J Appl Phycol (2015) 27:1213–1221
DOI 10.1007/s10811-014-0402-7

Bacillus amyloliquefaciens T1 as a potential control agent for cyanobacteria

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Received: 7 February 2014 / Revised and accepted: 8 September 2014 / Published online: 25 September 2014

Abstract Harmful cyanobacterial blooms, which force us to develop economical and environmental friendly ways for eutrophication control, are recognized as a nuisance that cause negative impacts on natural resources and humans. In the current study, strain *Bacillus amyloliquefaciens* T1 with anti-cyanobacterial activity against bloom-forming cyanobacterium *Microcystis aeruginosa* 905 was isolated from an eutrophication pond in Wuhan, China, and was identified by 16S ribosomal DNA (rDNA) sequence analysis. *B. amyloliquefaciens* T1 cell-free filtrate at a concentration of 2 % (*v*/*v*) showed a strong anti-cyanobacterial effect against *M. aeruginosa* 905 (initial cell density was 1.0×10^6 cells mL^{-1}), and a reduction of 99.4 % in cell number was observed after incubation for 6 days. The anti-cyanobacterial effect was in accordance with the spore number rather than the cell number of *B. amyloliquefaciens* T1, suggesting that *M. aeruginosa* 905 was indirectly affected by the secretion of anti-cyanobacterial active substances. In addition, the interactions of *B. amyloliquefaciens* T1 with other cyanobacteria and green algae indicated that *M. aeruginosa* 907, *M. aeruginosa* 908, *M. aeruginosa* 912, and *M. aeruginosa* 7806 were obviously suppressed, while *Anabaena flosaquae* 1092 and *Chlorella pyrenoidosa* 415 proved to be not susceptible. Effects of environmental factors showed that the best inhibition efficiency would be achieved at 30 °C and pH 9.0, and the solid *B. amyloliquefaciens* T1 agent of *B. amyloliquefaciens* T1 could selectively kill the wild cyanobacteria from a shallow eutrophic pond. Based on the results obtained from this study, the anti-cyanobacterial bacterium *B. amyloliquefaciens* T1 had a potential application for eutrophication control in the natural environment.

Keywords Harmful cyanobacterial blooms · Biological control · Anti-cyanobacterial effect · *Microcystis aeruginosa* · *Bacillus amyloliquefaciens* T1

Introduction

In recent years, eutrophication and harmful cyanobacterial blooms in lakes, rivers, and reservoirs have become a great concern on a global scale (Qin et al. 2006; Qu and Fan 2010; Tang et al. 2012). In particular, blooms caused by toxic cyanobacteria, such as *Microcystis*, *Anabaena*, *Oscillatoria*, and *Cylindrospermopsis*, result in the production of microcystin that affects ecosystem functioning and creates a significant water quality problem (Gan et al. 2012; Chislock et al. 2013; Paerl et al. 2013; Yang et al. 2014; Zhu et al. 2014). As a fast and efficient method, chemical agents such as copper sulfate, potassium permanganate, hydrogen peroxide, and ozone are used for eutrophication control (Miao et al. 2009; Qian et al. 2010; Fan et al. 2013; Matthijs et al. 2012). However, the use of chemical methods induces secondary pollution which is potentially dangerous in aquatic ecosystems (Qin et al. 2006; Qu and Fan 2010; Tang et al. 2012).

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Therefore, there is a need to explore ecologically safe ways to control harmful cyanobacterial blooms.

Early studies have shown that bacteria associated with harmful cyanobacterial blooms play an important role in regulating or terminating cyanobacterial blooms, and interactions between cyanobacteria and bacteria have gained increasing attention. A number of bacteria with the ability of inhibiting or biodegrading cyanobacteria have been found, including *Aquimarina* sp. (Chen et al. 2011), *Pseudomonas* sp. (Wang et al. 2005; Sakata et al. 2011), *Streptomyces* sp. (Hua et al. 2009; Kong et al. 2013a, b; Luo et al. 2013; Somdee et al. 2013), and *Bacillus* sp. (Ahn et al. 2003; Nakamura et al. 2003; Shi et al. 2006; Mu et al. 2007). It is widely recognized that the use of bacteria with anti-cyanobacterial activity represents an effective and environmental friendly option for the control of cyanobacterial and algal blooms (Lovejoy et al. 1998; Hua et al. 2009; Chen et al. 2011; Zhang et al. 2011; Kong et al. 2013a, b; Luo et al. 2013). In spite of the relatively large number of laboratory studies which have focused on isolation and identification of anti-cyanobacterial bacteria and their action modes, identification of anti-cyanobacterial active substances and the application of anti-cyanobacterial bacteria are limited. In addition, information regarding high efficient biodegradation of cyanobacteria and their potential application is very limited to date.

Given the importance of biological control for harmful cyanobacterial blooms, anti-cyanobacterial microorganisms, especially for the rapid and harmless biodegradation of *Microcystis* with microcystin toxin production, still need to be discovered. The aim of the present study was to isolate strains capable of biodegrading the cyanobacterium *Microcystis aeruginosa* 905, one of the predominant species involved in harmful cyanobacterial blooms in freshwater lakes of China. Four anti-cyanobacterial bacteria were obtained, and one of these with the most effective anti-cyanobacterial effect was identified by morphological characteristics and 16S ribosomal DNA (rDNA) gene sequence analysis, and the anti-cyanobacterial effects of the anti-cyanobacterial bacterium *Bacillus amyloliquefaciens* T1 on other cyanobacteria and green algae were studied as well. Additionally, the potential application of this anti-cyanobacterial bacterium was investigated.

Materials and methods

Microcystis aeruginosa 905, *M. aeruginosa* 907, *M. aeruginosa* 908, *M. aeruginosa* 912, *Anabaena flosaquae* 1092, and a green algae *Chlorella pyrenoidosa* 415 were obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences (Wuhan, China); *M. aeruginosa* 7806 was provided by Professor Brett Neilan from the School of Biotechnology and Biomolecular Sciences, University of New South Wales (Sydney, Australia). Before being used as inoculants, they were cultured for 7 days to reach the log phase, and the culture conditions were as follows: sterilized BG11 medium, 36 μmol photons m^{-2} s^{-1} white light, light/dark=14:10 h, and 25±1 °C (Kong et al. 2013a, b).

Isolation and screening of anti-cyanobacterial bacteria

Subsurface sediment samples were collected from the surface layer (0~20 cm) of an eutrophic pond in Wuhan, China. Sediment powder (10 g) was suspended in 90 mL sterile water and diluted to a series of concentrations. Approximately 0.1 mL of each dilution was spread on solid beef extract peptone plates and then cultured for 2~3 days at 37 °C (Shen et al. 2002). Colonies with different morphologies were selected and streaked on new agar plates to obtain purified isolates (Mu et al. 2007). A modified cyanobacterial growth inhibition bioassay was used to isolate anti-cyanobacterial bacteria according to our previous study (Kong et al. 2013b). In brief, 0.2 mL cell-free filtrate from each isolate (cultured at 37 °C with a shaking speed of 180 rpm for 48 h in beef extract liquid medium) was filtered through a 0.22 μm cellulose acetate membrane and was added into 10 mL BG_{11} medium containing *M. aeruginosa* 905 at an initial cell density of 1.0×10^6 cells mL^{-1} and was then cultivated for 7 days under the conditions described above. Positive strains were inoculated into inclined tubes and stored at 4 °C for further study.

Identification of anti-cyanobacterial bacterium T1

Gram staining and analysis of physiological and biochemical characteristics of T1 were performed according to the procedures described by Shen et al. (2002). For the 16S rDNA gene sequencing and phylogenetic analysis, strain T1 was incubated in LB medium at 37 °C for 24 h with a shaking speed of 170 rpm (Julkowska et al. 2005). The cells were collected by centrifugation at 7000×*g* for 5 min (4 °C). DNA extraction was performed by using the 3S DNA Isolation Kit V2.2 (Biocolor BioScience & Technology Co., Shanghai, China). Fragments of the 16S rDNA from the isolate were amplified by PCR using the primers 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′) (Mulder et al. 2011), and the amplified fragment was sequenced by AuGCT Biotech Co., Ltd. (Beijing, China) (Zhang et al. 2011). The BLAST procedure was performed using the database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

Cyanobacterial inhibition bioassays

Effects of the cell-free filtrate concentrations *Bacillus amyloliquefaciens* T1 was maintained at 4 °C in LB medium (Julkowska et al. 2005), and the fermentation broth was

prepared by incubating the seed culture at 37 °C for 36 h at a shaking speed of 170 rpm. Soon afterwards, cell-free filtrate was obtained by centrifuging the fermentation broth at 10,000×*g* for 10 min and then filtering through a 0.22-μm cellulose acetate membrane (Kong et al. 2013b). The anti-cyanobacterial effects were studied by adding the cell-free filtrate (0 to 5 %, *v*/*v*) to 95 mL *M. aeruginosa* 905 culture at an initial cell number of 1.0×10^6 cells mL^{-1}, brought to a final volume of 100 mL by the addition of beef extract liquid medium (Shen et al. 2002). A negative control was made by adding 5 mL beef extract liquid medium. All treatments and controls were in triplicate and incubated under the conditions described above. Cell numbers of *M. aeruginosa* 905 were counted every other day.

Effects of anti-cyanobacterial bacterium culture time To study the effect of anti-cyanobacterial bacterium culture time, *B. amyloliquefaciens* T1 was incubated in 200 mL LB medium at 37 °C and 170 rpm; 10 mL of the culture was sampled at 4- or 2-h intervals over the 36 h of incubation, and the cell and spore numbers of *B. amyloliquefaciens* T1 were determined. In addition, the removal efficiency of *M. aeruginosa* 905 for each sample was tested by adding *B. amyloliquefaciens* T1 cell-free filtrate at a concentration of 2 % (*v*/*v*).

Effects of B. amyloliquefaciens T1 on cyanobacteria and green algae The cyanobacteria *M. aeruginosa* 905, *M. aeruginosa* 907, *M. aeruginosa* 908, *M. aeruginosa* 912, and *A. flosaquae* 1092 and the green alga *C. pyrenoidosa* 415 were diluted to an initial cell density of 1.0×10^6 cells mL^{-1}, and *B. amyloliquefaciens* T1 cell-free filtrate at a concentration of 2 % (*v*/*v*) was added to evaluate the anti-cyanobacterial effects. Controls were made by adding 5 mL beef extract liquid medium. All treatments and controls were in triplicate and incubated under the conditions described above. Cell numbers of cyanobacteria or green algae were counted after incubating for 4 days.

Effects of environmental factors Environmental factors such as temperature and pH have an important influence on microbial degradation (Nakamura et al. 2003; Mu et al. 2009; Li et al. 2012). In consideration of the potential application of the anti-cyanobacterial bacterium for eutrophication control, the cyanobacterial suspensions (pH=7.0) were incubated at 25 and 30 °C, while to assess the effect of pH, the cyanobacterial suspensions were adjusted to pH 7.0, 9.0, and 10.0 and incubated at 25 °C. The pH was adjusted by adding 0.1 M NaOH or 0.1 M HCl. All treatments were performed with the addition of 2 % (*v*/*v*) cell-free *B. amyloliquefaciens* T1 filtrate, and the control was as previously mentioned. Cell numbers of *M. aeruginosa* 905 were counted after incubating for 4 days.

Application of solid B. amyloliquefaciens T1 agent In order to evaluate the anti-cyanobacterial effect of *B. amyloliquefaciens* T1 for eutrophication bioremediation, a solid *B. amyloliquefaciens* T1 agent was prepared as follows: *B. amyloliquefaciens* T1 fermentation broth was inoculated into liquid fermentation medium (molasses 5 %, corn starch 5 %, urea 0.1 %, KH_2PO_4 0.05 %, $MgSO_4$ 0.02 %, and pH 7.0~7.5) with an inoculation ratio of 10 % and was cultured at 37 °C for 24 h with 170 rpm, and afterwards, the semifinished product was transferred into a solid fermentation medium (corn starch 10 %, bran 20 %, rice husk 10 %, rape cake 20 %, and tortillas 10 %) with an inoculation ratio of 10 % and was cultured in an incubator (the humidity was about 85 %) at 37 °C for 48 h. Finally, the solid inoculants were dried to constant weight at 55 °C and the solid *B. amyloliquefaciens* T1 agent (1.20×10^{10} cells g^{-1}) was obtained.

The anti-cyanobacterial effect of solid *B. amyloliquefaciens* T1 agent on wild cyanobacteria (from a shallow eutrophic pond) was undertaken with different concentrations ranging from 0.1 to 1.0 mg L^{-1} and was incubated under the conditions mentioned above. The anti-cyanobacterial effect was observed by phase contrast microscope, and cell numbers were counted every other day.

Analytical methods

The cell number of anti-cyanobacterial bacterium was determined using the dilution method of plate counting, while for the determination of spore number, samples were pretreated in a water bath at 80 °C for 10 min and were then determined using the pour plate method. All the plates were incubated at 37 °C for 72 h (Shen et al. 2002). The cell number of cyanobacteria/algae was determined using a hemocytometer. Cell numbers of each treatment were determined three times, and the arithmetical mean (±SD) was obtained.

The removal efficiency of cyanobacteria/algae was calculated according to Eq. (1):

$$\text{Removal efficiency} = (1-C_t/C_0) \times 100\% \quad (1)$$

where C_0 and C_t are the cell number of the control and test group at initial and time *t*, respectively (Kong et al. 2013b).

Results

Isolation and identification of bacteria with anti-cyanobacteria activity

Numerous bacterial strains were isolated from the subsurface sediment samples and were then subjected to cyanobacterial inhibition bioassay for anti-cyanobacterial effects against the toxic cyanobacterium *M. aeruginosa* 905. As a result of

isolation and screening, four strains (R1, T1, A1, and L2) were selected for biodegradation experiments on the basis of their anti-cyanobacterial activity (Fig. 1). With an initial cell-free filtrate concentration of 5 % (*v*/*v*), the cyanobacterium *M. aeruginosa* 905 was biodegraded within 4 days with a removal efficiency of 94.7±5.8 % (strain R1), 97.1±4.3 % (strain T1), 92.2±3.8 % (strain A1), and 90.2±3.9 % (strain L2). Among the four strains, T1 was the most effective and further identified.

The cells of strain T1 were short rod shaped, and oval spores were observed in the cells, and the bacterium was Gram-positive. The strain could grow at 50 °C and pH 5.7 and utilize nitrate as an electron acceptor. However, growth under anaerobic conditions was negative. This bacterium was able to utilize glucose, xylose, arabinose, mannitol, lactose, and sucrose, but not glycerol (Table 1).

Additionally, the 16S rDNA sequences of strain T1 (1402 bp) were determined and submitted to the GenBank database (accession No. GU359043.1). A phylogenetic tree was constructed based on 16S rDNA sequences (Fig. 2) and similarity calculations following the phylogenetic analysis, showing that strain T1 was most closely related to *B. amyloliquefaciens* DSM7 with a 100 % similarity in nucleic acid sequence homology. Based on the results of the morphological and biochemical characteristics and 16S rDNA sequences, this anti-cyanobacterial bacterium was identified as *B. amyloliquefaciens* T1.

Anti-cyanobacterial effect of ***B. amyloliquefaciens*** T1 on ***M. aeruginosa*** 905

Cyanobacterial inhibition bioassay was carried out at various concentrations of the cell-free filtrate ranging from 0.5 to 5 % (*v*/*v*) to study the anti-cyanobacterial effects of *B. amyloliquefaciens* T1 on *M. aeruginosa* 905. A 1 % cell-free filtrate could effectively inhibit the growth of *M. aeruginosa* 905 with a removal efficiency of 60.3 % was observed on day 2 (Fig. 3). In the case of 0.5 % treatment group, the removal efficiency of *M. aeruginosa* 905 on day 2, 4, and 6 was only 16.3, 41.5, and 43.3 %, respectively, indicating that a 0.5 % concentration was not effective for *M. aeruginosa* 905 inhibition. More importantly, the cell density of treatment groups with 1 to 5 % cell-free filtrate addition was $0.85\pm0.091\times10^6$, $0.08\pm0.003\times10^6$, 0, and 0 cells mL^{-1} on day 6, giving a removal efficiency of 94.0, 99.4, 100, and 100 %, respectively. Moreover, no growth of *M. aeruginosa* 905 was observed over 1 week.

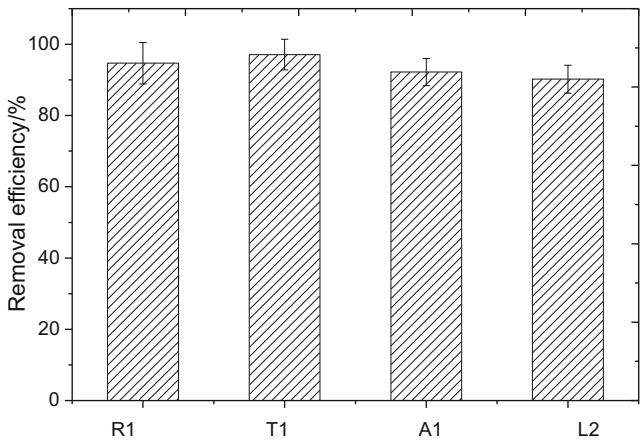

Fig. 1 Effects of four anti-cyanobacterial bacteria on *M. aeruginosa* 905 after incubation for 4 days. Each value is the mean±SD (n=3)

Table 1 Physiological and biochemical characteristics of bacterial strain T1

Characteristics	Response
Catalase test	+
Growth under anaerobic conditions	−
Methyl red test	+
Nitrate reduction	+
Growth at 50 °C	+
Growth at pH 5.7	+
NaCl tolerance	<7 %
Starch hydrolysis	+
Casein decomposition	+
Glucose	+
Xylose	+
Arabinose	+
Mannitol	+
Lactose	+
Glycerol	−
Sucrose	+
Gas from glucose fermentation	−
Citrate utilization	+

+, positive response; −, negative response

Anti-cyanobacterial bacterium culturing and cyanobacteria removal

Oval spores were produced when the *B. amyloliquefaciens* T1 was incubated in LB medium. In order to evaluate the effect of *B. amyloliquefaciens* T1 cell growth on *M. aeruginosa* 905, the time course for the anti-cyanobacterial bacterium cell growth and oval spore production was followed (Fig. 4). As the fermentation time went on, the cell number of *B. amyloliquefaciens* T1 increased sharply from $0.23\pm0.02\times10^8$ to $2.47\pm0.13\times10^8$ cells mL^{-1} in the first 20 h and then began a slow decline, whereas the spore number increased progressively with culture time, with a maximal production of $1.23\pm0.05\times10^3$ spores mL^{-1} after 26 h (Fig. 4). The *M. aeruginosa* 905 removal efficiency was 98.4±4.7 % and remained stable above 98 %. It was obvious that the removal efficiency was increased with increasing of spore number (from 16 to 26 h), suggesting

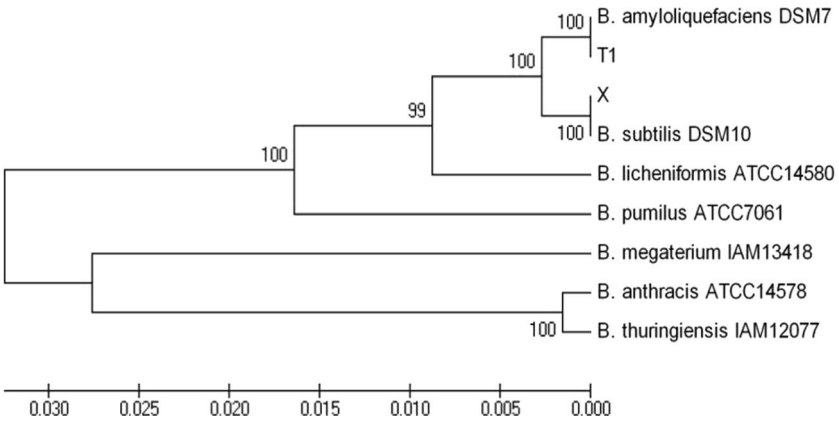

Fig. 2 The phylogenetic tree of strain T1

that the anti-cyanobacterial effect was better correlated with the spore number of *B. amyloliquefaciens* T1 rather than the cell number and that the anti-cyanobacterial active substances might be in the cell-free filtrate.

Anti-cyanobacterial effect of ***B. amyloliquefaciens*** T1 on other cyanobacteria and green algae

The effects of *B. amyloliquefaciens* T1 cell-free filtrate on other harmful cyanobacteria (*M. aeruginosa* 907, *M. aeruginosa* 908, *M. aeruginosa* 912, *M. aeruginosa* 7806, *A. flosaquae* 1092) and the green alga *C. pyrenoidosa* 415 were investigated over a period of 4 days (Fig. 5). Growth of all the *Microcystis* species was significantly inhibited by the *B. amyloliquefaciens* cell-free filtrate (ANOVA, $p<0.01$), achieving removal efficiencies of 76.9±3.1 % (*M. aeruginosa* 907), 78.2±2.2 % (*M. aeruginosa* 908), 72.9±3.0 % (*M. aeruginosa* 912), and 85.1±1.8 % (*M. aeruginosa* 7806). On the contrary, the growth of *A. flosaquae* 1092 was not affected, while the growth of *C. pyrenoidosa* 415 was promoted, reaching a cell density of $5.87\pm0.48\times10^6$ cells mL^{-1}, which was nearly 1.5 times than that of the control ($p<0.05$).

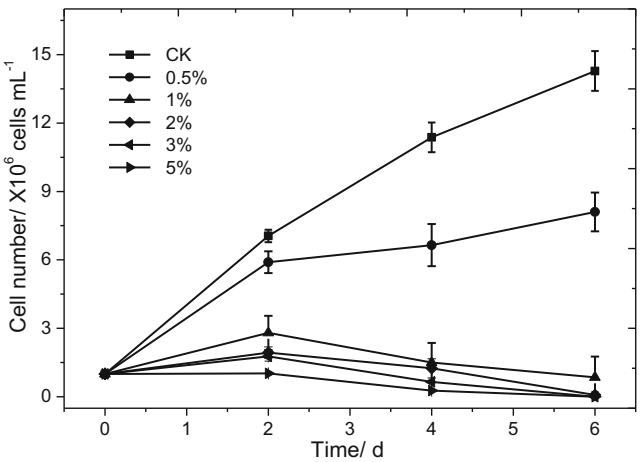

Fig. 3 Effect of cell-free filtrates of *Bacillus amyloliquefaciens* T1 on *M. aeruginosa* 905 in laboratory experiments. *CK* represents the control group. Each value is the mean±SD ($n=3$)

Effects of temperature and pH

Environmental factors show great influence on the growth of organisms and the biodegradation of cyanobacteria. In the control groups at 25 °C and pH 9.0, cell numbers of *M. aeruginosa* 905 were $9.54\pm1.31\times10^6$ and $9.27\pm0.81\times10^6$ cells mL^{-1}, respectively, which were much higher than the other conditions (Fig. 6). The results shown in Fig. 6 show that *B. amyloliquefaciens* T1 could be used at temperatures from 25 to 30 °C and pH from 7.0 to 10.0 with the best inhibition efficiency achieved at 30 °C and pH 9.0. Thus, the anti-cyanobacterial bacterium *B. amyloliquefaciens* T1 had the potential application for eutrophication control in the natural environment.

Application of solid ***B. amyloliquefaciens*** T1 agent

Aiming to confirm the anti-cyanobacterial effect of *B. amyloliquefaciens* T1, the growth of wild cyanobacteria from a shallow eutrophic pond was examined by adding solid *B. amyloliquefaciens* T1 agent at concentrations of 0.1, 0.5, and 1.0 mg L^{-1}. The result indicated that at 0.1 g L^{-1}, there was no anti-cyanobacterial effect, but higher concentrations showed a dose-dependent effect (Table 2). Within 24 h after the addition of solid *B. amyloliquefaciens* T1 agent, the cyanobacteria began to flocculate and the cyanobacterial suspension became yellow after incubation for 60 h, indicating that a dosage of 0.5 mg L^{-1} (or above) had a significant anti-cyanobacterial effect.

Control cyanobacteria and cyanobacteria with 0.5 mg L^{-1} solid *B. amyloliquefaciens* T1 agent were observed by phase contrast microscopy (Fig. 7). It was obvious that the control cells had an intact cell wall and the plasma membrane was close to the cell wall, demonstrating the integrity of cell structure (Fig. 7a). However, alterations in cyanobacterial cell

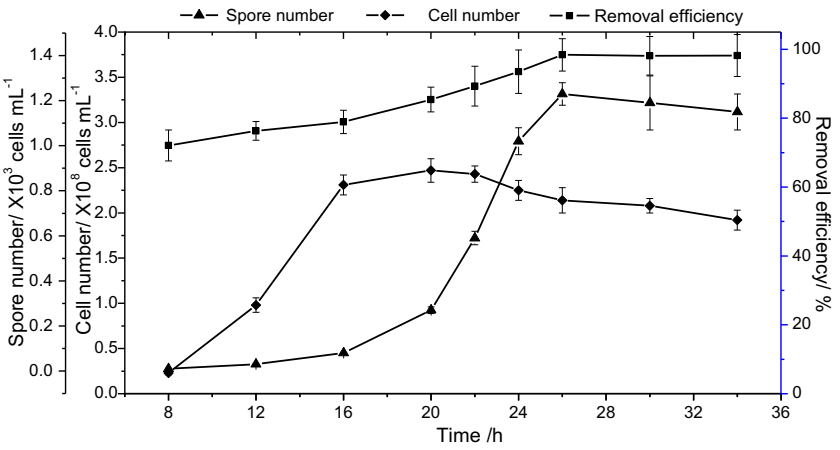

Fig. 4 Fermentation of *Bacillus amyloliquefaciens* T1 showing cell growth, oval spore production, and cyanobacteria removal efficiency. Each value is the mean±SD (n=3)

morphology were observed during solid *B. amyloliquefaciens* T1 agent exposure (Fig. 7b–d). Compared with the control, the cell membrane was gradually destroyed and the plasma membrane began to detach from the cells in some cases when it is exposed to solid *B. amyloliquefaciens* T1 agent for 60 h (Fig. 7b) and the cyanobacterial cells were absolutely destroyed (Fig. 7c), and the cell organelles were released after 80 h of incubation (Fig. 7d).

Discussion

Biodegradation is an important pathway for removing poisonous and harmful pollutants from the environment (Tang et al. 2012; Zhu et al. 2014). Therefore, screening and application of anti-cyanobacterial bacteria may be important to the eventually successful implementation for cyanobacteria inhibition. Hua et al. (2009) studied *M. aeruginosa* 905 biodegradation in conical flasks and showed that the culture broth of *Streptomyces* sp. NT0401 (5 %, *v*/*v*) could degrade nearly 90 % after incubation for 5 days. A recent study showed that *Streptomyces* sp. L74 cell-free medium (10 %, *v*/*v*) exhibited a 41.0 % algicidal rate on *M. aeruginosa* 905 on day 2 (Luo et al. 2013). *Bacillus* sp. strains also have been reported as *M. aeruginosa*-biodegrading bacteria in the past few years (Ahn et al. 2003; Nakamura et al. 2003; Shi et al. 2006; Mu et al. 2007). For example, *Bacillus cereus* demonstrated the ability of lysing the bloom-forming cyanobacterium *M. aeruginosa* and other cyanobacteria but required cell-cell contact (Shi et al. 2006). In the present study, *B. amyloliquefaciens* T1 was isolated from a eutrophic pond and the laboratory investigation demonstrated that only 1 % (*v*/*v*) of the cell-free filtrate was required to effectively suppress the growth of *Microcystis* (Fig. 3). In comparison with previous studies, the removal efficiency of 94.0 % obtained from our study indicated that *M. aeruginosa* 905 was more sensitive to *B. amyloliquefaciens* T1 than other *Bacillus* sp.

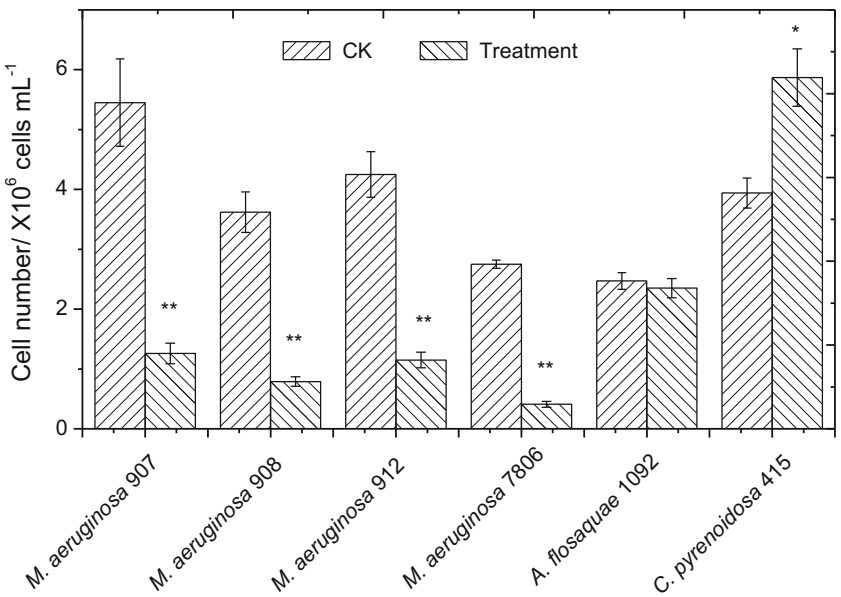

Fig. 5 Effects of *Bacillus amyloliquefaciens* T1 on cyanobacteria and green algae. *CK* represents the control group. *Asterisk* and *double asterisk* indicate a statistically significant difference of $p<0.05$ and $p<0.01$ when compared to the control. Each value is the mean±SD (n= 3)

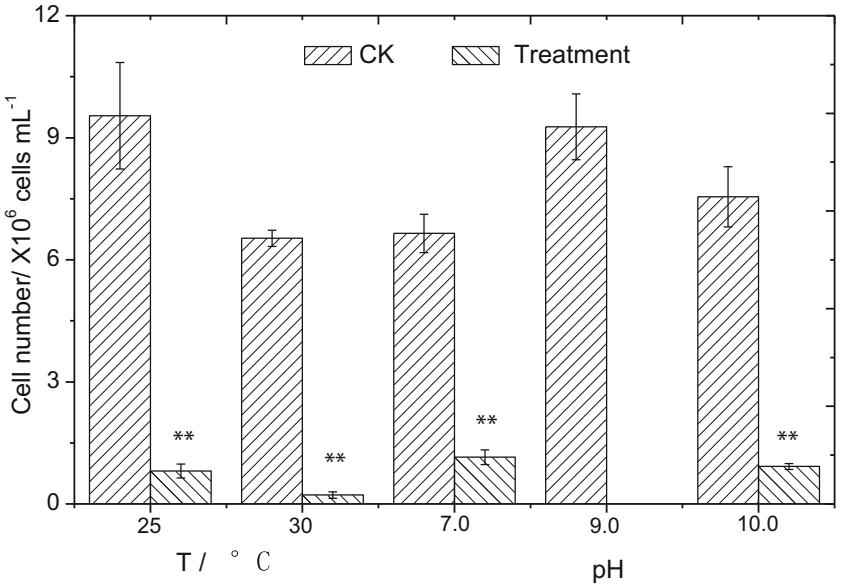

Fig. 6 Anti-cyanobacterial effects of *Bacillus amyloliquefaciens* T1 on *M. aeruginosa* 905 at different temperatures and pH. *CK* represents the control group. *Double asterisk* indicates a statistically significant difference of $p<0.01$ when compared to the control. Each value is the mean± SD ($n=3$)

strains (Ahn et al. 2003; Nakamura et al. 2003; Shi et al. 2006; Mu et al. 2007). The high sensitivity of *M. aeruginosa* 905 to *B. amyloliquefaciens* T1 may be attributed to the characteristics of secreta (Luo et al. 2013) or partly because of species differences within cyanobacteria and diatoms (Kang et al. 2005; Paul and Pohnert 2011). Although previous studies have indicated that harmful cyanobacteria or algae such as *M. aeruginosa* (Chen et al. 2011), *Aphanizomenon flos-aquae* (Shi et al. 2006), *Heterosigma akashiwo* (Wang et al. 2005), and *Karlodinium veneficum* and *Gyrodinium instriatum* (Pokrzywinski et al. 2012) could be inhibited by microorganisms, the removal effects of *M. aeruginosa* 905 in laboratory investigation by the strain *B. amyloliquefaciens* T1 was relatively more efficient compared with these studies.

Algicidal bacteria are intended to work selectively against cyanobacteria (Ahn et al. 2003; Chen et al. 2011; Kong et al. 2013a, b), marine diatoms (Paul and Pohnert 2011, 2013), dinoflagellates (Pokrzywinski et al. 2012), and *Phaeocystis* (Zheng et al. 2013). *Bacillus fusiformis*, an algicidal bacterium isolated from a sewage treatment plant, could suppress the growth of *M. aeruginosa*, *Chlorella*, and *Scenedesmus* (Mu et al. 2007). Our study revealed that the cell-free filtrate of *B. amyloliquefaciens* T1 also showed anti-cyanobacterial effects against other *Microcystis* strains such as *M. aeruginosa* 907, *M. aeruginosa* 908, *M. aeruginosa* 912, *M. aeruginosa* 7806, and *M. aeruginosa* 905 (Fig. 1). In contrast, *A. flosaquae* 1092 was not susceptible while growth of the green alga *C. pyrenoidosa* 415 was promoted by the addition of the cell-free filtrate. Similar results were obtained with the algicidal bacterium *Kordia algicida* which showed algicidal effects against the marine diatoms *Skeletonema costatum*, *Thalassiosira weissflogii*, and *Phaeodactylum tricornutum*, while *Chaetoceros didymus* was shown to be not susceptible (Paul and Pohnert 2011). Another study found that *Pseudomonas putida* HYK0203-SK02 appeared to have algicidal activity against a wide range of phytoplankton such as *Synechococcus hantzschii* (80.4 %), *M. aeruginosa* (70.0~71.4 %), *Aulacoseira granulata* (31.2 %), *Chlorella* sp. (30.3 %), and *Anabaena cylindrica* (41.6 %), whereas the diatom *Cyclotella* sp. was stimulated (Kang et al. 2005). Additionally, *P. putida* CH-22, isolated from Lake Chaohu of Anhui Province, China, also caused drastic reductions in *M. aeruginosa* (89.3 %), *Microcystis wesenbergii* (76.5 %), *Microcystis viridis* (70.3 %), *A. flos-aquae* (61.6~71.3 %), and *Chlorella ellipsoidea* (48.5 %) (Zhang et al. 2011).

Apart from the biodiversity of harmful cyanobacterial blooms in aquatic ecosystems, environmental factors such as temperature and pH influence the activity of anti-

Table 2 The anti-cyanobacterial effect of solid *Bacillus amyloliquefaciens* T1 agent

	Initial cell number (cells mL^{-1})	Final cell number (cells mL^{-1})	Removal efficiency (%)
CK	1.5×10^6	5.4×10^6	–
0.1 mg L^{-1}	1.5×10^6	3.6×10^6	34.3
0.5 mg L^{-1}	1.5×10^6	6.0×10^5	88.9
1.0 mg L^{-1}	1.5×10^6	0	100

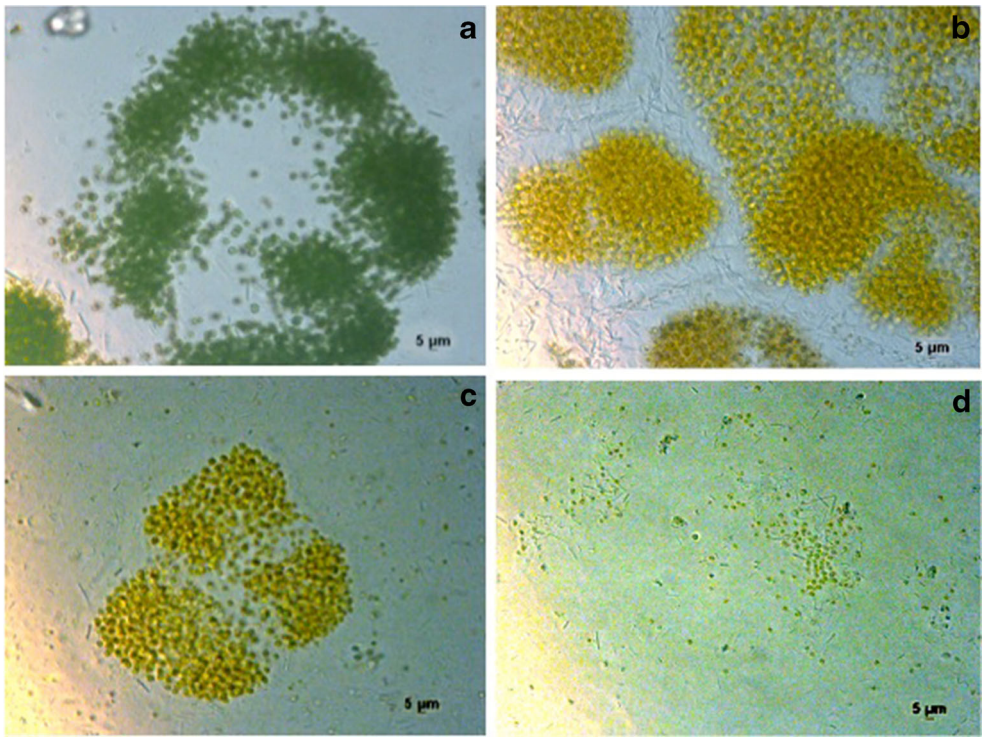

Fig. 7 Light micrographs showing various stages of the wild cyanobacterium treated with the solid *Bacillus amyloliquefaciens* T1 microbial agent (×400). **a** Control group, **b** 60 h of treatment, **c** 70 h of treatment, **d** 80 h of treatment.

cyanobacterial bacteria. *Ochrobactrum* sp. FDT5 was recently found to be effective at reducing the growth of *M. aeruginosa* at 30 °C and pH 7.6 (Mu et al. 2009). It was also reported that the removal efficiency of *M. aeruginosa* (in terms of Chl *a*) was 75.9, 83.6, and 78.3 % at pH 6.5, 7.5, and 8.5, respectively, demonstrating that pH 7.5 was the best for the anti-cyanobacterial effect of this bacterium (Li et al. 2012). However, compared with pH 7.5, the condition of pH 10.1 was beneficial for the anti-cyanobacterial bacterium *B. cereus* and 100 % lysis of *M. viridis* was attained in 24 h with the dosage of 1:1 (*v*/*v*) (Nakamura et al. 2003). Moreover, over the temperature range of 3 to 30 °C, both 25 and 30 °C were suitable for *M. viridis* removal (Nakamura et al. 2003). We also found that the optimal temperature and pH for *M. aeruginosa* 905 biodegradation by *B. amyloliquefaciens* T1 was 25 °C and pH 9.0.

As illustrated above, the elimination of harmful cyanobacterial blooms might depend on the population abundance and species composition of the cyanobacteria (Matthijs et al. 2012). Therefore, finding the right dosage is another critical issue for the potential application of *B. amyloliquefaciens* T1. The experiment using wild cyanobacteria from a shallow eutrophic pond indicated that a concentration of more than 0.5 mg L^{-1} solid *B. amyloliquefaciens* T1 agent would absolutely suppress the cyanobacterial growth, which was consistent with a previous report that the H_2O_2 concentration for field application was much higher than that in the laboratory study (Matthijs et al. 2012). Additionally, the reason for the higher dosage required could be due to the presence of *A. flosaquae* and *C. pyrenoidosa*, which were not biodegraded by *B. amyloliquefaciens* T1 (Fig. 5). On the basis of this information, we recommend the application a dosage of more than 1.0 mg L^{-1} solid *B. amyloliquefaciens* T1 agent for field samples from a wide range of *Microcystis*-dominated lakes or reservoirs. Although *B. amyloliquefaciens* T1 was isolated from an eutrophic pond, the side effects such as ecological risk on aquatic organisms should be considered; moreover, the released products from harmful cyanobacteria when biodegraded such as microcystins and nutrients should also be removed at the same time.

Previous studies on the action modes of anti-cyanobacterial bacteria showed that these were by direct and indirect interactions (Lovejoy et al. 1998; Mayali and Doucette 2002; Chen et al. 2011; Zheng et al. 2013). It was obvious that the anti-cyanobacterial substances were secreted by *B. amyloliquefaciens* T1 and existed in the cell-free filtrate, demonstrating an indirect interaction mode. However, we have not studied the anti-cyanobacterial mechanism, nor have we isolated and identified the anti-cyanobacterial substances and the potential toxicity caused by microcystin. Notwithstanding its limitations, this study does suggest that harmful cyanobacteria such as *M. aeruginosa* could effectively be inhibited by *B. amyloliquefaciens* T1.

Acknowledgments This study was financially supported by a grant from the National High Technology Research and Development Program of China (863 Program) (No. 2013AA102805-04) and the Science and Technology R & D Program of Wuhan, China (No. 201120637175-4).

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