Isolation of axenic cyanobacterium and the promoting effect of associated bacterium on axenic cyanobacterium

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Abstract

Background: Harmful cyanobacterial blooms have attracted wide attention all over the world as they cause water quality deterioration and ecosystem health issues. *Microcystis aeruginosa* associated with a large number of bacteria is one of the most common and widespread bloom-forming cyanobacteria that secret toxins. These associated bacteria are considered to benefit from organic substrates released by the cyanobacterium. In order to avoid the influence of associated heterotrophic bacteria on the target cyanobacteria for physiological and molecular studies, it is urgent to obtain an axenic *M. aeruginosa* culture and further investigate the specific interaction between the heterotroph and the cyanobacterium.

Results: A traditional and reliable method based on solid-liquid alternate cultivation is carried out to purify the xenic cyanobacterium *M. aeruginosa* FACHB-905. On the basis of 16S rDNA gene sequences, two associated bacteria named strain B905-1 and strain B905-2, are identified as *Pannonibacter* sp. and *Chryseobacterium* sp. with a 99% and 97% similarity value, respectively. The axenic *M. aeruginosa* FACHB-905A (*Microcystis* 905A) is not able to form colonies on BG_{11} agar medium without the addition of strain B905-1, while it grows well in BG_{11} liquid medium. Although the presence of B905-1 is not indispensable for the growth of *Microcystis* 905A, B905-1 has a positive effect on promoting the growth of *Microcystis* 905A.

Conclusions: The associated bacteria are eliminated by solid-liquid alternate cultivation method and the axenic *Microcystis* 905A is successfully purified. The associated bacterium B905-1 has the potential to promote the growth of *Microcystis* 905A. Moreover, the purification technique for cyanobacteria described in this study is potentially applicable to a wider range of unicellular cyanobacteria.

Background

The interactions between phototrophic phytoplankton and heterotrophic bacteria are considered to be an integral part of the algal/cyanobacterial life cycle. For example, diatoms and bacteria coexist in the ocean and coevolve in complex interactions that significantly modify each other's behavior and ultimately impact biogeochemical cycles [1-3]. This interaction plays an important role in photosynthesis and is therefore crucial for the metabolism of phototrophic phytoplankton. The relations between phototrophic phytoplankton and heterotrophic bacteria are much better understood compared with that between zooplankton and bacteria, and it is generally recognized that there are three different types of phototrophic phytoplankton and heterotrophic bacteria interactions: (i) bacteria and phototrophic phytoplankton form a mutualism relationship in which phytoplankton benefits from bacterial products such as nutrients, whereas bacteria profit from phytoplankton products such as extracellular polymeric substances [4]; (ii) bacteria and phototrophic phytoplankton form an antagonism relationship that the growth of phytoplankton is restricted or inhibited by bacteria through algal-bacterial/cyanobacterial-bacterial contact mechanism (direct interaction) or secretion of the extracellular antialgal/anticyanobacterial substances (indirect interaction) [5,6] and (iii) bacteria and phototrophic phytoplankton form a commensalism relationship that bacteria are loosely associated with

phytoplankton and may promote the growth and photosynthesis without having any negative effect, while phytoplankton grows well without the associated bacteria [7,8]. These scenarios may be dependent on the characteristics of phototrophic phytoplankton species, associated bacteria species and secreted substances of the associated heterotrophic bacteria [4].

Harmful cyanobacterial blooms (HCBs) in lakes, reservoirs and rivers have drawn great attention all over the world as microcystin-producing cyanobacteria cause animal and human health concerns [5,6,9]. *Microcystis aeruginosa*, a unicellular, photoautotrophic and gram-negative cyanobacterium that belongs to the genus *Microcystis*, division *Cyanophyta* is one of the most common and widespread bloom-forming cyanobacteria that secret toxins [5,6,10]. Previous studies show that the cyanobacterium is associated with a large number of bacteria, and these associated heterotrophic bacteria (heterotrophs) are considered to benefit from organic substrates released by the cyanobacterium [11-18]. In order to avoid the influence of heterotrophs for physiological and molecular studies, the purification of the axenic cyanobacterium (bacteria-free) is especially important as well as the understanding of its responses to the heterotrophs.

Various methods including UV irradiation, sonication, micropipette technique, phenol treatment, antibiotic treatment and lysozyme treatment have been used for cyanobacteria purification [19-25]. Previous study shows that treatment with antibiotics is a successful strategy to obtain axenic cyanobacteria cultures [1]. Additionally, solid medium is simple and useful for the growth and isolation of axenic *Microcystis* strains, in which way two axenic *Microcystis* strains are obtained [24,26]. Although the direct and indirect inhibiting effects of bacteria on cyanobacteria have been intensively studied [3,5,6,12,13,27] and the associated bacteria are potentially regarded to regulate cyanobacterium growth via extracellular amino acid monomers or other substances [5,6], the growth-promoting effects of heterotrophs on cyanobacterium have not received much attention. Apart from cyanobacterium purification, the growth-promoting effect of the heterotrophs on cyanobacterium is a significant aspect for understanding the interactions between heterotrophs and cyanobacteria. Therefore, the aim of the present study is to obtain an axenic *M. aeruginosa* culture and investigate the specific interaction between the heterotrophs and the cyanobacterium.

Results

Effects of antibiotics and lysozyme on the xenic cyanobacterium

1. *aeruginosa* 905 and 907 samples are curated by the Freshwater Algae Culture Collection of Institute of Hydrobiology (FACHB) as xenic consortia comprised of one *M. aeruginosa* strain and its associated heterotrophic bacteria. Antibiotics and lysozyme are commonly used to eliminate heterotrophic bacteria from cyanobacterial cultures [20,25,28]. In order to eliminate the heterotrophic contaminants, the sensitivities of the cyanobacterium and heterotrophs to antibiotics or lysozyme are investigated. Results indicate that these approaches are ineffective in completely separating the *Microcystis* from the heterotrophs (Table 1 and Fig. 1).

Table 1 Effects of antibiotics on heterotrophs and cyanobacterium

Antibiotics	Tetracycline	Cephalosporins	Kanamycin	Penicillins	Streptomycin
Antibacterial circle	3.3 cm	2.4 cm	—	—	1.8 cm
Inhibition efficiency	94.3 ± 3.5%	88.5 ± 4.1%	50.7 ± 2.4%*	5.6 ± 0.2%**	81.2 ± 3.9%**

* and ** represent a statistically significant difference of $p < 0.05$ and $p < 0.01$ when compared to the control.

Isolation and purification of the axenic culture

The colony forming process of cyanobacterium and heterotrophs on solid plates (BG_{11} agar medium) is observed by inverted phase contrast microscope, and the results are shown in Fig. 2. It is obvious that the heterotrophs colonies are much bigger than the cyanobacterium colonies, indicating the heterotrophs are grew much better compared with cyanobacterium. The cyanobacterium colony is formed when cultured for 15 d, although it is small; moreover, the cyanobacterial colonies are only found in 3 plates among the 20 replicate plates even after incubating for 20 d. Then the isolated cyanobacterial colonies are transferred into 6 test tubes with a Pasteur pipette under the microscope and incubated for 3 d. The result shows that 5 tubes become green, indicating the cyanobacterium is grown well. With several cycles of purification, the axenic *M. aeruginosa* FACHB-905A (*Microcystis* 905A) is obtained. Possible contamination such as heterotrophs is subsequently examined before and after the incubations, and the results reveal that there is no contamination. Then a molecular identification is carried out for the purified axenic cyanobacterium named as *Microcystis* 905A. The results indicate that *Microcystis* 905A presents the highest sequence similarity (99% of identity) with *M. aeruginosa* NIES-843, *M. aeruginosa* PCC 7820 and *M. aeruginosa* PCC 7806.

Identification of associated bacteria

Two gram-negative bacteria, named B905-1 and B905-2, are isolated from the xenic *M. aeruginosa* FACHB-905 (*Microcystis* 905). To identify the bacteria, phylogenetic analyses are performed using the 16S rDNA sequences. A total of 1367 bp of each of the two isolated strains is determined, and the 16S rDNA gene sequences obtained are subjected to GenBank BLAST search analyses [29]. Strain B905-1 is most closely related to *Pannonibacter phragmitetus* L-s-R2A-19.4 with a 99% similarity value, and strain B905-2 is most closely related to *Chryseobacterium* sp. with a 97% similarity value. With the same method, the xenic *M. aeruginosa* FACHB-907 (*Microcystis* 907) is diluted and then plated on the BG_{11} solid medium. After culturing under the culture conditions (Section "**Culture of cyanobacteria and heterotrophs**") for 15 ~20 d, the single cyanobacterial colonies are transferred into test tubes with a Pasteur pipette under the microscope and incubated for 3 d. The results show that 5 tubes become green, indicating the cyanobacterium is grown well. With several cycles of purification, the axenic *Microcystis* 907A and another heterotroph B907-1 are also successfully isolated, and B907 is identified as

Agrobacterium sp., which is most closely related to *Agrobacterium* sp. PNS-1 and *Agrobacterium albertimagni* C0008 with a 98% similarity value. The sequences of B905-1 and B907-1 are imported into the DNAMAN software V6 and aligned [30]. Phylogenetic tree is then constructed (Fig. 3) and it is further confirmed that strain B905-1 and B907-1 are closely related to *Pannonibacter* sp. and *Agrobacterium* sp., respectively.

Effect of associated bacteria on *Microcystis* 905A

The growth rates of xenic culture (*Microcystis* 905) and axenic culture (*Microcystis* 905A) are measured under both the static cultivation (without the shaking speed) and the shaking cultivation conditions (with the shaking speed of 150 rpm). Fig. 4 indicates that the generation time of axenic culture is 42.3 h (shaking cultivation) and 60.9 h (static cultivation), while the generation time of xenic culture is 33.6 h under the shaking cultivation and 45.3 h under the static cultivation, respectively. In addition, the generation time of xenic culture is much shorter than that of the axenic culture under the same cultivation condition, which demonstrates the photosynthetic efficiency of *Microcystis* 905 is much better. At the same time, the growth rates of both the xenic culture and axenic culture under the shaking cultivation condition are much faster than that under the static cultivation condition. These results point to the role of the heterotrophs in promoting the growth of *Microcystis* 905A.

Effect of heterotroph-cyanobacterium *ratio* on *Microcystis* 905A

To further study the effect of heterotroph B905-1 *on the growth of* axenic *Microcystis* 905A, a series of experiments that different initial cyanobacterial cell concentrations with the heterotroph -cyanobacterium *ratio* of 1:1, 1:10 and 1:100 are undertaken in BG_{11} liquid medium (Fig. 5). Compared with the control group (CK), the cyanobacterial cell numbers of 1:1 treatment group is slightly suppressed during the 21 d, while the 1:10 and 1:100 treatment groups show a remarkable increase, and they are increased with the extension of culture time. In addition, the highest cyanobacterial cell number for the treatment group of 1:10 and 1:100 is $(14.72 \pm 0.48) \times 10^6$ cell mL^{-1} and $(10.63 \pm 0.37) \times 10^6$ cell mL^{-1}, respectively (Fig. 5d), and both of them are obtained at the 21^{st} day. Obviously, the cyanobacterial cell numbers for the 1:10 treatment group are much higher than that for the 1:100 treatment group under the same conditions, and the reason may due to the higher concentration of the strain B905-1 that added at the beginning of the experiment. These results indicate the addition of heterotroph B905-1 has a positive promoting effect on the growth of *Microcystis* 905A.

The growth of axenic *Microcystis* 905A on BG_{11} agar medium with and without the addition of strain B905-1 is also investigated. For the treatments that with the addition of strain B905-1, the cyanobacterial colony of axenic *Microcystis* 905A becomes green after incubating for 20 days; while for the treatments that without the addition of strain B905-1, there is no cyanobacterial colony on BG_{11} agar medium. Moreover, the effects of different heterotroph -cyanobacterium ratio (1:1, 1:10 and 1:100) on the growth of cyanobacterium on BG_{11} agar medium are studied (Fig. 6). Interestingly, the *Microcystis* 905A is unable to grow in the treatment of 1:1 (Fig. 6b), but it grows well in both treatments of 1:10 and 1:100

(Fig. 6c and 6d). The results indicate high ratio of heterotroph-cyanobacterium (1:1) is not good for the growth of *Microcystis* 905A, which means when the initial concentrations of B905-1 and axenic *M. aeruginosa* are the same, the growth of *M. aeruginosa* on both BG_{11} liquid medium and BG_{11} agar medium is inhibited. Previous study shows BG_{11} can become carbon- or phosphate-limited in dense cultures for some cyanobacteria [31]. The growth of *Microcystis* 905A is best in the 1:10 condition indicates the C-P ratios influenced by B905-1 are best balanced, where the heterotroph produces an enhancing amount of CO_2, but doesn't consume too much phosphate in competition with *Microcystis* 905A.

In order to prove that the promoting effect is associated with the extracellular substances of strain B905-1, the effect of the cell-free filtrate of strain B905-1 on the growth of *Microcystis* 905A is carried out (Fig. 7). Results show that the cyanobacterium cell number of the treatment with the addition of the cell-free filtrate is 9.23 ± 0.56, 11.31 ± 1.85 and 22.14 ± 1.06 cell mL^{-1} after incubating for 4 d, 8 d and 12 d, respectively, and it is obviously higher than that with no cell-free filtrate. The axenic cyanobacterium grows much better with the addition of the cell-free filtrate again demonstrating strain B905-1 has the promoting effect on the growth of *Microcystis* 905A; moreover, the released substances of strain B905-1 with the promoting effect are apparently existed in the cell-free filtrate.

Discussion

HCBs occur around the world and are responsible for most aquatic environment pollution [9,10]. Researches of HCBs have been concentrated on the physical, chemical and bio-ecological methods for the control of cyanobacteria and the removal of nitrogen and phosphorous [5,9]. Little is known about the microbial community of cyanobacteria with heterotrophs and the interactions between them [2,3]. Previous studies demonstrate that the oxic cyanobacterial layer of eutrophic water is mainly composed by cyanobacteria and aerobic heterotrophic microorganisms, and the relationships between them are complicated [32,33]. Therefore, it is necessary to obtain the axenic *M. aeruginosa* from the complex microbial community and further research the interactions between cyanobacteria and heterotrophs.

Traditionally, cyanobacterial purification methods including antibiotic treatment and lysozyme treatment have been applied for eliminating heterotrophs from cyanobacteria and algae [20,21,25,28], and the purification effects depends on the concentrations and types of antibiotic or lysozyme [22-25]. With a series of antibiotic and lysozyme procedures, the axenic cyanobacteria such as *Anabaena flos-aquae*, *Aphanothece nidulans*, *Arthrospira platensis* and *Arthrospira* spp. are obtained [22,23]. While the sensitivities of xenic cyanobacterium *Microcystis* 905 to five antibiotics employed in the present study are quite different, in particular, four of the tested antibiotics have the inhibition effects on cyanobacterium growth. Furthermore, the lysozyme can inhibit both the cyanobacterium and heterotroph simultaneously (Table1 and Fig. 1), it is quite difficult to eliminate the heterotrophs from xenic *Microcystis* 905 culture by antibiotics treatment or lysozyme treatment methods. Researches indicate the bloom forming cyanobacteria in freshwater or seawater are more often occurred in nutrient-rich environments, and the cyanobacteria are surrounded by diverse communities of heterotrophic bacteria

[32-34]. The difficulty in obtaining the axenic *Microcystis* 905 is probably due to the lack knowledge of heterotrophs in xenic culture.

Heterotrophs can colonize within the enclosed region or directly adhere to the surface of a cyanobacterium colony [34]. By transferring and culturing xenic culture of *Arthrospira platensis* in fresh sterile medium, the axenic *A. platensis* is obtained by the technique of single-trichome manipulation performed with a microtrowel [35]. Considering the xenic *Microcystis* 905 can easily form single cyanobacterial colony on BG_{11} agar plate and the growth rates of *Microcystis* and heterotrophs are significantly different, heterotrophs are removed by solid-liquid alternate cultivation method and micropipette technique, which by picking and transferring the single cyanobacterial colony to BG_{11} liquid medium under the microscope. This method not only guarantees the minimum initial growth density of cyanobacterial cells, but also ensures the purity of cyanobacterial cells, thus results in the successful separation of the axenic *Microcystis* 905A. It is also successfully applied to purify other strain such as axenic *Microcystis* 907A. In spite of the traditional standard plate method based on solid-liquid alternate cultivation for obtaining axenic culture is time-consuming, the protocol that we have developed for purifying axenic *Microcystis* 905A culture maybe suitable for separating axenic strains from a commensal, and potentially syntrophic, symbiosis. These results indicate that this technique is at least applicable to unicellular cyanobacteria.

Molecular biological techniques such as denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization have been used to investigate the purity of cyanobacterial culture [17,31]. DGGE results suggest that a number of bacteria including α-*proteobacteria*, β-*proteobacteria*, γ-*proteobacteria*, *Bacteroidetes* and *Actinobacteria* have been detected in the cyanobacterial cultures, and the *Sphingomonadales* are the prevalent group among the *Microcystis*-associated bacteria [17]; in another study, the heterotrophs, for instance, *Aeromicrobium alkaliterrae*, *Halomonas desiderata* and *Staphylococcus saprophyticus* are also identified from the *Arthrospira platensis* culture [25]. The heterotrophic bacteria, such as α-*proteobacteria* and bacteria from the *Bacteroidetes*-group, are reported to associate with Diatoms in nature as well as in stock cultures [1]. We observe that the heterotrophs strain B905-1 and B905-2 are closely related to *Pannonibacter* sp. and *Chryseobacterium* sp., respectively. Besides the identification of heterotrophs, it seems that more attention should be paid to the interactions between heterotrophs and the cyanobacterium *M. aeruginosa*. It is suggested that the interaction between heterotrophs and cyanobacterium is symbiosis or parasitic [3,36], and the heterotrophs are difficult to isolate from cyanobacterium during the formation of cyanobacterial or algal colony [1,37].

Heterotrophs can enhance or suppress the growth of cyanobacteria, or even kill them [32,34]. To better understand the general interaction between heterotroph and cyanobacterium, the effect of the strain B905-1 on the cyanobacterium *M. aeruginosa* FACHB-905A is studied. It is showed that the growth rate of the xenic *Microcystis* 905 is much faster than that of the axenic xenic *Microcystis* 905A under both static cultivation and shaking cultivation conditions. The results indicate that the heterotroph B905-1 has a promoting effect on the growth of axenic *Microcystis* 905A. Inconsiderationof the initial cell number of *Microcystis* 905 is $(2.2 \pm 0.2) \times 10^6$ cell mL^{-1} and heterotroph B905-1 is $(0.64 \pm 0.07) \times 10^6$ cell mL^{-1}, it is

not surprising that the growth-promoting effect of the 1:10 treatment is much better than the 1:100 treatment. Interestingly, the *Microcystis* 905A is unable to form colonies in the 1:1 treatment group on BG_{11} agar medium. Although *M. aeruginosa* is a kind of photosynthetic bacterium (or autotrophic bacteria) and it grows well under the light with inorganic nutrients, which are supplied by BG_{11} liquid medium, it is not surprising that axenic *Microcystis* 905A could not divide at the heterotroph-cyanobacterium ratio of 1:1, as the heterotroph B905-1 can effectively compete nutrients with axenic *Microcystis* 905A.

The growth-promoting effect of heterotrophs on algae has recently been observed in other studies, for example, the growth of toxic dinoflagellate *Alexandrium fundyense* is promoted substantially by *Alteromonas* sp. [8], and the attached bacteria provide co-existing for diatom *Thalassiosira weissflogii* to form transparent exopolymer particles [4]. Interpretation of such phenomenon might be explained by the symbiotic interaction that the bacteria deliver vitamins for algae [38], or the addition of bacteria changes the available nutrient concentration such as extracellular organic carbon or dissolved organic matter [2,4,14,17,32]. In a previous study, the growth rate and metabolic products of *Shewanella putrfaciens*, *Brochothrix thermosphacta* and *Pseudomonas* sp. show a remarkable increase no matter cultured individually or in all possible combinations compared to the control cultures [39]. Difference from the above-mentioned microorganisms, axenic diatoms are unable to form biofilm when purified from bacteria [4]. Although the axenic *Microcystis* 905A grows well under the liquid culture condition, it could not form cyanobacterial colonies on the BG_{11} agar plate without the addition of strain B905-1, indicating the presence of heterotroph B905-1 is indispensable for the growth of axenic *Microcystis* 905A on BG_{11} agar plate. The different growth phenomenon of *Microcystis* 905A in solid and liquid BG_{11} medium is mainly attributed to the phosphate. It is reported that reactive oxygen species (ROS) were produced when phosphate was autoclaved together with agar, and total colony counts of *Gemmatimonas aurantiaca* in liquid medium (without agar) were remarkably higher than those grown on solid medium (with agar) [40]. In the same way, there may be some ROS produced in BG_{11} solid medium and the ROS is likely a contributing factor to the growth inhibition of *Microcystis* 905A. It is speculated that the heterotrophic bacterium B905-1 closely associated with cyanobacterium likely consume nutrients that released by *Microcystis* 905, and may also produce vitamins and other beneficial metabolites useful for cyanobacterial growth [33,34]. Nevertheless, the presence of strain B905-1 for the cyanobacterial colony formation mechanism needs to be further studied.

Previous study also indicates that the enhancement growth of axenic *Microcoleus chthonoplastes* PCC 7420 is upon the addition of a filtrate obtained from the closely related xenic culture of *Microcoleus* sp. M2C3, and the stimulated effect could be due to the release of certain growth factors and vitamins by associated aerobic heterotrophic microorganisms [32]. Most of the strains are able to secrete active substance to inhibit or enhance the growth of cyanobacteria [41]. Possible mechanisms may include various types of interactions from nutrient cycling to the production of growth-inhibiting and cell-lysing compounds [42]. Our results demonstrate that strain B905-1 has the potential to promote*Microcystis* 905A growth, whereas *Microcystis* 905A provides organic matter for associated bacterial proliferation. In

a comparable study it is pointed out that bacteria have the potential to control diatom growth, and their interactions are regulated by multiple signals involving common biomolecules such as proteins, polysaccharides and respective monomers [14]. In accordance with previous observations, we also find the associated bacterium has promoting effect on the growth of cyanobacterium *M. aeruginosa*. Increasing knowledge on molecular mechanisms of microbial interactions are crucial to better understand or predict nutrient and organic matter cycling in aquatic environment, and also to better understand the role of such associated bacterium for the formationmechanism of HCBs and eutrophication control.

Up to now, most studies on the interaction between heterotrophs and cyanobacteria are performed in pure cultures [33,34,41], and the growth of the axenic cyanobacteria is almost promoted by the heterotrophs [8,33,34]. However, the interaction can be profoundly different in nature, as most microbes are not axenic but grow together in communities. The complex communities or microbial networks often result in surprisingly coordinated multicellular behaviour, e.g. dinoflagellates can feed on associated bacteria and heterotrophs also attack and lysis the cyanobacteria [32]. Furthermore, the heterotrophs are considered as playing a significant role in carbon cycling and cyanobacterial photosynthesis [32]. All these studies suggest that the relationship between heterotrophs and cyanobacteria in nature is complex and manifold, further analysis is needed to have a full understanding of the microbial communities surrounding cyanobacteria.

Conclusions

Our results show that heterotrophs are eliminated by solid-liquid alternate cultivation method and the axenic *Microcystis* 905A is successful purified by means of picking and transferring the single cyanobacterial colony to BG_{11} liquid medium under the microscope; moreover, two heterotrophs, strain B905-1 and strain B905-2, are identified as *Pannonibacter* sp. and *Chryseobacterium* sp. with a 99% and 97% similarity value in the basis of 16S rDNA gene sequences. Further, strain B905-1 has the potential to promote the growth of *Microcystis* 905A. The purification technique for cyanobacteria described in this study is potentially applicable to a wider range of unicellular cyanobacteria.

Methods

Culture of cyanobacteria and heterotrophs

Xenic *Microcystis* 905 and *Microcystis* 907 used in this study are purchased from the FACHB, Chinese Academy of Sciences (Wuhan, China). Sterilized BG_{11} liquid medium or BG_{11} agar medium (with the agar concentration of 1.5%) is used as the main culture medium for both axenic and xenic *M. aeruginosa* [5,6,43]. Before being used as inoculants, cyanobacteria are cultured with 200 mL BG_{11} liquid medium in 500 mL Erlenmeyer flasks for 7 days to reach the log phase, and the culture conditions are as follows: 2000 lux white light, light: dark = 14 h: 10 h; 25 ± 1 °C [5,6]. Axenic *Microcystis* 905A is obtained by treating with antibiotics, lysozymes, and micropicking from *Microcystis* 905 culture.

Bacterial strains B905-1 and B905-2 are isolated from the culture solution of the cyanobacterium *Microcystis* 905. These two bacteria are routinely grown in TY liquid medium [44] at 28 ± 1 °C under aerobic conditions (with the shaking speed of 150 rpm). The cell-free filtrate of strain B905-1 is obtained by centrifuging the fermentation broth at 10,000×g for 10 min and then filtering through a 0.22 μm cellulose acetate membrane [5]. Stock cultures are kept at 4 °C, and working cultures are obtained from stock cultures through two transfers in appropriate TY liquid medium.

Isolation and purification of axenic culture

For the isolation and purification of axenic cultures, cyanobacterial cells are treated by the solid-liquid alternate cultivation method. The xenic cyanobacterium is diluted to different multiple from 10^{-1} to 10^{-8}, and the different multiple are inoculated onto sterile Petri dishes containing BG_{11} agar medium, respectively [45]. After incubating for 15 to 20 d under the culture conditions above, a single cyanobacterial colony is picked by a Pasteur pipette with the aid of a microscope, and then transferred into a test tube that containing 5 mL BG_{11} liquid medium. The purification result is checked as the test tube becoming green, and the testing method is as follows: 0.1 mL cyanobacterial culture from test tube is spread on the Luria-Bertani (LB) agar plate [44,46] and incubated at room temperature for 3 d or more to examine the existence of heterotrophs, the absence of heterotrophs indicates this cyanobacterial culture is axenic. After the purification, the axenic cyanobacterial colony is picked up by a Pasteur pipette, then transferred to Erlenmeyer flasks with BG_{11} liquid medium, and incubated at 25 ± 1 °C in a 14L/10D light-dark cycle. The purification procedure for axenic *Microcystis* is illustrated in Fig. 8.

Cyanobacterial inhibition bioassay

The antibiotics (including tetracycline, cephalosporin, kanamycin, penicillin and streptomycin) and lysozyme are purchased from Wuhan Dingguo biological technology Co., LTD. The sensitivities of heterotroph to tetracycline, cephalosporins, kanamycin, penicillins and streptomycin are evaluated by the filtering paper method with the final concentration of 100 μg mL^{-1} for each treatment [20]. The sensitivities of cyanobacterium to five antibiotics are carried out by adding antibiotic with the final concentration of 100 μgmL^{-1} in 250 mL sterilized Erlenmeyer flasks containing 100 mL xenic *Microcystis* 905 culture (the initial cyanobacterial cell number is 1.0×10^6 cell mL^{-1}), and the controls (CK) are added without any antibiotic. Effects of lysozyme on the cyanobacterium and heterotrophs are performed by adding lysozyme (the lysozyme is dissolved with sterile distilled water and then filtered through a 0.22 μm membrane) at final concentrations of 0, 1.0, 2.0, 5.0, and 10.0 mg mL^{-1} in 250 mL sterilized Erlenmeyer flasks containing 100 mL xenic *Microcystis* 905 culture (the initial cyanobacterial cell number is 1.0×10^6 cell mL^{-1}). Heterotrophs and cyanobacterial cell densities are determined after incubation for 2 d.

The growth curve of axenic *Microcystis* 905A and xenic *Microcystis* 905 are carried out at an initial cyanobacterial cell number of 1.0×10^6 cell mL^{-1}. Effects of heterotroph-cyanobacterium *ratio on the growth of four kinds of different initial* axenic cyanobacterial cell concentrations in BG_{11} liquid medium

are performed as follows: the axenic *Microcystis* 905A is firstly added in 250 mL sterilized Erlenmeyer flasks containing 100 mL BG_{11} liquid medium to keep the cyanobacterial cell number of 3.0×10^2, 3.0×10^3, 3.0×10^4 and 3.0×10^5 cell mL^{-1}, respectively, and then strain B905-1 (initial cell number is 2.73×10^7 cell mL^{-1}) is added according to heterotroph-cyanobacterium *ratio* of 1:1, 1:10 and 1:100, the controls (CK) are without the addition of strain B905-1. For the effects of heterotroph-cyanobacterium *ratio on the growth of* axenic *Microcystis* 905A on BG_{11} agar medium, the heterotroph (B905-1) and cyanobacterium (axenic *M. aeruginosa*) are mixed well in BG_{11} liquid medium, and the final heterotroph-cyanobacterium ratios of 1:1, 1:10 and 1:100 are performed by adding different amounts of bacterium into the 100 mL axenic *M. aeruginosa* culture with the initial cyanobacterial cell number of 1.0×10^4 cell mL^{-1}. The mixed suspensions are diluted to different multiples and then plated on the BG_{11} agar medium, each dilution gradient is repeated for three times.

The effect of cell-free filtrate of strain B905-1 on axenic *Microcystis* 905A is carried out by adding the cell-free filtrate (2%, v/v) into a 100 mL sterilized Erlenmeyer flask which containing initial axenic cyanobacterial cell number of 1.0×10^6 cell mL^{-1}. The cell-free filtrate is obtained by filtrating with the 0.22 μm cellulose acetate membrane. The negative control is made by adding the same amount of TY liquid medium into 100 mL cyanobacterial culture or BG_{11} agar plate.

All the experiments are performed under aseptic conditions, the controls (CK) and the treatments are replicated three times, and the arithmetical means (± SD) are used as the final results.

DNA extraction, sequencing and phylogenetic analysis

The isolated bacterial strains are identified based on 16S rRNA gene sequence analysis. Heterotrophs are prepared by incubating the seed culture at 37 °C with a shaking speed of 180 rpm for 20 h in sterilized LB liquid medium. The heterotroph cells are collected by centrifugation at 4000 rpm for 10 min (at 4 °C). DNA is extracted from the bacterial sample using the 3S DNA Isolation Kit V2.2 (Biocolor BioScience & Technology Co., Shanghai, China). Fragments of the 16S rDNA are amplified by PCR using the primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3'), and the amplified fragments are sequenced by AuGCT Biotech Co., Ltd. (Beijing, China) [17]. The BLAST procedure is used to search for sequence similarity in GenBank [29].

Analysis Methods

Bacteria cell density is determined by colony counting method. Samples are cultured on TY agar medium at 28 ± 1 °C for 48 h, and the colonies were counted. The cyanobacterium cell number is determined by hemocytometer using light microscopy (NIKON-YS100). The cell density or cell number of each sample is counted in triplicate, and standard error of the mean is calculated for all data. Statistical analysis is performed using Version 17.0 of SPSS for Windows (SPSS, Chicago, IL, USA) [6].

The generation time (G) of the cyanobacterium is calculated according to equation (1):

$$G = (t_2-t_1)/[3.322(\lg X_2-\lg X_1)] \quad (1)$$

where X_1 and X_2 are the cyanobacterium cell number at time t_1 and t_2, respectively.

The inhibition efficiency is calculated according to equation (2):

$$\text{Inhibition efficiency} = (1 - C_t/C_0) \times 100\% \quad (2)$$

where C_0 and C_t are the cyanobacterium cell number of the control and test group at time t, respectively[5,6].

Declarations

Ethics approval and consent to participate

This manuscript doesn't involve any human participants, human data, human tissue, individual person's data or animal experiment.

Consent for publication

Not Applicable.

Availability of data and materials

The data are presented within the manuscript and the cyanobacteria such as *M. aeruginosa* FACHB-905 and FACHB-907 used in this study could be purchased from the Freshwater Algae Culture Collection of Institute of Hydrobiology (FACHB), Chinese Academy of Sciences (Wuhan, China).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YK and LM conceived and designed the project. SG, JY, LJ and CZ performed the experiments. YK, LM and LS analyzed the data. LM, CZ and LS contributed reagents/materials/analysis tools. YK, SG and LM wrote the paper. All authors have read and approved the manuscript.

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Abbreviations

HCBs: Harmful cyanobacterial blooms

Microcystis 905A: The axenic *M. aeruginosa* FACHB-905A

Microcystis 905: The xenic *M. aeruginosa* FACHB-905

FACHB: Freshwater Algae Culture Collection of Institute of Hydrobiology

DGGE: denaturing gradient gel electrophoresis

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Figures

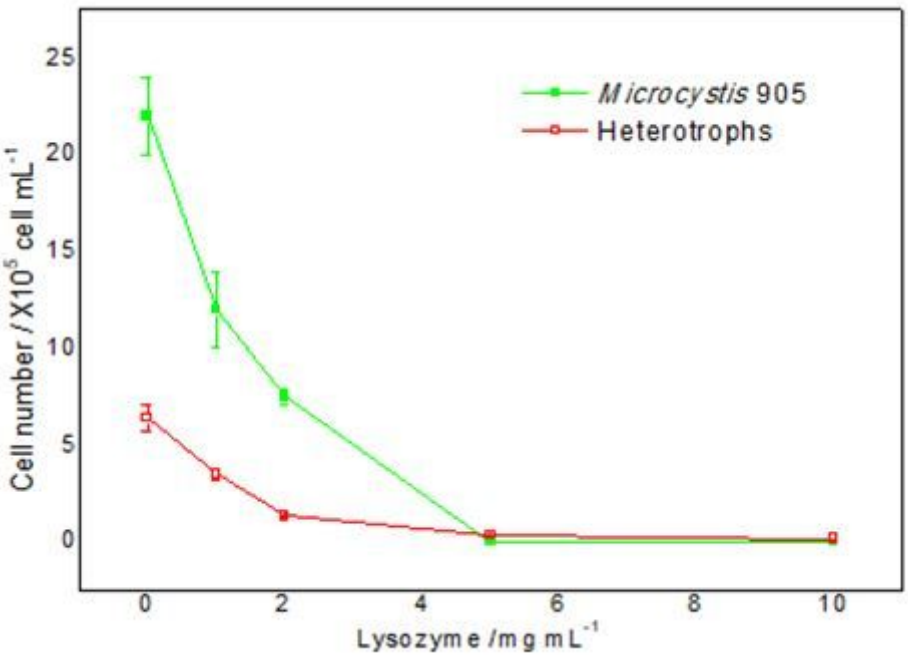

Figure 1

Effects of lysozyme on heterotrophs and Microcystis 905

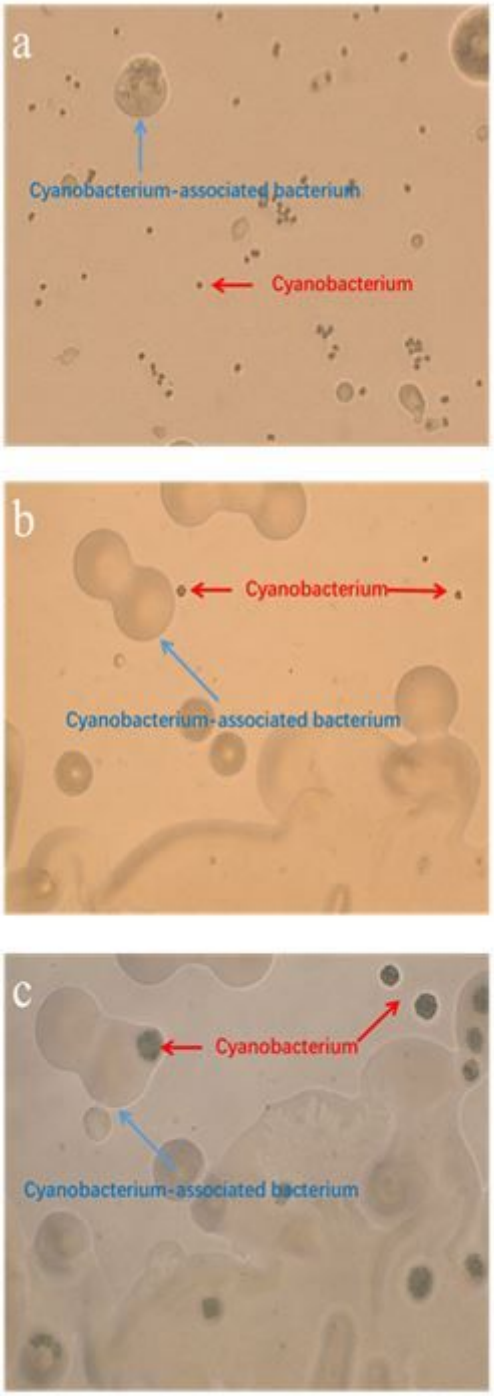

Figure 2

The growth of cyanobacterial and heterotrophic colonies (× 100). (a, b and c is the colonial morphology cultured for 1, 8 and 15 d, respectively)

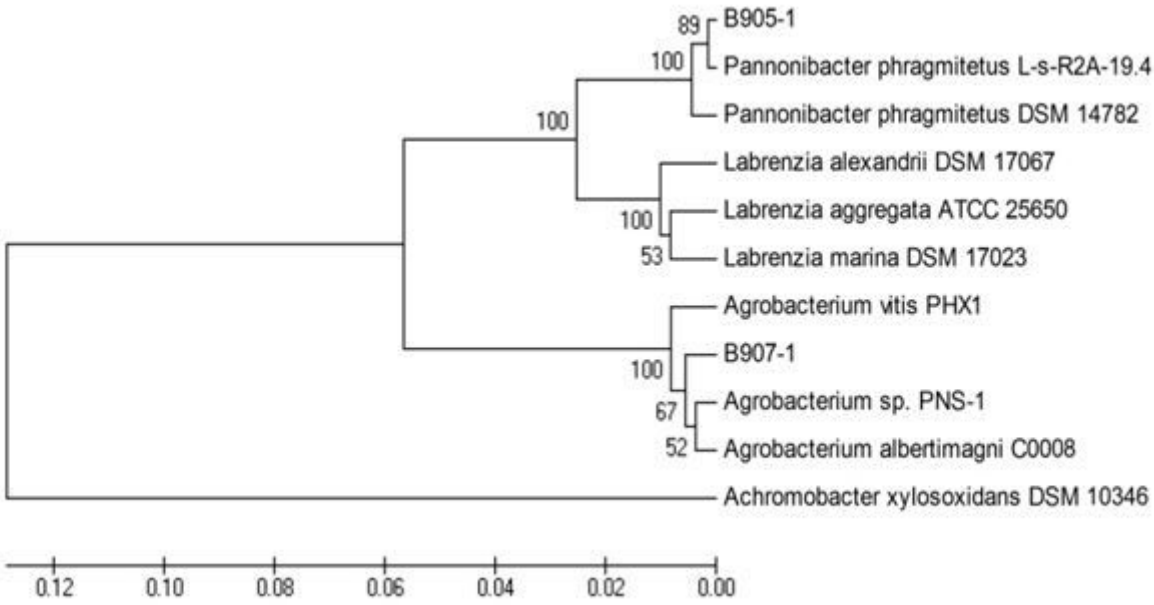

Figure 3

The phylogenetic tree of heterotrophs.

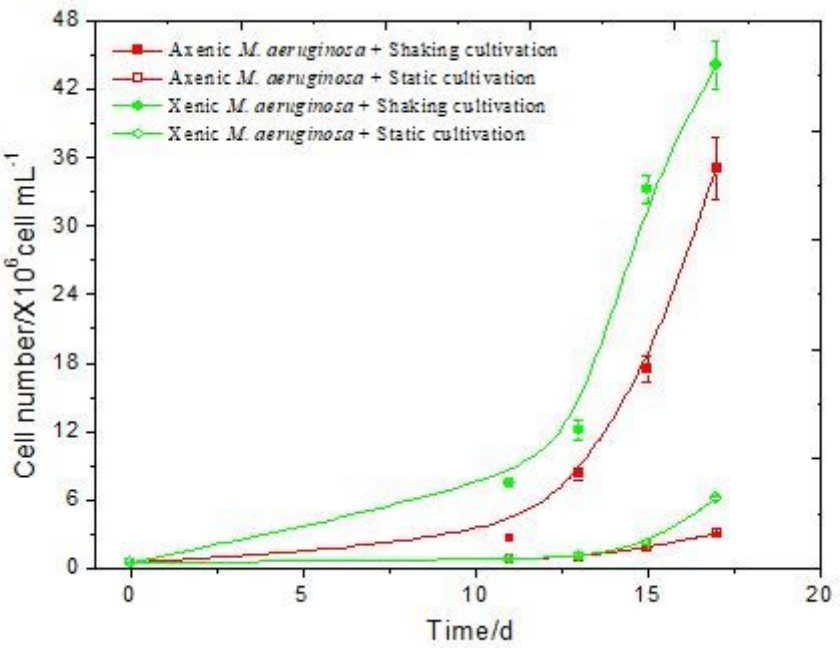

Figure 4

The growth curves of axenic Microcystis 905A and xenic Microcystis 905.

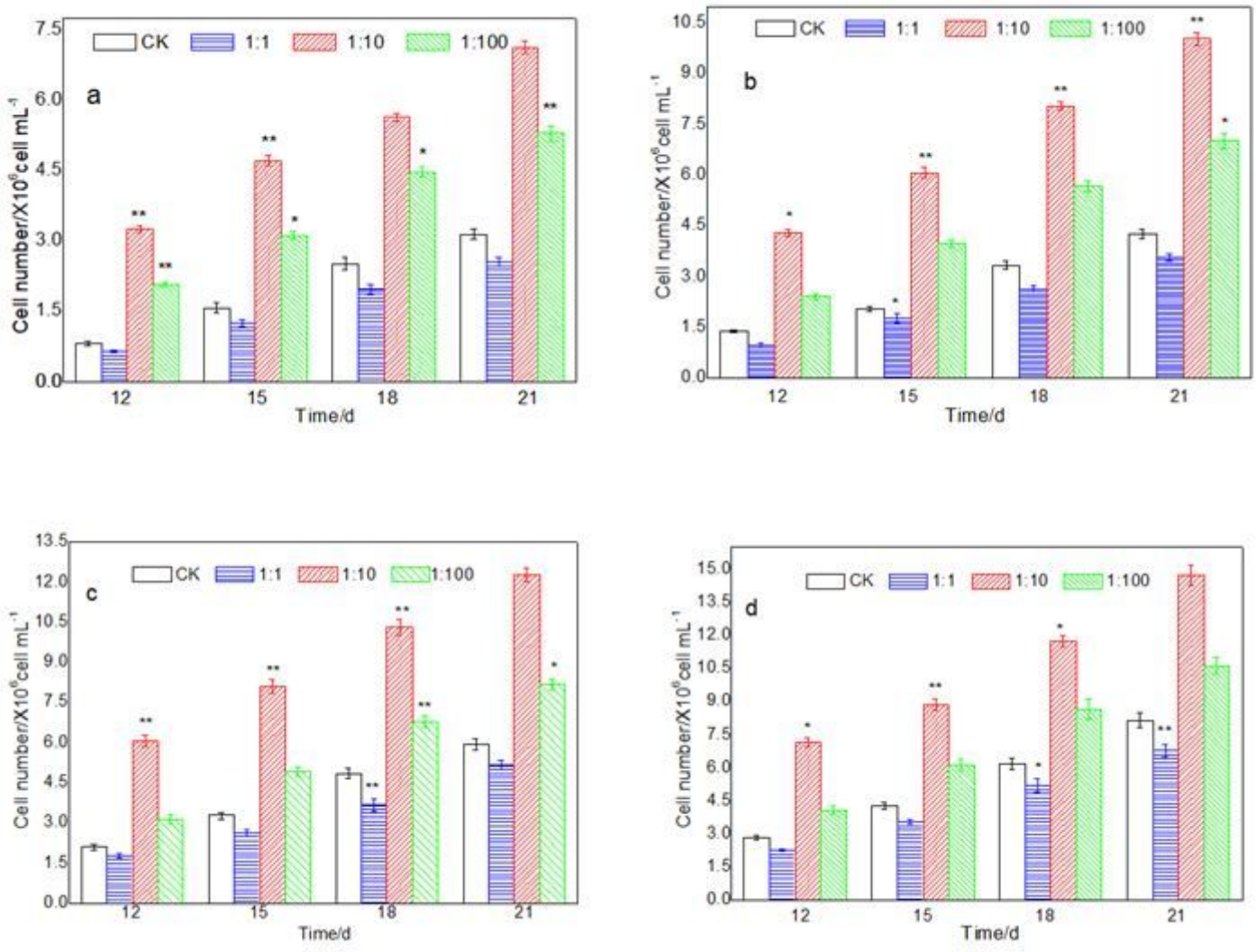

Figure 5

Effects of heterotroph-cyanobacterium ratio on axenic Microcystis 905A (a, b, c and d shows the initial cyanobacterial cell number of 3.0 × 102, 3.0 × 103, 3.0 × 104 and 3.0 × 105 cell mL-1, respectively). * and ** represent a statistically significant difference of $p < 0.05$ and $p < 0.01$ when compared to the control.

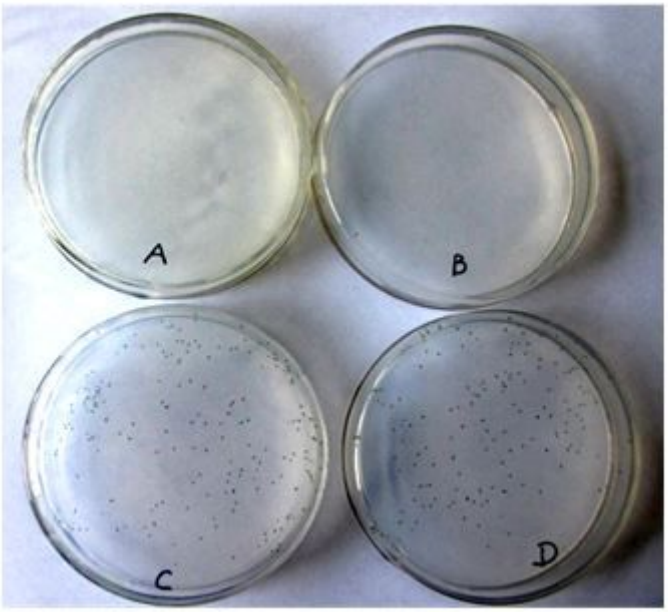

Figure 6

Effects of strain B905-1 on axenic Microcystis 905A cultured by plate. (a is the control without the addition of strain B905-1; b, c and d is the treatment with the addition of strain B905-1 at an initial cell number of 1.0 × 104, 1.0 × 103 and 1.0 × 102 cell mL-1, respectively)

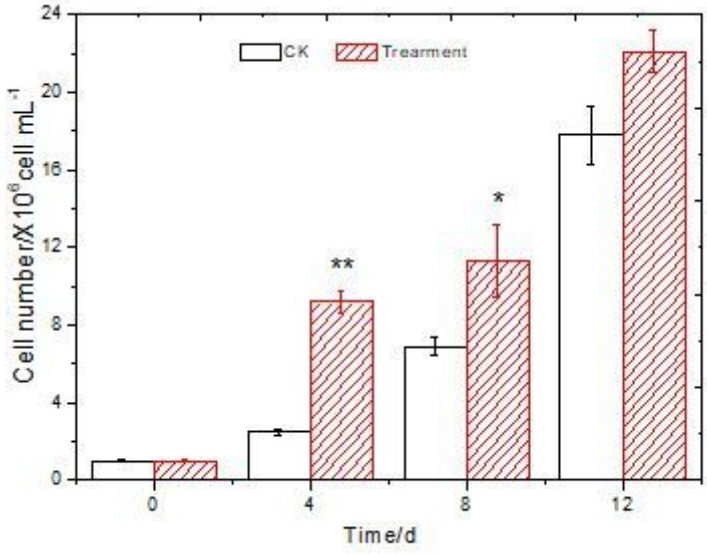

Figure 7

Effect of cell-free filtrate of strain B905-1 on axenic Microcystis 905A. * and ** represent a statistically significant difference of $p < 0.05$ and $p < 0.01$ as compared with the control.