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Biodegradation of microcystin-LR by an amino acid-degrading anaerobic bacterium

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ABSTRACT

Recent studies have proved that microcystins (MCs) can be degraded by sediment under anaerobic conditions, suggesting that anaerobic biodegradation is an important pathway to remove MCs in water treatment. In this study, the potential for biodegradation of microcystin-LR (MCLR) by an amino acid-degrading anaerobic bacterium ALA-1 was investigated. Under mesophilic conditions (20, 25 and 30°C), MCLR was dropped from 4 mg/L to below the detection limit in 10 d without lag phase. While at cold temperatures (10 and 15°C), MCLR degraded completely in 14 and 19 d with a delay time reaching 6 and 8 d, respectively. Under alkaline conditions (pH 8.0, 9.0), MCLR was degraded completely within 10 d without a lag phase. While MCLR was degraded completely within 12 d under neutral condition (pH 7.0). A lag time of 8 and 20 d was needed prior to the onset of MCLR degradation under two slightly acidic conditions (pH 6.0, 5.0), respectively, and MCLR was completely consumed until day 22 and day 38. In addition, extra carbon or nitrogen sources had no significant effect on the degradation ability of ALA-1. Linearized MCLR (*m*/*z* 1,013.3729, $C_{49}H_{77}N_{10}O_{13}$), tetrapeptide (*m*/*z* 616.1208, $C_{32}H_{48}N_4O_8$) and Adda (*m*/*z* 332.0186, $C_{20}H_{30}NO_3$), commonly known as aerobic biodegradation products of MCLR, were detected as the intermediate products. The mlrA gene homologue, known for its degradation potential of MCLR, was absent in strain ALA-1, implying that other enzymes which were not encoded by the mlrA gene cluster might have been involved in metabolizing MCLR in the present study.

Keywords: Anaerobic bacterium; Microcystin-LR; Biodegradation products; Enzymatic degradation; mlrA gene

1. Introduction

Microcystins (MCs) are a group of cyclic heptapeptides consisting of seven amino acids produced by cyanobacteria, which are widely distributed in eutrophic water [1,2]. Most of them are chemically stable compounds and are resistant to regular physical and chemical factors [3]. Their potent hepatotoxicity and tumour promotion activity have posed risks to ecosystem and human health. The toxicity mainly occurs through inhibitory activity against protein phosphatases [4]. Nearly 80 variants have been isolated and identified in cyanobacterial blooms, among which microcystin-LR (MCLR) is the most common variant.

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Since chronic exposure to low concentrations of MCs may promote tumour growth, the MCs in drinking water are of great concern. They cannot be well eliminated by conventional water treatment procedures. It needs more costly treatments to remove MCs such as advanced oxidation and activated carbon adsorption, which have certain limitations, both in terms of formation of toxic intermediates and economical restrict application in production [5]. It seems that biodegradation with the participation of environmental bacteria appears to be the main pathway that leads to the decrease of MCs in eutrophic water environment, and biodegradation process is considered as a potential method to get rid of MCs contamination from water bodies [6,7].

Aerobic biodegradation of MCs, as a major attenuation mechanism for MCs in the environment, has been widely studied. The degradation rate is affected by several environmental factors such as temperature [8], pH value [9] and nutrient conditions [10]. One approach for MCLR biodegradation has been expounded, which consists of sequential enzymatic hydrolyses of the peptide bonds of Arg–Adda, Ala–Leu and Adda–Glu to produce linear MCLR, a tetrapeptide and Adda [11,12]. The authors determined that these intermediate products were less active than the parent MCLR using protein phosphatase inhibition and mouse bioassays. It suggests that biodegradation by aerobic MCs-degrading bacteria is a safe and practical elimination pathway for MCs in water. Moreover, Bourne et al. [11] identified a gene cluster including mlrA, mlrB, mlrC and mlrD in *Sphingomonas* sp.. The mlrA gene is able to encode an enzyme responsible for the hydrolytic cleaving of the cyclic structure of MCLR, which is the first and most important step.

Unlike aerobic degradation, anoxic or anaerobic degradation has been regarded as a negligible attenuation mechanism for MCs in the environment. Nevertheless, the study of Chen et al. [13] showed that MCs can be rapidly decomposed under anoxic conditions by indigenous microbial community in lake sediments. Adda was produced and accumulated during the anoxic degradation of MCs, and no toxic product was detected in this process. These findings suggest that anoxic biodegradation process may have a more important role in MCs removal than previously thought. Moreover, the intermittent hypoxia and anaerobic environment exist extensively in the eutrophic water body and sediments, especially when there are serious algal bloom. Anaerobic degradation can be an important pathway to remove MCs in water treatment. However, little information is available about the anoxic or anaerobic degradation of MCs and its mechanisms until now. No further study on anaerobic degradation of MCs was reported.

Since MCs are a group of cyclic heptapeptides containing several amino acid groups, an amino acid-degrading bacterium ALA-1 [14] was thus selected in this study to degrade MCLR. Strain ALA-1 was originally isolated from anaerobic lagoon of a dairy waste water treatment plant, sequenced for its 16S rRNA and deposited in a GenBank (accession number AF 069287). It has been proved to be able to degrade MCLR effectively under anaerobic condition in the preliminary experiment. In this study, the anaerobic MCLR degradation process by ALA-1 was further investigated. Scanning electron microscope (SEM) image of ALA-1 cells was applied to show the cell morphology. The effects of initial inoculum, temperature, pH value, extra carbon and nitrogen source on the degradation of MCLR were also investigated. And MCLR degradation rates for different experimental conditions were quantified by fitting a first-order exponential degradation curve. The position of degradation enzyme which is responsible for the MCLR degradation was investigated through enzymatic degradation experiments. The degradation product was identified by time-of-flight mass spectrometry (TOF-MS). PCR amplification was performed to testify whether strain ALA-1 contains mlrA gene. Results from this study will provide insights into the role of anaerobic degradation on the fate of MCs.

2. Materials and methods

2.1. Materials and reagents

ALA-1 (DSM 12261) was purchased from the Deutsche Sammlung von Mikroorganismen, Germany, and pre-incubated by inoculation in PY medium (5 g of peptone, 2.5 g of yeast extract per 1,000 mL at pH 7.2). Standard MCLR for analysis was purchased from Sigma–Aldrich (St. Louis, MO, USA). Crude MCLR was extracted from lyophilized *Microcystis aeruginosa* cells in methanol and water under conditions previously documented by Hu et al. [15]. The lyophilized *Microcystis aeruginosa* cells were purchased from the fresh algae culture collection of the Institute of Hydrobiology, Chinese Academy of Sciences. The purity of MCLR obtained was ≥90% as determined by high-performance liquid chromatography (HPLC). The purified MCLR was concentrated and stored at −20˚C. HPLC-grade methanol bought from Tedia Company (Incorporated, Fairfield, OH, USA) was used as the extraction solvent and HPLC mobile phase. All other chemicals were of analytical grade. The mineral salt (MS) medium used for ALA-1 and degradation

contained (g/L) NaCl 0.1; $MgSO_4{\cdot}7H_2O$ 0.5; KH_2PO_4 0.5; K_2HPO_4 0.5; $CaCl_2$ 0.02; $FeSO_4{\cdot}7H_2O$ 0.01. The final pH of MS medium was adjusted to 7.0 by HCl and NaOH. Medium solution, pipette tips and Erlenmeyer flasks sealed with rubber stoppers were autoclaved at 120˚C for 20 min for sterilization before use.

2.2. Control of anaerobic conditions

Anaerobic conditions were provided by an anaerobic glove incubator (YQX-II, Shanghai Xinmiao Medical Equipment Manufacturing Company Limited). High purity nitrogen was used to replace air of the incubator. Then, use activated palladium particles (506HT, Dalian Kelian New Technology Development Company) and silica gel (Shanghai Puzhen Biological Science and Technology Company Limited) to remove residual oxygen and to keep incubator dry. Meilan indicator (Beijing Zhongxi Scientific Instruments and Sci-tech Company Limited) was applied to determine whether the incubator was under anaerobic conditions. If the indicator had no change of colour, the glove incubator was in good anaerobic conditions. If the indicator changed to blue instead, it showed that the anaerobic conditions were not guaranteed. During the experiments, the nitrogen was injected continuously into the incubator. All operations of this study were done in the anaerobic glove incubator.

2.3. Design of MCLR anaerobic biodegradation experiments

Different treatments were as follows: first, ALA-1was incubated at two initial inoculums (10 and 20%) with MS medium containing crude MCLR extract. Second, ALA-1 was incubated with MS medium containing crude MCLR extract at various incubation temperatures, such as 10, 15, 20, 25 and 30˚C. Then ALA-1 was incubated with MS medium containing crude MCLR extract at 30˚C but with different pH values, such as 5.0, 6.0, 7.0, 8.0 and 9.0. The influence of amended nutrients on anaerobic biodegradation of MCLR by ALA-1 was also investigated by addition of glucose (100 mg/L), $NaNO_3$ (100 mg/L) and mixture of glucose (50 mg/L) and $NaNO_3$ (50 mg/L) at 30˚C. Control group was prepared in the same way without amended nutrients. An aliquot (300 μL) of samples was withdrawn at intervals. The concentrations of MCLR in supernatants were detected using HPLC. All experiments were carried out in duplicate.

2.4. Crude enzyme preparation

Cultures of the bacterial strain were inoculated into YP medium and incubated overnight at 30˚C. The cultures were harvested in the late logarithmic phase and centrifuged at 4˚C to obtain the thallus. The supernatant was passed through a sterile 0.22 mm filter to obtain extracellular enzyme. Then, an equal volume of 50 mM potassium phosphate buffer (pH 7.0) was added. The cells were resuspended and filtered, and the supernatant was discarded. This washing protocol was repeated three times. The resuspended cells were sonicated in an ice bath with microson cell disrupters (25 W). The cell debris was pelleted by centrifugation at 15,000 rpm for 10 min, and the supernatant was passed through a sterile 0.22 mm filter to obtain intracellular enzyme.

2.5. Enzyme degradation assays

ALA-1 was incubated with MS medium containing crude MCLR extract at initial inoculums of 20%. The same biomass of bacteria solution was used to obtain intracellular and extracellular enzymes as the way documented by Bourne et al. [11]. Enzyme assays were performed at final concentrations of 2 mg/L using obtained enzymes and MCLR. All assay mixtures were incubated at 30˚C. Samples (300 mL) were removed periodically, inactivated in a 95˚C water bath for 10 min, and then analyzed by HPLC.

2.6. Analysis of MCLR and degradation products

Analysis of MCLR was performed using an Agilent 1100 HPLC machine. Briefly, the crude extract was thawed in room temperature and centrifuged at 12,000 rpm for 10 min at 4˚C. The supernatant were applied to a conditioned SPE cartridge (SepPak C18, Waters). The cartridge was first washed with 5 mL methanol followed by 5 mL distilled water. Impurities were eluted with 2 mL methanol, and MCLR was eluted with 2 mL 80% (v/v) methanol. The eluate was analyzed by HPLC kept at 40˚C. The mobile phases were composed of 0.1% (v/v) trifluoracetic acid aqueous solution and methanol, which were blended at a rate of 45:55 over 25 min. The flow rate was 1 mL/min. The eluent was passed through a variable wavelength detector operated at 238 nm and calculated against a standard curve with MCLR (Sigma–Aldrich, USA).

The degradation product was analyzed using an Agilent 1200 HPLC combined with an Agilent 6224 TOF-MS with a mass range from m/z 100 to 1,200 in positive electrospray ion (ESI) mode. The degradation product was applied to a Sep-pak octadecyl silane (ODS) cartridge (500 mg/6 mL) preconditioned with methanol and water. The cartridge was rinsed with

10 mL of 20% methanol (v/v) solution and eluted with 10 mL of 100% methanol. The eluate was stored at −20℃ before analysis. Then, the sample of purified degradation product was dissolved in 45:55 water–methanol solutions containing 0.1% formic acid. The used analytical column was Zorbax Extend C_{18} column (2.1 × 50 mm, 1.8 μm, Agilent, USA). The mobile phase was at a flow rate of 0.1 mL/min. The column temperature was maintained at 40℃, and the injection volume was 2 μL. The ESI–TOF-MS parameters were set as follows: gas temperature, 320℃; fragmentor voltage, 150 V; capillary voltage, 3,500 V; flow of drying gas, 10 L/min; and nebulizer pressure, 40 psi.

2.7. Detection of the mlrA gene

Two or three single colonies of ALA-1 were picked from PY medium and cracked at 94℃ for 3 min. Then the product was centrifuged at 3,000 rpm for 1 min to remove cell debris. The supernatant was employed as DNA template in the next PCR process. The ultra pure water was used as a negative control, and an aerobic MC-degrading bacterium X-1 isolated in the laboratory was used as a positive control.

The primers employed for the amplification of mlrA (accession number AF411068) fragment in this experiment were MF (5′-GACCC GATGT TCAAG ATACT-3′) and MR (5′-CTCCT CCCAC AAATC AGGAC-3′) [16]. The volume of reaction system was 25 μL containing (μL): 10 × PCR buffer 2.5, $MgCl_2$ (25 mmol/L) 2.5, dNTP (10 mmol/L) 2.5, MF 0.5, MR 0.5, DNA template (100 ng/μL) 1, TaqDNA polymerase 0.5 andsterilization ultrapure water 10. The DNA template was the last one to add into the sterile PCR tube. All operations were done on the ice to ensure active TaqDNA polymerase and substrates. The PCR ran with initial denaturation at 95℃ for 1 min, 30 thermal cycles of denaturation for 20 s at 94℃, annealing for 30 s at 60℃, extension for 30 s at 72℃ and a final elongation for 10 min at 72℃. The product was stored at −20℃ before electrophoresis detection [9].

2.8. Statistical analysis

In order to compare degradation rates for different experimental conditions, MCLR degradation was quantified by fitting a first-order exponential degradation curve to the measured [17], normalized values (c/c_0; see Eq. (1), with x being the concentration as a function of time (mg/L), x_0 the residual concentration (mg/L), A the initial concentration (mg/L), k_1 the degradation rate (d^{-1}) and y time (d)). In the presence of a lag time, the curve was fitted to the data at the end of the lag time.

$$x = x_0 + Ae^{-k_1^* y} \quad (1)$$

Half-life ($T_{1/2}$) was calculated based on the obtained degradation rate according to the following equation.

$$T_{1/2} = \ln 2/k_1 \quad (2)$$

3. Results and discussion

3.1. Cell morphological observation

SEM image of ALA-1 cells (Fig. 1) showed the cell morphology. The cells were 1.0 ~ 2.0 μm in length and 0.2 ~ 0.4 μm in width, and were slightly curved to rod-shaped. The cell morphology of ALA-1 is similar to most of the known separation and purification aerobic degradation bacteria, which are rod shaped without spores and gram negative [9].

3.2. Effects of environmental factors on anaerobic MCLR biodegradation

3.2.1. Effect of initial inoculums

MCLR was completely degraded within 9 d at both initial inoculums (10 and 20%). When strain ALA-1 was inoculated at initial inoculums of 10%, a lag time of 6 d was needed prior to the onset of MC degradation. While when strain ALA-1 was inoculated at initial inoculums of 20%, MCLR was degraded completely without obvious lag phase (figure not shown). The values of OD_{600} detected by spectrophotometer after 9 d at two initial inoculums were about

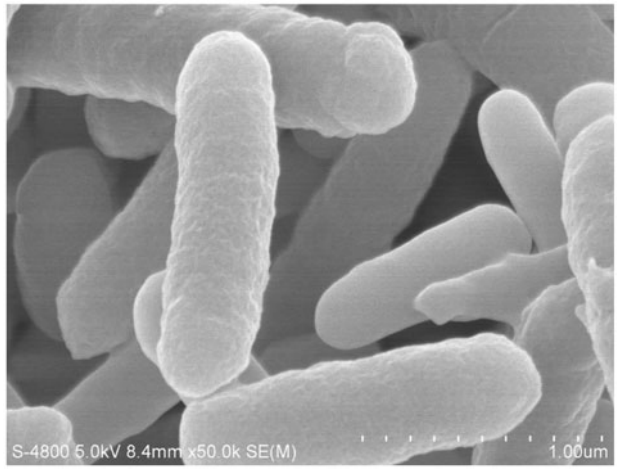

Fig. 1. Cell morphology of ALA-1 cells was observed under SEM.

Table 1
Summary of experimental conditions and results of anaerobic biodegradation of MCLR by strain ALA-1. The error is given as the variation coefficient

Test conditions	k_1 (d^{-1}) (%)	$T_{1/2}$ (d) (%)
Temperature (℃)		
10	0.049 ± 6	14.2 ± 6
15	0.075 ± 3	9.3 ± 3
20	0.103 ± 2	6.7 ± 2
25	0.110 ± 2	6.3 ± 2
30	0.120 ± 4	5.8 ± 4
pH		
5	0.026 ± 8	26.7 ± 6
6	0.043 ± 5	16.2 ± 5
7	0.116 ± 3	6.0 ± 0
8	0.150 ± 2	4.6 ± 2
9	0.158 ± 2	4.4 ± 0
Extra carbon and nitrogen source		
None (control)	0.124 ± 2	5.6 ± 1
Glucose (100 mg/L)	0.120 ± 2	5.8 ± 1
$NaNO_3$ (100 mg/L)	0.112 ± 2	6.2 ± 2
Glucose (50 mg/L) and $NaNO_3$ (50 mg/L)	0.132 ± 2	5.2 ± 1

Note: k_1 is the degradation rate of MCLR, and $T_{1/2}$ is half-life of MCLR degradation.

0.01 and 0.02. The low values of OD_{600} at two initial inoculums suggest that strain ALA-1 grew slowly under anaerobic condition and the degradation rate was relatively fast.

3.2.2. Effect of temperature

The biodegradation curves of MCLR at various incubation temperatures showed a common trend, but the degradation rates were strongly dependent on the incubation temperature. The degradation rates of MCLR from 4 mg/L to below the detection limit were 0.049, 0.075, 0.103, 0.110 and 0.120 d^{-1} at 10, 15, 20, 25 and 30℃ (Table 1). As shown in Fig. 2, the lag periods were 6 and 8 d at 15 and 10℃, while there are no lag periods at 20, 25 and 30℃. And, MCLR was completely consumed within 10 d under these mesophilic conditions, while MCLR was completely removed within 19 and 14 d at 10 and 15℃.

Although anaerobic degradation occurred at all temperatures tested, a higher rate was observed at higher temperatures (20, 25 and 30℃) than at lower temperatures (10 and 15℃) (Fig. 2 and Table 1). These results suggest that strain ALA-1 involved in MCLR degradation is likely to be mesophilic bacteria. Similar effects of temperature on aerobic MCs degradation have also been reported. Wang et al. [18] discovered that the aerobic biodegradation rate of MCLR in a GAC column was higher at 30℃ than at 22℃. Park et al. [8] reported that the degradation rate of MCs by a strict aerobic bacterium was strongly dependent on temperature, and the highest degradation rate occurred at 30℃.

3.2.3. Effect of pH

As shown in Table 1, the degradation rates of MCLR from 2 mg/L to below the detection limit were

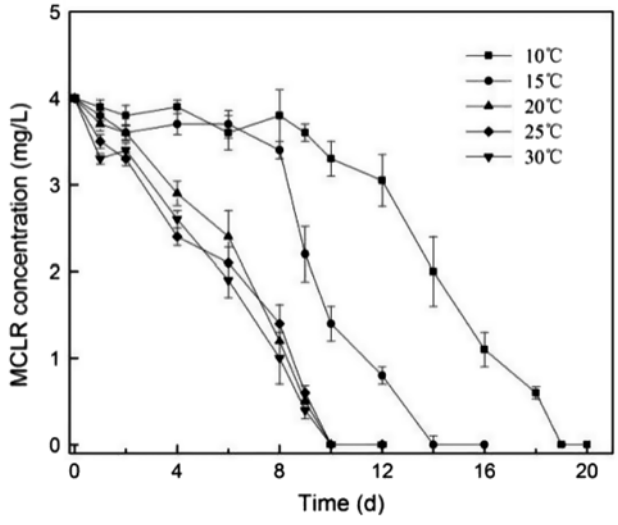

Fig. 2. Degradation of MCLR with strain ALA-1 at various incubation temperatures, 10℃ (■), 15℃ (●), 20℃ (▲), 25℃ (◆) and 30℃ (▼). Error bars represent the range of duplicate microcosms.

0.026, 0.043, 0.116, 0.150 and 0.158 d^{-1} with various pH values at 5.0, 6.0, 7.0, 8.0 and 9.0, respectively. At pH 7.0, 8.0 and 9.0, MCLR was degraded completely within 12 d without lag phase. While a lag time of 8 and 20 d was needed before the onset of MCLR degradation at pH 6.0 and 5.0, and MCLR was completely consumed until day 22 and day 38 (Fig. 3).

The results above imply that ALA-1 prefers to degrade MCLR in alkaline rather than acidic environments. Since the actual water body is closer to neutral or alkaline environment, it is promising to apply this strain for the removal of MCs through anaerobic systems such as biological sand filter and submerged constructed wetland. Similarly, Chen et al. [9] discovered MCLR and MCRR could be completely degraded by aerobic strain EMS at pH 7.0–9.0. But during the same time, the MCs were far to consumed at pH 5.0–6.0. Okano et al. [19] found that the MCs degradation activity of the bacterium C-1 reached the peak between pH 6.52 and 8.45 but the highest point was detected at pH 10.0. These results further illustrate that most MC-degrading bacteria prefer to degrade MCLR under alkaline and neutral conditions.

3.2.4. Effect of extra carbon and nitrogen source

From the statistics given in Table 1, it can be seen that the degradation rates of MCLR from 2 mg/L to below the detection limit are 0.124, 0.120, 0.112 and 0.132 d^{-1} with addition of nothing, glucose (100 mg/L), NO_3-N ($NaNO_3$ 100 mg/L) and mixture of glucose (50 mg/L) and $NaNO_3$ (50 mg/L), respectively. Meanwhile, Fig. 4 shows that MCLR was completely degraded within 11 d without lag time in the control group and groups with extra carbon or nitrogen source. Moreover, MCLR was completely consumed within 10 d in the group with extra carbon and nitrogen source simultaneously.

The results of glucose addition are similar to the findings reported by Chen et al. [13] who reported that glucose addition had no statistically significant effect on the anoxic biodegradation of MCLR by sediment. But Holst et al. [20] found that glucose addition significantly stimulated MCs degradation under anoxic conditions in sediment slurries, and attributed this result to the stimulation of bacterial activity by glucose. More recently, Eleuterio and Batista [21] demonstrated that addition of a carbon source (acetate) significantly repressed the degradation of MCLR by MCs-degrading bacterium isolated from the biofilter. The contradictory results may be due to the following reasons. Carbon source may have an intricate role in the biodegradation process. On the one hand, additional carbon source, such as glucose, may stimulate bacterial activity [20] by providing essential energy for MCs degrading bacteria. On the other hand, an extra carbon source may inhibit bacteria from degrading MCs because MCs and glucose both can be a carbon source [8,20]. Moreover, accumulation of the metabolic product of

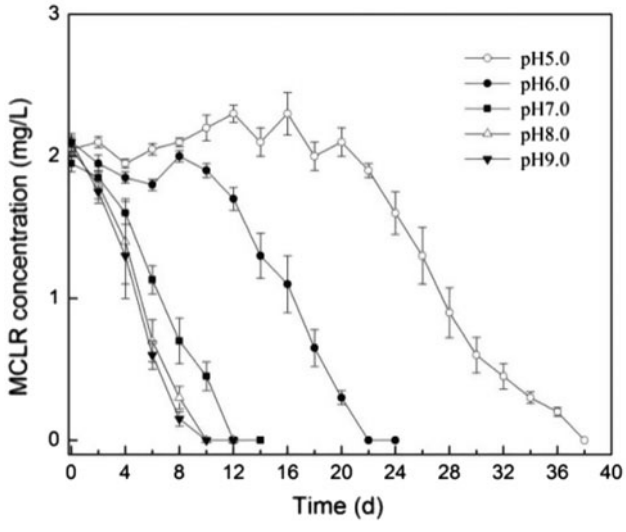

Fig. 3. Degradation of MCLR with strain ALA-1 under various pH conditions, 5.0 (○), 6.0 (●), 7.0 (■), 8.0 (△) and 9.0 (▼) at 30℃. Error bars represent the range of duplicate microcosms.

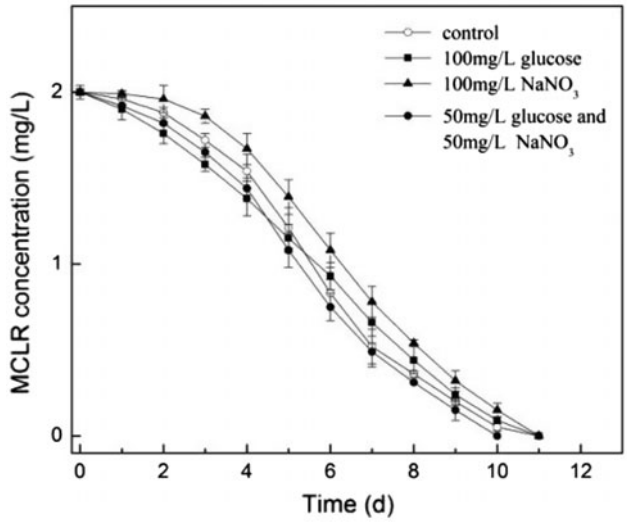

Fig. 4. Effect of extra carbon or nitrogen source amended on the anaerobic biodegradation of MCLR at 30℃, control group (○), 100 mg/L glucose (■), 100 mg/L $NaNO_3$ (▲), 50 mg/L glucose and 50 mg/L $NaNO_3$ (●). Error bars represent the range of duplicate microcosms.

glucose (e.g. lactic acid) can reduce bacterial viability through changing the pH [10], thereby influencing MCs biodegradation.

The result of NO_3-N addition (1.18 mM) differs from those of previous works. Holst et al. [20] found that NO_3-N (5 mM) addition significantly stimulated MCs degradation under anoxic conditions, and suggested that this process was coupled to denitrification. But Chen et al. [13] reported that NO_3-N (0.026 mM) addition significantly retarded MCs degradation under anoxic conditions and suggested that the inhibition of degradation was due to the increasing redox potential by nitrate addition rather than due to increasing the nitrogen source. If the same coupling occurred in our experiment, NO_3-N addition should have the same effects because our NO_3-N content was far higher than that of 0.026 mM in the study of Chen et al. [13] and lower than that of 5 mM in the study of Holst et al. [20]. The fact that no stimulation or apparent inhibition was observed in our experiment suggests that the pathways for the anaerobic biodegradation of MCs are different between sediment and pure bacterial isolate. Nitrogen source may also have an intricate role in the biodegradation process. The definite effect of extra nitrogen source may depend on the concentration of nitrate.

3.3. MCLR degradation by strain ALA-1 and its crude enzyme

MCLR was consumed to below the detection limits within 10 h by the intracellular enzymes, while extracellular enzymes showed no degradation activity within 85 h (figure not shown). Enzyme degradation assays showed that the intracellular enzymes were mainly responsible for MCLR degradation process. The intracellular enzymes of strain ALA-1 degraded MCLR faster than the ALA-1 cells. The results are similar to the aerobic bacteria reported by Bourne et al. [11].

HPLC chromatograms of MCLR degradation by the strain ALA-1 were obtained (Fig. 5(A)). The peak of MCLR decreased gradually during the degradation process, and the major peaks at 6.3, 7.3 and 7.9 min of retention time were observed in the medium as intermediate degradation products. The peak at 6.3 min of retention time was first observed at 1 h and then the other two peaks were observed at 10 h on the HPLC chromatograms. The disappearance of all the peaks in 85 h indicated the complete degradation of MCLR by strain ALA-1. HPLC chromatograms of MCLR degradation by the intracellular enzyme of strain ALA-1 were also obtained (Fig. 5(B)). The retention times of the major peaks

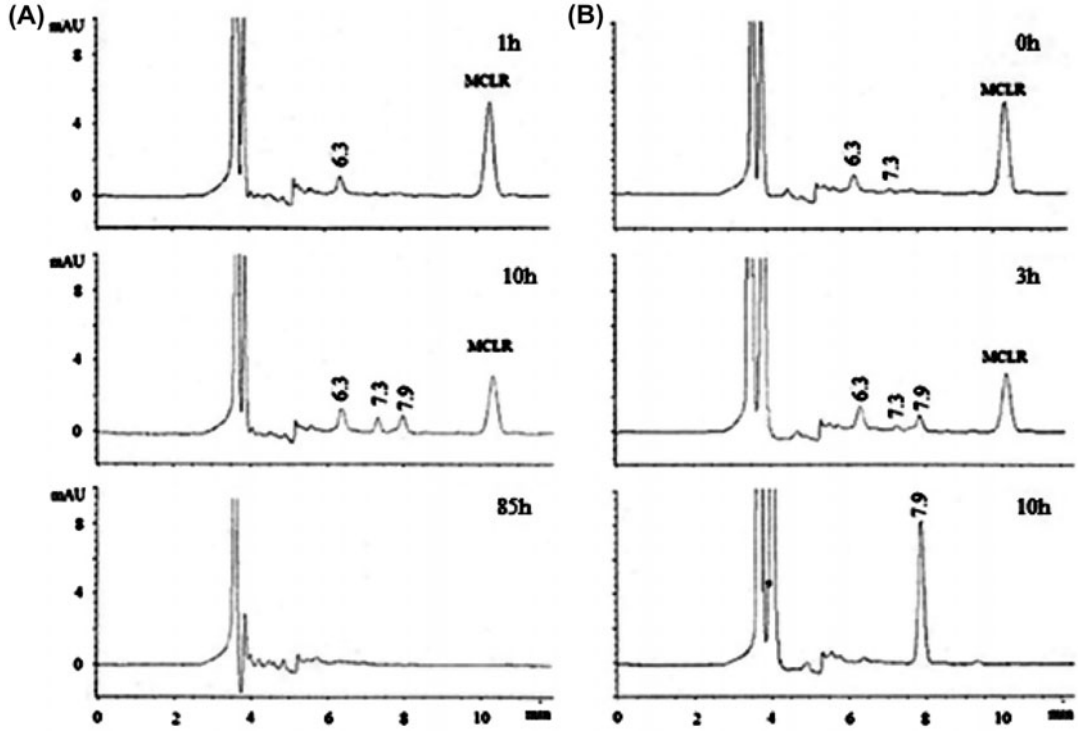

Fig. 5. HPLC chromatograms of MCLR degradation by strain ALA-1 (A) and by the intracellular enzyme of strain ALA-1 (B). Peaks at 6.3, 7.3 and 7.9 min of retention time showed intermediate products of MCLR.

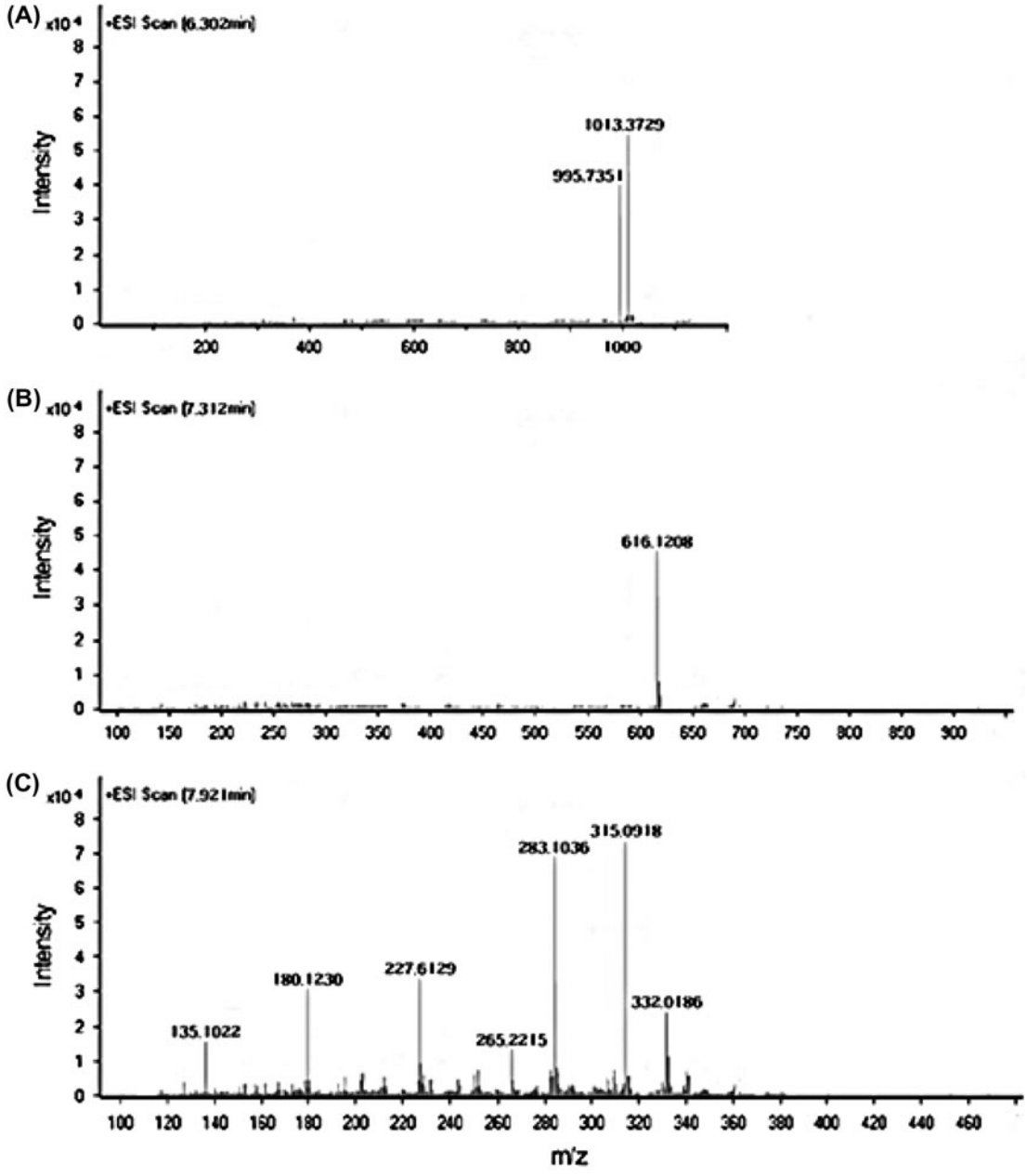

Fig. 6. ESI–TOF-MS spectra of the degradation products detected at 6.302 min of retention time (A), 7.312 min of retention time (B) and 7.921 min of retention time (C). The low peaks are not related to MCLR degradation products and less than 10% of relational intensity.

in Fig. 5(B) and (A) corresponded closely, which suggested that the intermediate degradation products were the same. When other peaks disappeared in 10 h, the peak at 7.9 min of retention time accumulated up to the largest (Fig. 5(B)). Furthermore, the amount of intermediate products in the MCLR degradation by anaerobic bacterium differed from that in the aerobic degradation of MCs [9,12].

3.4. Identification of intermediate degradation products

The MCLR and their degradation products were further identified using the HPLC–TOF-MS system (Fig. 6). The first intermediate degradation product exhibited a protonated molecular ion at *m*/*z* 1,013.3729 and 995.7351(Fig. 6(A)). MS data were analyzed using MacQuan software version 1.6: one at *m*/*z* 1,013.3729 ($C_{49}H_{77}N_{10}O_{13}$) was a precursor ion ($[M + H]^+$) and

the other at *m/z* 995.7351 ($C_{49}H_{75}N_{10}O_{12}$) was loss of a water ([M + H-H2O]$^+$). The product ions were consistent with those in the MS spectra, linearized MCLR, which were reported as the aerobic degradation product of MCLR [9,12]. The second intermediate degradation product ions were at *m/z* 616.1208 ($C_{32}H_{48}N_4O_8$) (Fig. 6(B)). The ion ([M + 2H]$^+$, *m/z* 616.1208) was similar to the loss of Arg-MeAsp-Leu from linearized MCLR. The product ion was identical to those in the MS spectra, tetrapeptide, which is recognized as the intermediate degradation products of the aerobic bacterial strains ACM-3962 and B-9 [9,12]. The third intermediate degradation product ions were at *m/z* 332.0186, 315.0918, 283.1036, and 265.2215. One of them, at *m/z* 332.0186 ($C_{20}H_{30}NO_3$), was a precursor ion ([M + H]$^+$), and other accompanying ions, at *m/z* 315.0918 ($C_{20}H_{27}O_3$) (loss of an ammonia [M + H-NH_3]$^+$), 283.1036 ($C_{19}H_{23}O_2$) and 265.2215 ($C_{19}H_{21}O$) (further loss of a methanol [M + H-NH_3-MeOH]$^+$ and a water [M + H-NH_3-MeOH-H_2O]$^+$), were fragment ions. The product ions were consistent with those in the ESI-MS spectra, Adda, which is identified as both of the anoxic [13] and aerobic degradation product [22,23] of MCLR. These results indicated that the intermediate degradation products of MCLR produced successively by ALA-1 under anaerobic condition were linearized MCLR, tetrapeptide and Adda.

In the present study, three intermediate degradation products were identified as linearized MCLR, tetrapeptide and Adda using ESI–TOF-MS. To the best of our knowledge, this is the first report identifying linearized MCLR, tetrapeptide and Adda as intermediate degradation products of MCLR by an anaerobic bacterial strain because they have previously been detected as degradation products of MCLR by aerobic *Sphingopyxis* sp., *Sphingomonas* sp. and *Bordetella* sp. [12,22,23]. Furthermore, Adda has been detected as a degradation product of MCLR by anoxic sediment of Lake Dianchi [13]. In their study, except for Adda, no other intermediate degradation products containing Adda residue, such as linearized MCLR and tetrapeptide, were observed under anaerobic and anoxic conditions, which implied that they may be rapidly degraded by peptidases after they were produced [13,24]. In this study, the strain completely degraded the MCLR within 85 h, while Adda was more resistant to subsequent biodegradation by the intracellular enzymes, which resulted in the accumulation of Adda in anaerobic cultures (Fig. 5(B)). The purified Adda does not show acute toxicity in mouse bioassays or inhibition to protein phosphatase activity, although it has been proved that Adda residue is critical for MCs toxicity [12]. No other products containing Adda residue were detected by the 10 h mark in the Fig. 5(B). Therefore, it can be concluded that the anaerobic degradation by ALA-1 is efficient for the detoxification of MCs. Further work is needed to determine the practical application of ALA-1 in various types of water bodies contaminated with MCs, since the intermittent hypoxia and anaerobic environment exist extensively in the eutrophic water body and sediments.

The MCLR degradation pathway for the strain ALA-1 was similar to that for the isolated *Sphingomonas* sp. B-9 and ACM-3962, in which two identical intermediates of MCLR degradation products (linear MCLR and tetrapeptide) were detected, whereas no Adda was observed [11,24]. Within the genome of the first isolated MC-degrading bacterium, *Sphingomonas* sp. ACM-3962, Bourne et al. [11,25] identified a gene cluster, mlrA, mlrB, mlrC and mlrD, involved in the degradation of MCLR. Particularly, mlrA was the first gene involved in cleaving the cyclic structure of MC. To verify whether the mechanism of MCLR degradation by ALA-1 under anaerobic condition was similar to that by *Sphingomonas* strain ACM-3962, further experiment for detection of the mlrA gene was carried out as follow.

3.5. Detection of the mlrA gene

Sense and antisense primers for mlrA amplification were located on the position 891–911 on mlrA gene of

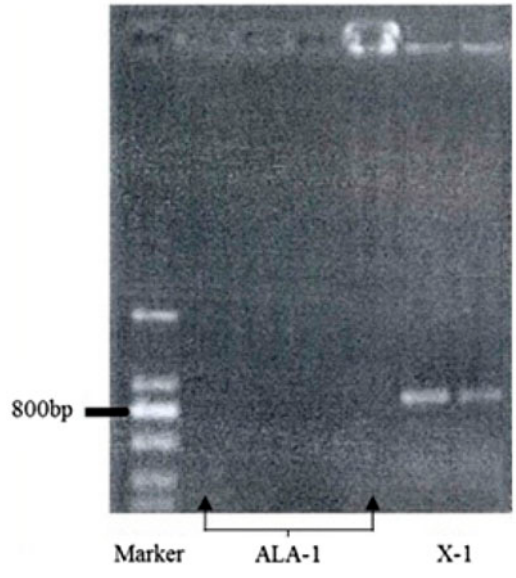

Fig. 7. Detection of mlrA gene fragment by PCR in strain ALA-1. The size of the amplified PCR product was detected in agarose gel (0.8%). An aerobic MC-degrading bacterium X-1 isolated in the laboratory was used as a positive control.

the ACM-3962 strain according to the report of satio et al. [16]. No band with the expected size (800 bp) was detected by PCR in strain ALA-1, while the band was detected in positive control group (Fig. 7). The best condition of PCR amplification of ALA-1 degradation gene was explored according to existing conditions of the PCR amplification mlrA gene. To optimize the PCR amplification conditions, the initial denaturation time (2, 4 and 8 min), annealing temperature (56, 58, and 62˚C), amount of template (diluted 1 × 2 × 3) and cycles (35, 40, 45 cycles) were changed, respectively. The results were the same as the findings above, suggesting that there was no mlrA gene in ALA-1.

Various studies have designed PCR assays on aerobic biodegradation for the detection of the mlrA gene [16,26,27], the first important gene involved in cleaving the cyclic structure of MC. However, no further study on anaerobic biodegradation of MCs was reported until now. In present study, no mlrA gene homologue was detected in ALA-1, indicating that there may be other enzymes which were not encoded by the mlr gene cluster capable of metabolizing MCLR in this degradation pathway, or there may be other separate pathways for MCs degradation. Chen et al. [13] extracted DNA from the anoxic sediment slurries and then used it as a template for PCR targeting the MC degrading genes, mlrA mlrB, mlrC and mlrD, respectively. But the amplification products for these four genes were not obtained. Manage et al. [28] were also unable to detect the *mlr* genes in their isolates under aerobic conditions, suggesting that there may be other pathways (or possibly genes) involved in MC degradation. Taking the degradation process by the intracellular enzymes of strain ALA-1 under anaerobic condition into account, it was different from the degradation process by the intracellular enzymes of aerobic bacteria that Adda was produced and accumulated. The reasons for the result may be that: (i) there were other enzymes which were not encoded by the mlr gene cluster which can be capable of metabolizing MCLR in this degradation pathway and (ii) some mlrA gene homologous genes might be present but the sequences at the primer sites were different. In order to assess this proposition, further research is in progress to identify the possible degradation gene of ALA-1. And, assays are in progress to isolate and identify the new bacteria which can degrade MCs under anoxic and anaerobic conditions. It may provide a new explanation for the MCs degradation pathway and enhance the theoretical foundation for the study of the enzymatic pathway for biodegrading MCs.

4. Conclusions

This study further investigated the potential for anaerobic degradation of MCLR by ALA-1. The main conclusions drawn from this study are as follows:

(1) MCLR could be rapidly degraded at 20, 25 and 30˚C, while the degradation of MCLR was slow at low temperatures (10, 15˚C) with a 6 d or 8 d lag time before degradation, respectively. MCLR was easily degraded under neutral and alkaline (pH 7.0, 8.0, 9.0) conditions and was degraded completely within 12 d without obvious lag phase. In addition, extra carbon or nitrogen sources had no significant effect on the degradation ability of ALA-1.
(2) The intracellular enzymes were mainly responsible for MCLR degradation process. Linearized MCLR (*m*/*z* 1,013.3729, $C_{49}H_{77}N_{10}O_{13}$), tetrapeptide (*m*/*z* 616.1208, $C_{32}H_{48}N_4O_8$) and Adda (*m*/*z* 332.0186, $C_{20}H_{30}NO_3$), commonly known as biodegradation products of MCLR degradation by isolated aerobic bacteria, were detected as the intermediate products of MCLR degradation by an anaerobic bacterium.
(3) No mlrA gene homologue was detected in strain ALA-1, suggesting that there were other enzymes which were not encoded by the mlr gene cluster capable of metabolizing MCLR in this degradation pathway.

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