A Fungus Capable of Degrading Microcystin-LR in the Algal Culture of Microcystis aeruginosa PCC7806

Article *in* Applied biochemistry and biotechnology · December 2011

DOI: 10.1007/s12010-011-9486-6 · Source: PubMed

Appl Biochem Biotechnol (2012) 166:987–996
DOI 10.1007/s12010-011-9486-6

A Fungus Capable of Degrading Microcystin-LR in the Algal Culture of *Microcystis aeruginosa* PCC7806

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Received: 28 June 2011 / Accepted: 30 November 2011 /
Published online: 15 December 2011

Abstract Microcystins (MCs) are a family of natural toxins produced by cyanobacteria (blue-green algae). Microbial degradation is considered an efficient method for eliminating cyanobacteria and MCs in environmental conditions. This study examines the ability of *Trichaptum abietinum* 1302BG, a white rot fungus, to degrade microcystin-LR in the harmful algal culture of *Microcystis aeruginosa* PCC7806. Results showed that microcystin-LR could not be detected by high-performance liquid chromatography after 12 h in algal culture incubated with the fungus. There were also high activities of catalase and peroxidase in algal culture incubated with the fungus. However, similar to the control, they decreased to normal levels after 72 h. Meanwhile, the micronucleus test in the toxicity studies revealed that the degraded algal culture had low toxicity.

Keywords Cyanobacteria · *Trichaptum abietinum* 1302BG · Microcystin-LR · Micronucleus

Introduction

Harmful algal and cyanobacterial blooms have been found in eutrophic fresh water bodies all over the world. Algal blooms can alter the physical properties of water (color, taste, and odor) and pose significant negative impact on aquatic environments, aquaculture, and public health. They can also produce, excrete, and release highly toxic compounds; microcystins (MCs), in particular, can make water unsuitable for use and consumption [22]. MC is a family of hepatotoxins produced by freshwater cyanobacterial blooms responsible for liver failure in wild animals, livestock, and aquatic life [5]. Furthermore, exposure to hepatotoxin-contaminated

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water can cause animal illnesses and death [2]. They have a general structure comprising five D-amino acids with minor structural variations and a pair of variable L-amino acids. The most commonly studied and most toxic analogue is microcystin-LR (MC-LR) (L and R represent the variable amino acids leucine and arginine), which has an LD_{50} value of 0.05 mg kg^{-1} in mice by intraperitoneal injection [5, 27].

The toxic effects and mechanism of MCs in animals have been well studied. The commonly accepted toxic mechanism of MCs is their ability to inhibit protein phosphatase type-1 and 2A [25]. There has been evidence suggesting that oxidative stress is involved in MC toxicity [23, 26]. A possible second toxic factor of MCs is the formation of reactive oxygen species during their biotransformation. To prevent cellular damage, cells have developed a protective system involving antioxidative enzymes, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). These enzymes play a pivotal role in protection against oxidative damage and adjustment processes of metabolic pathways [26, 33].

Since algal blooms and toxic MC-producing cyanobacteria threaten human health and life, an effective method for the regulation of the occurrence of algal blooms and their toxic metabolites must be established [13]. Although many strategies, such as biomanipulation and algicides, have been evaluated for the removal of algal blooms or cyanobacterial toxins in a lake, no suitable method has been developed. The occurrence of cyanobacteria is difficult to regulate through conventional methods [6]. An effective method for simultaneous removal of MCs and harmful algal cells is also necessary.

In recent years, there has been increasing interest in white rot fungi with the capability to degrade various environmental pollutants, including dyes. Many studies have demonstrated that white rot fungi are able to decolorize textile dyes and remove some complex structured toxins, such as xenobiotics, lignin, and dyestuffs, by their extracellular lignolytic enzyme system [3].

The present investigation is designed to study the possible degradative abilities of a white rot fungus, *Trichaptum abietinum* 1302BG (TA-1302), on MC-LR in algal culture. Changes in the activities of detoxifying enzymes (SOD, POD, and CAT) involved in fungal metabolism, and the metabolites will also be determined to confirm detoxification during the biotransformation of the toxin.

Materials and Methods

Cyanobacterial Strains and Cultivation

MC-producing strain *Microcystis aeruginosa* PCC7806 (Mpc) was originally collected by the Pasteur Culture Collection of Cyanobacteria in France from the Freshwater Algae Culture Collection of the Chinese Academy of Sciences, Wuhan, China.

The growth medium of Mpc was BG11 [30]. The stock and experimental cultures of Mpc were kept at 25 °C under a 12:12 h (light/dark) cycle at approximately 90 μmol photons $m^{-2}s^{-1}$ [15].

Fungal Isolation, Culture, and Maintenance

The fungus strain of TA-1302 was isolated from the soil of bamboo forests in Hangzhou, China. A piece of round fungal mycelia (2%) from a PDB solid-plate (2%) were inoculated into 9-cm diameter plate containing 15 mL liquid potato dextrose broths in stationary position. After

7 days of cultivation, mycelial pellicles were used for liquid degradation systems. Fungal isolates were kept at 4 °C after initial screening for use in later experiments [11].

Degradation Test of TA-1302 on MC-LR

M. aeruginosa PCC7806 culture (100 mL) was transported into 250-mL flask that was previously autoclaved (120 °C, 20 min). Each mycelial pellicle of TA-1302 prepared on a Petri dish was inoculated into a flask and cultivated on a shaking incubator (25±2 °C, 125 rpm). All experiments were conducted in triplicate.

The temperature of Mpc culture was 25±2 °C and pH was 7.0. MC-LR concentration of Mpc culture was determined by high-performance liquid chromatography (HPLC). Samples for testing MC-LR were conducted at 0, 4, 8, 12, 18, 24, 36, 48, and 72 h. The initial algal cell density of Mpc determined by a hemocytometer under microscope was $1–8\times10^5$ cells mL^{-1}, and chlorophyll-a concentration was 800–1,000 μg L^{-1}. Algal culture without fungus and BG11 medium with fungus served as negative and positive controls, respectively.

HPLC Analysis

Collected water samples (2 mL) were run through a pre-washed 0.45-μm Millipore membrane filter. The filtrate was then passed through disposable 1-g C_{18} cartridges (Supelco) attached to a vacuum chamber at a rate of approximately 5 mL min^{-1}. Cartridges were prepared by washing with 5 mL methanol and 5 mL pure water beforehand. Cartridges were then washed with 0.05% (*v/v*) trifluoroacetic acid (TFA) in acetonitrile solution. After concentration of eluates using a rotary evaporator at 35 °C, residuates were redissolved in 1 mL 50% methanol and stored at −20 °C until HPLC analysis. Obtained samples were analyzed on a Dionex UltiMate 3000 (Dornierstrasse, Germany) equipped with CHROMELEON chromatography management system and a UV–vis detector set at 238 nm. A C_{18} Discovery HS (Supelco) column (5 μm, 4.6×250 mm, Dionex, Sunnyvale, CA, USA) was utilized. Gradient elution with a mixture of mobile phase A (0.05% (*v/v*) TFA in methanol solution) and mobile phase B (0.05 M KH_2PO_4, pH 3.0 adjusted with phosphoric acid) was used. Phase A was linearly increased from 52.5% to 55% in 0–15 min, 55% to 60% in 15–25 min, held steady at 25–27 min, and then finally brought back to 52.5%. Column oven temperature was 40 °C, and flow rate was 1 mL min^{-1} [1, 12].

Antioxidant Enzyme Activity Assays

About 5 mL degraded culture was centrifuged at 12,000×*g* at 4 °C for 30 min. Supernatants were stored at 4 °C prior to use.

CAT activity was detected according to Cakmak and Marschner [4] with some modifications. Reaction mixture with a total volume of 3 mL contained 1.9 mL 50 mM sodium phosphate buffer (pH 7.0) and 1 mL 0.2% H_2O_2. Reaction was initiated by the addition of 0.1 mL enzyme extract, and activity was determined by measuring the initial rate of disappearance of H_2O_2 at 240 nm. Activities for CAT were expressed in enzyme units (U), where one enzyme unit was defined as a change of 0.01 absorbance min^{-1} mL^{-1}.

POD activity was determined by measuring the rate of increase in absorbance at 470 nm of a mixture containing 1 mL 50 mM sodium phosphate buffer (pH 7.0), 0.95 mL 0.2% 2-methoxyphenol, 1 mL 0.2% hydrogen peroxide, and 0.05 mL enzyme extract. Activities for POD were also expressed in units, where 1 U was defined as a change of 0.01 absorbance min^{-1} mL^{-1}.

SOD activity assay was based on the method described by Giannopotitis and Ries [16]. One unit of enzyme activity was defined as the amount of enzyme required to cause a 50% inhibition of the rate of nitro blue tetrazolium reduction measured at 560 nm.

Toxicity Studies

The formation of micronuclei in root tips has been widely described and used as a bioassay for the evaluation of toxicity of environmental pollutants. To check the toxicity of test culture after decomposition treatment, a toxicity test using *Vicia faba* root tip micronucleus assay was performed. Dry *V. faba* seeds previously stored at 4 °C were soaked for 4 h in deionized water [17, 24, 32]. Seedlings with 15–20 mm root length were collected for experimental use. Roots were treated with algae culture and degraded algae culture. Treatment exposure time was 4 h, followed by a 24-h recovery period. Deionized water was used as negative control. After treatment, root tips were cut and fixed overnight in Carnoy's solution (1:3 glacial acetic acid/ethanol) at 4 °C and transferred into 70% ethanol for storage. They were then hydrolyzed in 1 N HCl at 60 °C for 12–15 min. Three slides were prepared for each of the three seeds. After staining of the root tips with 1% aceto-orcein, micronucleus frequencies of interphase cells were scored at ×1,000 magnification. Three thousand cells per tip were counted.

Statistical Analysis

The constant potential concentration loss over time was represented by the exponential equation:

$$\mathrm{Ln}(x_t/x_0) = -kt$$

Where x_0 is the original concentration of MC, x_t is the amount of MC remaining after time t, and k is the MC decomposition coefficient (per hour).

All calculations were performed in triplicate, and the mean values were presented. ANOVA was conducted by SPSS PC+13.

Results

Changes in Concentration of MC-LR

Degradation efficiencies of MC-LR were obtained from the MC-LR levels determined by HPLC of samples. MC-LR concentrations constantly increased within 18 h in the control culture and then decreased within 24 h. After 24 h, MC-LR concentrations became stable at 0.087±0.002 (Fig. 1). In the test sample, MC-LR concentrations decreased very quickly within 12 h compared with the control (Fig. 1). Removal rate of MC-LR was 48.22% at 8 h. After 12 h, MC-LR concentration was approximately zero in the test culture. First-order exponential decay rate was approximately 0.0927 h^{-1}. There were significant differences in MC-LR concentrations compared with the control ($p<0.001$).

To observe the efficacy of MC degradation by TA-1302, HPLC chromatograms of the samples at different treatment durations were compared (Fig. 2). As shown in Fig. 2b, the chromatogram of MCs processed for 72 h presented one peak because of MC-LR at reservation time of 25.8 min in the control, while MC-LR was completely degraded by

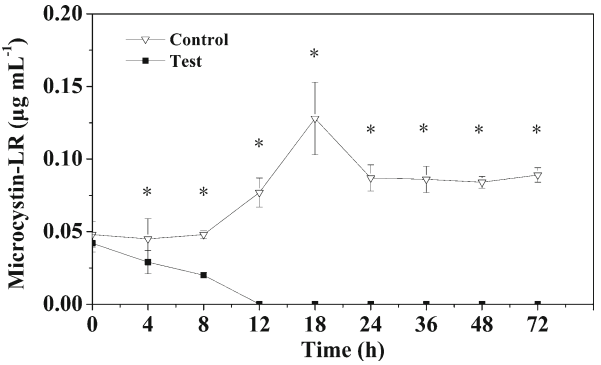

Fig. 1 Changes in MC-LR concentration in *M. aeruginosa* PCC7806 (Mpc) culture during incubation with TA-1302. Data are mean±SD from three independent assays. Significant differences between the control and TA-1302 treatment are indicated by *asterisk* ($p<0.001$)

TA-1302 in the test culture. One new peak was detected at 72 h in the test culture, and the peaks were eluted at 13.5 min (Fig. 2c).

Effects of MC-LR on Antioxidant Enzymes

CAT activity increased gradually after incubation of TA-1302 in Mpc culture. CAT activity in test culture was significantly higher than that in the negative and positive control after 18 h, and was 15-fold of the values in the negative and positive controls (Fig. 3). There were significant differences compared with the negative and positive controls ($p<0.01$). Prolongation of treatment to more than 18 h resulted in a dramatic decrease in CAT activity to levels that were not significantly different compared with the control.

Enhancement of POD activity in the incubated Mpc culture was detected upon initiation of TA-1302 treatment. Significant differences between the treatment and the control ($p<0.05$) were found at 8-, 12-, and 24-h durations (Fig. 4). Further prolongation of treatment resulted in decreased POD activity that was not significantly different from the control.

Significant increase in SOD activity was found in three different treatments after 4 h (Fig. 5). However, there were no significant differences in test culture compared with that in negative and positive control cultures.

Toxicity of Degraded Algal Culture

Results of *V. faba* root micronucleus test of three samples, negative control, positive control, and test culture, are presented in Fig. 6. No significant increase in micronucleus frequency was recorded in the test culture compared with the negative control (Fig. 6), while a significant increase in micronucleus frequency was observed in roots exposed to the positive control compared with the test culture and negative control. Micronucleus frequency of the positive control was approximately 11-fold of the test culture and tenfold of the negative control.

Discussion

Previous studies have reported that high chemical and biological stability were hazardous properties of MC-LR in addition to its toxicity [5]. They report that some co-existing bacteria can lyse the cyanobacterial cells and that some can more effectively degrade MCs

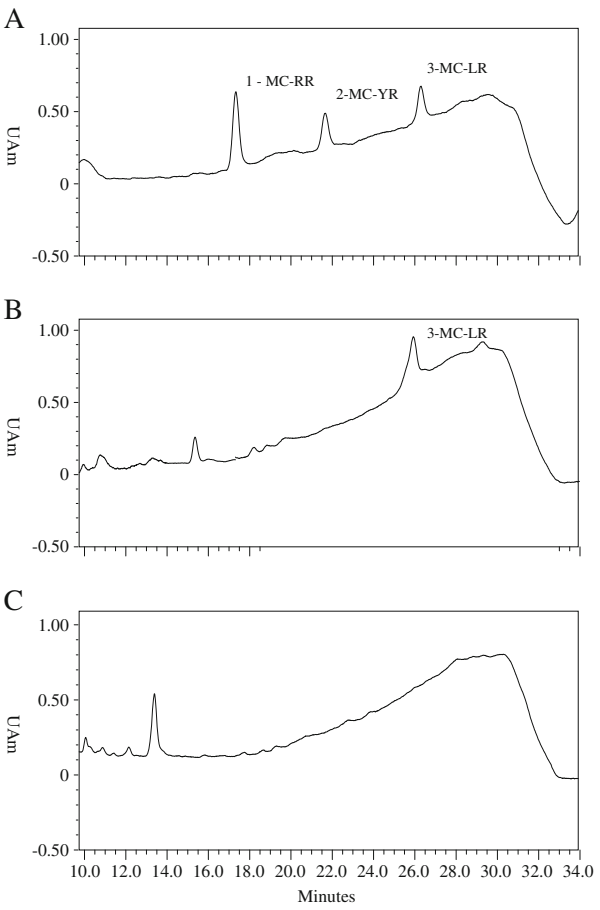

Fig. 2 Chromatograms showing the decreasing MC-LR peak from the Mpc culture. **a** Standard samples of MC-LR; **b** positive control after 72 h; and **c** test culture after 72 h

compared with other treatments such as sunlight, heat, and adsorption, under natural conditions [10, 20]. Some bacterial strains capable of breaking down cyclic MC molecules were have been isolated by several authors [14, 21]. However, degradation rates and lag-phase presence or duration can differ markedly under various conditions. Occurrence of lag-phase (3–21 days) and longer degradation periods (6–25 days) in waters of different origins have also been documented [7, 8, 20]. In the present study, extracellular MC-LR could not be detected in Mpc culture incubated with the fungus after 8 h. Results show a relatively rapid and efficient way to remove extracellular MC-LR from *M. aeruginosa* PCC7806. MC-LR simultaneously exists inside cyanobacterial cells and in its surrounding water, so that both pools of MC-LR should be degraded simultaneously for complete removal of MC-LR. In our previous study, we suggested that the chlorophyll-a concentrations and the biomass of Mpc culture became approximately zero at 36 h after incubation [18, 19]. Results of this study demonstrated that white rot fungus (TA-1302) can also effectively degrade extracellular MC-LR from *M. aeruginosa* PCC7806. Therefore, it suggests that the fungus can remove the MC-LR completely and reduce the biomass of algal culture simultaneously.

Furthermore, MCs can induce oxidative stress in vitro culture of plant cells and primary cultured rat [9]. To avoid oxidative damage, plants possess the antioxidative enzymes CAT,

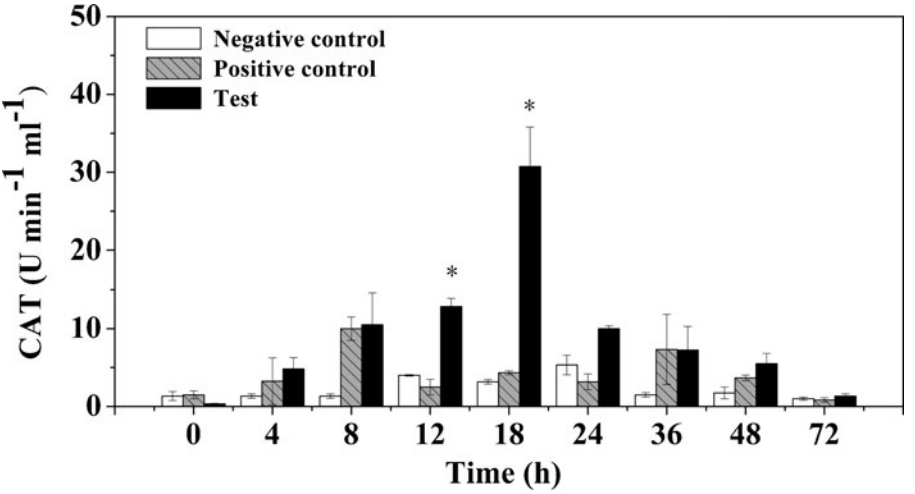

Fig. 3 Changes in catalase (CAT) activity of incubated Mpc culture. Negative control, algal culture without TA-1302; positive control, BG11 medium incubated with TA-1302; test, algal culture incubated with TA-1302. Data are mean±SD from three independent assays. Significant differences between the controls and test are indicated by *asterisks* ($p<0.01$)

POD, and SOD. Yin et al. showed that, in MC-RR-treated cell, there was very high POD and SOD activity while CAT activity was very low [33]. Results of this study show very high activity of CAT and POD (Figs. 3 and 4) and no significant difference in SOD activity between test culture and control (Fig. 5). When test culture treatment was prolonged; CAT and POD activity decreased to levels that were not significant difference from the control. The decline in CAT and POD activities may be due to the effective degradation of MC-LR by TA-1302.

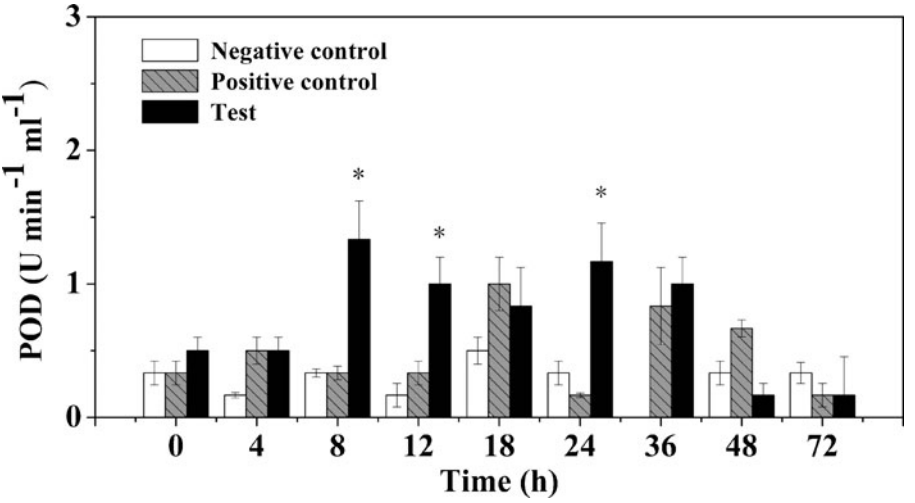

Fig. 4 Change in peroxidase (POD) activity of incubated Mpc culture. Negative control, algal culture without TA-1302; positive control, BG11 medium incubated with TA-1302; test, algal culture incubated with TA-1302. Data are mean±SD from three independent assays. Significant differences between the controls and test are indicated by *asterisks* ($p<0.05$)

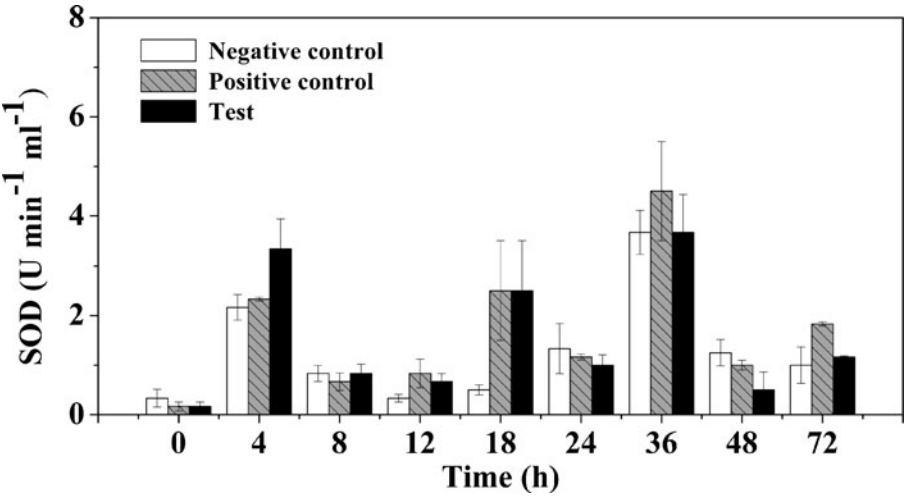

Fig. 5 Change in superoxide dismutase (SOD) activity of incubated Mpc culture. Negative control, algal culture without TA-1302; positive control, BG11 medium incubated with TA-1302; test, algal culture incubated with TA-1302. Data are mean±SD from three independent assays

Previous studies have found that degradation products, such as linearized MC-LR, tetrapeptide, and Adda, are essentially non-toxic. This strongly indicates that microbial degradation using MC-degrading bacteria is effective for the detoxification of MCs [31]. The present study also found that the degradation products of MC-LR by TA-1302 were low-toxic compared with the positive control, which concurs with previous works. However, degradation products of MC-LR by the fungus have not been detected and identified. Future studies focusing on the degradation products and biodegradation comparisons with MC-degrading bacteria strains from different reference cultures should be done to gain new insights into the degradation of MC-LR.

Frequent reports of toxic cyanobacterial bloom occurrences worldwide are alarming, and it is growing as quickly as the emergence of the eutrophic state in some natural and artificial water bodies. The exceptional metabolic versatility of white rot fungi presents a potential resource for bioremediation of recalcitrant MC molecules. However, the ecological effects of the fungus to natural water bodies could not be what we expected. Although microbial and phytotoxicity studies of the extracted metabolites after dye degradation by white rot fungi support their ecofriendly nature [28, 29], a mesocosm test and assessment of the ecological effect should be done prudent before the application.

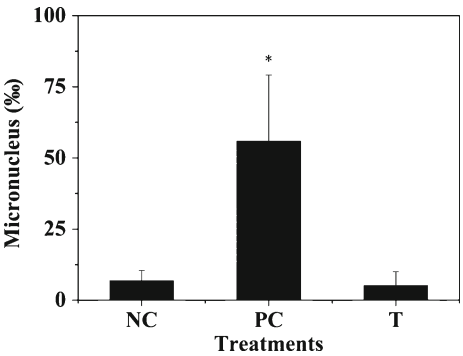

Fig. 6 Micronucleus frequency values of *V. faba* roots exposed to different algal treatments. *NC* negative control, deionized water; *PC* positive control, algal culture without TA-1302 after 72 h; *T* test culture, algal culture incubated with TA-1302 after 72 h. Data are mean±SD from three independent assays. Significant differences between the control and TA-1302 addition are indicated by *asterisks* ($p<0.01$)

Conclusion

This study investigated the efficacy of *T. abietinum* 1302BG-degrading microcystin-LR in the algal culture of *M. aeruginosa* PCC7806. The results showed that microcystin-LR could be degraded by *T. abietinum* 1302BG in the algal culture incubated with the fungus. Meanwhile, the micronucleus test in the toxicity studies revealed that the degraded algal culture had low toxicity.

Acknowledgments This work was financial supported by Project of National Basic Research Program of China (2008CB418004) and National Science Foundation of China (30870419).

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