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Growth inhibition of the cyanobacterium *Microcystis aeruginosa* and degradation of its microcystin toxins by the fungus *Trichoderma citrinoviride*

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ABSTRACT

Harmful cyanobacterial blooms are recognized as a rapidly expanding global problem that threatens human and ecosystem health. Many bacterial strains have been reported as possible agents for inhibiting and controlling these blooms. However, such algicidal activity is largely unexplored for fungi. In this study, a fungal strain kkuf-0955, isolated from decayed cyanobacterial bloom was tested for its capability to inhibit phytoplankton species in batch cultures. The strain was identified as *Trichoderma citrinoviride* Based on its morphological characteristics and DNA sequence. *Microcystis aeruginosa* co-cultivated with living fungal mycelia rapidly decreased after one day of incubation, and all cells completely died and lysed after 2 days. The fungal filtrate of 5-day culture also exhibited an inhibitory effect on *M. aeruginosa*, and this inhibition increased with the amount of filtrate and incubation time. Conversely, green algae and diatoms have not been influenced by either living fungal mycelia or culture filtrate. Interestingly, the fungus was not only able to inhibit *Microcystis* growth but also degraded microcystin produced by this cyanobacterium. The toxins were completely degraded within 5 days of incubation with living fungal mycelia, but not significantly changed with fungal filtrate. This fungus could be a potential bioagent to selectively control *Microcystis* blooms and degrade microcystin toxins.

1. Introduction

Occurrence of cyanobacterial blooms in many large freshwater lakes has become more frequent worldwide. In Particular, *Microcystis aeruginosa* proliferates rapidly and accumulates in water bodies causing significant adverse impacts on aquatic environments and public health (Hua et al., 2009). This is due to the ability of this species to produce microcystin toxins (Codd et al., 2005) which are associated with allergies, irritation reactions, gastroenteritis, liver diseases, and tumors (Bell and Codd, 1994; Dawson, 1998). Many strategies including physical, chemical and biological methods have been adopted to reduce and remove these nuisance blooms and their toxins in water supplies all over the world. However, high treatment costs (Hua et al., 2009) and secondary pollutants formed as a result of using chemical algicides (Jeong et al., 2000; Mason, 2002) make these methods undesirable.

Biological methods, on the other hand, are considered to be a more economical and environment-friendly way to control cyanobacterial blooms (Jia et al., 2010a) and degrade their cyanotoxins (Mohamed and Alamri, 2012). Biological control agents such as viruses, bacteria, fungi and heterotrophic flagellates were found to play a major role in

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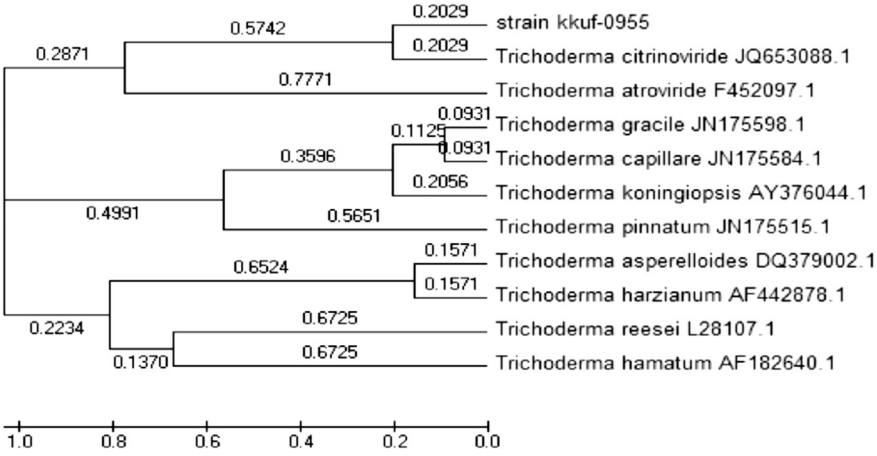

Fig. 1. Phylogenetic relationship between the *T. citrinoviride* strain kku-0955 and other ITS sequences of published strains. In the phylogenetic tree, kku-0955 and *T. citrinoviride* were clustered together as one clade Segments corresponding to an evolutionary distance of 0.005 are shown with bars. Accession numbers for sequences are as shown in the phylogenetic tree.

the regulation and termination of harmful algal blooms (Sigee, 2005; Mohamed and Al-Shehri, 2013). Most biological studies have focused mainly on screening cyanophages and bacteria for their ability to control and degrade harmful cyanobacteria in aquatic environments (Ahn et al., 2003; Mayali and Azam, 2004; Kang et al., 2005; Mu et al., 2007; Dillon and Parry, 2008; Alamri and Mohamed, 2013). However, little attention has been paid to fungi with algicidal activity on cyanobacteria and other harmful algae. Some studies have reported an antagonistic activity of some antibiotic producing fungi toward cyanobacteria (Redhead and Wright, 1980). Other studies demonstrated that fungal strains that produce no antibiotics, particularly white rot fungi, can inhibit the growth of cyanobacteria species (Jia et al., 2010a,b 2011; Wang et al., 2010). Moreover, a recent study by Jia et al. (2012) revealed the ability of *Trichaptum abietinum* 1302BG, a white rot fungus, to degrade microcystin-LR in the harmful algal culture of *M. aeruginosa* PCC7806. It is, therefore, necessary to screen more fungal species of different groups for their algicidal activity to differentiate the strong aligicidal species to be used as an efficient bioagents against harmful algal and cyanobacterial blooms. In this study, we investigated the ability of other fungal species, *Trichoderma citrinoviride* to inhibit the growth of the toxic cyanonabacterium *M. aeruginosa* and to degrade its microcystin toxins as a contribution to the knowledge of algicidal fungi.

2. Materials and methods

2.1. Fungal isolation and identification

The fungus *T. citrinoviride* was isolated from decayed *Microcystis* bloom collected from a Saudi eutrophic lake on yeast extract malt extract agar medium (YMA) containing; 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 5 g L^{-1} peptone, 10 g L^{-1} glucose, 0.033 g L^{-1} rose bengal and 0.1 g L^{-1} chloramphenicol. The fungal mycelia were transferred into 50 mL glass flask containing 25 mL liquid medium. After 5 d of cultivation, the mycelial pellicles were used as inocula for algicidal experiments. The fungus was identified preliminarily based on the morphological and cultural characteristic. The identification was confirmed by molecular identification. The fungal genomic DNA extraction was

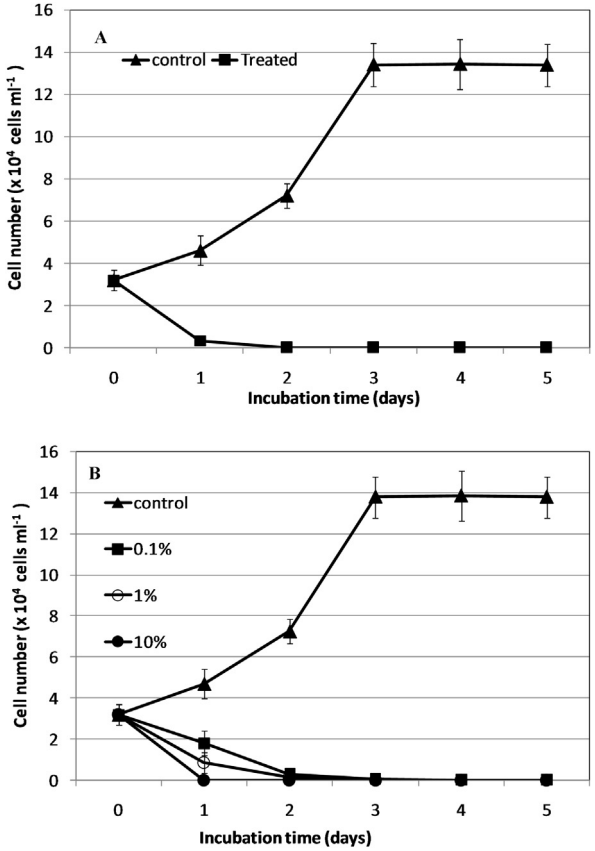

Fig. 2. Changes in the growth (cell density) of *Microcystis aeruginosa* treated with the mycelia (A) and extract (B) of *T. citrinoviride*.

carried out using the Qiagen DNeasy Plant Mini Kit protocol according to the manufacturer's instructions. The ITS region of fungal DNA was amplified using the fungal specific primer set: ITS1-F (CTTGGT CAT TTA GAG GAA GTA A) and ITS4 R (TCC TCCGCT TAT TGA TAT GC) as described by White et al. (1990). PCR reaction was performed in a final volume of 50 μL containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, each dNTPs at a concentration of 0.2 mM, 1.25 IU of Taq polymerase, each primer at a concentration of 0.2 mM and 1 μL of DNA template. The amplification was carried out by PCR under the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min; final extension at 72 °C for 10 min and holding at 4 °C. The amplified products were examined by electrophoresis in 1.5% agarose gels in TAE buffer. Then, the PCR product was purified and sent for sequencing to Macrogen company Korea. The ITS sequence of fungus isolate was used for a BLAST search in the EMBL/GenBank database. The sequence of the isolate was further aligned and compared to published ITS region sequences searched with the taxonomy browser of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and retrieved from GenBank.

2.2. Phytoplankton strains and cultivation

Microcystin producing strain *M. aeruginosa*, *Ankistrodesmus* sp., *Chlorella* sp. and the diatom *Nitzschia* sp., were previously isolated from an eutrophic Saudi lake (Mohamed and Al-Shehri, 2009). *M. aeruginosa* strain used

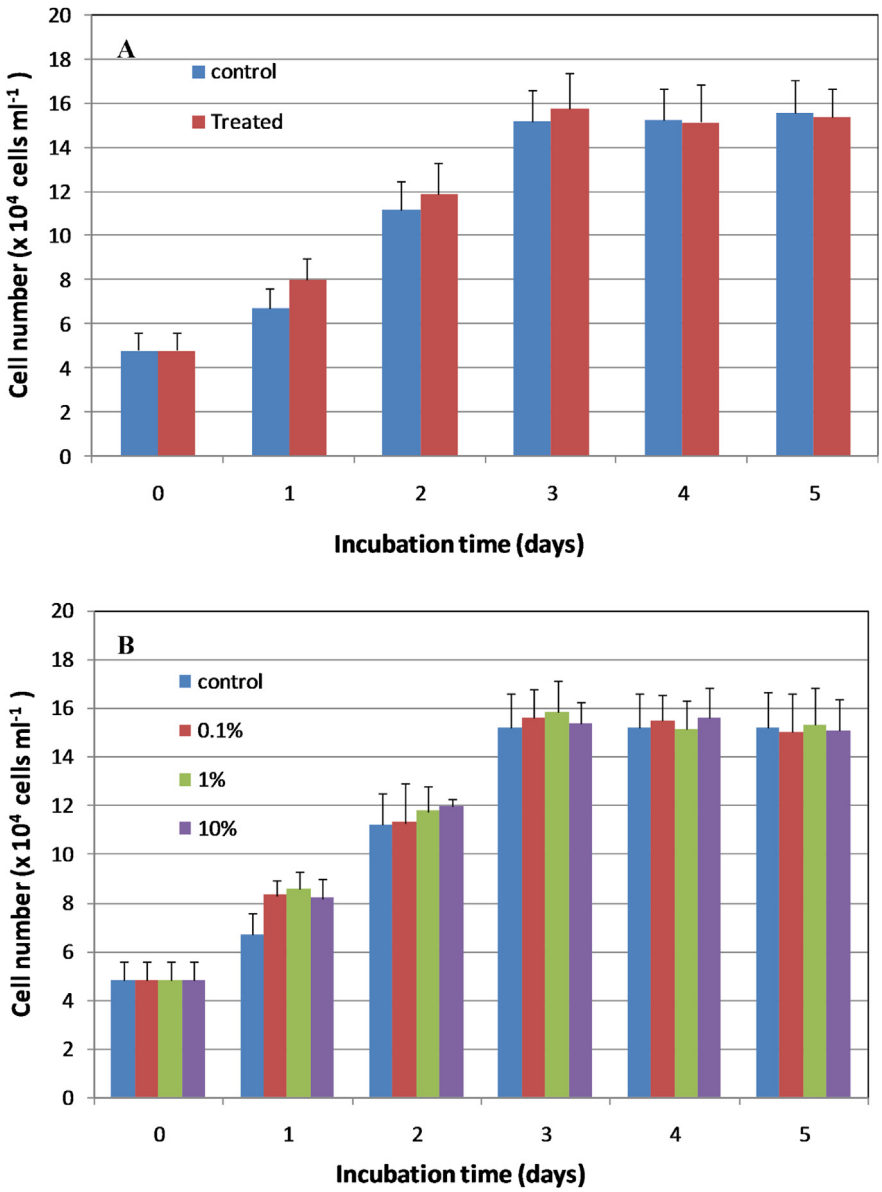

Fig. 3. Changes in the growth (cell density) of *Ankistrodesmus* sp. treated with the mycelia (A) and extract (B) of *T. citrinoviride*.

in the experiments was reported to produce 3.04 mg microcystins g^{-1} dry weight as determined by HPLC (Al-Shehri, 2010). All cyanobacteria and algae were grown axenically in BG11 medium at 25 ± 2 °C under continuous illumination at approximately 90 μmol photons m^{-2} s^{-1} (Rippka et al., 1979) and used at the exponential phase for inoculation in the algicidal activity experiments. An aliquot of each algal culture was transferred separately into a 250 mL flask containing 100 mL BG-11 medium to give an initial algal cell density of 3.2×10^4 cells mL^{-1} for *M. aeruginosa*, 4.2×10^4 cells mL^{-1} for *Ankistrodesmus*, 2.8×10^4 cells mL^{-1} for *Chlorella* sp., and 3.2×10^4 cells mL^{-1}, 1.6×10^4 cells mL^{-1} for *Nitzschia* sp. Each reserved mycelial pellicle was inoculated into a flask and co-cultivated with each alga under the same conditions outline above for 5 days. The dry weight of each fungal inoculum was 65.3 mg. The growth of *M. aeruginosa* and algae measured as a cell density was determined every day by a hemocytometer under a microscope. Algal culture without fungus and BG11 medium with fungus served as negative and positive controls, respectively. At the end of the experiment, the mycelial pellicles in completely lysed algal cultures were harvested by polycarbonate filters, rinsed with sterilized deionized water, and oven dried. The filtrate of this lysed culture was filtered through 0.2-μm Millipore membrane filter and added at a dilution of 10% (V/V) to each 250 mL flask containing 100 mL BG-11 medium and algal inocula at the same initial density

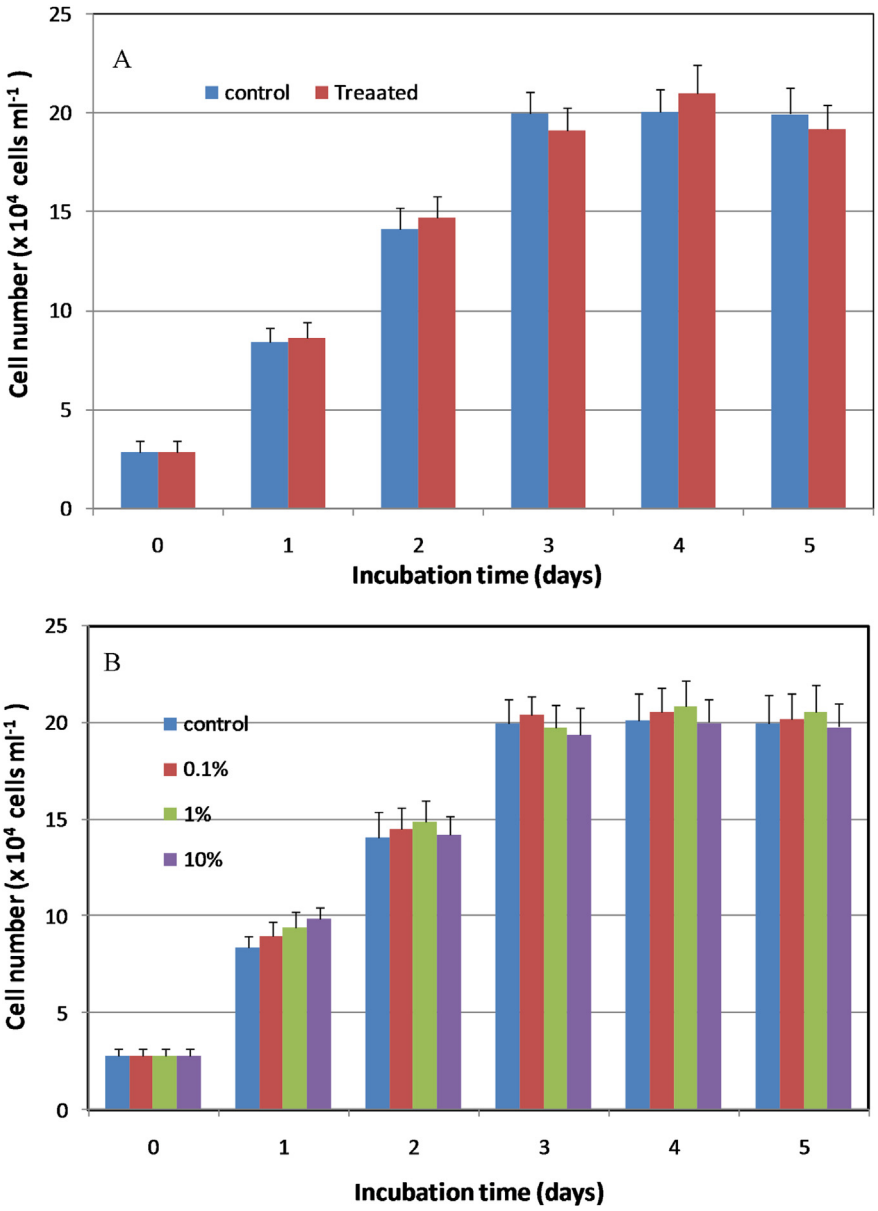

Fig. 4. Changes in the growth (cell density) of *Chlorella* sp. treated with the mycelia (A) and extract (B) of *T. citrinoviride*.

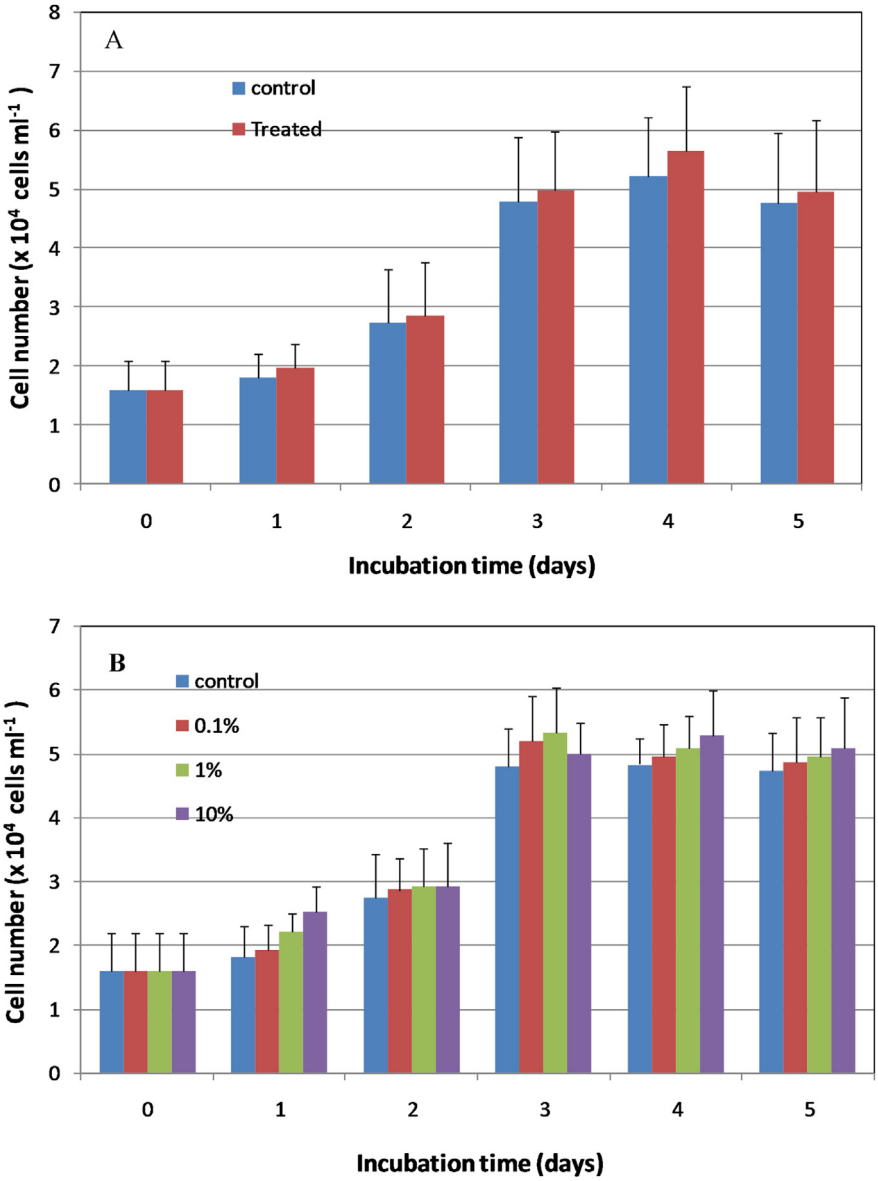

Fig. 5. Changes in the growth (cell density) of the diatom *Nitzschia* sp. treated with the mycelia (A) and extract (B) of *T. citrinoviride*.

mentioned above. Algal culture without fungal filtrate was used as negative controls. The cell density of cyanobacterial and algal cultures was monitored daily by a heamocytometer under a microscope. All the experiments were conducted in triplicate.

2.3. Microcystin degradation

To investigate the ability of the fungus *T. citrinoviride* or its filtrate to degrade microcystin toxin released in fungal-cyanobacterial cultures, an aliquot of *Microcystis* culture with fungus or fungal filtrate were taken daily, centrifuged at 12000× *g* for 10 min. BG-11 medium containing only fungal filtrate without microcystins was used to test the interference of compounds in fungal filtrate with ELISA assay. The microcystin concentration was then determined in the supernatant by Enzyme-linked immunosorbent assay (ELISA) according to Carmichael and An (1999) using the commercial kit, Microcystin-ADDA ELISA kit purchased from Abraxis (Warminster, PA).

To investigate the potential adsorption of microcystins on the fungal cell wall, a known weight (250 mg, close to the highest value of fungal dry mass obtained during co-cultivation with *M. aeruginosa*) of heat-inactivated fungal mycelia was added to a 250-ml conical flask containing BG-11 medium and microcystin crude extract at a concentration of 100 μg L^{-1}. A flask containing BG-11 medium and heat-inactivated fungal mycelia without microcystin was used as a control. The experiment was run in triplicate. The flasks were incubated at the same conditions of *Microcystis*

lysis experiment for 5 days. Microcystin concentrations were monitored daily by ELISA as outlined above.

3. Results

Trichoderma is one of complex fungal groups that are difficult to identify solely based on morphological characters. Thus, to confirm the identity of the fungus kku-0955 at the molecular level, partial sequencing of ITS region was amplified by PCR with ITS1 and ITS4 primers. Fungal rDNA-ITS sequences for the kku-0955 strain were compared with the sequences of ITS region in GenBank database by means of BLAST search. Results showed that the ITS sequence of the isolated strain was highly homologous to *T. citrinoviride*, with 98% sequence similarity. To confirm the position of the strain kku-0955 in phylogeny, a number of sequences representative some *Trichoderma sp*. were selected from Genbank database for the construction of a phylogenetic tree. As shown in Fig. 1, the phylogenetic tree indicated that strain kku-0955 and *T. citrinoviride* shared one clade cluster. Therefore, the strain kku-0955 was identified as *T. citrinoviride*. Recently, *T. citrinoviride* was identified based on ITS sequencing and phylogenetic analysis (Huh et al., 2011).

Among different fungal strains isolated from decayed *Microcystis* bloom, a strain kkuf-0955 showed a selective inhibitory activity towards the cyanobacterium *M. aeruginosa* rather than other algal groups such as chlorophyceean and diatom species. During the first day of co-cultivation with living fungal mycelia, the growth (cell density) of *M. aeruginosa* sharply and rapidly decreased compared to control ($F = 13.6$, $df = 1$, $p = 0.014$), and all *Microcystis* cells completely died within 2 days (Fig. 2A). The fungal filtrate of 5-day culture also exhibited an inhibitory effect on *M. aeruginosa*, and this inhibition varied and increased with the increase in the amount of filtrate ($F = 12.6$, $df = 3$, $p < 0.001$) (Fig. 2B). Additionally, the growth inhibition of *Microcystis* cells by fungal filtrate depended on the incubation time. The longer the incubation time the strong the growth inhibition. In contrast to their effects on the cyanobacterium *M. aeruginosa*, neither living fungal mycelia nor culture filtrate had an inhibitory activity towards green algae (Figs. 3 and 4). The cell density of the green algae *Ankistrodesums* ($F = 2.5$, $df = 1$, $p = 0.1$) and *Chlorella* ($F = 2.2$, $df = 1$, $p = 0.1$) under fungal treated conditions did not vary significantly from control cultures ($P < 0.05$). However, the cell density of the diatom *Nitzschia* showed a significant increase in cultures treated with fungal filtrate ($F = 5.56$, $df = 3$, $p = 0.01$) (Fig. 5). After co-cultivation for 5 days with algal cultures, the dry weight of fungal mycelia co-incubated with *Microcystis* culture increased significantly ($F = 227226$, $df = 1$, $P < 0.001$) compared to its initial weight (Table 1). Conversely, the mycelial dry weights co-incubated with *Ankistrodesmus*, *Chlorella*, and *Nitzschia* did not significantly change compared to their initial weights ($F = 1.6–6.3$, $df = 1$, $P = 0.1–0.3$).

The results of ELISA showed an increase in microcystin concentrations released into the medium of *Microcystis* cultures incubated with *T. citrinoviride* mycelia compared to control cultures (without fungus) ($F = 78$, $df = 2$, $p = 0.01$). The highest microcystin concentrations were obtained in the culture medium after 2 days incubation, coinciding with the complete lysis of *Microcystis* cells (Figs. 2 and 6). Thereafter, these released microcystins decreased sharply in the medium of fungal-treated cultures and became undetectable at day 5 ($F = 650.8$, $df = 5$, $p < 0.001$). This was opposed to control cultures which showed an increase in microcystin concentrations released into the medium along all the incubation period. On the other hand, microcystin concentrations released into the medium of treated cultures with fungal filtrate did not significantly change along the incubation period ($F = 0.6$, $df = 9$, $P < 0.8$) (Fig. 6). The concentration of microcystins incubated with heat-inactivated fungal cells did not reveal any significant change ($F = 0.7$, $df = 5$, $p = 0.64$) along the experiment period (Table 2).

Table 1
Weight of the fungal mycelia after co-incubation with different algal species for 5 days.

Algal species	Mycelial dry weight (mg)	
	0 day	5 days
Microcystis aeruginosa	65.3 ± 0.05	231 ± 0.6
Anksitrodesmus sp.	65.3 ± 0.05	65.1 ± 0.17
Chlorella sp.	65.3 ± 0.05	65.8 ± 0.12
Nitzschia sp.	65.3 ± 0.05	65.5 ± 0.14

4. Discussion

The results of the present study clearly demonstrated the ability of the fungus *T. citrinoviride* isolated from decayed algal bloom to inhibit and destroy the toxic cyanobacterium *M. aeruginosa*. Previously, some species of *Trichoderma* including *T. atroviride*, *T. spirale*, *Trichoderma virens* isolated from the top soils and litter of the forest floor of Zijin Mountain (Nanjing, China), showed no or weak algicidal activity with algal cell removal efficiency (0–33%) when co-cultivated with *M. aeruginosa* for 120 h (Han et al., 2011). Our strain (*T. citrinoviride* kkuf-0955) removed 89.7%

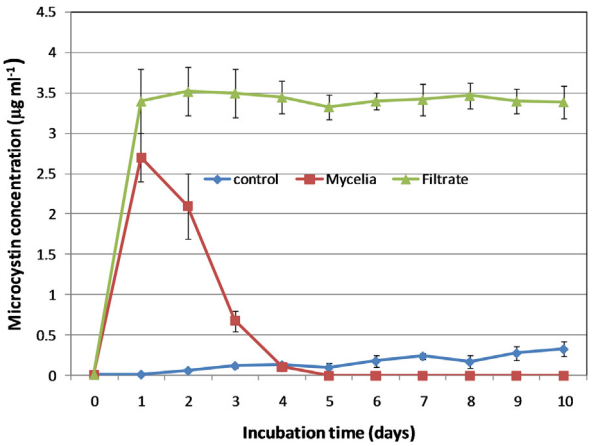

Fig. 6. Changes in concentrations of extracellular microcystins in the medium of *M. aeruginosa* cultures during incubation with mycelia and extracts of *T. citrinoviride*.

Table 2
Changes in concentrations of microcystins during incubation with heat-inactivated cells of *T. citrinoviride*.

Initial microcystin	Remaining microcystin concentrations ($\mu g\ l^{-1}$)				
($\mu g\ l^{-1}$)	Day 1	Day 2	Day 3	Day 4	Day 5
Control (100)	100 ± 0.1	99.9 ± 0.2	100.2 ± 0.1	100 ± 0	99.8 ± 0.3
100	99.8 ± 0.3	99.7 ± 0.2	100.1 ± 0.2	99.6 ± 0.3	99.8 ± 0.1

of *Microcystis* cells within 24 h and the cell removal efficiency was 100% after 48 h. Therefore, our strain can be compared to other strong algicidal fungi such as *Irpex lacteus* T2b, *Trametes hirsuta* T24, *Trametes versicolor* F21a, and *Bjerkandera adusta* that removed more than 95% of the algal cells after 120 h of co-incubation, in particular the fungus *T. versicolor which* completely eliminated algal cells within 30 h (Han et al., 2011). Other studies reported the ability of white rot fungi for eliminating all *Microcystis* cells within 48 h by *T. abietinum* 1302BG (Jia et al., 2010b), and 39 h by *Lopharia* spadicea (Wang et al., 2010). It has been noticed that most of the strong algicidal fungi reported so far belong to the order Polyporales of the phylum Basidiomycota. By contrast, the present study is the first to add a strong algicidal fungus *T. citrinoviride* of a phylum Ascomycota. The algal cells were also lysed by algicidal bacteria for 144–240 h with removal rate of 70–90% (Uribe and Espejo, 2003; Amaro et al., 2005). By comparison, our study along with other previous works showed that the fungi could remove all the test algal cells within 48 h, indicating that it is faster and more efficient than the algicidal bacteria. Most previous studies have shown that algicidal fungi inhibit or lyse algal cells indirectly through production of diffusible extracellular substances (Nakagiri and Ito, 1997; Jenkins et al., 1998). However, recent studies revealed that algicidal fungi have preying ability on the algal cells, as the algal cells are encased with a mucous membrane secreted by the fungal mycelia, and finally degraded by the fungus directly (Jia et al., 2010b). In the present study, the addition of *T. citrinoviride* cultural filtrate inhibited the growth of *M. aeruginosa*, this fungus degraded cyanobacterial cells through the excretion of algicidal compounds into the medium rather than direct attack. The dry weight of *Trichoderma* highly increased in *Microcystis* cultures upon decaying all cells after 2 days of co-cultivation. This suggests that this fungus decomposed and utilized the constituents of *Microcystis* cells for its growth. Previously, Han et al. (2011) found that the biomasses of strong algicidal fungi slightly increased, when most cyanobacterial cells disappeared after 60 h of co-incubation. Jia et al. (2011) also reported that the white rot fungi, *T. abietinum* and *Lopharia spadicea* can use cyanobacterial bloom material as a glucose substitute in Martin medium with an increase in the dry weight of the mycelial pellicles.

In addition to inhibition and decaying *Microcystis* cells, *T. citrinoviride* was able to degrade microcystin toxins released into the medium after the decay of *Microcystis* cells. To our knowledge, this is the second study of microcystin degradation by fungi. The first one was made by Jia et al. (2012) who demonstrated that the white rot fungus *T. abietinum* 1302BG completely degraded extracellular MC-LR after 12 h after in *M. aeruginosa* culture incubated with the fungus. In our study, complete microcystin degradation by *T. citrinoviride* occurred after 72 h. The longer time for complete microcystin degradation by *T. citrinoviride* could be due to the high concentration of microcystin concentration (2.7 $\mu g\ ml^{-1}$) in the medium to which this fungus was exposed, compared to lower concentration (0.05 $\mu g\ ml^{-1}$) used for *T. abietinum*. What is noticed here in the present study is that microcystin degradation did not occur when incubated with fungal filtrate or heat-inactivated fungal cells indicating that microcystin could not be adsorbed on the fungal cell wall and is degraded only by living fungal cells, which could use this toxin as a carbon source for their growth. Many studies isolated some bacterial strains capable of breaking down cyclic microcystin molecules and used it as a carbon source (Hyenstrand et al., 2003; Kato et al., 2007). However, our results along with those of previous studies (Jia et al., 2012) show that fungal strains rapidly degrade microcystins (12–72 h) than bacterial strains (6–25 days) (Cousins et al., 1996; Christoffersen et al., 2002).

Taken the results of this study, the fungus *T. citrinoviride* could be used as a cyanobacterial bloom control agent. However, this study was performed on representatives of each algal group. Therefore, further studies on the effect of this fungus or its filtrate on the whole planktonic community in mesocosm experiments are needed. Although terrestrial fungi do not multiply well in a natural body of water, our study showed that the fungal extract or filtrate can selectively inhibit cyanobacterial growth rather than other algal species. Therefore, these extracted metabolites could be sprayed directly on the blooms in the natural environment without any effect on other beneficial algae. Otherwise, the fungus could be brought into contact with cyanobacteria by settling fungus in a natural setting at a buoyancy neutral level and water should be circulated as suggested by Jia et al. (2010a).

Conflict of interest

The authors declare that there is any conflict of interest.

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