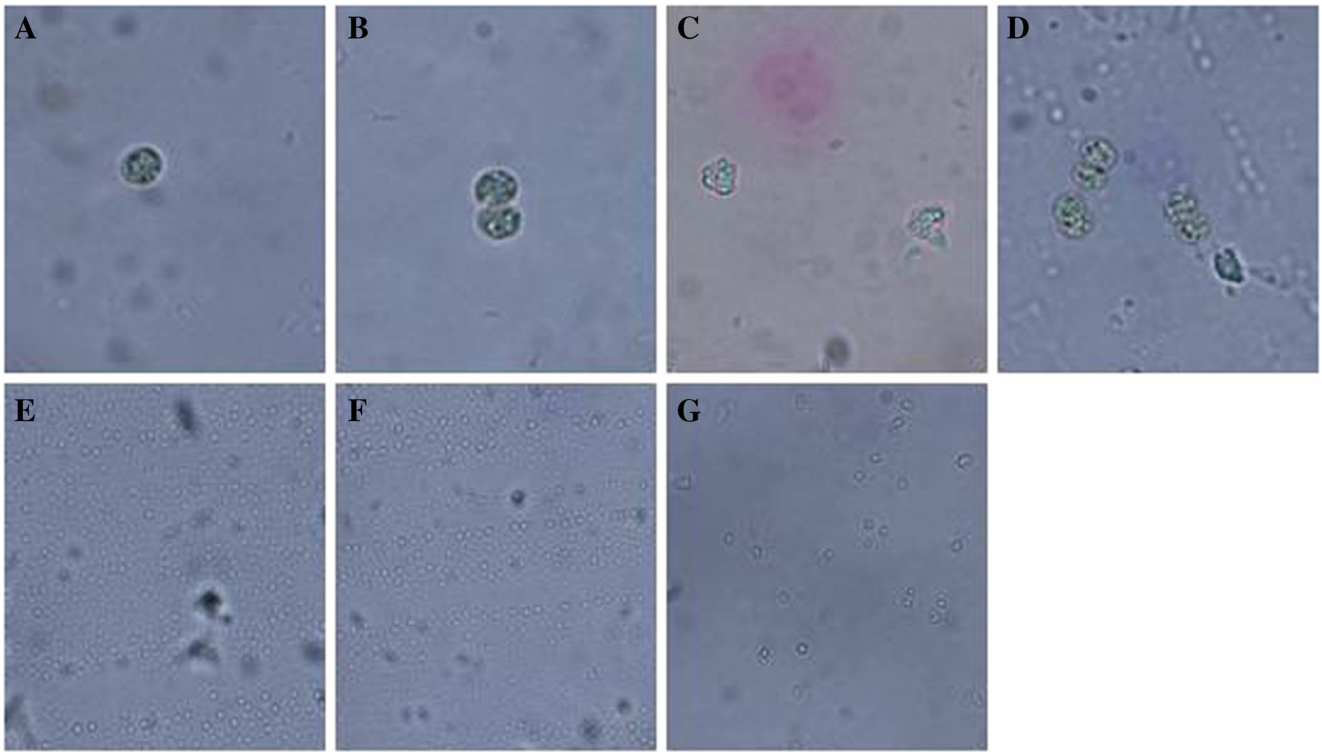


Fig. 4 Morphological changes of *Microcystis aeruginosa* 9110 and *Synechococcus* sp. BN60 treated with S-2A (or S-2B) (10 μg ml^{-1}) for 24 h, respectively. **a** Normal *Microcystis aeruginosa* 9110 cell, **b** normal *Microcystis aeruginosa* 9110 cells during cell division, **c** *Microcystis aeruginosa* 9110 cells treated with S-2A, **d** *Microcystis aeruginosa* 9110 cells treated with S-2B, **e** normal *Synechococcus* sp. BN60 cell, and **f** *Synechococcus* sp. BN60 cells treated with S-2A, **g** *Synechococcus* sp. BN60 cells treated with S-2B

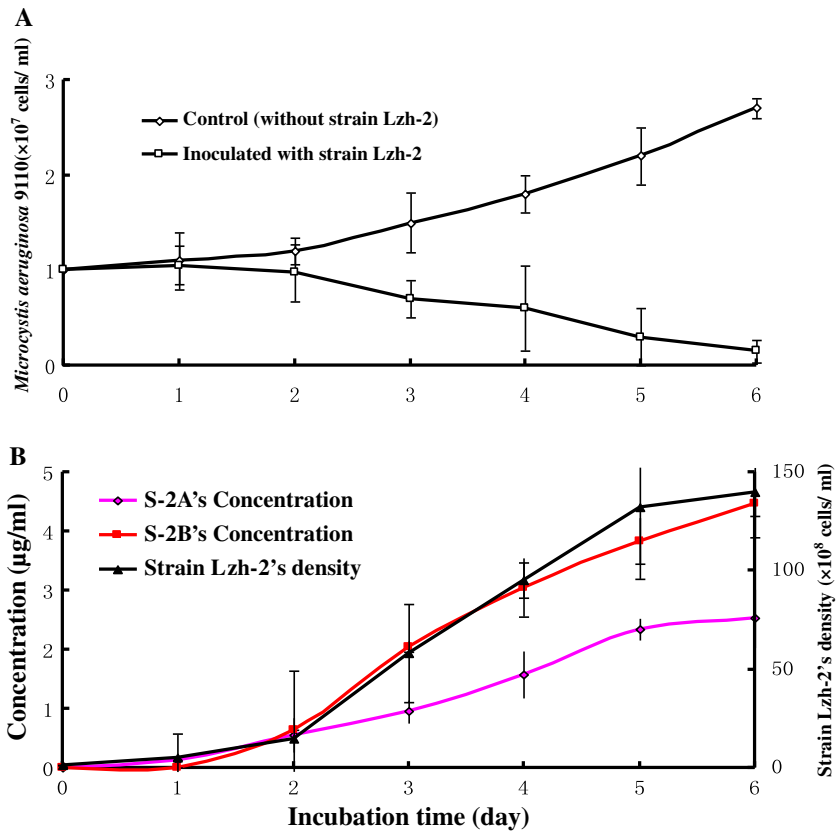


Fig. 5 Dynamics of cell density of *Microcystis aeruginosa* 9110, cell density of algicidal strain Lzh-2, and concentration of S-2A and S-2B in the cocultures of *Microcystis aeruginosa* 9110 and algicidal strain Lzh-2. **a** Inoculated with Lzh-2 (*square*). Initial concentration of Lzh-2 in the cocultures 1×10^8 cells/ml; control (without strain Lzh-2) (*diamond*) and **b** concentration of S-2A (*filled triangle*) and S-2B (*filled square*); cell density of strain Lzh-2 (*filled triangle*). Experiments were performed in triplicate. *Error bars* represent the SD

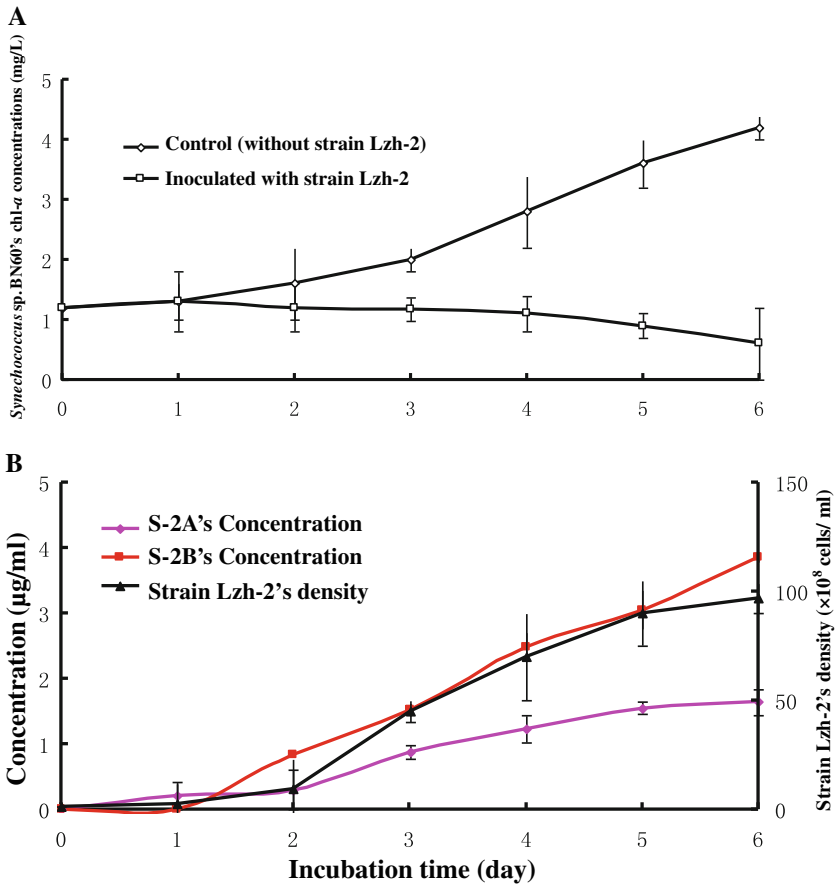


Fig. 6 Dynamics of chlorophyll-*a* (chl-*a*) concentrations of *Synechococcus* sp. BN60, cell density of algicidal strain Lzh-2, and concentration of S-2A and S-2B in the cocultures of *Synechococcus* sp. BN60 and algicidal strain Lzh-2. **a** Inoculated with Lzh-2 (*square*). Initial concentration of Lzh-2 in the cocultures 1×10^8 cells/ml; control (without strain Lzh-2) (*diamond*) and **b** concentration of S-2A (*filled diamond*) and S-2B (*filled square*); cell density of strain Lzh-2 (*filled triangle*). Experiments were performed in triplicate. *Error bars* represent the SD

while the chl-*a* concentrations of *Synechococcus* sp. BN60 was almost the same as the control. With the strain Lzh-2' density going up to 1.0×10^9 cells/ml at the end of the second day, S-2B was detected (0.83 μg/ml), resulting in the increase of algicidal activity. At the end of the sixth day, strain Lzh-2' density was up to 1.0×10^{10} cells/ml and the concentrations of S-2A and S-2B reached to 1.64 and 3.86 μg/ml, respectively, while the algicidal activity increased to 85 %.

Discussion

Microcystis and *Synechococcus* are the two most dominant microorganisms in cyanobacteria blooms in Lake Taihu (Ye et al. 2011), and *Shewanella* sp. Lzh-2 not only showed strong algicidal activity against *M. aeruginosa* and *Synechococcus* sp., but also significantly inhibited the growth of several other cyanobacterial species isolated from cyanobacterial blooms in Lake Taihu (Table 1). These results indicated that *Shewanella* sp. Lzh-2 has potential for use in the control of outbreaks of cyanobacterial blooms in Lake Taihu. When compared to other algicidal bacteria isolated from fresh water, such as *Pseudomonas fluorescens* HYK0210-SK09 from Paldang Reservoir of Korea (Jung et al. 2008) and *Exiguobacterium* A27 (Tian et al. 2012), *P. aeruginosa* R219 (Ren et al. 2010), and *Aeromonas* sp. LTH-1 (Yang et al. 2013) from Lake Taihu, *Shewanella* sp. Lzh-2 exhibited similar or stronger algicidal activity against *M. aeruginosa* (data on SI Table. S1). *Shewanella* sp. is reported for the first time as a cyanobacteria-lysing bacterium in the present study, and the results presented herein increase our knowledge about *Shewanella* sp. in aquatic environments.

Isatin, which is the commercial name for 2,3-indolinedione, has been shown to have a physiological effect on wood decay fungi (Bach and Magee 1962; Jarrahpour et al. 2007; Kupinic et al. 1979; Ogata et al. 2003; Pandeya et al. 1999). In a previous study, isatin secreted by a marine bacterium Pseudomonas C55a-2 (Taizo et al. 2011) was found to have algicidal activity against *Chattonella marina*, which is known to be one of the most noxious red-tide organisms to cause toxic blooms in many parts of the world (Kim et al. 2009). The present study is the first to report that isatin has algicidal activity against cyanobacteria in freshwater.

Isatin, which is also a pigment, shares some characteristics with prodigiosin which is a well-known anti-algal pigment secreted by marine bacteria (Jeong et al. 2005; Kwon et al. 2010; Nakashima et al. 2006a; Nakashima et al. 2006b). Specifically, prodigiosin and isatin both have algicidal activity against *C. marina* (Nakashima et al. 2006a; Taizo et al. 2011) with LD50 values of 8.5 μg/ml (Nakashima et al. 2006a) and

10 μg/ ml (Taizo et al. 2011), respectively. In this study, the LD50 value of isatin against *M. aeruginosa* was about 12.5 μg/ml, indicating that isatin and prodigiosin have similar algicidal activity. Prodigiosin could inhibit cell division of *C. marina* in previous report (Nakashima et al. 2006a), and isatin was found to influence *M. aeruginosa* 9110's cell division in the present study (Fig. 4d), which means that they may have the same algicidal mechanism.

Few studies about hexahydropyrrolo[1,2-a]pyrazine-1,4-dione have been conducted, even though it is known to inhibit family 18 chitinases (Houston et al. 2004). In this study, hexahydropyrrolo[1,2-a]pyrazine-1,4-dione at a level of 10 μg ml^{-1} did not lead to the morphology change of *Synechococcus* sp. BN60 (Fig. 4f) and did not inhibit the growth of *Synechococcus* sp. BN60 even when its concentration went up to 100 μg/ml (Fig. 3a), which means that it has no algicidal effects against *Synechococcus* sp. BN60. On the other hand, hexahydropyrrolo[1,2-a] pyrazine-1,4-dione (LD50 value=5.7 μg/ml) has stronger algicidal activity against *M. aeruginosa* 9110 than 2, 3-indolinedione (isatin) (LD50=12.5 μg/ml). This is the first study to report that hexahydropyrrolo[1,2-a]pyrazine-1,4-dione has algicidal activity against *Microcystis aeruginosa*.

Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione had no algicidal effects against *Synechococcus* sp. BN60, but had strong algicidal activity against *M. aeruginosa* 9110 (LD50 value= 5.7 μg/ml) (Fig. 3a), while 2, 3-indolinedione had both algicidal effects against *M. aeruginosa* 9110 (LD50 value= 12.5 μg/ml) and *Synechococcus* sp. BN60 (LD50 value= 34.2 μg/ml) (Fig. 3b). In general, both algicidal substances had stronger algicidal activity against *M. aeruginosa* 9110 than against *Synechococcus* sp. BN60. As shown in Figs. 5 and 6, the cell density of strain Lzh-2 in the cocultures of *M. aeruginosa* 9110-strain Lzh-2 was higher than that in the cocultures of *Synechococcus* sp. BN60-strain Lzh-2 during the cocultivation process, while the concentration of the two algicidal compounds in the cocultures of *M. aeruginosa* 9110-strain Lzh-2 was also higher than that in the cocultures of *Synechococcus* sp. BN60- strain Lzh-2, which could explain why the algicidal activity of *Shewanella* sp. Lzh-2 against *M. aeruginosa* 9110 was higher than that against *Synechococcus* sp. BN60.

As shown in Figs. 5 and 6, hexahydropyrrolo[1,2-a]pyrazine-1,4-dione was not detected until strain Lzh-2's cell density was higher than 5.3×10^8 cells/ml and 2.6×10^8 cells/ml in the two cocultures, respectively, and isatin was not detected until strain Lzh-2's cell density was higher than 1.5×10^9 cells/ml and 1.0×10^9 cells/ml in the two cocultures, respectively. Approximately, the algicidal activity, the concentration of S-2A and S-2B, and the cell density of Lzh-2 were positively related to each other from the first day to the sixth day. It suggested that the algicidal activity was dependent on the concentration of algicidal compounds and the cell density of algicidal bacteria during the cocultivation process.

Both hexahydropyrrolo[1,2-a]pyrazine-1,4-dione and 2, 3-indolinedione are biodegradable (Grant et al. 1988; Zhang et al. 2007), which is an important characteristic for environment-friendly use. Overall, the strain Lzh-2 and its two algicidal substances have the potential for use as a bioagent in controlling cyanobacterial blooms in Lake Taihu.

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