Isolation, culture and identification of microcystis in source water☆

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Abstract

Objective. To isolate microcystis in the main source water of Zhengzhou city and identify the toxicity. *Methods*. The algae cell was isolated by 96-microbiological assay. The phycocyanin intergenic spacer region (PC-IGS) and the microcystin synthetase gene B (mcyB) were detected by whole-cell PCR. Toxicity of microcystin was identified by ELISA. *Results.* Single cell clones were acquired successfully, and the whole-cell PCR results were positive of PC-IGS and mcyB. Compared to the reported mcyB of *Microcystis aeruginosa* in GenBank, the homology of gene sequence was 99%, which suggested that the three microcystis from the source water were cyanobacteria and toxigenic. The contents of the three microcystis powder were 1.07 μg/mg, 4.70 μg/mg, 0.47 μg/mg, respectively. *Conclusion.* 96-microbiological assay is a simple, fast and accurate method which can isolate algae cells from bloom water successfully. The microcystis isolated from the main source water in Zhengzhou city were blue-green algae which could produce microcystin. [Life Science Journal. 2007; 4(2): 28 – 32] (ISSN: 1097 – 8135).

Keywords: microcystis; isolation; culture; 96- microbiological assay; whole-cell PCR; ELISA

1 Introduction

With the aggravation of the source water pollution, a great quantity of industrial sewage and sanitary sewage has been discharged into the water, which has increased the content of the nitrogen and phosphorus in the water rapidly, and bloom is exploding frequently all over the world threatening human health[1,2]. The main pollutant of bloom is all kinds of toxin of algae, and microcystin which is released by *Microcystin aeruginosa*, *Anabaena flosaquae, Oscillation aeruginosa, nostoc* and so on is the most threatening. Microcystin is a kind of intracellular toxin[3,4], which is investigated widely. In order to get the information of pollution state of the source water in Zhengzhou city, the author selected the Xiliu lake and the Huayuankou reservior which were the main source water of local area for sampling.

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2 Materials and Methods

2.1 Collection of algae sample and culture

The sampling sites were the Xiliu lake and the Huayuankou reservior. The algae was collected by using the No.25 plankton net. The culture medium was BG-11, and illumination was 2500 LUX, the ratio of light and hazy was 12 hours *vs.* 12 hours, and the temperature was (25 ± 1)ºC.

2.2 Isolation and culture of the microcystis

The preincubated solution was diluted to 3 cells/ml, and then 300 μl solution was delivered to 96-microbiological assay. The 96-microbiological assay was put into the illumination incubator until obvious color was seen. Then the plate was observed under inverted microscope and the monoplast solution was delivered to a test tube for growth. The passage should be made every 7 days.

2.3 Bionomics of the microcystis

The morphology of microcystis was observed under light microscope. 5 ml solution was verted to a cuvette and the optical density was determined with 680 nm

wavelength. The optical density was determined at the same time of each day. The calculation formula of cell division velocisity was $k = 3.322/(n-1)\log(OD_n/OD_1)$, and n was the day number. The formula of the generation time of microsystis was $Td = 24/k$, and the unit was hour.

2.4 Identification of microcystis toxicity

2.4.1 Genes of PC-IGS and mcyB were detected by whole-cell PCR. The algae solution which was in exponential growth phase was 1×10^6 cells/ml. And 1.5 ml of the solution was centrifuged for 5 minutes at 13,000 rpm. It was washed by sterile distilled water for 3 times and was diluted by 1.5 ml sterile water. The steps should be repeated for several times. The last supernatant was used as the template of whole cell PCR. The positive control was the No. 940 microcystis which was bought from the Chinese Academy of Science, Hydrobiont Research Center (Wuhan, China) and the negative control was the sterile double distilled water.

The primers of mcyB were 5'-AGGAACAAGTTGCAC AGAATCCGCA-3' (p_1), 5'-ACTAATCCCTATCTAAAC ACAGTAACTCA-3' (p_2); and primers of PC were 5'-GGC TGCTTGTTTACGCGACA-3' (p_1), 5'-CCA GTA CCA CCA GCA ACT AA-3' (p_2). Both of the primers were synthesized by Augct Biotechnology Co, Ltd (Beijing, China).

All PCRs were performed in a 30 μl reaction solution containing 10× buffer, 25 mmol/L $MgCl_2$, 2.5 mmol/L dNTP, forward and reverse primers 7.5 pmol respectively, 3U Taq DNA polymerase, 10 μl template. The reaction procedure contained predenaturation for 6 minutes at 94ºC, followed by 35 cycles at 94ºC for 30 seconds, 55ºC for 1 minute, 72ºC for 1 minute and 72ºC for 8 minutes. The equipment of PCR amplification was Biometra T-Gradient. All PCRs' products were analyzed by electrophoresis in 1.2% agarose using TAE buffer.

2.4.2 The mcyB sequence of PCR amplifying production. The cloning and sequencing was finished by TaKaRa Biotechnology (Dalian, China) Co, Ltd after the PCR amplification of mcyB was purified. The sequencing primer of mcyB were 5'-AGG AAC AAG TTG CAC AGA ATC CGC A-3' (p_1), 5'-ACT AAT CCC TAT CTA AAC ACA GTA ACT CA-3' (p_2); and the para-primers of PC were 5'-GGCTGCTTGTTTACGCGACA-3' (p_1), 5'-CCAGTA CCACCAGCAACTAA-3' (p_2). The sequence was made in the positive direction .

2.4.3 Extraction and determination by ELISA. 80 mg algae powder was disolved by 20 ml 5% acetic acid, and was disintegrated for 30 minutes by ultrasonic, mixed for 30 minutes, centrifuged at 4ºC under 10,000 rpm for 10 minutes, and collected the supernatant named as No.1. The rest was mixed for 30 minutes after added into 20 ml 80% methanol, centrifuged for 10 minutes under the similarity condition, and collected the supernatant named as No.2. The No.3 supernatant was got by the same procedures. All of the supernatant was mixed together and rotary evaporation to near-dried at 70ºC under 100 rpm, then the microcystin was dissolved with 1 ml sodium chloride, centrifuged for 10 minutes at 10,000 rpm and filtrated by the 0.45 μm membrane in the end. The ultimate toxin was diluted by 80,000 and 100,000 times and determined by using Microcystin ELISA kit (Chinese Academy of Science, Wuhan).

3 Results

3.1 Morphology of microcystis

There were 3 types of microcystis that were isolated successfully, containing 1 type from Xiliu lake, 2 types from the Huayuankou reservior, and were named as XLH, BM1, and BM2 respectively (Figure 1 A, B, C). XLH grew aggregating and the shape of the cells were globular or anomalistic. The volume was big with a gum theca and the distribution of the cytoplasm was uneven. The color of BM1 under the microscope was blue-green, and it had a gum theca. The distribution of the cytoplasm was uneven too, the volume of which was smallest of the three. The color of BM2 under microscope was green and BM2 had gum theca with the uneven distribution. The strains of the microcystis from the Huayuankou reservior were globular and grew monocellular.

3.2 Growth curve of microcystis

From Figure 2 we can see that the cycle of the three kinds of microcystis was about two weeks. The growth velocity of BM2 was higher than the others. Their growth curve was "S" shape and the highest velocity was from the eleventh day to the thirteenth day.

3.3 Cleavage velocity and the generation

As we can see from Table 1, the mean cleaving velocities of the three kinds of microcystis were 0.396, 0.199, 0.345 respectively. The geneation time of BM2 was 121 hours which was the longest of the three. The denominatort means the monitoring time and k means the cleaving velocity and Td means the generation time.

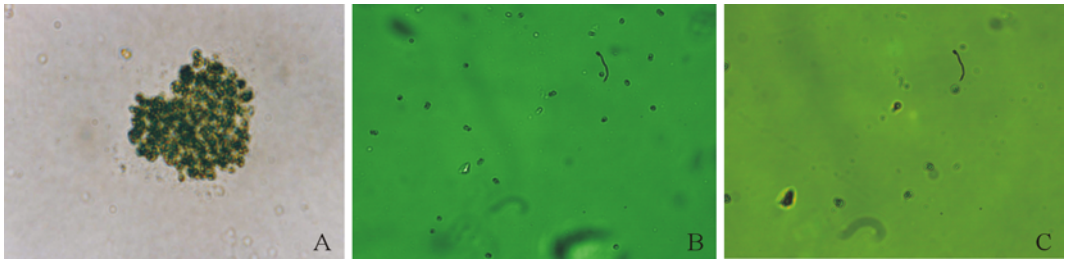

Figure 1. A: the microcystis of XLH; B: the microcystis of BM1; C: the microcystis of BM2.

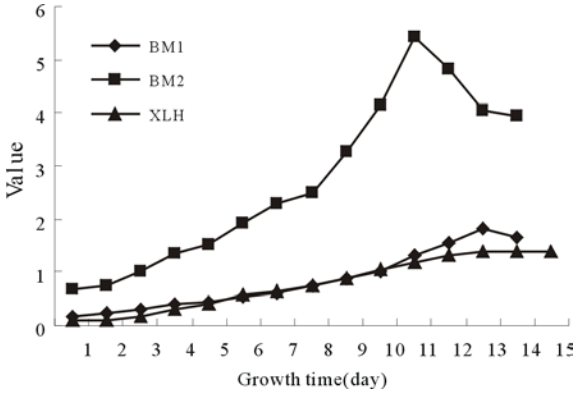

Figure 2. Growth curve of microcystis.

Table 1. Time of cleavage velocity and generation

Number	OD_0	OD_n	t_{n-1} (d)	k	Td (h)
BM1	0.182	1.635	12	0.396	61
BM2	0.690	3.940	12	0.199	121
XLH	0.100	1.388	11	0.345	70

3.4 Results of the amplification of whole cell PCR

As we can see from the Figure 3, the length of the primer amplification products were 350 bp and 700 bp, which were as the same the length as the aim genes' and we can see that all of the microcystises that researched could release microcystin.

3.5 The mcyB sequence of PCR production

3.5.1 Sequence of XLH

1 GGTATTTTTG AGGACAAAAT TAACCTATCA
ACAGTTAAAT AACCGGGGCA ATCAGTTAGC
61 TCACTGTTTA CGAGATAAGG GTGTAAATCC
AGAAAGTTTA GTCGGGATTT TTATGGAGCG
121 TTCCCTAGAG ATGGTCATCG GTTTATTAGG
GATATTAAAA GCCGGGGGAG CTTATGTACC
181 TTTAGATCCG GATTATCCTA CCGAGCGCTT
GGGGGATATC CTCTCAGATT CGGGTGTTTC
241 TTTGGTGTTA ACTCAGGAAT CTTTAGGGGA
TTTTCTTCCC CAAACTGGGG CTGAGTTACT
301 GTGTTTAGAT

Results of sequencing indicated: length of the gene was 310 bp, and there was only one restriction enzymes of *Eco*RV at the 217th base. The sequence was submitted to GenBank using the BLAST, and the gene number given by GenBank was EF216874, and compared to the reported mcyB of *Microcystis aeruginosa* No.899316, 92109242, 14486356 in GenBank, the homology of gene sequence was 98%.

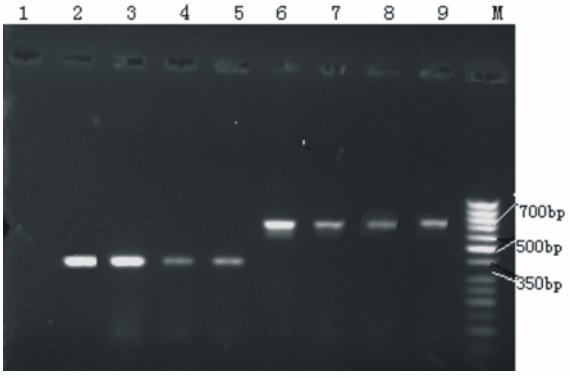

Figure 3. Spectrum of PCR amplification. M: Marker; Lane 1: negative control-mcyB; Lane 2: BM1-mcyB; Lane 3: BM2-mcyB; Lane 4: XLH-mcyB; Lane 5: positive control-mcyB; Lane 6: BM1-PC-IGS; Lane 7: BM2-PC-IGS; Lane 8: XLH-PC-IGS; Lane 9: positive control-PC-GS.

3.5.2 Sequence of BM1

1 GGGGAGAATC CCCTAAAGAT TCCTGAGTTA
ACACCAAAGA AACACCCGAA TCTGAGAGGA
61 TATCCCCCAA GCGCTCGGTA GGATAATCCG
GATCTAAAGG TACATAAGCT CCCCCGGCTT
121 TTAATATCCC TAATAAACCG ATGACCATCT
CTAGGGAACG CTCCATAAAA ATCCCGACCA
181 AACTTTCTGG AACTACACCC TTATCTCGTA
AACAGTGAGC TAACTGATTG CCCCGGTTAT
241 TTAACTGTTG ATAGGTTAAT TTTTGTCCTT
CAAAAATAAC CGCTATTCCC TGCGGATTCT
301 GTGCAACTTG TTCA

The sequencing results indicated: the length of BM1 was 314 bp and the code-star was the second base. There was only one restriction enzymes of *Eco*RV in the 61th base. The sequence was submitted to GenBank using the BLAST, and the gene number given by GenBank was EF216872 with the protein number ABP04110. Compared to the reported mcyB of *Microcystis aeruginosa* No.92109242, 28804617, 28804615 in GenBank, the homology of gene sequence was 99%.

3.5.3 The sequence of BM2

1 GGGGAGAATC CCCTAAAGAT TCCTGAGTTA
ACACCAAAGA AACACCCGAA TCTGAGAGGA
61 TATCCCCCAA GCGCTCGGTA GGATAATCCG
GATCTAAAGG TACATAAGCT CCCCCGGCTT
121 TTAATATCCC TAATAAACCG ATGACCATCT
CTAGGGAACG CTCCATAAAA ATCCCGACCA
181 AACTTTCTGG AACTACACCC TTATCTCGTA
AACAGTGAGC TAACTGATTG CCCCGGTTAT
241 TTAACTGTTG ATAGGTTAAT TTTTGTCCTT
CAAAAATAAC CGCTATTCCC TGCGGATTCT
301 GTGCAACTTG TTC

The sequencing results indicated: the length of BM1 was 313bp and the code-star was the first base. There was only one restriction enzymes of *Eco*RV in the 61th base. The sequence was submitted to GenBank using the BLAST, and the gene number given by GenBank was EF216873 with the protein number ABP04111. Compared to the reported mcyB of *Microcystis aeruginosa* No.92109242, 28804617, 28804615 in GenBank, the homology of gene sequence was 99%, and the basic radical was the same to BM1 except one in the 8th site.

3.6 Results of ELISA

The normo-concentration extent of the Microcystis ELISA kit was 0.1 – 2.5 ng/ml MC-LR and the determined results were contained in the extent after diluted. We can see from Table 2, all of the microcystises could release microcystin, and BM2 could release more than the others.

Table 2. Determination results of microcystin

Cell strain	OD_{450}	Concentration (mg/ml)	Quantity of powder (μg/mg)
BM1	0.0760	0.3635	1.07
BM2	0.0785	0.1688	4.70
XLH	0.0715	0.3510	0.47

4 Discussion

4.1 Isolation technics of 96-microbiological assay

The problem of the frequent explosion of bloom has evoked attention worldwide. Successful isolation of all kinds of algaes was considerable in bioresearch, biological effect, and toxic mechanism. We used 96-microbiological assay in the isolation of microcystis for the first time, and had isolated three kinds of microcystis successfully integrating the ultimate dilution method. Our method not only decreases the difficulty but increases the achievement ratio. It is a simple, easy-operated, new, highly effective method and do a good base for the bioresearch and removing research of algae toxin.

4.2 Whole cell PCR in microcystis

The phycocyanin gene is in the genome of blue-green algae, and its intergenic spacer region (PC-IGS) between α and β subunit is highly conservative. Blue-green algae gemera can be identified according to the PCR amplification production and the enzyme digestion results if the primers are designed according to the PC-IGS sequence, but the toxic character can't be identified[7]. Microcystin synthetase gene(mcy) has 10 gymnatremoid reading frame in 2 reverse transcription operon(mcy A – C and mcy D – J). Only microcystin contains this sequence[8], toxingenic character can be identified according to the PCR amplification production with the primer designed according to mcy. On these grounds, we designed 2 couple primers according to PC and MB, and amplified the gene using whole cell PCR of microcystis from Xiliu lake and Huayuankou reservior. The results displayed that the whole-cell PCR results were masculine of PC-IGS and mcyB, which demonstrated that the three kinds of microcystis could generate microcystin.

4.3 ELISA in determination of microcystis toxin

There are many methods of determining microcystin up to now, biochemistry and chemical analysis are the main methods. With the preparation of Microcystin antibody, ELISA could be a simple, fast, sensitive method. We diluted the microcystin 80,000 and 100,000 times to ensure the results could be contained in the standard concentration. The determination result displayed that all of the three kinds of microcystis could generate microcystin and the producing toxin capability was strong. On the base of the above, the author presumes that the further study should be done to the condition of source water polluted by algae, also to the poisoning effect and toxic mechanism.

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