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

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Abstract

In order to avoid the influence of associated bacteria on the target cyanobacteria for physiological and molecular studies, a traditional and reliable method based on solid-liquid alternate cultivation is carried out to purify the non-axenic cyanobacterium Microcystis 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. To better investigate the general interaction between the bacterium and the cyanobacterium, the effect of strain B905-1 on the axenic cyanobacterium M. aeruginosa FACHB-905A is studied. Results indicate that the axenic M. aeruginosa FACHB-905A could not form colonies on BG11 agar medium without the addition of strain B905-1, while it grows well in BG11 liquid medium. Although the presence of B905-1 was not indispensable for the growth of M. aeruginosa FACHB-905A, B905-1 had a positive effect on promoting the growth of M. aeruginosa FACHB-905A. The purification technique for cyanobacteria described in this study is potentially applicable to a wider range of filamentous cyanobacteria.

Introduction

The interactions between phytoplankton and bacteria in aquatic environments have been proposed to be an integral part of the algal/cyanobacterial life cycle (for example, diatom species), and it contributes significant role for photosynthesis thus being important for the metabolism of phytoplankton [1–3]. The relations between heterotrophic bacteria and phytoplankton are much better understood, and it is generally recognized that there are three different types of phytoplankton-bacteria interactions: (i) bacteria and phytoplankton form a commensalism 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 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 phytoplankton form a parasitism relationship that bacteria are loosely associated with phytoplankton and may promote the growth and photosynthesis without having any negative effect, however, phytoplankton grows well without the associated bacteria [7, 8]. These scenarios may be dependent on the characteristics of phytoplankton species, associated bacteria species and secreted substances of the associated bacteria [4].

Harmful algal blooms (HABs) 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]. One of the most common and widespread bloom-forming cyanobacteria that secrets toxins is Microcystis aeruginosa [5, 6, 10], which is a unicellular, photoautotrophic and gram-negative cyanobacterium that belongs to the genus Microcystis, division Cyanophyta [5, 10]. Previous studies show that the cyanobacterium is wrapped by a large number of associated bacteria, and these associated bacteria were considered to benefit from organic substrates released by the cyanobacterium [11–18]. In order to avoid

the influence of cyanobacterium-associated bacteria for physiological and molecular studies, isolation of the axenic cyanobacterium (bacteria-free) is especially important as well as understanding its responses to the associated bacteria.

Various methods including UV irradiation, sonication, micropipette technique, phenol treatment, antibiotic treatment and lysozyme treatment have been described 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, and 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], the growth-promoting effects of cyanobacterium-associated bacteria on cyanobacterium have not received much attention. Apart from cyanobacterium purification, the growth-promoting effect of the cyanobacterium-associated bacteria on cyanobacterium is a significant aspect for understanding the interactions between bacteria and cyanobacteria. Therefore, the aim of the present study is to obtain an axenic M. aeruginosa culture and investigate the specific interaction between the cyanobacterium-associated bacterium and the cyanobacterium.

Materials And Methods

Culture of cyanobacteria and bacteria

Microcystis aeruginosa FACHB-905 and FACHB-907 used in this study are purchased from the Freshwater Algae Culture Collection of Institute of Hydrobiology (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 non-axenic *M. aeruginosa* [5,6,28]. 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 *M. aeruginosa* FACHB-905A is obtained by treating with antibiotics, lysozymes, and micropicking from *M. aeruginosa* FACHB-905 culture.

Bacterial strains B905-1 and B905-2 are isolated from the thallus surface of the cyanobacterium *M. aeruginosa* FACHB-905. These two bacteria are routinely grown in TY liquid medium [29] 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 non-axenic cyanobacterium is diluted to an appropriate desaturation multiple, and inoculated onto sterile Petri dishes containing BG_{11} agar medium [30]. 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 spreaded on the Luria-Bertani (LB) agar plate [29,31] and incubated at room temperature for 3 d or more to examine the existence of associated bacteria, the absence of associated bacteria 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 M. aeruginosa FACHB-905 is illustrated in Fig. 1.

Fig. 1 Purification procedure for axenic culture of *M. aeruginosa* FACHB-905.

Cyanobacterial inhibition bioassay

The antibiotics including tetracycline, cephalosporin, kanamycin, penicillin and streptomycin are purchased from Wuhan Dingguo biological technology Co., LTD. The sensitivities of cyanobacterium-associated bacterium 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]. Effects of lysozyme on the cyanobacterium and associated bacteria 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 non-axenic *M. aeruginosa* FACHB-905 culture (the initial cyanobacterial cell number is 1.0×10^6 cell mL^{-1}). Cyanobacterium-associated bacteria and cyanobacterial cell densities are determined after incubation for 2 d.

The growth curve of axenic *M. aeruginosa* FACHB-905A and non-axenic *M. aeruginosa* FACHB-905 are carried out at an initial cyanobacterial cell number of 1.0×10^6 cell mL^{-1}. Effects of bacterium-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 *M. aeruginosa* FACHB-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 bacterium-cyanobacterium *ratio* of 1:10 and 1:100, the controls (CK) are without the addition of strain B905-1. For the effects of bacterium-cyanobacterium *ratio on the growth of* axenic *M. aeruginosa* FACHB-905A on BG_{11} agar medium, the initial cyanobacterial cell number is 1.0×10^4 with the final bacterium-cyanobacterium *ratio* of 1:1, 1:10 and 1:100.

The effect of cell-free filtrate of strain B905-1 on axenic *M. aeruginosa* FACHB-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 and axenic *M. aeruginosa* FACHB-905A are identified based on 16S rRNA gene sequence analysis. Cyanobacterium-associated bacteria 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; while axenic *M. aeruginosa* FACHB-905A is cultured for 7 days in sterilized BG_{11} liquid medium under the condition described in Section "Culture of cyanobacteria and bacteria". Cyanobacterium and associated bacteria cells are collected by centrifugation at 4000 rpm for 10 min (at 4 °C). DNA is extracted from the bacterial or cyanobacterial 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.

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):

$$G = (t_2\text{-}t_1)/[3.322(\lg X_2\text{-}\lg X_1)] \qquad (1)$$

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):

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

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

Results

The sensitivities of the cyanobacterium and associated bacteria to antibiotics

Antibiotics are commonly used to eliminate heterotrophic bacteria from cyanobacterial cultures [20,25,32]. In order to eliminate the heterotrophic contaminants, the sensitivities of the cyanobacterium and associated bacteria to antibiotics are investigated. Table 1 indicate that tetracycline, cephalosporin and streptomycin have obvious inhibiting effect on strain B905-1 with the antibacterial circle of 3.3, 2.4 and 1.8 cm, respectively, while kanamycin and penicillin have a negative inhibiting effect. In addition, the inhibition efficiency of tetracycline, cephalosporin, kanamycin, penicillin and streptomycin on the growth of *M. aeruginosa* FACHB-905 is 94.3 ± 3.5%, 88.5 ± 4.1%, 50.7 ± 2.4%, 5.6 ± 0.2% and 81.2 ± 3.9%, respectively (Fig. 2). It is demonstrated that *M. aeruginosa* FACHB-905 is significantly inhibited by all of the tested antibiotics except penicillins.

Table 1 Effects of antibiotics on cyanobacterium-associated bacterium 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.

Fig. 2 The effects of antibiotics on the growth of *M. aeruginosa* FACHB-905. * and ** represent a statistically significant difference of $p < 0.05$ and $p < 0.01$ when compared to the control.

Effects of lysozyme on the cyanobacterium and associated bacteria

To obtain the axenic culture, the effects of lysozyme on the cyanobacterium and associated bacteria are investigated. The results demonstrate that both *M. aeruginosa* FACHB-905 and associated bacteria are reduced with the increasing of lysozyme concentration; moreover, the reduction of *M. aeruginosa* FACHB-905 is much more obvious (Table 2). As the lysozyme concentration increases from 1.0 to 10.0 mg mL^{-1}, the cyanobacterium cell number decreases from $(1.2 \pm 0.2) \times 10^6$ to less than 10^4 cell mL^{-1}, and the associated bacteria cell density decreases from $(0.35 \pm 0.03) \times 10^6$ to $(1.3 \pm 0.04) \times 10^4$ cell mL^{-1}. Results indicate that the associated bacteria may not be removed even though 10.0 mg mL^{-1} of lysozyme is added into the non-axenic culture.

Table 2 Effects of lysozyme on the growth of cyanobacterium-associated bacteria and cyanobacterium

Lysozyme (mg mL^{-1})	*M. aeruginosa* FACHB-905 (cell mL^{-1})	Cyanobacterium-associated bacteria (cell mL^{-1})
0	$2.2 \times 10^6 \pm 0.2 \times 10^6$	$0.64 \times 10^6 \pm 0.07 \times 10^6$
1.0	$1.2 \times 10^6 \pm 0.2 \times 10^6$	$0.35 \times 10^6 \pm 0.03 \times 10^6$
2.0	$0.75 \times 10^6 \pm 0.04 \times 10^6$	$0.13 \times 10^6 \pm 0.01 \times 10^6$
5.0	N^a	$3.2 \times 10^4 \pm 0.16 \times 10^4$
10.0	N^a	$1.3 \times 10^4 \pm 0.04 \times 10^4$

N^a represents not detected.

Isolation and purification of the axenic culture

The colony forming process of cyanobacterium and associated bacteria on solid plates (BG_{11} agar medium) is observed by inverted phase contrast microscope, and the results are shown in Fig. 3. It is obviously that the associated bacteria colonies are much bigger than the cyanobacterial colonies, indicating the associated bacteria are grew well than 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. The isolated cyanobacterial colonies are transferred into 6 test tubes and incubated for 3 d. The result shows that 5 tubes become green, indicating the cyanobacterium is grown well. However, the associated bacterium could still be detected. After two cycles of purification, only cyanobacterial colonies are observable on the BG_{11} solid medium. Possible contamination such as associated bacteria 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 *M. aeruginosa* FACHB-905A. The results indicate that *M. aeruginosa* FACHB-905A presented the highest sequence similarity (99% of identity) with *M. aeruginosa* NIES-843, *M. aeruginosa* PCC 7820 and *M. aeruginosa* PCC 7806.

Fig. 3 The growth of cyanobacterial and associated bacterial colonies (× 100). (a, b and c was the colonial morphology cultured for 1, 8 and 15 d, respectively)

Identification of associated bacteria

Two gram-negative bacteria, named B905-1 and B905-2, are isolated from the non-axenic *M. aeruginosa* FACHB-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. 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, another associated bacterium B907-1 is also successfully isolated from *M. aeruginosa* FACHB-907, and it 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. Phylogenetic tree is then constructed (Fig. 4) and it is further confirmed that strain B905-1 and B907-1 are closely related to *Pannonibacter* sp. and *Agrobacterium* sp., respectively.

Fig. 4 The phylogenetic tree of cyanobacterium-associated bacteria.

Effect of associated bacteria on *M. aeruginosa* FACHB-905A

The growth rates of non-axenic culture (*M. aeruginosa* FACHB-905A) and axenic culture (*M. aeruginosa* FACHB-905A) are measured during both the static cultivation (without the shaking speed) and the shaking cultivation conditions (with the shaking speed of 150 rpm). Fig. 5 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 non-axenic culture is 33.6 h under the shaking cultivation and 45.3 h under the static cultivation, respectively. In addition, the generation time of non-axenic culture is much shorter than that of the axenic culture under the same cultivation condition, which demonstrates the photosynthetic efficiency of *M. aeruginosa* FACHB-905 is much better. At the same time, the growth rates of both the non-axenic 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 associated bacteria in promoting the growth of *M. aeruginosa* FACHB-905A.

Fig.5 The growth curves of axenic *M. aeruginosa* FACHB-905 and xenic *M. aeruginosa* FACHB-905A.

Effect of bacterium-cyanobacterium *ratio* on *M. aeruginosa* FACHB-905A

To further study the effect of associated bacterium B905-1 *on the growth of* axenic *M. aeruginosa* FACHB-905A, a series of experiments that different initial cyanobacterial cell concentrations with the

bacterium-cyanobacterium *ratio* of 1:10 and 1:100 are undertaken in BG_{11} liquid medium (Fig. 6). Compared with the control group, the cyanobacterial cell numbers of treatment groups show a remarkable increase, while there is no obvious difference for the initial cyanobacterial cell number from 3.0×10^2 to 3.0×10^5 cell mL^{-1}. It is $(5.63 \pm 0.08) \times 10^6$, $(6.07 \pm 0.15) \times 10^6$, $(8.11 \pm 0.25) \times 10^6$ and $(11.75 \pm 0.25) \times 10^6$ cell mL^{-1} for the treatment group of 1:10, while it is $(4.47 \pm 0.11) \times 10^6$, $(3.98 \pm 0.11) \times 10^6$, $(4.92 \pm 0.18) \times 10^6$ and $(8.65 \pm 0.44) \times 10^6$ cell mL^{-1} for that of 1:100 after incubating for 18 d, respectively. In addition, the higher cyanobacterial cell numbers of $(14.72 \pm 0.48) \times 10^6$ cell mL^{-1} for the treatment group of 1:10 and $(10.63 \pm 0.37) \times 10^6$ cell mL^{-1} for the treatment group of 1:100 are obtained at the day 21, respectively (Fig. 6d). These results indicate the addition of associated bacterium B905-1 has a positive promoting effect on the growth of *M. aeruginosa* FACHB-905A; besides, the bacterium-cyanobacterium *ratio* of 1:10 is much more suitable for promoting the growth of *M. aeruginosa* FACHB-905A than that of 1:100.

Fig. 6 Effects of bacterium-cyanobacterium ratio on axenic M. aeruginosa FACHB-905A (a, b, c and d show the initial algal 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 ** represent a statistically significant difference of $p < 0.05$ and $p < 0.01$ when compared to the control.

The growth of axenic *M. aeruginosa* FACHB-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 *M. aeruginosa* FACHB-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 bacterium-cyanobacterium ratio (1:1, 1:10 and 1:100) on cyanobacterium division are studied (Fig. 7). Interestingly, the *M. aeruginosa* FACHB-905A is unable to grow in the treatment of 1:1 (Fig. 7b), but it grows well in both treatments of 1:10 and 1:100 (Fig. 7c and 7d). The results indicate high ratio of bacterium-cyanobacterium (1:1) is adverse to the growth of *M. aeruginosa* FACHB-905A.

Fig. 7 Effects of strain B905-1 on axenic *M. aeruginosa* FACHB-905A cultured by plate. (a was the control without strain B905-1; b, c and d show the treatments with the addition of strain B905-1 at an initial cell number of 1.0×10^4, 1.0×10^3 and 1.0×10^2 cell mL^{-1}, respectively)

In order to prove that the promoting effect was associated with the extracellular substances of strain B905-1, the effect of the cell-free filtrate of strain B905-1 on the growth of *M. aeruginosa* FACHB-905A is carried out (Fig. 8). 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 *M. aeruginosa* FACHB-905A; moreover, the released substances of strain B905-1 with the promoting effect apparently exist in the cell-free filtrate.

Fig. 8 Effect of cell-free filtrate of strain B905-1 on axenic *M. aeruginosa* FACHB-905A. * and ** represent a statistically significant difference of $p < 0.05$ and $p < 0.01$ as compared with the control.

Discussion

HABs occur around the world and are responsible for most aquatic environment pollution. It is necessary to obtain the axenic M. aeruginosa from the complex microbial community for its physiological and molecular studies. Many recent researches have indicated that cyanobacterial purification methods such as antibiotic treatment and lysozyme treatment can remove associated bacteria within several days [20, 21, 25, 32]. It is indeed that most of the antibiotics such as imipenem (100 μg mL^{-1}), neomycin (100 μg mL^{-1}) and cycloheximide (20 μg mL^{-1}) are highly effective for eliminating associated bacteria. For example, the axenic Arthrospira platensis SAG 21.99 culture is obtained within 3 d using antibiotic treatment [25]. A series of physical and chemical procedures are also applied to obtain the axenic cyanobacterium Arthrospira sp., and it is found that cefoxitin (76.9 μg mL^{-1}) is the most effective antibiotic [23].

The effects of antibiotics on the growth of M. aeruginosa also have been investigated by previous studies. It is demonstrated that the growth of M. aeruginosa is concerned with the types and concentrations of antibiotics. For instance, the cefradine promotes the growth of M. aeruginosa at concentrations of lower than 3.0 mg/L, while it induces a negative influence at higher concentrations (above 3.0 mg/L) [33]. Liu et al. (2012) observes that the 50 percent effective concentrations (EC_{50}) of spiramycin and amoxicillin to M. aeruginosa are 1.15 and 8.03 mg/L, respectively, indicating spiramycin is more toxic than amoxicillin according to the cyanobacterium growth [34]. These results are in accordance with the present study that the sensitivities of cyanobacterium M. aeruginosa FACHB-905 to five antibiotics are quite different. Moreover, four of the tested antibiotics have the inhibition effects on cyanobacterium growth. Similar to our results, the streptomycin caused a significant reduction in M. aeruginosa with the EC_{50} of 0.28 mg/L [35].

Apart from the antibiotics treatment, the lysozyme treatment is also used for the induction of axenic culture. For instance, axenic cultures of Anabaena flos-aquae and Aphanothece nidulans are obtained by

using the lysozyme treatment [22]. As the antibiotics and lysozyme employed in our study can inhibited both the cyanobacterium and associated bacterium simultaneously (Table 1, Table 2 and Fig. 1), it is quite difficult to eliminate the associated bacteria from non-axenic M. aeruginosa FACHB-905 culture by antibiotics treatment or lysozyme treatment methods. Considering the non-axenic M. aeruginosa FACHB-905 can easily form single cyanobacterial colony on BG_{11} agar plate, associated bacteria 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 M. aeruginosa FACHB-905A. 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 M. aeruginosa FACHB-905A culture maybe suitable for eliminating bacteria that attach strongly to the cyanobacteria or are embedded in the extended cyanobacteria cell surface.

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, 36]. DGGE results suggested 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 associated bacteria, 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 associated bacteria strain B905-1 and B905-2 are closely related to Pannonibacter sp. and Chryseobacterium sp., respectively. Besides the identification of associated bacteria, more general interactions seem to occur. It has been suggested that the interaction between bacteria and cyanobacterium is symbiosis or parasitic [3, 37], and the associated bacteria are difficult to isolate from cyanobacterium during the formation of cyanobacterial or algal colony [1, 38]. Moreover, there are few literature with respect to the relationship between cyanobacterium and associated bacteria, although most of the studies describe the anti-cyanobacterial effects of bacteria on M. aeruginosa [5, 6].

To better understand the general interaction between associated bacterium 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 non-axenic M. aeruginosa FACHB-905 is much faster than that of the axenic M. aeruginosa FACHB-905A under both static cultivation and shaking cultivation conditions. The results indicate that the associated bacterium has a promoting effect on the growth of axenic M. aeruginosa FACHB-905A. In consideration of the initial cell number of M. aeruginosa FACHB-905 and associated bacterium is $(2.2 \pm 0.2) \times 10^6$ and $(0.64 \pm 0.07) \times 10^6$ 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 M. aeruginosa FACHB-905A is not able 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 M. aeruginosa FACHB-905A could not divide at the bacterium-cyanobacterium ratio of 1:1, as the associated bacterium can effectively compete nutrients with axenic M. aeruginosa FACHB-905A.

The growth-promoting effect of bacteria 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 maybe explained by the symbiotic interaction that the bacteria deliver vitamins for algae [39], or the addition of bacteria changes the available nutrient concentration such as extracellular organic carbon or dissolved organic matter [2, 4, 14, 17, 36]. 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 [40]. Different from the above-mentioned microorganisms, axenic diatoms aren't able to form biofilm when purified from bacteria [4]. Although the axenic M. aeruginosa FACHB-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 associated bacterium B905-1 is not indispensable for the growth of axenic M. aeruginosa FACHB-905A on BG_{11} agar plate. Nevertheless, the presence of strain B905-1 for the cyanobacterial colony formation mechanism needs to be further studied.

Previous study also indicate that the enhancement of M. chthonoplastes PCC 7420 growth upon the addition of a filtrate obtained from the closely related non-axenic culture of Microcoleus sp. M2C3 could be due to the release of certain growth factors and vitamins[36]. Most of the strains are able to secrete active substance to inhibited or enhanced the growth of cyanobacteria[42]. Possible mechanisms may include various types of interactions from nutrient cycling to the production of growth-inhibiting and cell-lysing compounds[43]. Our results demonstrate that strain B905-1 has the potential to promote M. aeruginosa FACHB-905A growth, whereas M. aeruginosa FACHB-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. Since associated bacteria potentially regulate cyanobacterium growth via extracellular amino acid monomers or other substances [5, 6], they could be regarded as ecological important microbial gardeners to successfully distribute cyanobacterium strains in nature [14]. 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 formation mechanism of HABs and eutrophication control.

In conclusion, our results show that associated bacteria are eliminated by solid-liquid alternate cultivation method and the axenic M. aeruginosa FACHB-905A is successful purified by means of picking and

transferring the single cyanobacterial colony to BG_{11} liquid medium under the microscope; moreover, two cyanobacterium-associated bacteria, 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 M. aeruginosa FACHB-905A.

Abbreviations

HABs
Harmful algal blooms
FACHB
Freshwater Algae Culture Collection of Institute of Hydrobiology
EC_{50}
The 50 Percent Effective Concentrations
DGGE
denaturing gradient gel electrophoresis

Declarations

Acknowledgments

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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).

Authors' contributions

Conceived and designed the project: Yun Kong and Lihong Miao. Performed the experiments: Suqin Gao, Jing Yu Lipeng Ji and Chi Zeng. Analyzed the data: Yun Kong, Lihong Miao and Lirong Song. Contributed reagents/materials/analysis tools: Lihong Miao, Chi Zeng and Lirong Song. Wrote the paper: Yun Kong, Suqin Gao and Lihong Miao.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not Applicable.

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.

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Figures

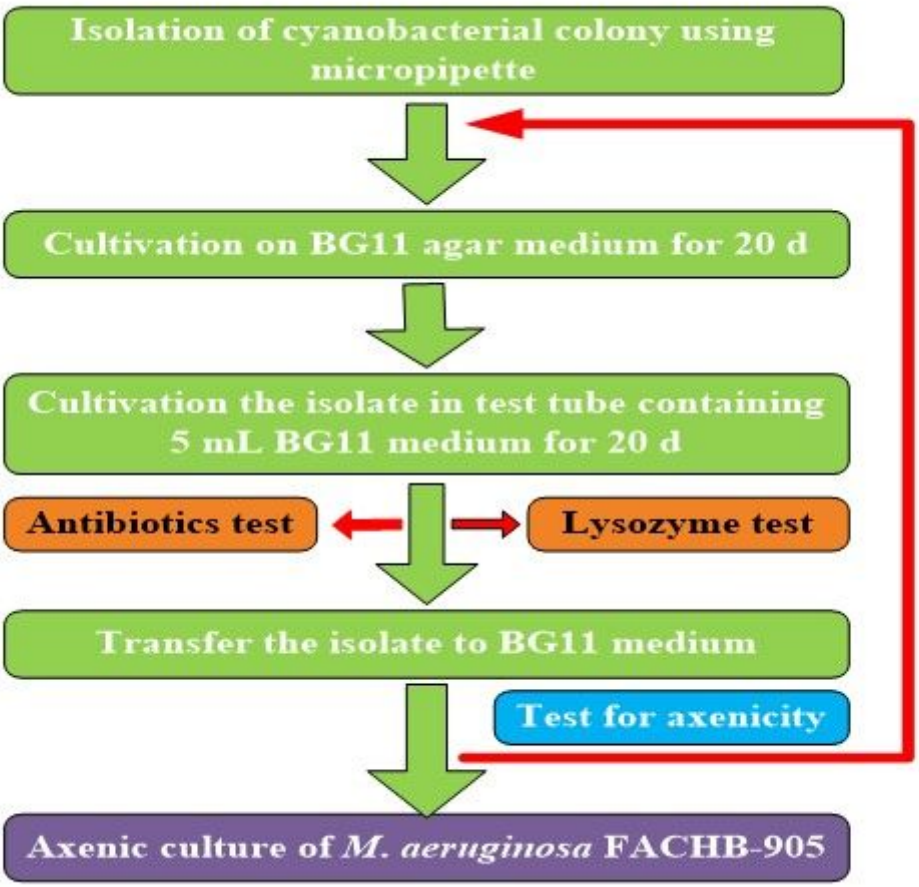

Figure 1

Purification procedure for axenic culture of M. aeruginosa FACHB-905.

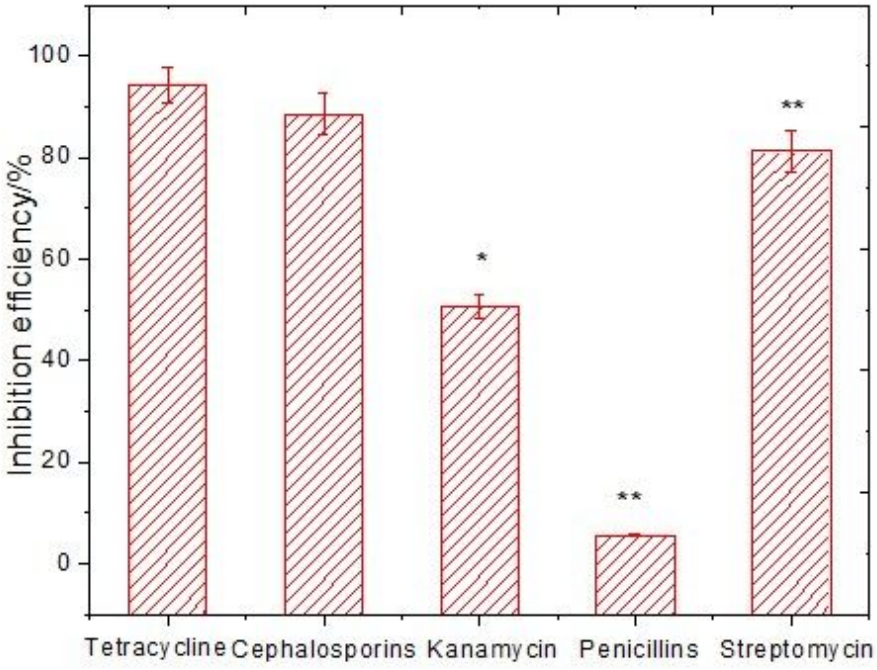

Figure 2

The effects of antibiotics on the growth of M. aeruginosa FACHB-905. * and ** represent a statistically significant difference of $p < 0.05$ and $p < 0.01$ when compared to the control.

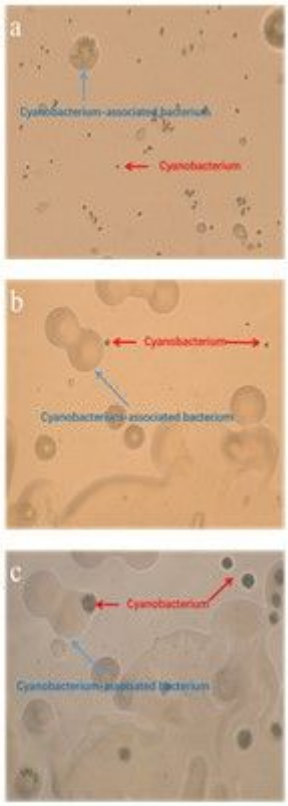

Figure 3

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

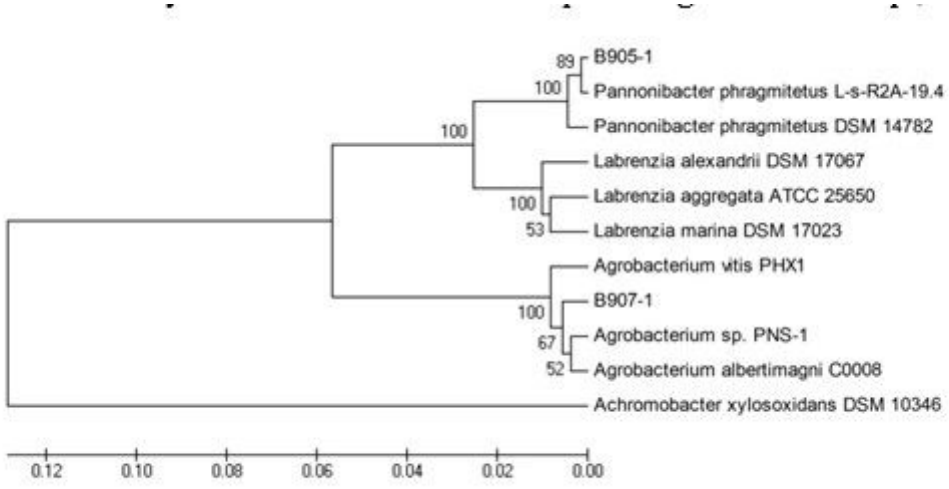

Figure 4

The phylogenetic tree of cyanobacterium-associated bacteria.

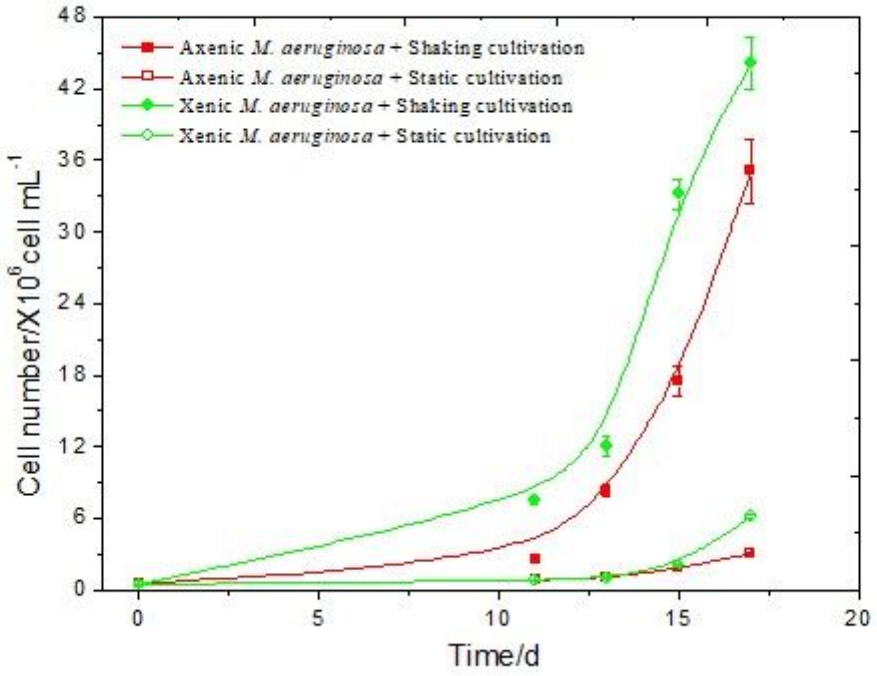

Figure 5

The growth curves of axenic M. aeruginosa FACHB-905 and xenic M. aeruginosa FACHB-905A.

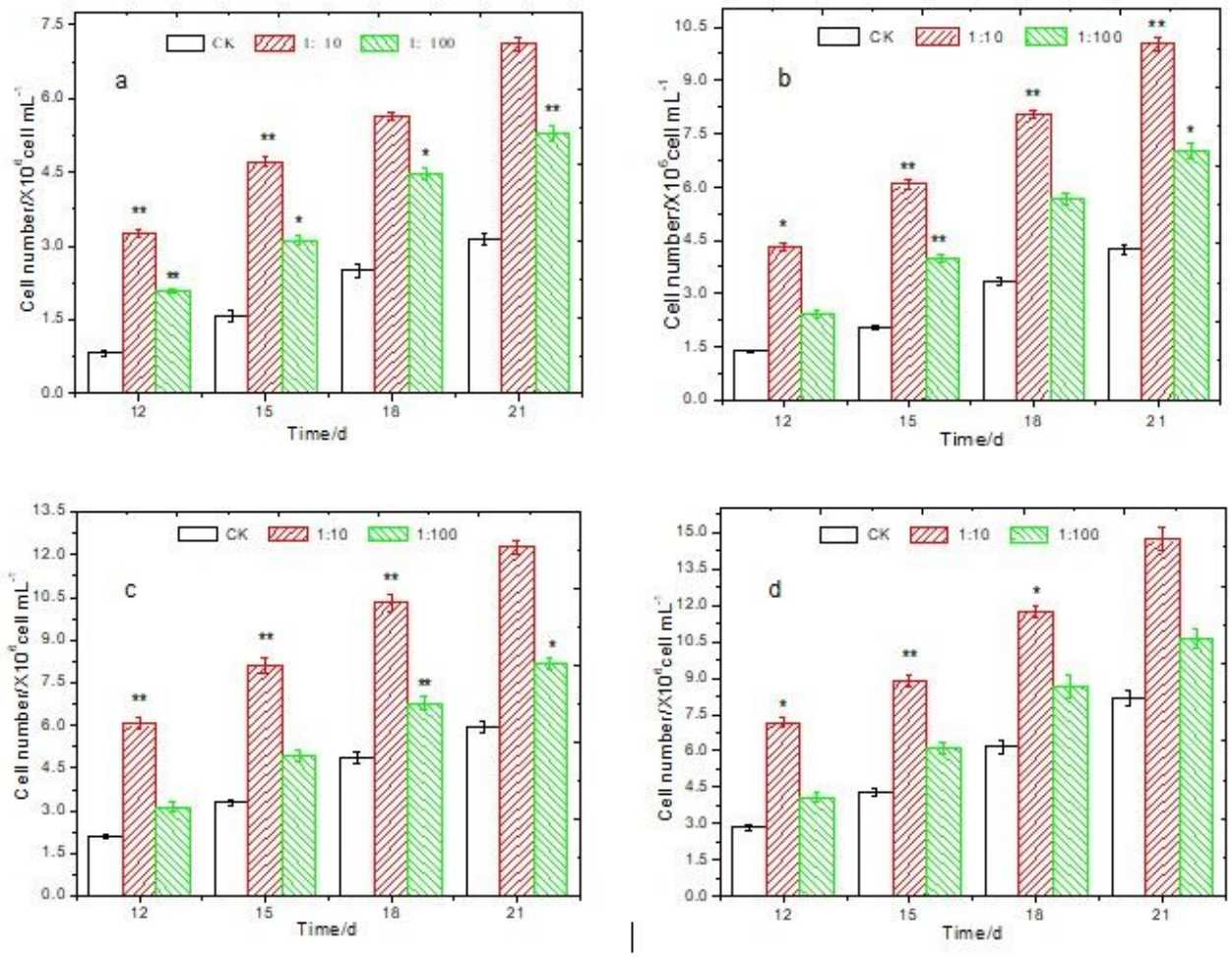

Figure 6

Effects of bacterium-cyanobacterium ratio on axenic M. aeruginosa FACHB-905A (a, b, c and d show the initial algal 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.

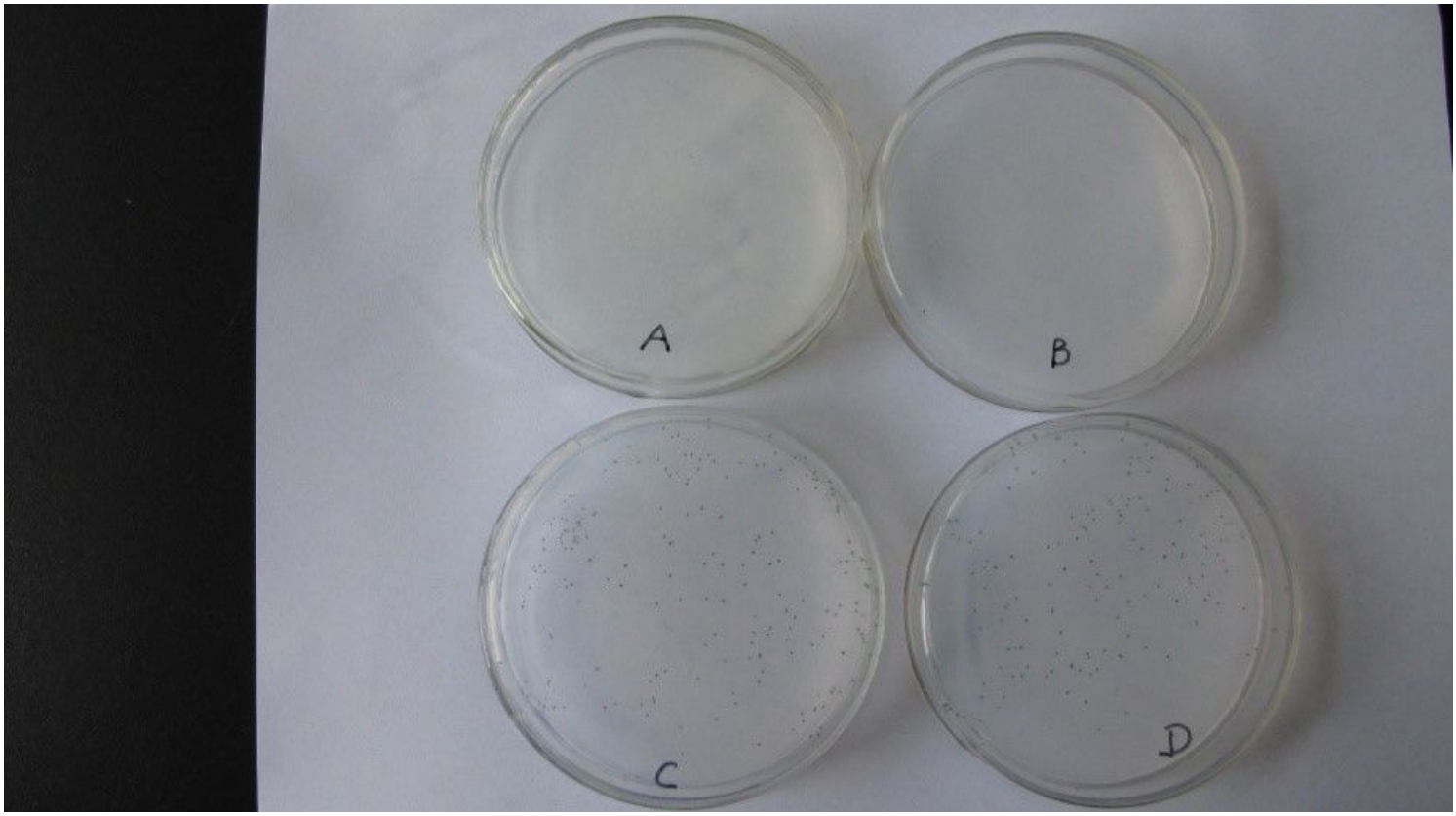

Figure 7

Effects of strain B905-1 on axenic M. aeruginosa FACHB-905A cultured by plate. (a was the control without strain B905-1; b, c and d show the treatments 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)

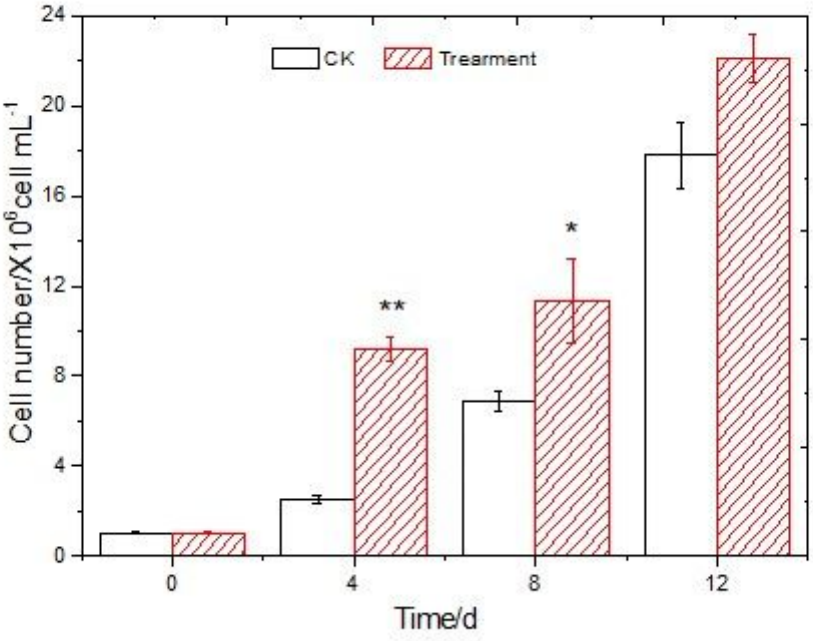

Figure 8

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