Journal of Hazardous Materials 183 (2010) 176–181

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

The efficacy and mechanisms of fungal suppression of freshwater harmful algal bloom species

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ARTICLE INFO

Article history:
Received 3 December 2009
Received in revised form 26 June 2010
Accepted 5 July 2010
Available online 2 August 2010

Keywords:
Cyanobacteria
Trichaptum abietinum 1302BG
Preying ability

ABSTRACT

Microorganisms have attracted worldwide attention as possible agents for inhibiting water blooms. Algae are usually indirectly inhibited and degraded by secretion from microorganisms. In this study, algal cultures *Microcystis aeruginosa* (Ma) FACH-918, *Microcystis flos-aquae* (Mf) FACH-1028, *Oocystis borgei* (Ob) FACH-1108, and *M. aeruginosa* PCC 7806 were co-cultured with the fungus strain *Trichaptum abietinum* 1302BG. All algal cells were destroyed within 48 hours (h) of co-incubation. Scanning electron microscope and transmission electron microscope observation revealed that the fungal strain had preying ability on the algal cells. The mechanism may be that the algal cells were encased with a mucous membrane secreted by the fungal mycelia, and finally degraded by the fungus directly.

1. Introduction

Harmful algal blooms, and cyanobacterial blooms, in particular, have been observed in eutrophic fresh waters all over the world, causing significant adverse impacts on aquatic environments, aquaculture and public health. Recently, interactions between microorganisms and algal bloom species have been considered an important factor in regulating both the microorganism and algal population dynamics. Extensive research investigated bloom mitigation methods, including phosphorus sequestration [1] and bacterial [2] and cyanophage lysis of the algal cells [3], while fewer studies have looked into the algicidal ability of fungi on algal bloom species.

Previous studies have indicated that the suppressive effect on the algal species could be due to secretion by other microorganism during the incubation. The interaction between the microorganism and algae was always indirect. Moreover, these studies have shown that the microorganism has limited applicability, and some toxic wastes have been found in these studies [4,5].

In this study, we demonstrate the preying ability and mechanism of *Trichaptum abietinum* 1302BG, a white-rot fungus, on algal bloom species.

2. Materials and methods

2.1. Cyanobacterial strains and cultivation

The microcystin-producing strain *M. aeruginosa* PCC7806 (Mpc) used in our experiment was originally collected by the Pasteur Culture Collection of Cyanobacteria in France. *Microcystis aeruginosa* (Ma), *Microcystis flos-aquae* (Mf) and *Oocystis borgei* (Ob) isolated from Taihu, China, were provided by the Freshwater Algae Culture Collection of the Chinese Academy of Sciences, Wuhan, China. The algal cell density of four strains used for inoculation was $(1–6) \times 10^5$ cells ml^{-1}.

The growth medium of all cultures was BG11: $NaNO_3$ 150 mg, K_2HPO_4 4 mg, $MgSO_4{\cdot}7H_2O$ 7.5 mg, $CaCl_2{\cdot}2H_2O$ 3.6 mg, $Na_2SiO_3{\cdot}9H_2O$ 5.8 mg, citric acid 0.6 mg, Ferric ammonium citrate 0.6 mg, EDTA 0.1 mg, Na_2CO_3 2 mg, A_5 solution + Co 0.1 ml, distilled water 99.9 ml (A_5 solution: H_3BO_3 286 mg, $MnCl_2{\cdot}4H_2O$ 181 mg, $ZnSO_4{\cdot}7H_2O$ 22 mg, $CuSO_4{\cdot}5H_2O$ 7.9 mg, $Na_2MoO_4{\cdot}2H_2O$ 3.9 mg, distilled water 100 ml), pH 7.0. All stock and experimental cultures were conducted at 25 °C under a 12:12 h (L:D) cycle at approximately 90 μmol photons m^{-2} s^{-1}. In order to reduce any influence brought about by minor differences in photon irradiance, the flasks were shaken manually twice each day and rearranged randomly [6,7].

2.2. Fungal isolation, culture and maintenance

The fungus strain *T. abietinum* 1302BG (TA-1302) was isolated from the soil of bamboo forests (Hangzhou, China,) on the lowest and the highest positive MPN dilutions of rose bengal agar

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doi:10.1016/j.jhazmat.2010.07.009

medium (5 g L^{-1} peptone, KH_2PO_4 1 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.05 g L^{-1}, 10 g L^{-1} dextrose anhydrous, 18.5 g L^{-1} agar, 0.033 g L^{-1} rose bengal, 0.1 g L^{-1} chloramphenicol). Single fungal mycelia (2%) were transferred from rose bengal agar inoculated into 9 cm diameter plate containing 15 ml liquid potato dextrose broths (PDB) in stationary position. After 7 d of cultivation, the mycelial pellicles were used for the degradation test. All fungal isolates were kept at 4 °C after initial screening for use in later experiments [8].

2.3. Degradation test of TA-1302 on algae

Each 100 ml of the culture, Ma, Ob, Mf, Mpc, was transported into 250 ml flask that was previously autoclaved (120 °C, 21 min). Each reserved mycelial pellicle prepared on a plate was inoculated into a flask and cultivated on a shaking incubator (25 ± 2 °C and 125 rpm). The reserved mycelial pellicles were harvested by polycarbonate filters (25 mm in diameter, 0.22 μm in mesh), rinsed with sterilized deionized water, and dried using a Freezone-6, Labconco freeze dry system. The dry weight of each inoculum was 71.03 ± 0.32 mg. All the experiments were conducted in triplicate.

The water samples of cultured algae looked deep green with a noxious smell. Water temperature was 25 ± 2 °C and pH was 7.0. The algal cell density was (1–6) × 10^5 cells ml^{-1} and the chlorophyll-a concentration was 700–1000 μg L^{-1}. Algal cell density was determined by a hemocytometer under a microscope. The chlorophyll-a in the water sample was extracted with 90% acetone after removing the mycelia and measured with a UV-2100 spectrophotometer at λ = 630, 663 nm. Samples for algal cell counts were conducted at 0, 4, 8, 12, 18, 24, 36, and 48 h. The system without the fungus served as control for all tests.

2.4. Lipid peroxidation

Lipid peroxidation was determined by the amount of malondialdehyde (MDA), a product of lipid peroxidation, with the thiobarbituric acid method [9].

2.5. Biomass of mycelial pellicle

The mycelial pellicle was filtered by polycarbonate filters (25 mm in diameter, 0.22 μm in mesh) at 0, 4, 8, 12, 18, 24, 36 and 48 h, rinsed with sterilized deionized water, and dried using a freeze dry system (FREEZONE-6, LABCONCO). The weight of each dried mycelial pellicle sample was measured. The system without the algae (BG11) served as control.

2.6. Algae lysis

To test the algae-lysing mode of the TA-1302, dead mycelial pellicle (121 °C, 20 min), the reused mycelial pellicle which had been used once and the culture liquid filtered with the filter (25 mm in diameter, 0.22 μm in mesh), were added into 100 ml fresh algal culture, respectively. The algal cell density was determined by a hemocytometer under a microscope.

2.7. Scanning electron microscopy

The mycelial pellicle was filtered with polycarbonate filters (25 mm in diameter, 0.22 μm in mesh) at 0, 4, 8, 12, 18, 24, 36 and 48 h, washed with sterilized water and dried using a freeze dry system (FREEZONE-6, LABCONCO). The processed samples were coated with platinum using a sputter coater (EMS-1010, HITACHI). The samples were then examined at The Modern Analysis Center, Nanjing University, China, using a SEM-3400, HITACHI [10].

2.8. Transmission electron microscopy

To examine the mechanism of TA-1302 preying on the algae, the mycelial pellicle was filtered by polycarbonate filters (25 mm in diameter, 0.22 μm in mesh) at 0, 4, 8, 12, 18, 24, 36 and 48 h, then washed with deionized water. The original biopsy was prepared for electron microscopy by fixation in 4% glutaraldehyde in PBS and post-fixation in 1% buffered osmium tetroxide, dehydrated using a serial dilution of acetone, and then embedded in Epon-812 (R). Half-micron to one-micron sections were ultrathin sectioned and stained with uranyl acetate and lead citrate-nitrate. Micrographs were obtained using a HITACHI H-600 electron microscope [11].

2.9. Statistical analysis

All determinations were carried out in triplicate and the mean values were presented. One-way analysis of variance was conducted by the SPSS PC+13.

3. Results

3.1. Changes in algal cells during incubation

In the first 8 h, the amount of algal cells decreased quickly. The removal rates were 45.65%, 70.18%, 90.96% and 81.56% for Ma, Ob, Mf and Mpc, respectively (Fig. 1). After 12 h, a constant decrease of the algal cell densities was found in all algae. The cell densities of four algae reached 0 within 48 h. Significant differences were found compared with the control ($P < 0.001$).

3.2. Changes in lipid peroxidation

MDA contents, as a measure of membrane lipid peroxidation, increased in the test sample cultures of four algae compared with the control (Fig. 2).

In the first 4 h, no significant differences of MDA contents were found between the four algae and the control. After 8 h, the MDA contents increased quickly. Significant differences of MDA contents were found between test samples and control.

3.3. The mass of mycelial pellicle

The mass of mycelial pellicle co-incubated with algal culture generally increased (Table 1). Significant differences were found between each algal culture and the control ($P < 0.001$).

3.4. Degradation mechanism of the algal cells

Fig. 3 and Table 2 show that the fresh algal cells of Mpc were degraded by reused mycelia; however, there was no influence on the algal cells with dead mycelial pellicle and filtered medium. The results were statistically the same for other algae.

In TEM examination, Fig. 4A revealed that the algal cells adsorbed to the mycelial networks of TA-1302. The single algal cell was in physical contact with the mycelial surface in the 8 h test samples (Fig. 4B). As can be seen in Fig. 4C, the algal cells were enveloped with the membrane. The membrane was secreted by the mycelial surface of TA-1302 in the 24 h test samples. In comparison, Fig. 4D showed that the algal cells surrounded with membrane were lysed at approximately 48 h.

In SEM examination, Fig. 5A revealed that algal cells were adsorbed by the mycelial networks of TA-1302. The transverse section of single algal cell was closed to the mycelial surface from the 4 h test samples (Fig. 5B). As can be seen in Fig. 5C, the algal cells were enveloped with the membrane. The algal cells were still

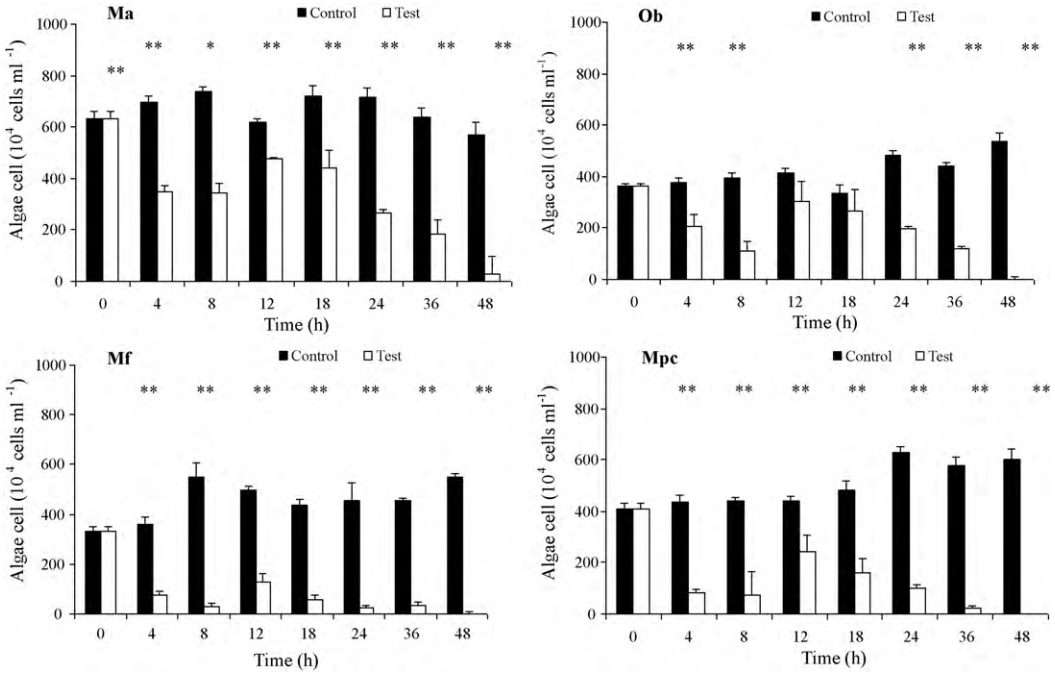

Fig. 1. Changes in cells of four algae during the incubation treated with TA-1302. Data are mean ± SD from three independent assays. Significant differences between the control and TA-1302 addition are indicated by $^{*}P < 0.01$; $^{**}P < 0.001$.

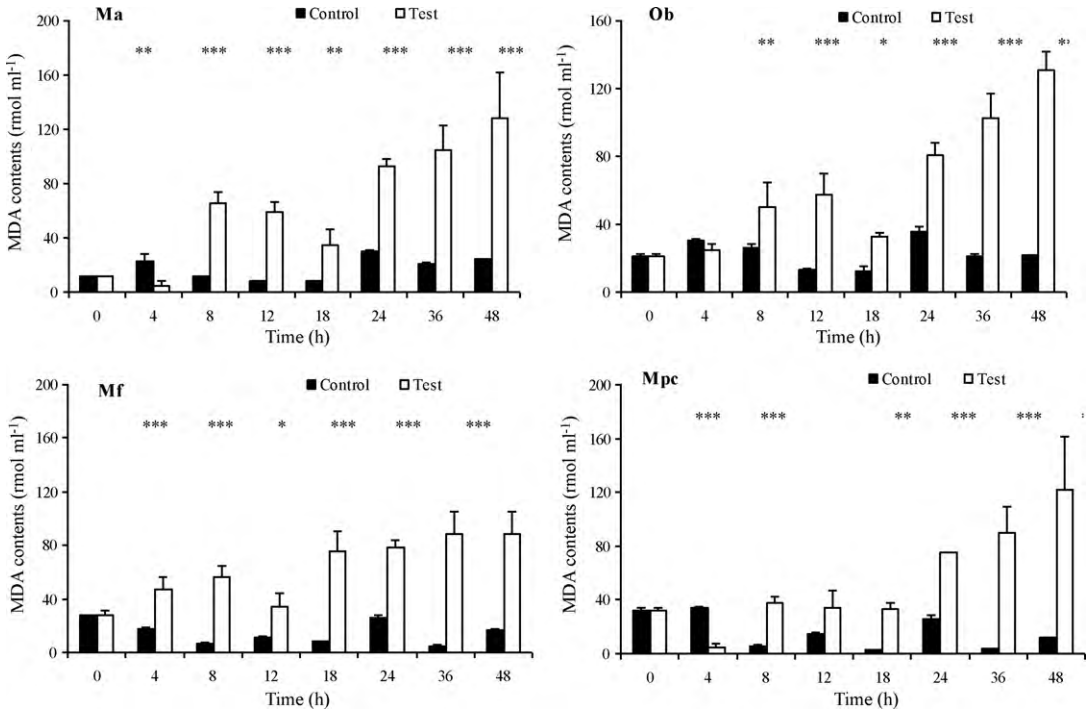

Fig. 2. Changes in MDA during incubation of four algae with TA-1302. Data are mean ± SD from three independent assays. Significant differences between the control and TA-1302 addition are indicated by $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 1
Mycelial pellicle mass of TA-1302 co-incubated with four algae and BG11 medium (g per mycelial pellicle).

Time (h)	Treatments				
	BG11	Ma	Ob	Mf	Mpc
0	0.059 ± 0.009[a]	0.077 ± 0.009[a]	0.072 ± 0.005[a]	0.079 ± 0.005[a]	0.066 ± 0.004[a]
48	0.068 ± 0.006[a]	0.113 ± 0.004[b]	0.361 ± 0.070[b]	0.105 ± 0.008[b]	0.552 ± 0.105[b]

The data in table indicates means ± SD ($n = 3$).
[a] Denotes no significant difference.
[b] Denotes significant difference at $P < 0.001$.

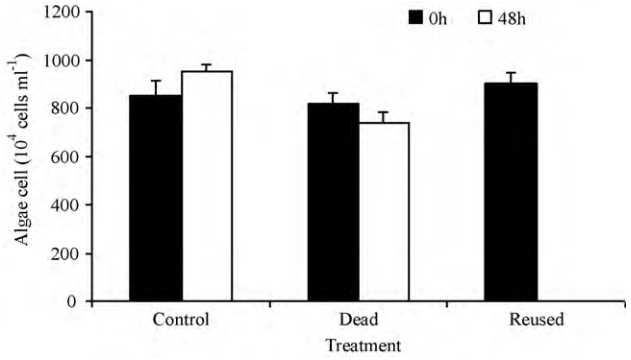

Fig. 3. Amount of algal cells for Mpc after treatments: Dead mycelial pellicle (121 °C, 20 min), reused mycelial pellicle (after one time degradation of algae).

Table 2
Number of algae cells for Mpc between control and addition of filtered medium (cells ml^{-1}).

Time (h)	Control	Filtered medium
0	958 ± 23[a]	962 ± 40[a]
48	978 ± 50[a]	1066 ± 25[a]

The data in table indicates means ± SD (n = 3).
[a] Denotes no significant difference.

intact at 24 h. In comparison, Fig. 5D showed that the algal cells surrounded with membrane were lysed by 48 h. The cell membrane of algal cell had been lysed and the cellular organ of algal cells had become vague. Notably, a small number of round, unwalled pellet structures were observed (Fig. 5D).

4. Discussion

The antagonistic activity of fungus towards blue-green algae was observed in 1978 [4]. However, the preying ability of fungus on algae has apparently not been pursued in detail [12]. Moreover, most recently studies focused on the algicidal bacteria. Generally, the algal cells were lysed by algicidal bacteria for 144–240 h and the removal rate of chlorophyll-a was approximately 70%-90% at the suitable bacterial density [13,14]. However, our study showed that the fungus 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 the algal cells are inhibited indirectly by microorganisms. Redhead and Wright [4] reported that the ability of fungus to inhibit and destroy live blue-green algae was associated with their production of diffusible extracellular substances. They suggested that the diffusible extracellular substances was β-lactam antibiotic cephalosporin C. Recent investigations on algicidal bacteria have suggested that the algal cells are indirectly inhibited and lysed by the secretion of algicidal bacteria [13,15]. The compounds produced by algicidal bacteria are heat tolerant, unstable in acidic condition and could be easily synthesized regardless of variation in temperature, salinity or initial pH for bacterial growth. Other studies have shown that the algal cells are lysed only by a fibrous glycocalyx or directly invaded after physical contact [16,17].

However, our studies showed a different way to degrade algal cells. The results suggest that the observed effects were due to the direct preying capability of TA-1302 on Mpc, Mf, Ma and Ob algal cells. First, the suppressive effect on the algal species could have been due to secretion by the fungus during the incubation, but the control conditions indicated that the presence of live fungus in co-culture was required for the destruction of algal cells.

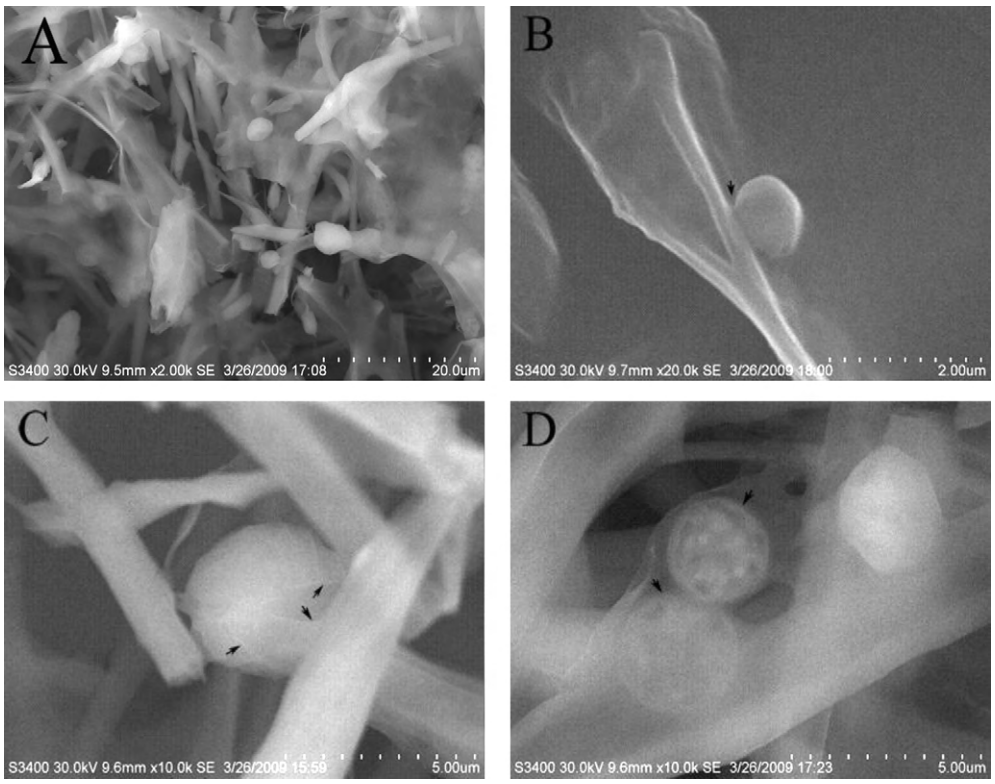

Fig. 4. Ultrastructure of Mpc cells during treatment with TA-1302 (SEM). (A) Algal cells attached to the mycelia of TA-1302 (after 4 h) and (B) detail showing attachment of algae to the mycelial surface of TA-1302 indicated by arrowheads (after 8 h) and (C) algal cells were surrounded by mucous membrane produced by TA-1302 indicated by arrowheads (after 24 h) and (D) algal cells were lysed (after 48 h). Bar = 20 μm for (A), bar = 2 μm for (B), and bar = 5 μm for (C) and (D).

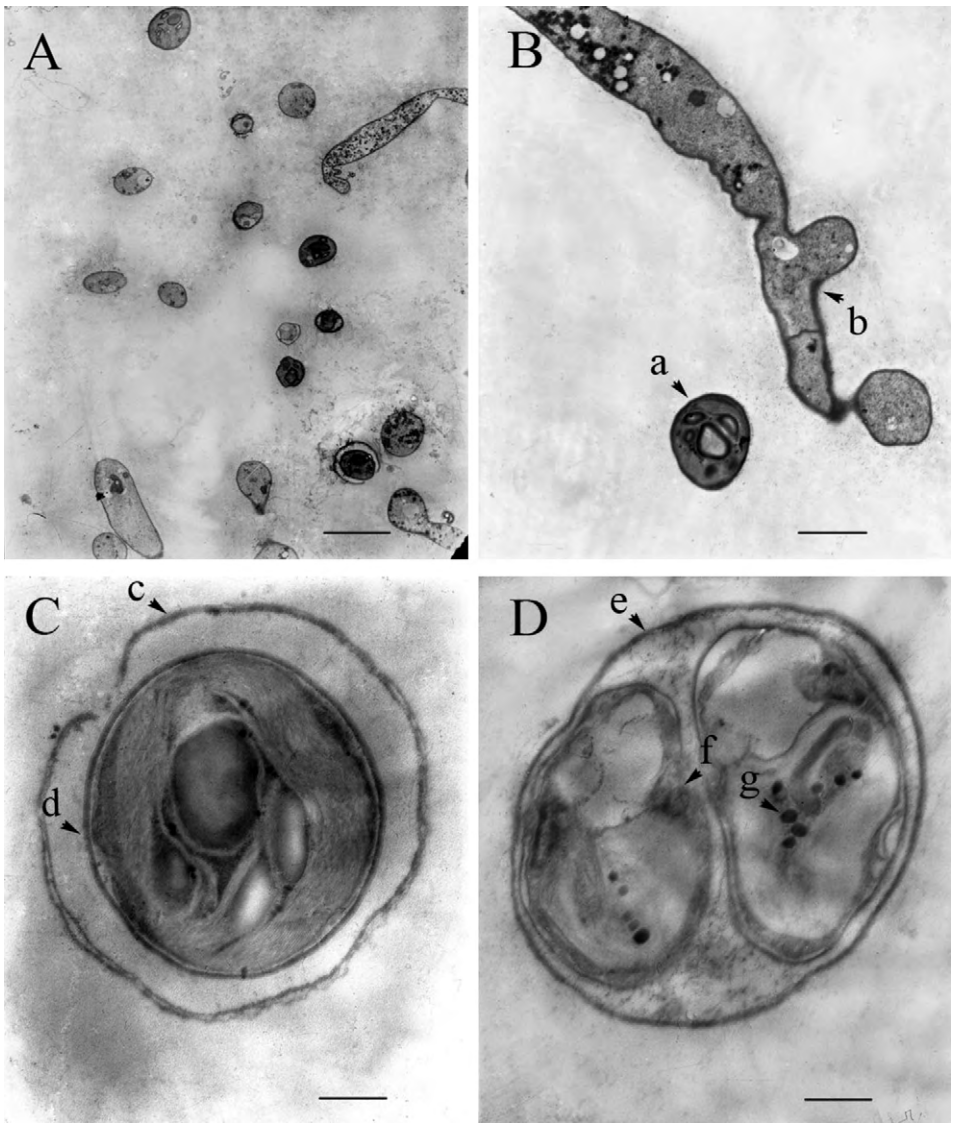

Fig. 5. Changes in ultrastructure of Mpc cells during treatment with TA-1302 (TEM). (A) The transverse section of algal cells absorbed to the mycelial networks of TA-1302 and (B) after 8 h treatment, a transverse section of unchanged single algal cell close to the mycelial surface of TA-1302 indicated by arrowheads (a and b) and (C) after 24 h treatment, a transverse section of changed single algal cell (d) surrounded by mucous membrane (c) and (D) after 48 h treatment, disruption of the plasma membrane (f) of algal cell, round, unwalled algal cell pellet structures (g) and a transverse section of the lysed cells (e), respectively. Bar = 5000 nm for (A), bar = 2000 nm for (B), bar = 500 nm for (C) and (D).

We also observed that the filtered medium and dead mycelial pellicle did not degrade the fresh algal cells of Mpc. These findings indicated the algal cells were degraded directly by TA-1302. Second, SEM and TEM observation of the co-culture processes further confirmed that the disappearance of algal cells in the co-culture was due to the preying ability of TA-1302 (Figs. 4 and 5). Moreover, the MDA assays indicated that MDA content was significantly higher in the test samples than in the controls. The results showed that compared with the control, MDA content in the test sample was significantly higher. It implied that the polyunsaturated fatty acids of membranes were being damaged since MDA is the oxidative product of unsaturated fatty acids. Both of the above evidences supported the preying ability of TA-1302 on the algal cells.

Our study suggests that there may be three stages of the algal degradation. Stage one, the algal cells physically contacted with the mycelia of TA-1302 under a shaking condition. Stage two, the algal cells were surrounded with mucous membrane secreted by the mycelia of TA-1302. The mycelial pellicle emerged some green color. Stage three, the algal cells were degraded. The color of the mycelial pellicle was restored from green to white at approximately 48 h. This result indirectly showed that the algal cells that came in contact with the mycelial networks were degraded last.

Cyanobacterial blooms are detrimental to public life, particularly to economic and social development [13,18]. Controlling or eliminating blooms remains a problem. The fungal strain has potential for use as an algal bloom control agent in natural setting, but additional data are needed before a mesocosm test is warranted or prudent. Despite the strain has potential utilization, fungal propagation and suppression of algae in natural water bodies need further works. To bring the fungus into contact with and suppress algae, fungus in a natural setting should be settled at a buoyancy neutral level and water should be circulated. This work is being seriously considered as one of our future studies.

Elimination of blooms using the fungus is desirable. However, the ecological effects of the fungus to natural water bodies could not be what we expected. Although terrestrial fungus does not multiply well in a natural body of water, the possibility that the fungus bloom in natural setting and become another type of harm-

ful bloom still exists. Furthermore, the food chains were destroyed because of the environmental deterioration of the water bodies when the cyanobacterial blooms happened [19,20]. In our study, all four algae were suppressed by the fungus. The fungus is likely to suppress all types of algae, regardless of whether it is "good" or "bad". Therefore, the ecological effect to the food chain should also be assessed before it is applied. Moreover, this white-rot fungus belongs to Phylum Basidiomycota, while the vast majority of known mycotoxin-producing fungi belong to the class Ascomycota [20]. Microbial and phytotoxicity studies of the extracted metabolites after dye degradation by white-rot fungi support their ecofriendly nature [21,22]. From the biosafety point of view, however, small-scale experiments and assessment of the ecological effect should be done strictly before the application. This will also be another work in our future research.

5. Conclusion

This study investigated the efficacy and mechanisms of *T. abietinum* 1302BG preying on different algae. The results reveal that the algal culture, *M. aeruginosa* FACH-918, *M. flos-aquae* FACH-1028, *O. borgei* FACH-1108 and *M. aeruginosa* PCC 7806 were destroyed within 48 h when co-cultured with the fungus. SEM and TEM observations show that there might be three stages for the mechanism of fungus preying on the algal cell. In stage one, the algal cells physically contacted with the mycelia of TA-1302 under the shaking condition. In stage two, the algal cells were surrounded with mucous membrane secreted by the mycelia of TA-1302. Finally, in stage three, the algal cells were digested by the fungus.

Acknowledgements

This study was financial supported by Project of National Basic Research Program of China (2008CB418004) and National Science Foundation of China (30870419, 40971151).

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