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Biodegradation of Microcystins by Bacillus sp. strain EMB

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Abstract

A bacterium strain EMB capable of degrading microcystin-RR and microcystin-LR was isolated from heap of algae in hudai town. Based on phylogenetic analysis of the 16S rRNA gene sequence, the strain EMB is identified as a member of the Bacillus sp. Strain EMB is able to completely remove 2.99mg/L of MC-RR and 2.15mg/L of MC-LR within 24h. Further research points out that mlrA, microcystin-degrading gene detected in Sphingomonas sp. MJ-PV, also exists in the genome of strain EMB and probably mediates the microcystins degradtion.

Keywords: microcystin; biodegradation; 16S rRNA; mlrA

1. Introduction

Toxic cyanobacterial blooms and cyanotoxins in eutrophic lakes, rivers, and reservoirs have been reported during the last two decades all over the world [1,2]. Microcystins (MCs), produced by species of cyanobacteria including Microcystis, Anabaena, Nostoc and Planktothrix, have been most extensively associated with toxicoses and the focus of many studies in recent years [3]. MC-RR and MC-LR are the highest of contents and detected frequency. Microcystins show a potent hepatotoxicity and tumor-promoting activity by inhibition of the protein phosphatases 1 and 2A [4], and have caused the death of livestock and wildlife and illness and even death in humans [5]. A toxic incident involving the death of 50 people occurred in Brazil in 1996 due to microcystins in the water used for hemodialysis [6]. Potential chronic toxicity from microcystins led the WHO to establish a guideline value of 1μ g L^{-1} as a maximum concentration of microcystin-LR in drinking water [7].

Many methods have been examined in order to remove these microcystins from water. Traditional water treatment methods of chemical coagulation, flocculation and sand filtration are often effective in removing cyanobacterial cells but are not effective in removing and destroying cyanobacterial toxins [8].

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Compared with these methods biological treatment can be considered as a feasible option for effective removal of the microcystins, and some microcystin-degrading organisms have been identified as *Sphingomonads* [9-11].

In present study, a MCs-degrading strain, designated as EMB, was isolated from Wuxi Taihu in China. Further research on the characterization of the MCs-degradation by this strain was performed.

2. Materials and methods

2.1Toxins and other reagents

Standard MC-RR and MC-LR were purchased from Sigma- aldrich. All reagents used were of analytical grade or HPLC grade.

2.2Crude microcystins extract from lyophilized Microcystis aeruginosa cells

Microcystis cells for purification of microcystin were collected from taihu wuxi during August 2008. Samples were concentrated by plankton net and lyophilized and stored at -20℃ until purification of microcystin was performed.

0.2g of lyophilised cyanobacterial cells were extracted with 10mL of 80:20 methanol:water by sonication for 1h and centrifugation(12000 rev/min 10 min 4℃). Rotary evaporation was employed to eliminate methanol of the supernatants. The resulting extract was metered volume to 5mL with distilled water. Then transferred pH value of the extract to 3-4 and centrifugation (12000 rev/min 10 min 4℃) for another time, followed passed through a 0.2 μm cut-off Acrodisc® syringe filter (Pall Corporation, Saint-Germain-en-Laye, France). Subsequently, the pH value of extract was returned to 7. After being autoclaved, the extract was metered volume to 5mL with sterile water and stored at 4°C until used.

2.3 Isolation of EMB strain

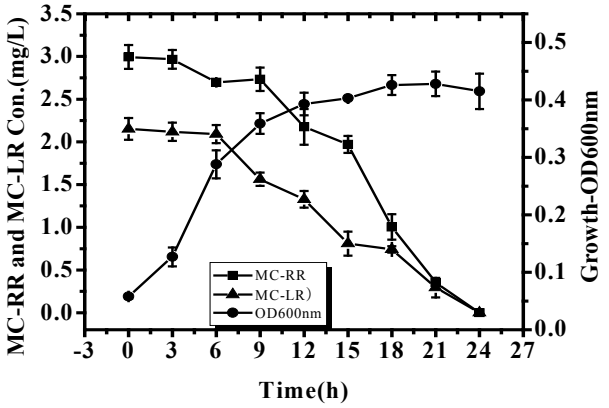

Fig.1. Degradation of MC-RR and MC-LR over 24h by the strain EMB isolated from Taihu. The initial concentration of MC-RR and MC-LR were adjusted to 2.99 mg/L and 2.15 mg/L of the EBB medium at 30℃. Initial optical density (OD600nm=0.058)

Soils samples were collected from heap of algae in hudai town of Wuxi, China, from April 2008 to September 2008. 10g of soil samples were suspended in sterilized water (90 ml) which shook in constant temperature (30°C, 120rev/min) for 1h, followed standing for 30min. 5mL of the supernatants were subjected to 50ml Enrich Broth of Bacteria (EBB) with crude microcystins as candidate carbon and nitrogen resource. After shaking in constant temperature (30°C, 120rev/min) for 5days, 5mL of the resulting solution were inoculated to 50mL EBB again in a constant temperature shaker (30°C, 120rev/min) for 5 days. Repeating the same operation 5 times later, the incubated medium was spread on Isolation Medium (IM) with crude microcystins as candidate carbon and nitrogen resource. Single colonies from these plates were transferred to EBB Medium, and the Microcystin-RR and Microcystin-LR of Crude Microcystins degradation was monitored by HPLC. Among the tested colonies, strain EMB was selected for its high microcystin-degrading capability. The OD value at 600 nm was used as index to the viable bacteria count of strain EMB.

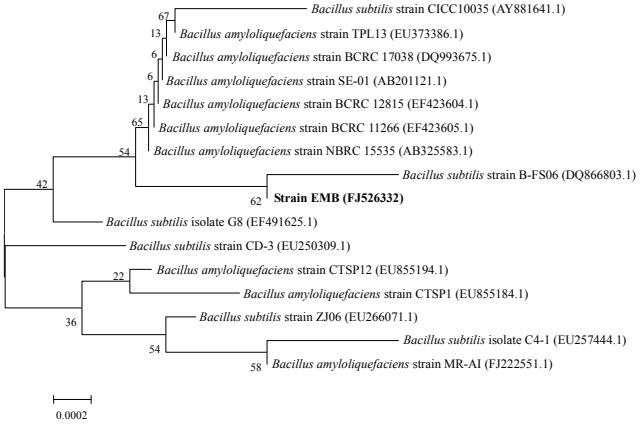

Fig 2. Phylogenetic tree indicating the position of strain EMB within the radiation of related species of the Bacillus. The value on each branch is the estimated confidence limit (expressed as a percentage) for the position of the branch as determined by a bootstrap analysis

2.4 Determination of microcystins

The sub-samples were thawed to room temperature and centrifuged (12,000rev/min, 10 min, 4°C). The supernatants were applied to a conditioned SPE-cartridge (SepPak C18, Waters). The cartridges were firstly washed with 5 ml of 100% methanol and 5 ml of distilled water. Impurities were eluted with 2mL 20% methanol and microcystins were eluted with 2 ml 70% methanol. The eluates were analysed by Aglient HPLC 1100 system equipped with Waters ODS-C18 column (250×4.6 mm, 5 mm particle size) kept at 40°C. The mobile phase was Milli-Qs water containing 0.05% (v/v) trifluoracetic acid (Sigma) and methanol, with a rate 45:55 over 25 min. The flow rate used was 1mL/min. The column eluent was passed through a variable wavelength detector (VWD) operated at 238nm and calculated against a standard curve with MC-RR and MC–LR (Sigma). The variant of MC-RR or MC-LR were respectively quantified as MC-RR or MC–LR equivalents.

2.5 PCR and sequencing

Total DNA of EMB was isolated by using bacterial genomic DNA extraction Kit (SBS Genetech Co., Ltd. Shanghai) according to the manufacturer's instructions. PCR amplification of partial 16S rDNA

fragments was carried out by using primers as described by Saito [12]. After an initial denaturation step of 94℃for 5 min, 40 cycles were performed for the PCR, consisting of denaturation at 94℃for 30 s, primer annealing at 52℃ for 10 s, and elongation at 72℃ for 1min and stay 10min at 72℃.The 16S rDNA fragment was sequenced in the JINSITE Technology Limited Company (Nanjing, China). The sequence determined was blasted in NCBI neuclecotide database and a phylogenic tree topology was performed with the closest 24 cultured sequences by the Cluster X software and Mega 4.1 software.

The primers employed for the amplification of *mlrA* fragment in this experiment were 5'-GACCCGATGTTCAAGATACT-3' (sense) and 5'-TTAATCTTCATGCTGCTAGGAGC-3' (antisense). PCR was performed as follows: 95 ºC for 5 min, 35 cycles at 94 ºC for 30 s, 52 ºC for 30 s, 72 ºC for 60 s, and a final extension step at 72 ºC for 10 min. The size of the amplified PCR product (*mlrA*) was detected in agarose gel (1.5%).

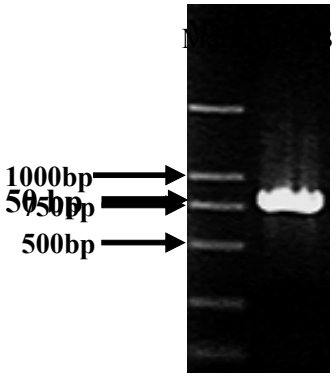

Fig. 3. Detection of mlrA gene fragments by PCR in strain EMB

3. Results

3.1 Isolation of Microcystin-Degrading Bacteria

Among 12 strains isolated from heap of algae in hudai town of wuxi, China, strain YSY-08 was shown to high-performance degrade MC-RR and MC-LR. MC-RR and MC-LR with each initial concentration of about 0.67mg/L and 1.56 mg/L respectively were completely biodegraded by the strain EMB in 24h (Fig. 1).

3.2 Phylogenetic Analysis

To obtain more definitive information on the taxonomic and phylogenetic position of strain EMB, we determined its 16S rRNA gene sequence and compared this with the sequences available from the GenBank databases .On-line homology search showed that, among established species, the 16S rDNA sequence of strain EMB was most similar to the sequence of *Bacillus*（99%Similarity) among the sequences of the established species. Evolutionary distances were calculated for a dataset that consisted of the sequence of strain EMB and 15 other sequences as an outgroup. A rooted phylogenetic tree was reconstructed on the basis of the distance matrix data thus obtained (Fig. 2).It could be concluded that

present data merely demonstrated strain EMB as a member of the *Bacillus sp*. rather than a specific species.

3.3 Detection of the mlrA gene from the microcystin degrading bacterium EMB

The results of detection of the *mlrA* gene by PCR could be observed from Fig.3. Band of the expected size (approximately 800 bp) were detected in the strain EMB by the PCR, which pointed out that *mlrA* homologues existed in this strain.

4. Discussion

Production of microcystin by cyanobacteria in aquatic environments has been well investigated. Degradation of MC-LR by gram-negative bacteria, *Sphingomonas* sp., *Paucibacter toxinivorans*, *Sphingosinicella microcystinivorans*, and *Pseudomonas aeruginosa* has been reported[13-16]. To our knowledge, no *Bacillus* strain was reported with MCs-degrading activity. Our study revealed that *Bacillus* strain EMB could efficiently remove microcystins. Further research pointed out *mlrA* homologues, which was proved to be associated with the MCs-degrading potential of bacterial strains [12], also existed in strain EMB. It is interesting that no *mlrA* gene was found in the present genome database of Bacillus strains, and its appearance was probably due to the horizontal transferring of *mlrA* gene from other genus. Strain EMB could degrade microcystins completely in 24h which showed a high rate and did not show any lag phase during the degradation of microcystin. *Bacillus* is friendly with environments and adaptable to adversity, which is beneficial application in practical surroundings.

Further studies are necessary to characterize the nature and toxicity of intermediate and final metabolites.

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