Enhanced and continued degradation of microcystins using microorganisms obtained through natural media

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ABSTRACT

Microorganisms isolated through artificial media are often unsustainable in biodegrading microcystins (MCs) in natural water. Here we studied alternative approaches to isolate MCs-degrading bacteria using natural media. In comparison to two species (MS-1 and MS-2) isolated from artificial media and the failure of bacterial colonies formation using water extracts of sediment (10%, w/v), five colony species (WC-1 to WC-5) appeared using concentrated water extracts of sediment that is 10-fold enhancement of nutrient level. In the simulated biodegradation test in Lake Taihu water with continuous supply of MCs, a lag phase of 6 days was required for MS-1 and M-2 to degrade 13% and 15% of the added MC-RR and MC-LR, respectively, whereas the lag phase was only 3 days with approximately 44% and 31% removal of the added MC-RR and MC-LR by WC-1 to WC-5. During the continuous supply experiment, degradation of MCs by MS-1 and MS-2 stopped after 3 days, while degradation of MCs by WC-1 to WC-5 lasted continuously throughout the 18 day test period with 2 to 6-fold enhancement of removal rate. 16S rRNA gene sequences and phylogenetic analysis indicated the potential to amplify species of MCs-degrading bacteria when natural media were used. The results suggested that the increased adaptability of bacteria obtained through concentrated natural media was responsible for the enhanced and continued biodegradation under simulated natural water conditions.

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1. Introduction

Cyanobacteria-dominated harmful algae blooms (cyano-HABs) in eutrophic lakes, reservoirs, and recreational waters have been reported worldwide (Pan et al., 2006; Chen et al., 2006). Some genera, such as *Microcystis*, can produce a wide range of toxins, including a family of hepatotoxins called microcystins (MCs) (Botes et al., 1984), which are the most frequently encountered cyanotoxins. More than 70 variants of MCs have been isolated and identified, among which MC-RR and MC-LR are considered the most common and dangerous variants (Chen et al., 2010). Since MCs are known to be tumor promoters to liver and colon (Zhao et al., 2011; Zhang et al., 2009), they pose a risk to human or animal health when surface waters are used as a drinking water source and/or for recreational purposes.

It is reported that approximately 80–90% of total MCs remain in intact algae cells (Hart et al., 1998; Otten et al., 2012). Lake Taihu has frequently cyano-HAB for many years, and the detected maximal concentration of dissolved MCs in Lake Taihu water is $15.6 \mu\text{g L}^{-1}$ (Xu et al., 2008; Song et al., 2007). In other cases, toxin concentrations of $>140 \mu\text{g L}^{-1}$ MC-LR have been reported in natural lake waters (Barrington et al., 2013). These values far exceed the provisional

guideline value of $1.0 \mu\text{g L}^{-1}$ of MC-LR in drinking water recommended by World Health Organization. On the other hand, it is also well recognized that biodegradation is one of the elimination pathways to reduce its concentration in natural waters and sediment (Chen et al., 2008, 2010). However, in severe cyano-HABs lakes, dramatic increased concentration of MCs after the cyano-HAB may exceed the endurance of indigenous MCs-degrading microorganisms (Gheraout et al., 2010; Cai et al., 2010). Therefore, the application of exogenous MCs-degrading bacteria may become an important engineering measure for the elimination of MCs.

The isolation of MCs-degrading bacteria and the biodegradation of MCs under laboratory conditions have been reported before (Tsuji et al., 2006; Valeria et al., 2006; Zhang et al., 2010). These studies improve our understanding on biodegradation mechanisms and on degradation abilities of different strains (Bombach et al., 2010). However, the degradative abilities of these bacteria are often depressed under field conditions (Markiewicz et al., 2011; Megharaj et al., 2011). This is partly attributed to the fact that laboratory studies are performed in artificial media (mostly mineral salts media) with optimized nutrients and culture conditions, high concentrations of bacteria and the absence of competition with indigenous bacterial populations. When microorganism communities are collected from MCs contaminated sites, these microorganisms often experience enrichment and isolation in artificial medium where MCs are supplied as the sole carbon and nitrogen source (Janssen et al., 2002). The different conditions of growth requirements between artificial media and the natural environment may lead to the

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degeneration of their adaptability in the environment (Markiewicz et al., 2011). Besides, the number and diversity of reported strains isolated from artificial media are still limited (Edwards et al., 2008) and the majority of them is identified as *Sphingomonad* (Tsuji et al., 2006; Valeria et al., 2006). In addition, potential degradative capacity, as assessed by studies performed in laboratory experimental systems, is often not fully realized in contaminated environments since field conditions are often characterized by fluctuating substrate concentrations resulting from heterogeneous source distributions (Alexander, 1985; Vroblesky and Chapelle, 1994). Microbes in the field may have experienced a different substrate exposure history from a continuous cyanohab rather than only one time toxins addition in many lab tests (Zhang et al., 2010; Park et al., 2001).

To bypass the bottleneck of microorganism isolation, measures are taken to modify the isolation media or procedure, including the use of agar media with little or no added nutrients and relatively long periods of incubation (Stevenson et al., 2004) and the diluted nutrient broth as growth medium (Janssen et al., 2002). These methods allow cultivation of previously uncultivated microorganism and have been successfully used in biodegradation test under laboratory conditions. However, the lack of successful biodegradation in natural environment by these pure cultures makes the investigation of new approaches to bacteria isolation a challenge. So far it is not clear how to provide the conditions necessary to enable growth of incubated bacteria under natural conditions. To our knowledge, it is still not clear how to isolate MC degrading bacteria from natural media.

Here, we report a method for isolation of MCs-degrading bacteria from MCs contaminated sediment using natural media. Water extracts of sediment and concentrated water extracts of sediment were compared to evaluate the influence of nutrients level on bacterial isolation. 16S rRNA gene sequencing was employed for identification of the obtained pure cultures. Simulated biodegradation experiments in Lake Taihu water with continuous supply of MCs were conducted to evaluate the potential of continuous biodegradation ability of MCs in natural water. The purpose of the study is to develop an alternative approach by which MCs-degrading bacteria can be selectively isolated, with enhanced and continuous MCs removal efficiency in natural waters.

2. Material and methods

2.1. Standards and reagents

The MCs standard (MC-RR and MC-LR) was purchased from Taiwan Algal Science Inc. (purity $\geq 95\%$ by HPLC). MCs used for biodegradation experiment were prepared according to the method described previously (Yan et al., 2004a,b). Deionized water was prepared using a Milli-Q filtration system (Millipore, Bedford, MA, USA).

2.2. Bacteria isolation

The microorganism communities were collected from the sediment in Meiliang Bay at Taihu Lake in August, 2010. After removal of carpolite and shells with sterile tweezers, 10 g sediment was added to 100 mL sterile deionized water. Microorganisms within the sediment were detached by vortexing for 30 min and the supernatant was collected. The steps for isolation of the wild microorganisms in the supernatant, namely enrichment and isolation, were performed in sterile flasks containing the following five groups of enrichment or isolation media spiked with filter sterilized $5 \mu\text{g mL}^{-1}$ MC-RR and $2 \mu\text{g mL}^{-1}$ MC-LR. Isolation through mineral salt medium (MS medium) was conducted when the medium was used as both enrichment and isolation media for the purpose of comparison with the natural media. Pore water, water extracts of sediment and concentrated water extracts of sediment were used as both enrichment and isolation media. The utilization of water extracts of sediment as enrichment media and concentrated water extracts of sediment as isolation media were concurrently

evaluated. All media were prepared in triplicates. MS medium was prepared as described before (Valeria et al., 2006; Zhang et al., 2010). Water extracts of sediment were prepared by vortexing 50 g sediment in 500 mL deionized water, centrifuged at 9000 rpm and sterilized by filtration through $0.22 \mu\text{m}$ cellulose ester membrane. The concentrated water extracts of sediment were prepared through evaporation 90% of water in the water extracts of sediment at 40°C , followed by filtration through $0.2 \mu\text{m}$ membrane. Pore water was prepared by centrifugation of sediment at 12,000 rpm for 30 min, followed by filtration through $0.22 \mu\text{m}$ membrane. The characterization of the media and Lake Taihu water is presented in Table 1.

Enrichment cultures were generated using the five groups of enrichment media containing $5 \mu\text{g mL}^{-1}$ MC-RR and $2 \mu\text{g mL}^{-1}$ MC-LR in 50 mL flasks inoculated with 1 mL supernatant mentioned above at 25°C with shaking at 120 rpm in the dark. Enriched microorganisms were obtained from serial subcultures by transferring 1 mL of the resulting solution to new flasks containing enrichment media and incubated for 2 days, such operations were repeated at 25°C for 3 times. To isolate MCs-degrading bacteria, 200 μL samples of the enriched cultures were transferred and spread on five different groups of solid isolation media supplemented with $5 \mu\text{g mL}^{-1}$ MC-RR and $2 \mu\text{g mL}^{-1}$ MC-LR as carbon and nitrogen sources, and incubated at 25°C in the dark. The average colony numbers, growth periods and total colonies species in triplicates of each media were recorded until insignificant changes were observed. On the basis of size, color and morphology, single colonies from these plates were selected as candidate MC-degrading strains and transferred to nutrient agar medium to obtained pure cultures.

2.3. Biodegradation in MS medium

Autoclaved MS medium (70 mL) containing the pure isolates with initial bacteria density of $3.54 \times 10^5 \text{ CFU mL}^{-1}$ was incubated in sterilized 100 mL flask. Each flask was spiked with filter sterilized MCs to initial concentrations of $207.57 \mu\text{g L}^{-1}$ for MC-RR and $171.84 \mu\text{g L}^{-1}$ for MC-LR. The control was prepared in autoclaved MS medium with the same level of MCs. For each treatment, 1.0 mL of well-mixed sample was collected everyday for MC-RR and MC-LR analysis with sterile needle and syringe. This is to evaluate the MCs' degradation ability of the individual isolates obtained at different strategies.

Table 1

The characterization of media and Lake Taihu water.

Component	Concentrations			
	Water extracts of sediment	Concentrated water extracts of sediment	Pore water	Lake Taihu water
TN (mg L^{-1})	1.93 ± 0.12	5.82 ± 1.02	3.71 ± 1.13	2.32 ± 0.65
TP (mg L^{-1})	0.12 ± 0.02	0.37 ± 0.10	0.25 ± 0.05	0.28 ± 0.07
NH_4^+-N (mg L^{-1})	0.64 ± 0.05	2.71 ± 0.54	1.62 ± 0.05	0.33 ± 0.04
NO_3^--N (mg L^{-1})	0.03 ± 0.01	1.04 ± 0.03	0.08 ± 0.02	0.16 ± 0.04
PH	7.37 ± 0.75	7.41 ± 0.96	7.69 ± 0.94	7.62 ± 0.91
DO (mg L^{-1})	7.52 ± 1.04	3.84 ± 1.09	5.86 ± 1.05	9.20 ± 1.48
TOC (mg L^{-1})	1.08 ± 0.53	3.56 ± 0.68	1.93 ± 0.54	2.21 ± 0.19
Mg^{2+} (mg L^{-1})	0.18 ± 0.05	1.24 ± 0.03	0.98 ± 0.07	0.14 ± 0.08
Ca^{2+} (mg L^{-1})	0.15 ± 0.05	1.76 ± 0.13	0.77 ± 0.02	0.05 ± 0.01
Fe^{2+} (mg L^{-1})	0.12 ± 0.03	0.73 ± 0.07	0.63 ± 0.05	0.06 ± 0.01
Zn^{2+} (mg L^{-1})	ND	0.12 ± 0.02	ND	ND
K^+ (mg L^{-1})	0.03 ± 0.01	0.23 ± 0.03	0.20 ± 0.01	ND
Na^+ (mg L^{-1})	0.02 ± 0.01	0.16 ± 0.01	ND	0.03 ± 0.01
Cu^{2+} (mg L^{-1})	ND	0.08 ± 0.01	0.02 ± 0.01	ND
Mn^{2+} (mg L^{-1})	ND	0.02 ± 0.01	0.01 ± 0.01	ND
Cl^- (mg L^{-1})	ND	ND	ND	ND
SO_4^{2-} (mg L^{-1})	0.05 ± 0.01	0.17 ± 0.04	0.09 ± 0.01	ND
PO_4^{3-} (mg L^{-1})	0.05 ± 0.01	0.13 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
MCs ($\mu\text{g L}^{-1}$)	0.02 ± 0.01	0.46 ± 0.03	0.54 ± 0.01	0.44 ± 0.04

TN: total nitrogen; TP: total phosphorus. MCs (microcystins) here contain MC-RR and MC-LR. ND: not detected. Standard errors are displayed ($n = 3$).

2.4. Simulated biodegradation in Lake Taihu water with continuous supply of MCs

This experiment was conducted in 700 mL filtered (0.45 μm) Lake Taihu water spiked with 207.57 $\mu\text{g L}^{-1}$ MC-RR and 171.84 $\mu\text{g L}^{-1}$ MC-LR. Bacteria obtained at different strategies were incubated in the flasks for 18 days with initial bacteria density of 3.54×10^6 CFU mL^{-1} . To obtain MCs-degrading bacteria free system as control-1, the collected Lake Taihu water was autoclaved. Control-2 was prepared in natural water without the addition of exogenous MCs-degrading bacteria. Continuous supply of MCs was conducted for each treatment through addition of 2 mL filter sterilized MCs into the water every 3 days after sampling. A volume of 2.0 mL well-mixed sample was collected with sterile needle and syringe every 3 days for MC-RR and MC-LR analysis. Growth of bacteria in flasks was determined by the plate count method in solid MS medium where MCs were added as the sole carbon and nitrogen sources. The removal rates of MCs were assessed by calculating the percentage of MCs removed in each treatment compared with the detected MCs in control-1 as follows: $[(\text{concentration of MCs in control-1} - \text{concentration of MCs in treatments}) / (\text{concentration of MCs in control-1})] \times 100\%$.

2.5. Identification of MCs-degrading bacteria

The sequence of bacterial 16S rRNA gene was determined and compared with the sequences available from NCBI-BLAST database to get definitive information on the phylogenetic position. The genomic DNA was isolated by Tiangen Genomic DNA Isolation kit (Tiangen Biotech Co., Ltd. Beijing, China). Each phylogenetic tree was obtained from comparison of the obtained isolates sequences with closely related NCBI sequences. The tree was constructed using a neighbor-joining analysis. The numbers indicate the present bootstrap support based on a neighbor-joining analysis of 1000 re-sampled datasets. Sequence accession numbers used for phylogenetic analysis are shown in parentheses following the species name. The primer set 27F-1492R was used in PCR amplification. Sequencings were conducted in Sangon Biotech Co., Ltd. Shanghai, China. Similarity searches were conducted through the National Center for Biotechnology Information BLAST network service (Blastn). The alignment data were then used for neighbor-joining analysis with 1000 bootstrap replicates.

2.6. Chemical analytical methods

Mg^{2+} , Ca^{2+} , Fe^{2+} , Zn^{2+} , K^{+} , Na^{+} , Cu^{2+} and Mn^{2+} were analyzed by ICP-OES (Optima 2000, PerkinElmer Co. U.S.A.). TOC was measured by the total organic carbon analyzer (phoenix 8000, Tekmar-Dohrmann Co.). Cl^{-} and SO_4^{2-} were determined by Metrohm 861 Ion chromatography (Switzerland, Metrohm Co.). Ultra-performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) was used to determine the concentration of MCs. Sample separation was conducted by Acquity Ultra Performance LC system (Waters, USA) and Acquity UPLC BEH130 C18 column (i.d. 2.1 mm $\times$ 50 mm, particle size 1.7 μm , pore size 130 Å) (Waters). The column oven was kept at 30 $^{\circ}\text{C}$ and the injection volume was 10 μL . The flow rate was 0.2 mL min^{-1} . Water and acetonitrile (containing 0.05% formic acid) were used as mobile phases, the organic phase was linearly increased from 45% to 60% in 2.5 min, then increased to 100% in 1.5 min and held for 0.5 min, finally brought back to 45% and held for 3 min until the next injection. The mass spectrometer was operated in positive mode electrospray ionization in multiple-reaction monitoring mode. The capillary voltage was maintained at 3.8 kV. The cone voltage was 50 V. Source temperature and desolvation gas temperature were held at 120 $^{\circ}\text{C}$ and 350 $^{\circ}\text{C}$, respectively. The flow of the desolvation gas and cone gas was 600 and 50 L h^{-1} , respectively. The pressure of the collision chamber was kept at 3.0×10^{-3} mbar. Satisfactory recoveries ranging between 91.0 and 103.2% were obtained by

the developed method. The limit of detection (LODs, based on signal-to-noise ratio of 3) and limits of quantification (LOQs, based on signal-to-noise ratio of 10) were 0.05 and 0.16 $\mu\text{g L}^{-1}$ for MC-RR, 0.012 and 0.04 $\mu\text{g L}^{-1}$ for MC-LR, respectively. Masslynx software workstation was used for the UPLC-MS/MS instrument control, data acquisition and data processing.

2.7. Statistics analysis

A one-way ANOVA was applied to determine if significant differences existed between isolates during biodegradation. A significance was assumed when $P < 0.05$.

3. Results

3.1. Growth of bacteria in different media

The growth period required for microorganisms to form colonies in MS and natural media showed significant differences. Artificial MS medium enabled fast formation of bacteria colonies within 3 days, however, with only two bacterial species (marked as strains MS-1 and MS-2, Table 2). No growth of bacteria was recorded when the water extractions from sediment were directly used as isolation media within 20 days. The utilization of concentrated water extracts of sediment and pore water enabled the cultivation of bacteria in 15 days. Of the four groups of natural media, the utilization of concentrated water extracts of sediment for isolation of bacteria, which were enriched in water extracts of sediment, showed significantly more species and colony numbers within shortest growth period. As a result, the two bacterial strains isolated through MS medium (marked as strains MS-1 and MS-2) and five strains enriched in water extracts of sediment and then isolated in concentrated water extracts of sediment (marked as strains WC-1 to WC-5) were pure-cultured.

3.2. Biodegradation ability in MS media

The concentration of MC-RR and MC-LR remained constant in the control during the experimental period, but were remarkably reduced with the treatment of the seven strains irrespective of the isolation strategies (Fig. 1). The removal efficiency increased over time to approximately 100% on day 6 for MC-RR with the incubation of the seven strains (Fig. 1a), of which WC-4 was the most efficient bacteria where MC-RR was degraded to below the detection limit in two days, illustrating substantial difference with other six strains. MC-LR were completely removed within 6 days and all the tested bacteria were able to reduce an initial concentration of 171.84 $\mu\text{g L}^{-1}$ MC-LR to below the WHO guideline value of 1.0 g L^{-1} for drinking water, although a longer time of lag phase was required for WC-2 (Fig. 1b).

3.3. Biodegradation under simulated natural water conditions

Continuous supply of MCs resulted in linear increase of MCs in the control-1 and the concentration reached 1263.45 $\mu\text{g L}^{-1}$ MC-RR and 1025.76 $\mu\text{g L}^{-1}$ MC-LR after 18 days (Fig. 2). In the MS-1 and MS-2

Table 2
Comparison of the results obtained through artificial and natural media.

Enrichment media	MS	WES	WES	CWES	PW
Isolation media	MS	WES	CWES	CWES	PW
Average growth period (d)	3	ND	6	15	17
Average colony numbers (CFU mL^{-1})	3.6×10^5	ND	5.4×10^5	2.2×10^4	4.4×10^5
Total colony species	2	ND	5	2	3

MS: mineral salt medium. WSE: water extracts of sediment. CWSE: concentrated water extracts of sediment. PW: pore water. ND: no detected.

Fig. 1. Removal efficiency of MC-RR (a) and MC-LR (b) by the seven strains isolated through artificial and natural media. MS-1 and MS-2: bacteria isolated through mineral salt medium. WC-1 to WC-5: bacteria enriched in water extracts of sediment (10%, w/v) and then isolated in concentrated water extracts of sediment. MC: microcystin. Initial concentration of MC-RR and MC-LR is $207.57 \mu\text{g L}^{-1}$ and $171.84 \mu\text{g L}^{-1}$, respectively. Data are expressed as mean $\pm$ SD of triplicates.

incubated systems, degradation of MCs occurred on day 6 with 13.47% and 15.26% reduction of MC-RR and MC-LR compared to control-1, respectively (Fig. 2), whereas a clear improvement on the removal of MCs was observed for strains WC-1 to WC-5 incubated systems since day 3, with approximately 44.38% and 31.23% reduction of MC-RR and MC-LR, respectively. In the MS-1 and MS-2 incubated systems, despite up to 53.68% and 71.34% reduction of MC-RR and MC-LR was achieved after 18 days, they were demonstrated to be ineffective to degraded MCs due to the distinguishable difference with 59.16% and 68.61% reduction of MC-RR and MC-LR by indigenous bacteria in control-2 (Fig. 2). With regard to strains WC-1 to WC-5, up to 89.75% reduction of MC-RR (WC-1) were observed on day 18, of which WC-4 and WC-5 reduced 98.41% and 92.95% MC-RR (Fig. 2a), 96.25% and 92.73% MC-LR (Fig. 2b), respectively, and showed remarkably higher removal efficiency than other strains.

Continuous supply of MCs caused significant enhancement of MCs degradation in WC-1 to WC-5 incubated systems and the effects were quantitatively confirmed by the measurement of removal rate, which were increased from up to $11.52 \mu\text{g L}^{-1} \text{d}^{-1}$ on day 3 to $69.01 \mu\text{g L}^{-1} \text{d}^{-1}$ on day 18 for MC-RR, and $9.56 \mu\text{g L}^{-1} \text{d}^{-1}$ to $54.81 \mu\text{g L}^{-1} \text{d}^{-1}$ for MC-LR, respectively. Though 2 to 3-fold enhancement of MCs removal rate was observed for MS-1 and MS-2 incubated systems, the removal was suggested to be accomplished by indigenous bacteria in control-2.

The number of MCs-degrading bacteria in control-1 showed no changes during the experiment (Fig. 3). With the same initial bacteria density of $3.54 \times 10^6 \text{ CFU mL}^{-1}$, total MCs-degrading bacteria amount, including incubated bacteria and indigenous ones that enriched from continuous supply of MCs, showed 1000-fold enrichment during the 18 days in MS-1 and MS-2 incubated systems. Constant increase of indigenous MCs-degrading bacteria in control-2 was observed and the amount showed no substantial difference with that in MS-1 and MS-2 after 18 days. In contrast, the population in WC-1 to WC-5 incubated system showed up to 1000-fold enrichment since day 3 and the biomass maximally reached $7.4 \times 10^{12} \text{ CFU mL}^{-1}$ on day 18 (Fig. 3).

3.4. Identification of MCs-degrading microorganisms

Based on the biodegradation performance of the seven strains in MS medium simulated natural water conditions, WC-4 and WC-5 were demonstrated to be most efficient in MCs' biodegradations. The 16S rRNA gene sequences were aligned with the sequences deposited in

the GenBank database using a BLAST search and the results showed that WC-4 shared 99% identity with *Pseudomonas* sp. An 18, WC-5 shared 99% identity with *Acinetobacter* sp. dcm5A, with GenBank accession No. KC871534 and KC871535, respectively (Fig. 4).

4. Discussion

4.1. Influence of nutrient condition on bacteria isolation

A typical way for microbe isolation is to collect samples of soil, sediment or water from the environment, followed by enrichment and isolation of microorganism communities in artificial media (Valeria et al., 2006; Zhang et al., 2010). The fact that bacteria isolated through artificial media often show limit removal efficiency or encounter failure in natural environment, urges researchers to optimize the isolation strategy, such as the use of agar media with little or no added nutrients and relatively long periods of incubation (Stevenson et al., 2004). Janssen et al. (2002) use diluted nutrient broth as growth medium. In our study, no colonies were shown with direct application of water extracts of sediment (10%, w/v) after 20 days (Table 2), evaluations of the nutrient condition revealed that the sediment extraction was characterized by $1.9 \text{ mg L}^{-1} \text{ TN}$ and rather low or even missed mineral salts (Table 1). It was recognized that microorganism from the environment had a requirement for components from the media (Kenters et al., 2011). It might be the poor nutrient condition that was responsible for the failure of bacteria cultivation through water extracts of sediment extracts. After improvement of the nutrient level through evaporation, 6–11 fold improvement of nutrient level was achieved (Table 1), which resulted in successful bacterial cultivation in concentrated sediment extracts (Table 2). In the traditional isolation process, MCs were supplied as the sole carbon and nitrogen source (Valeria et al., 2006; Zhang et al., 2010), resulting in a quick elimination of MCs and enrichment of the target bacteria. The longer growth period and fewer colonies species in the pore water and concentrated water extract of sediment may be due to the extra nitrogen and carbon source (Table 1), which agreed well with the report that exogenous nutrients led to slow bacteria growth (Kenters et al., 2011; Markiewicz et al., 2011). It was hard to prepare natural media with the equivalent nutrients level as artificial media during microorganism isolation. Our studies indicated that the limitation of poor nutrient condition may be diminished by the application of low and then relatively high nutrients media (also referred to as feast-famine). Enrichment in water extracts

Fig. 2. Biodegradation of MC-RR (a) and MC-LR (b) with the incubation of MC-degrading bacteria in Lake Taihu water under continuous supply of MCs every 3 days. The Taihu water is initially spiked with $207.57 \mu\text{g L}^{-1}$ MC-RR and $171.84 \mu\text{g L}^{-1}$ MC-LR. Equivalent amount of MCs is added after every sampling. Control-1: MCs added in autoclaved Lake Taihu water without adding exogenous bacteria. Control-2: MCs added in natural water without incubation of exogenous MCs-degrading bacteria. MS-1 and MS-2: isolated through mineral salt medium. WC-1 and WC-5: enriched in water extracts of sediment and then isolated in concentrated water extracts of sediment. MC: microcystin. Data are expressed as mean $\pm$ SD of triplicates.

of sediment followed by isolation in concentrated water extracts of sediment led to fast proliferation of bacteria within 5 days in the present study, which stood in contrast to previous studies that some bacteria convert to non-culturable condition due to the energy starvation (Surono et al., 2008). When the enrichment process was performed in water extracts of sediment with nutrient deficiency, bacteria experienced starvation incubation, resulting in induced cultivation in media with relatively higher nutrients, experience of such selective pressure avoided the time-consuming purification and incubation for selective isolation (Wittlich et al., 2001). In addition, a number of research groups had performed microorganism isolation with MCs served as sole carbon

and nitrogen source (Valeria et al., 2006; Zhang et al., 2010) and they believed other substrates may be preferentially degraded, leading to depression of the target substrate (Markiewicz et al., 2011), while some other reports found that utilization of the target substrate were not prevented by additional carbon source. This was supported by Edwards et al. (2008) who demonstrated the utilization of MCs by bacteria in river and subsequently enriched them by direct addition of MCs in the river water (Manage et al., 2009). In our studies, the TOC and TN concentrations of the media ranged from 1.08 to 5.82 mg L^{-1} , which were significantly higher than some isolation process (Zhang et al., 2010), resulted in delay but not inhibition of bacteria growth.

Fig. 3. The total amount of microcystins-degrading bacteria (including the incubated and indigenous bacteria) with the incubation of microcystins-degrading bacteria in Lake Taihu water under continuous supply of MCs. Control-1: confirmation of the aseptic condition during the experiment. Control-2: demonstration of the enrichment and proliferation of indigenous MCs-degrading bacteria in lake water. MS-1 and MS-2: isolated through mineral salt medium. WC-1 and WC-5: enriched in water extracts of sediment and then isolated in concentrated water extracts of sediment. Data are expressed as mean $\pm$ SD of triplicates.

4.2. The effect of isolation strategy on bacteria adaptability in natural water

With the same initial concentration of $207.57 \mu\text{g L}^{-1}$ MC-RR and $171.84 \mu\text{g L}^{-1}$ MC-LR, the dramatic decrease of MCs by MS-1 and MS-2 in MS media (Fig. 1) disappeared when the isolates were incubated in Lake Taihu water, particularly during the first 3 days (Fig. 2). This suggests that laboratory obtained degradation rates are limited when transferred to natural waters. Conversely, strains WC-1 to WC-5 showed continued and enhanced biodegradation, indicating stronger degradation capacity and higher MCs' endurance. Adaptation was recognized as changes in the microbe that leads to increase of the biodegradation rate or maximal biodegradable concentration of given substrates (Markiewicz et al., 2011). The dramatically reduced quantity of MCs by strains WC-1 to WC-5 on day 3, along with the remarkable enrichment of MCs-degrading bacteria in the simulated test (Fig. 3), indicated a stronger adaptability compared with the reported adaptation after 8 months of incubation (Aelion et al., 1987). Bacteria enriched from water extracts of sediment experienced selective pressure and subsequent isolation in concentrated water extracts of sediment, which showed close nutrient condition, component and PH with the real environment, led to adaptation of the re-incubated microbes in the water. With regard to bacteria obtained through MS media, the enrichment and isolation process were conducted in optimized nutrient conditions without the competition with indigenous microorganism, made the isolates sensitive in natural water. The reason for poor successful biodegradation in field test was also attributed to the fact that the laboratory test did not reveal the real environmental condition

Fig. 4. Phylogenetic relationships between strains WC-4, WC-5 and closely related bacteria for 16S rRNA sequences from GenBank, NCBI analysis. Each phylogenetic tree is obtained from comparison of the obtained isolates sequences with closely related NCBI sequences. The tree is constructed using a neighbor-joining analysis. The numbers indicate the present bootstrap support based on a neighbor-joining analysis of 1000 re-sampled datasets. Sequence accession numbers used for phylogenetic analysis are shown in parentheses following the species name.

including the availability of substrates, nutrients, and electron acceptors (Bogan and Sullivan, 2003; Greenwood et al., 2009), and did not enable a continuous substrate flux since the experiment was carried out when target substrates were added initially only once. In our study, simulated biodegradation was performed with continuous supply of MCs in Lake Taihu water and the number of bacteria in flasks where strains WC-1 to WC-5 were incubated showed substantial increase with a lag phase of less than 3 days. Whereas it was not until day 6 that MS-1 and MS-2 incubated system showed approximately 13% and 15% reduction of MC-RR and MC-LR, respectively, however, the degradation ability was lost from day 9 onward as revealed by the undistinguishable difference with control-2 (Fig. 2). Along with the abrupt increase of indigenous MCs-degrading bacteria since day 6 (Fig. 3), we concluded that 6 days are required for MS-1 and MS-2 to adapt the natural water and the indigenous bacteria dominated MCs' removal.

Previous studies suggested that the effect of extra carbon source on removal of MCs was a strain-specific and strain-dependent property. Nybom et al. (2008) revealed that MCs' removal was enhanced when extra carbon source was added to the solution, whereas Ishii et al. (2004) found that strains scarcely degraded MC-RR in the presence of extra carbon and nitrogen source. In our study, the concentration of TOC and total nitrogen of the water reached 2.21 mg L^{-1} and 2.32 mg L^{-1} in the lake water (Table 1), which far exceed the MCs level of $207.57 \text{ } \mu\text{g L}^{-1}$ MC-RR and $171.84 \text{ } \mu\text{g L}^{-1}$ MC-LR. The constant proliferation of total MCs-degrading bacteria in MS-1 and MS-2 incubated system (Fig. 3) along with the insignificant removal of MCs with control-2 (Fig. 2), suggested the utilization of other organic material rather than MCs as carbon and nitrogen sources, indicated the loss of degradative ability in natural water. In addition, a huge number of

microorganisms existed in natural environment and it was reported that competition among microorganisms may result in the dominance of one species in a relatively short time (Hsu et al., 1981; Yoon et al., 1977). In terms of similar pattern of MCs' degradation and bacterial growth with control-2, it was safe to conclude that MS-1 and MS-2 had failed in the competition with indigenous bacteria in our study.

4.3. The effects of continuous MCs supply on the biodegradative ability of bacteria

So far, assessment of biodegradation potential had mainly been conducted when MCs are added only once (Valeria et al., 2006; Zhang et al., 2010), though it is informative, this experimental system is often not fully realized in contaminated environments with continuous substrate flux (Alexander, 1985; Bollmann et al., 2010). In this study, we simulated the dramatically increase and continuous flux in MCs after collapse of cyano-HABs by adding the MCs continuously to the experimental system. In addition to the total degraded amount, the rates of biodegradation were enhanced for strains WC-1 to WC-5, which were confirmed by the fact that a maximum of 6-fold enhancement of removal was observed for both MC-RR and MC-LR (Fig. 2). The enhancement of the biodegradation may be attributed to the improved bacterial survival, persistence and degradative activities with exposure and subsequent re-exposure of MCs (Lamberts et al., 2008; Loh and Wang, 1997), which agreed well with approximately 10^7 -fold proliferation of bacteria in the present study (Fig. 3). Biodegradation activities were inducible with continuous exposure of substrates (Lamberts et al., 2008), however, with respect to MS-1 and MS-2 incubated system, though reduction of MC-RR and MC-LR were observed (Fig. 2), the ineffectiveness of the

incubated bacteria were demonstrated as insignificant changes of MCs concentration were shown with control-2, indicating that the induced degradation strongly depended on bacterial species, isolation strategy, as well as the solution where the biodegradation occurred. With 6 times addition of MCs every 3 days, the total amount of toxins can reach 1263.45 and 1025.76 $\mu\text{g L}^{-1}$ of MC-RR and MC-LR apart from degradation, and was significantly lower than the concentration of total nitrogen and TOC in the water (Table 1). The proliferation of bacteria in MS-1 and MS-2 indicated that metabolization of total nitrogen and TOC, rather than MCs, as nitrogen and carbon source occurred. Therefore, the induced and enhanced biodegradation for strains WC-1 and WC-5 was achieved not only from continuous supply of MCs, but also from the preferential utilization of nitrogen and carbon source. It was difficult to conclusively explain the reasons for the failure of MCs biodegradation by artificial media obtained bacteria in lake water, nevertheless, it was possible to postulate that the following considerations had affected the biodegradative activities: i) the poor adaptability in natural water, ii) the failure to compete with indigenous bacteria, iii) the poor endurance of the isolates to MCs when continuous supply is maintained, and iv) the utilization of other organic material rather than MCs as carbon and nitrogen sources.

4.4. The effect of natural media on diversification of MCs-degrading bacteria

So far the number and diversity of reported MCs-degrading bacteria are still very low (Edwards et al., 2008) and the majority of them have been identified as “*Sphingomonads*” (Tsuji et al., 2006; Valeria et al., 2006). The reason for limited diversity is partly attributed to the failure of some bacteria to form colonies on the artificial media (Eilers et al., 2000). Of the bacteria enriched from water extracts of sediment and then isolated from concentrated water extracts of sediment, WC-4 and WC-5 were genetically close to *Pseudomonas* sp. An 18 and *Acinetobacter* sp. dcm 5A (Fig. 4), respectively. Despite the report of MCs degrading previously (Lee et al., 2006; Takenaka and Watanabe, 1997), the simultaneously obtained two strains of “non-*Sphingomonads*” indicated the potential to amplify of member of MCs-degrading bacteria from environment through natural media. Aimed at amplifying the member of cultivated microorganism, Hahn et al. (2004) developed a filtration-acclimatization method which enabled the isolation and cultivation of an important fraction of the bacterial diversity. Kaeberlein et al. (2002) indicated that uncultivated microbes might grow if provided with the chemical components of their natural environment. The reported strategy in our study, which was characterized by chemical free process, extended approaches to amplify the member of MCs-degrading bacteria.

4.5. Implication for MCs removal from eutrophic water

The occurrence of cyano-HABs and the production of associated MCs have been recorded worldwide. Measures were taken to reduce the MCs in natural water but with limited efficiency (Song et al., 2009). Although biodegradation are supposed to be an attractive way, degradation of MCs by artificial media obtained bacteria often encounter failure. In the present study, strains WC-1 to WC-5 in Lake Taihu water lead to substantially reduction of MCs, showed potential to persist in the environment for extended periods of time. With respect to the operational stability and cell washout of direct application of bacteria in natural water, we previously find that *Ralstonia solanacearum* trapped in carbon nanotube show great removal efficiency of MCs in laboratory conditions (Yan et al., 2004a,b), however, the ecotoxicity of carbon nanotube and the lack of biodegradation in natural environment make the application of such strategy challenging. Investigations on the application of the obtained bacteria that trapped in an eco-friendly material in MCs involved water are in progress. Moreover, relatively shorter period of lag phase of these bacteria provides a potential way to reduce the MCs levels in terms of dramatically increased MCs after collapse of extraordinary

cyano-HABs, particularly with continuous MCs flux. However, biodegradation of MCs in natural water is also affected by initial MCs' concentration, biological, physical and chemical properties of water environment, and these aspects will be further investigated when using these strains for MCs' removal in natural waters.

5. Conclusion

In this study we evaluated water extracts of sediment and pore water for probable isolation of MCs degrading bacteria. The results suggested that nutrients condition affected the cultivation of microorganisms when water extracts of sediment was served as media. Compared with bacteria isolated through traditional MS medium, MCs-degrading bacteria isolated through this natural media exhibited enhanced MCs removal efficiency and stimulated MCs removal rate in Lake Taihu water under continuous supply of MCs.

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