Microcystin-LR biodegradation by *Sphingopyxis* sp. USTB-05

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Abstract A promising bacterial strain for biodegrading microcystin-LR (MC-LR) as the sole carbon and nitrogen source was successfully isolated from Lake Dianchi, China. The strain was identified as *Sphingopyxis* sp. USTB-05, which was the first isolated MCs-biodegrading *Sphingopyxis* sp. in China. The average biodegradation rate of MC-LR by *Sphingopyxis* sp. USTB-05 was 28.8 mg·L⁻¹ per day, which was apparently higher than those of other bacteria reported so far. The optimal temperature and pH for both strain USTB-05 growth and MC-LR biodegradation were 30°C and 7.0, respectively. The release of MC-LR from the cyanobacterial cells collected from Lake Guishui and the biodegradation of MC-LR by both strain and cell-free extract (CE) were investigated. The results indicated that MC-LR with the initial concentration of 4.0 mg·L⁻¹ in water was biodegraded by *Sphingopyxis* sp. USTB-05 within 4 d, while MC-LR with the initial concentration of 28.8 mg·L⁻¹ could be completely removed in 3 h by CE of *Sphingopyxis* sp. USTB-05 containing 350 mg·L⁻¹ protein. During enzymatic biodegradation of MC-LR, two intermediate metabolites and a dead-end product were observed on an HPLC chromatogram. Moreover, the similar scanning profiles of MC-LR and its metabolic products indicate that the Adda side-chain of MC-LR was kept intact in all products.

Keywords microcystin-LR(MC-LR), biodegradation, *Sphingopyxis* sp, USTB-05, cell-free extract

1 Introduction

With the increase in the discharge of wastewater containing high nitrogen and phosphorus into rivers and lakes, harmful cyanobacteria blooms (HCB) have become a growing environmental problem in the world [1–3]. The ratios of eutrophic lakes are 53%, 28%, 48%, and 41% in

Europe, Africa, North America, and South America and increase to 54% and 80% in the Asian-Pacific region and China [4], respectively. HCB not only destroy the natural ecological system of lakes and rivers but also produce many types of cyanobacteria toxins of which microcystins (MCs) are the most dangerous [5]. In previous studies, we found that HCB in Lake Dianchi in Yunnan Province of China could produce a large amount of MCs [6], and MCs were also detected in Lake Guishui in Beijing, China. MCs are usually retained in algal cells during the growth, but these intracellular toxins are eventually released into water bodies, which can cause severe health risks to animals and humans.

MCs are a group of monocyclic heptapeptide hepatotoxins produced by species of cyanobacteria including *Microcystis*, *Anabaena*, *Nostoc*, and *Oscillatoria* [7,8]. More than 60 different analogues of MCs have been isolated from natural blooms or laboratory cultures of cyanobacteria [9] in which the most common and toxic analog is microcystin-LR (MC-LR) [10–13]. (2S,3S,8S,9S)-3-amino-9-methoxy-2,4,6-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) as one of the constituent amino acids in MC-LR is essential for the characteristic biologic activities of MCs [14,15].

The stable cyclic structure of MCs has presented many challenges to traditional water treatment technologies [16] where conventional processes, such as adsorption [17], ozone oxidation [18], photo-degradation [14], and electrochemical oxidation [19], have limited effect on their removal [11]. Aerobic biodegradation has been considered to be the main attenuation mechanism for MCs, and this process was reported to occur in diverse ecosystems, such as rivers [12], sand filters [11], reservoirs [20,21], coastal lagoons [22], lakes [17,23,24], and sewage effluent [25]. Bacterial strains of *Sphingopyxis* sp. [11], *Sphingomonas* sp. [8,15,20,21], *Pseudomonas aeruginosa* [23] *Burkholderia* sp. [22], and *Paucibacter toxinivorans* [26], isolated from Australia reservoirs, Japan lakes, a Brazilian coastal lagoon, and Finland lakes were found to have the capabilities of biodegrading MCs, respectively. Most of

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the strains reported belong to genus *Sphingomonas*, such as ACM-3962 [7], Y2 [8], LH21 [8], B-9 [15], 7CY [20], and CBA4 [21]. *Sphingomonas* sp. Y2 was reported to have the highest degradation rate of $5.4 \text{ mg} \cdot \text{L}^{-1}$ per day for MC-LR [8,22,24]. On the other hand, Holst et al. [27] and Chen et al. [28] investigated the potential for anoxic biodegradation of microcystin and the effects of environmental factors on the process through a series of well-controlled microcosm experiments.

As to the biodegradation mechanisms for MC-LR, the previous studies focused on the biodegradation pathways of *Sphingomonas* sp. [7,15,29,30]. MC-LR is subject to microbial degradation to provide three types of products, linearized MC-LR (Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg), tetrapeptide Adda-Glu-Mdha-Ala, and Adda [7]. Further, intact nontoxic Adda was first isolated from MC-LR by degradation using a B-9 strain as one of the final products [15]. In addition, Hashimoto et al. [29] identified the following MC-degrading products for cell preparation of strain B-9: a tetrapeptide, Adda-Glu-Mdha-Ala, tripeptides Adda-Glu-Mdha, Glu-Mdha-Ala, and Arg-MeAsp-Leu, a dipeptide, Glu-Mdha, and amino acids Adda, Arg, and methylamine derived from Mdha. However, less information is provided on the biodegradation and pathway of MC-LR directly released from the cyanobacterial cells by *Sphingopyxis* sp.

In this study, we isolated a bacterial strain *Sphingopyxis* sp. USTB-05 from Lake Dianchi, which showed a stronger capability to biodegrade MC-LR than those of other isolated bacterial strains reported previously. Rapid biodegradation was found for MC-LR directly released from the cyanobacterial cells collected from Lake Guishui in China. Furthermore, two intermediate metabolites and dead-end products were observed on the HPLC chromatogram during the biodegradation of MC-LR by cell-free extract of *Sphingopyxis* sp. USTB-05, which is very important from the standpoint of both basic research and its application in the efficient removal of MCs from the source of drinking water.

2 Materials and methods

2.1 Strain and chemicals

A bacterial strain used in this study for the biodegradation of MC-LR was isolated from the sediment of Lake Dianchi in China using MC-LR as the sole carbon and nitrogen sources. Standard MC-LR with a purity of 95% was purchased from Sigma Chemical, Co, and all other chemicals used were of analytical grade. The cyanobacterial cells collected from the surface of Lake Guishui on July 15, 2009, were prepared in 50 mM PB solution (pH 7.0) containing wet cells of $35 \text{ g} \cdot \text{L}^{-1}$, and an MC-LR biodegrading bacterial strain was inoculated to investigate the release and biodegradation of MC-LR.

2.2 Media and cultural conditions

The basic medium for the isolation and culture of bacterium consisted of 1.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g KH_2PO_4 , 4.0 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 20 mg CaCl_2 , 5 mg FeSO_4 , 5 mg ZnCl_2 , 5 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.5 mg CuCl_2 , and 1000 mL distilled water. MC-LR with different initial concentrations was added to the medium, and the initial pH was adjusted to 7.0 with $1.0 \text{ mol} \cdot \text{L}^{-1}$ NaOH or HCl.

The bacterium was inoculated in the sterilized medium and grown in a 50-mL flask containing 10 mL of liquid medium. The culture condition was at 30°C with a shake rate of 200 rpm. The optical density at 680 nm ($\text{OD}_{680 \text{ nm}}$) was measured to represent bacterial growth.

2.3 Isolation and identification of MC-degrading strain

The supernatant fluid of sediments taken from Lake Dianchi was inoculated into the culture medium containing $30 \text{ mg} \cdot \text{L}^{-1}$ MC-LR at 30°C with a shake rate of 200 rpm for isolating MC-LR biodegrading bacterial strains. After three consecutive transfers, the cultures were diluted and spread onto solid plates. Bacterial colonies grown on the plates were picked up and inoculated into liquid medium containing MC-LR of $30 \text{ mg} \cdot \text{L}^{-1}$ to test their biodegradation capabilities of MC-LR, respectively, which were repeated several times until a pure bacterial strain was isolated.

An isolated bacterial strain capable of biodegrading MC-LR was identified by 16S rDNA sequence analysis, which was selectively amplified by PCR (Eppendorf-5331) with two universal primers (Pf: 5'-AGAGTTTGATCATGGCT-CAG-3'; Pr: 5'-CTACGGTTACCTTGTTACGAC-3'). PCR reactions were performed for 5 min at 94°C , then cycled 30 times (94°C for 1 min, then 50°C for 1 min, and, finally, 72°C for 3 min), and then extension reactions were carried out for 10 min at 72°C . The amplified DNA was sent to the Beijing Sunbiotech, Co, in China for sequencing.

2.4 Preparation of cell-free extract (CE)

The bacterial cells were harvested by centrifugation at $12000 \text{ r} \cdot \text{min}^{-1}$ for 10 min at 4°C , followed by washing twice with 50 mM potassium phosphate buffer (PB, pH 7.0). The cells were resuspended in 20 mL PB solution and sonicated with an output power of 400 W for 10 min at 4°C . The cell debris was removed by centrifugation at 15000 rpm for 20 min, and the supernatant was saved as CE. The protein concentration of CE was determined with the Bradford method [31]. The reactions containing the mixture of CE and MC-LR in PB solution were performed at the temperature of 30°C with a shake rate of 200 rpm. The samples of MC-LR taken at various time points were measured by HPLC.

2.5 Analysis of MC-LR

MC-LR was measured using a High Performance Liquid Chromatography (HPLC) system (Shimadzu LC-10ATVP, Shimadzu, Co, Japan) with an UltraViolet (UV) Diode Array Detector at 239 nm using a column of Waters μ Bondapak C₁₈ (300 $\times$ 3.9 mm). The mobile phase was 40% (v/v) acetonitrile-water solution containing 0.03% (v/v) of trifluoroacetic acid with flow-rate of 1.0 mL $\cdot$ min⁻¹. The calibration curve was established between the peak areas and the concentration of MC-LR: the curve was used to calculate the unknown concentration of MC-LR in the experiments.

3 Results and discussion

3.1 Isolation and identification of MC-degrading strain

Using MC-LR as the sole carbon and nitrogen sources, an MC-LR biodegrading bacterial strain named USTB-05 was successfully isolated, which formed a circle yellow colony on solid medium (Fig. 1). The observation under a microscope indicated that the cells of strain USTB-05 were gram-negative and short-rod with Gram Stain (Fig. 2). The strain could grow in a pH range from 6.0 to 11.0 and also on the agar medium with a maximum NaCl of 2%. It had no antibiotic resistance to any of the eight antibiotics including tetracycline, streptomycin, kanamycin, amphotericin, chloromycetin, gentamycin, spectinomycin, and erythromycin.

Fig. 1 Colonies of strain USTB-05 grown on solid medium

The relationship of strain USTB-05 to other closely related members is shown in Fig. 3. The analysis results of 16S rDNA sequence showed that strain USTB-05 appeared to be closely related to *Sphingopyxis* sp. Based on the phylogenetic analysis of 16S rDNA sequences, the isolated bacterial strain was identified and named as *Sphingopyxis* sp. USTB-05. The complete 1453 bp sequences of strain USTB-05 16S rDNA have been

Fig. 2 Cell shape of strain USTB-05 under microscope ($\times$ 1000)

deposited in the GenBank database under accession number EF607053.

3.2 Biodegradation of MC-LR by *Sphingopyxis* sp. USTB-05

At 30°C with a shake rate of 200 rpm, the biodegradation of MC-LR and the growth of *Sphingopyxis* sp. USTB-05 are shown in Fig. 4. The growth process of strain USTB-05 underwent a lag phase in the first 8 h, exponential phase between 8 h to 24 h, and stationary phase after 24 h. With the growth of strain USTB-05, the MC-LR concentration slowly decreased from 0 h to 8 h and sharply declined from 8 h to 24 h. The maximum biodegradation rate appeared at the most rapid growth phase of USTB-05, and the initial MC-LR of 28.8 mg $\cdot$ L⁻¹ was completely removed within 24 h.

Some bacterial strains, such as *Sphingopyxis* sp. [11], *Sphingomonas* sp. [8,15,20,21,29], and *Pseudomonas aeruginosa* [22] for the MCs biodegradation were successfully isolated from the surface water or sediment of HCB lakes. *Sphingomonas* sp. Y2 was reported to have the highest biodegradation rate of 5.4 mg $\cdot$ L⁻¹ MC-LR per day [8,22,24]. A very promising bacterial strain isolated and identified as *Sphingopyxis* sp. USTB-05 was the first isolated bacterial strain of *Sphingopyxis* capable of biodegrading MC-LR found in China (Fig. 3). *Sphingopyxis* sp. USTB-05 indeed had a stronger capability to biodegrade MC-LR than those bacterial strains reported previously, and the initial MC-LR of 28.8 mg $\cdot$ L⁻¹ could be completely removed within one day (Fig. 4).

3.3 Effects of pH and temperature on USTB-05 growth and MC-LR biodegradation

The MC-LR biodegradation by *Sphingopyxis* sp. USTB-05 under the different initial pHs and temperatures was investigated (Fig. 5). It indicated that the MC-LR biodegradation was promoted at initial pHs of 7.0 and 9.0 but

Fig. 3 Phylogenetic tree of *Sphingopyxis* sp. USTB-05

Fig. 4 Growth of *Sphingopyxis* sp. USTB-05 and biodegradation of MC-LR

was apparently inhibited at an initial pH of 5.0 (Fig. 5(a)). It also showed an optimal growth temperature of 30°C (with a range from 25°C to 37°C) for the MC-LR biodegradation (Fig. 5 (b)). Generally, HCB appeared in an alkaline water body at 30°C, and the isolated *Sphingopyxis* sp. USTB-05 was suitable to the environment of high pH and mesophilic temperature, so the MC-LR biodegradation was significantly enhanced under the conditions of an alkaline environment and mesophilic temperature.

3.4 Release and biodegradation of MC-LR

Figure 6 indicates that the initial MC-LR of 4.0 mg·L⁻¹

increased to about 5.6 mg·L⁻¹ within 2 d and then kept stable until day 5 in the presence of cyanobacterial cells collected from Lake Guishui, which reflects the release of MC-LR from the cyanobacterial cells into water. However, in the presence of *Sphingopyxis* USTB-05, the MC-LR concentration decreased rapidly to nil at day 4, which indicated that *Sphingopyxis* USTB-05 can quickly remove MC-LR presented in water initially and that released from cyanobacterial cells within 4 d. This is very important in the efficient removal of MC-LR from water.

3.5 Biodegradation of MC-LR by CE of *Sphingopyxis* sp. USTB-05

Figure 7 shows the MC-LR biodegradation rates by the crude enzymes of *Sphingopyxis* sp. USTB-05 containing protein concentrations of 70, 210, and 350 mg·L⁻¹, respectively, which were much higher than that by *Sphingopyxis* sp. USTB-05 cells (Fig. 4), resulting in the initial MC-LR of 28.8 mg·L⁻¹ being completely eliminated within 12, 5, and 3 h, respectively. MC-LR could be biodegraded by both cells and CE of *Sphingopyxis* sp. USTB-05, which is in agreement with the earlier reports by Bourne et al. [7] and Ishii et al. [30]. The results suggested that the CE of MC-biodegrading bacterium is also valuable to efficiently eliminate MC-LR from drinking water.

In the presence of CE containing 70 mg·L⁻¹ protein and 28.8 mg·L⁻¹ MC-LR, the MC-LR biodegradation products were further investigated using HPLC profiles over time (Fig. 8). The retention time of the MC-LR peak was 10.1 min (Fig. 8(a)). After reaction for 90 min, the peak of

Fig. 5 Effects of pH (a) and temperature (b) on the biodegradation of MC-LR

Fig. 6 Biodegradation of MC-LR remained in water and released from cyanobacterial cells collected from Lake Guishui by *Sphingopyxis* sp. USTB-05

Fig. 7 Biodegradation of MC-LR by CE of *Sphingopyxis* sp. USTB-05 with different protein concentrations

MC-LR decreased and three peaks of products A, B, and C at retention times 3.6 min, 6.1 min, and 8.6 min appeared, respectively (Fig. 8(b)). The peaks of MC-LR and product A decreased, while those of product B and C increased at 180 min, (Fig. 8(c)). At the time of 720 min, only peak C could be observed, while all the peaks of MC-LR and the products of A and B disappeared (Fig. 8(d)). This showed that at least three products were produced during the biodegradation of MC-LR catalyzed by the enzymes of *Sphingopyxis* USTB-05; the products of A and B were intermediate products, and product C was a final biodegradation product.

Bourne et al. [7] and Haradi et al. [15] found that linear MC-LR and a tetrapeptide ($\text{NH}_2\text{-Adda-Glu-Mdha-Ala-OH}$) were the first and second products of MC-LR biodegradation catalyzed by the enzymes of *Sphingomonas* sp. and *Sphingomonas* sp. B-9, respectively. However, Haradi et al. [15] reported that Adda was one of the final products of MC-LR biodegradation by another

Sphingomonas sp. isolated. Furthermore, Hiroshi et al. [30] observed three products from the biodegradation of MC-LR by *Sphingomonas* sp.7CY, which indicated that the biodegradation pathways of MC-LR by various bacterial strains might be different. Here, we found two intermediates and one dead-end product clearly on the HPLC profile (Fig. 8), which is very important in the further study of the biodegradation pathway of MC-LR.

The 4, 6-conjugated diene of Adda amino acid side chain in MCs contributed to the characteristic spectrum and was an important determinant of MC-LR in HPLC analysis [7]. MC-LR showed a λ_{max} of 239 nm, while the λ_{max} of products A, B, and C were all at or near 239 nm (Fig. 9); moreover, the UV spectra of all products were very similar to that of MC-LR. This indicates that the Adda side-chain was kept intact following enzymatic modification of MC-LR by CE of *Sphingopyxis* sp. USTB-05. The biodegradation pathway of MC-LR by *Sphingopyxis* sp. USTB-05 is being studied now.

Fig. 8 HPLC profiles for biodegrading MC-LR by CE of *Sphingopyxis* sp. USTB-05 with time course. (a) 0 min; (b) 90 min; (c) 180 min; (d) 720 min

Fig. 9 Absorbance profiles of MC-LR and its biodegradation products in the ultraviolet wavelength from 200 to 300 nm, respectively

$350 \text{ mg} \cdot \text{L}^{-1}$ protein. Initial MC-LR of $4.0 \text{ mg} \cdot \text{L}^{-1}$ in water, which was produced by the cyanobacterial cells collected from Lake Guishui, was totally eliminated by *Sphingopyxis* sp. USTB-05 within 4 d. During the biodegradation of MC-LR catalyzed by crude enzymes, two intermediate and a dead-end products of MC-LR had been observed on a HPLC chromatogram. The similar scanning profiles of MC-LR and its metabolic products at the ultraviolet wavelength from 200 to 300 nm indicated that the group of Adda in MCs was kept intact, which is very important for the efficient removal of MC-LR from the drinking water source.

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4 Conclusions

Sphingopyxis sp. USTB-05 is a MCs-biodegrading strain isolated from the sediment of Lake Dianchi, China. The initial MC-LR of $28.8 \text{ mg} \cdot \text{L}^{-1}$ could be completely biodegraded within 24 h. The optimal temperature and pH for the MC-LR biodegradation are 30°C and 7.0, respectively. Furthermore, the initial MC-LR of $28.8 \text{ mg} \cdot \text{L}^{-1}$ could be completely removed at 3 h by its CE containing

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