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Isolation and identification of amino acids secreted by *Bacillus amyloliquefaciens* T1 with anti-cyanobacterial effect against cyanobacterium *Microcystis aeruginosa*

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ABSTRACT

Eutrophication caused by harmful cyanobacteria is becoming more and more serious all over the world. Biological control of harmful cyanobacterial blooms (HCBs) such as anti-cyanobacterial microorganism is considered to be a safe, economical and effective method as its environmental friendly characteristics and species specificity. *Bacillus amyloliquefaciens* T1, which isolated from a a eutrophic pond in Wuhan, China, shows remarkable anti-cyanobacterial activity against bloom-forming cyanobacterium *Microcystis aeruginosa* 905. In the current study, the anti-cyanobacterial action mode of strain T1 is investigated, and the anti-cyanobacterial compounds secreted by T1 are purified after extraction with organic solvents. Additionally, the anti-cyanobacterial compounds are further analyzed using Fourier-transform infrared spectroscopy (FTIR) and identified with high-performance liquid chromatography-mass spectrometry (HPLC-MS). Results indicate that the anti-cyanobacterial active components are existed in the cell-free filtrate of *B. amyloliquefaciens* T1 and the anti-cyanobacterial activity on *M. aeruginosa* is achieved by indirect interactions. The FTIR absorption spectra reveals that the O–H, –C=C–, C–C, C–O bonds and the aliphatic chains are existed in fraction A2. The purified anti-cyanobacterial active compounds are identified as Lys and Phe with HPLC-MS. The anti-cyanobacterial activity assay indicates the purified amino acids have good anti-cyanobacterial effects against *M. aeruginosa* with the inhibition efficiency of 94.1% ± 3.5% and 80.8% ± 4.3% for Lys and Phe, respectively. It is speculated that amino acids secreted by *B. amyloliquefaciens* T1 may be used as a biological agent and are potentially useful for controlling HCBs.

Keywords: Bacillus amyloliquefaciens T1; Anti-cyanobacterial action mode; Anti-cyanobacterial active substances; Amino acids; Harmful cyanobacterial blooms

1. Introduction

Harmful cyanobacterial blooms (HCBs) caused by toxic cyanobacteria (including *Microcystis*, *Anabaena*, *Oscillatoria* and *Cylindrospermopsis*) result in the significant water quality problem and affect the ecosystem function [1]. As *Microcystis aeruginosa* is a dominant and cosmopolitan bloom-forming species causing such problems due to the production of microcystin [2], a still increasing number of

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studies have dealt with possible ways to reduce, prevent and counter the HCBs (especially cyanobacteria) in recent years [3,4]. In consideration of the environmental friendly and species specificity characteristics of biological methods, biotic interactions between cyanobacteria and bacteria, particularly inhibitory interactions, have gained increasing attention [5,6].

With the aim of finding microbial technology for controlling HCBs, laboratory studies have evaluated the anti-cyanobacterial bacteria for their effectiveness on a broader range of cyanobacteria [6–9]. For instance, strain *Streptomyces* sp. KY-34 inhibits the growth of *M. aeruginosa* and the removal efficiency is 81.2% after 8 d exposure [6]; and strain *Bacillus* sp. Lzh-5 is also proved to possess strong algicidal activity against *M. aeruginosa* [8]. Furthermore, the anti-cyanobacterial action modes of the anti-cyanobacterial bacteria are achieved by direct interaction (cyanobacterial-bacterial contact mechanism) and indirect interaction (through the extracellular secretion of anti-cyanobacterial substances) [10–12]. For example, a large amount of microorganisms has been found to secrete extracellular substances with intense inhibition effects on *M. aeruginosa* [5,7,10,13], and the anti-cyanobacterial action modes of these microorganisms are indirect interaction.

Apart from the researches on anti-cyanobacteria isolation/identification and cyanobacterial-bacterial interactions, the isolation and identification of anti-cyanobacterial compounds has also been studied [7–9,12–14], and these anti-cyanobacterial compounds can be summarized as enzymes [10], antibiotics [9,12], amino acids [14,17], proteins [15], peptides [16], surfactants [18], and pigments [19]. Although the genus *Bacillus* is reported to be accounted for the strains showing lytic activity against cyanobacteria [7,20], and the anti-cyanobacterial compounds produced by *Bacillus* also have been investigated [21,22], while few studies demonstrate that amino acids produced by *Bacillus* could inhibit the growth of cyanobacteria [23].

Our preliminary study indicates that *Bacillus amyloliquefaciens* T1, isolated from a eutrophic pond in Wuhan, China, has excellent anti-cyanobacterial activities on harmful cyanobacteria [24], and the removal efficiency of 94.0% indicates that *M. aeruginosa* 905 is more sensitive to *B. amyloliquefaciens* T1 than other *Bacillus* sp. [20–22]. Hence, it is of great significance to study the anti-cyanobacterial action mode and to isolate and identify the anti-cyanobacterial active substances of *B. amyloliquefaciens* T1 on *M. aeruginosa*. In the present study, a detailed investigation of the anti-cyanobacterial action mode of *B. amyloliquefaciens* T1 is conducted, and the anti-cyanobacterial compounds secreted by strain T1 are purified after extracting with organic solvents. Additionally, the anti-cyanobacterial compounds are further analyzed using Fourier-transform infrared spectroscopy (FTIR) and identified with high-performance liquid chromatography-mass spectrometry (HPLC-MS).

2. Materials and methods

2.1. Cyanobacterium and anti-cyanobacterium culturing

Microcystis aeruginosa FACHB-905 is purchased from the Freshwater Algae Culture Collection of Institute of Hydrobiology (FACHB), Chinese Academy of Sciences (Wuhan, China). Before used as inoculants, it has been cultured for 7 d to reach the log phase, and the axenic culture conditions are as following: sterilized BG11 medium [6]; 36 μmol photons m^{-2} s^{-1} white light, light:dark = 14 h:10 h; 25°C ± 1°C [23].

The anti-cyanobacterium used in this study is isolated from a eutrophic pond in Wuhan, China. It is identified as *B. amyloliquefaciens* T1 by the morphology and homology researches based on 16S rDNA gene sequencing (GenBank no. GU359043.1) in our previous study [24]. The *B. amyloliquefaciens* T1 is kept at 4°C with LB agar medium [25]. Bacterial suspension or fermentation liquid of *B. amyloliquefaciens* T1 is prepared by incubating the seed culture with LB liquid medium at 37°C with a shaking speed of 170 rpm for 36 h.

2.2. Preparation of frozen substances

The fermentation liquid (5 L) of *B. amyloliquefaciens* T1 is collected by centrifugation at 5,000 × *g* for 15 min and then filtered through a 0.22 μm cellulose acetate membrane; finally, the filtrate is concentrated with a rotavapor. The concentrated solution is evaporated using rotary evaporator (BUCHI, Switzerland) under the temperature of 45°C and with the rotate speed of 30 rpm, and the final condensed substances are placed in the refrigerator for 24 h at –80°C. The frozen substances are completely dried to a powder via freeze drying and used for further study.

2.3. Isolation and purification of anti-cyanobacterial products

It is recognized that silica gel column chromatography (CC) is generally used to isolate active substances [26,27]. The isolation and purification procedures of anti-cyanobacterial compounds from *B. amyloliquefaciens* T1 are shown in Fig. 1. The frozen substances (solid frozen powder, 2,000 mg) are extracted with 100 mL chloroform by shaking with hands for more than 30 min at room temperature (the extracted step is repeated four times), followed by concentration and drying under reduced pressure with the evaporator at 30°C, and the solvents are removed by vacuum filtration.

The fractions with anti-cyanobacterial effects are screened with the cyanobacterial inhibition bioassay noted below (Section 2.4), and the $CHCl_3$ fraction (A1) with the potential anti-cyanobacterial effect is subjected to silica gel (200~300 mesh) and isolated by silica gel CC with 150 ml of chloroform-methanol (2:1, 1.5:1, 1:1 and 1:2, v/v), respectively. Four fractions (G1, G2, G3 and G4) without solvent are obtained. The G2 fraction is further separated by silica gel CC with 150 mL chloroform-methanol (2:1), and A2 fraction without solvent is obtained, and then A2 fraction is further separated by the thin layer chromatography (TLC) [26], and isolated and identified by HPLC-MS.

2.4. Cyanobacterial inhibition bioassay

The anti-cyanobacterial effect of strain *B. amyloliquefaciens* T1 is tested using a classic cyanobacterial growth inhibition bioassay [6]. Five mL LB liquid medium, bacterial suspension or the cell-free filtrate (obtained by filtering the bacterial suspension or fermentation liquid with a 0.22 μm cellulose acetate membrane) is added into 250 mL

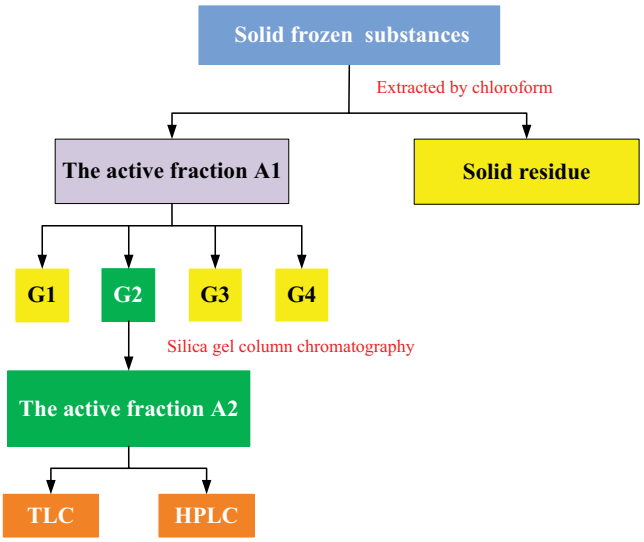

Fig. 1. Schematic diagram of isolation and purification of the anti-cyanobacterial compounds from *Bacillus amyloliquefaciens* T1.

sterilized Erlenmeyer flasks containing 95 mL *M. aeruginosa* culture (initial cyanobacterial density is 1.0×10^6 cells mL^{-1}), respectively. For the control group, five mL BG11 liquid medium is added. All the samples and controls are incubated under the pre-set conditions described in Section 2.1 and the cyanobacterial cell number is determined every day during the incubation.

The anti-cyanobacterial ability of each fraction is modified slightly. In brief, *M. aeruginosa* is inoculated into 10 mL BG11 liquid medium in a 20 mL glass vial with the initial cyanobacterial density of 1.0×10^6 cells mL^{-1}. Five mg of each fraction is dissolved into 1 mL methanol as stock solution, while for the anti-cyanobacterial effect experiments of amino acids on *M. aeruginosa*, 5 mg of A2 or commercial amino acids (Lys, Phe and Trp is 5 mg, respectively; amino acids mixture of Lys+Phe, Lys+Trp, Phe+Trp and Lys+Phe+Trp is 5 mg with the ratio of 1:1, 1:1, 1:1 and 1:1:1, respectively) is dissolved into 1 mL methanol as stock solution, and the small amount of each stock solution (2%, v/v) are added into the glass vial and then cultivated under the pre-set conditions described in Section 2.1.

All experiments are performed in three replicates including control and treatment groups and the arithmetical means (±SD) are obtained. Statistical analysis is performed using Version 17.0 of SPSS for Windows (SPSS, Chicago, IL, USA).

2.5. Analytical methods

2.5.1. Determination of anti-cyanobacterial activity

The cell number of *M. aeruginosa* is determined by hemocytometer using light microscopy (NIKON-YS100). The determination of each sample is repeated 3 times and standard error of means is calculated for all data.

The inhibition efficiency is calculated according to Eq. (1):

$$\text{Inhibition efficiency} = \left(1 - \frac{C_t}{C_0}\right) \times 100\% \quad (1)$$

where C_0 and C_t are the cyanobacterium cell number of the control and test group at time t, respectively [24].

2.5.2. FTIR analysis

FTIR analysis is used to characterize the major functional groups of organic matters and to predict the major components [28]. A2 fraction obtained by freeze-drying is analyzed for the structural and chemical characteristics. KBr is mixed with the fractions with the ratio of 100:1 and the FTIR spectra of the mixture are obtained by scanning with FTIR-8900 spectrometer (SHIMADZU, Japan).

2.5.3. HPLC-MS analysis

The procedures of sample pretreatments of anti-cyanobacterial fraction A2 for isolating anti-cyanobacterial substances by HPLC (Waters, Milford, MA, USA) are in accordance with previous study with minor modification [26,27]. A2 sample (2 mg) is dissolved in 1 mL methanol solution and then filtered through a 0.22 μm organic membrane filter, and the filtrate is collected with an autosampler vial. A ZORBAX Eclipse SB-C18 column (4.6 mm × 150 mm, 5 μm) (Agilent, Palo Alto, CA, USA) is used for reversed-phase separation, using a mobile phase of water-methanol (3/17, v/v) flowing at 1.0 mL min^{-1}. The injection volume for all samples is 20 μL and the spectrophotometric detection wavelength is 220 nm, the testing time is 20 min.

Desorption electrospray ionization (DESI) is carried out to identify the anti-cyanobacterial compounds and MS conditions are as following: negative ionization mode, ion source temperature 100°C, capillary temperature 350°C, sprays voltage 3.6 kV, capillary voltage 20 V, voltage multiplier 650 V; shielding gas flow 450 L h^{-1}, auxiliary gas flow 50 L h^{-1}. Molecular weight data is obtained from m/z 100 to 1,000 in full MS scan mode.

3. Results

3.1. Anti-cyanobacterial effect of strain B. amyloliquefaciens T1

To determine the anti-cyanobacterial effect of strain *B. amyloliquefaciens* T1, *M. aeruginosa* cultures are incubated in the presence of the bacterial suspension and the cell-free filtrate, respectively (Fig. 2). It is showed that the LB liquid medium has no inhibition effect on *M. aeruginosa*, while both the bacterial suspension and the cell-free filtrate of *B. amyloliquefaciens* T1 have excellent anti-cyanobacterial activity against *M. aeruginosa*. In the presence of the cell-free filtrate, the inhibition efficiency of *M. aeruginosa* is 100% on the 4th day; while for the bacterial suspension group, the cell density of *M. aeruginosa* is $5.0 \pm 0.3 \times 10^4$ cells mL^{-1} on the 4th day. The results indicate the anti-cyanobacterial effect of the cell-free filtrate is much better than that of the bacterial suspension, which means the anti-cyanobacterial active components secreted by strain *B. amyloliquefaciens* T1 are mainly existed in the cell-free filtrate.

3.2. Isolation and purification of anti-cyanobacterial products

To isolate the anti-cyanobacterial active compounds, solid frozen powder (2,000 mg) are extracted with 100 mL

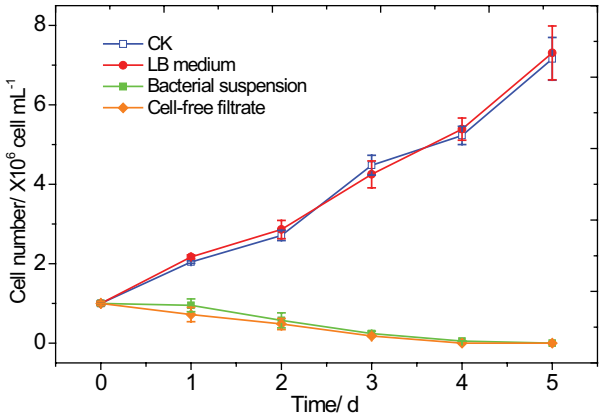

Fig. 2. Anti-cyanobacterial effect of *Bacillus amyloliquefaciens* T1 on *M. aeruginosa*.

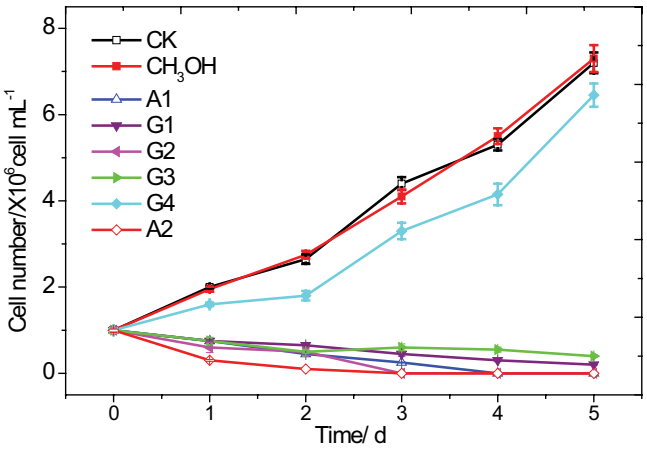

Fig. 3. Anti-cyanobacterial effect of isolated fractions on *M. aeruginosa*.

chloroform (this step is repeat four times), and the major anti-cyanobacterial fraction (A1, 45 mg) is obtained after extraction. This active fraction is vacuum-concentrated and subjected to silica gel CC and then four major fractions, namely, G1 (6 mg), G2 (7 mg), G3 (9 mg), and G4 (8 mg) are yielded (Table 1). It is found that the growth inhibition efficiency of the G1, G2 and G3 fraction on *M. aeruginosa* is 94.3%, 100% and 89.6% on the 4th day, respectively, while it is only 21.7% for G4 fraction (Figs. 3 and S1, S2). The results suggest that the anti-cyanobacterial activity of G2 fraction is remarkable, therefore, it is chosen for further purification. The G2 fraction is collected and applied in a second round of silica gel CC with chloroform-methanol of 2:1. After purification twice by silica gel CC, the eluate with anti-cyanobacterial activity is combined and named as A2. In addition, fraction A2 is further investigated by the TLC analysis, and four main substances, which should be further separated with HPLC are found (Fig. 4).

Table 1
Yields of isolated fractions

Fraction	Weight/mg	Yield/%
Solid frozen powder	2,000	
Light yellow solid	1,300	65.00
A1	45	2.25
G1	6	0.30
G2	7	0.35
G3	9	0.45
G4	8	0.40
A2	2.5	0.13

3.3. Functional group characteristics of anti-cyanobacterial compounds

To illustrate the characteristics of the major functional groups of anti-cyanobacterial active compounds in fraction A2, FTIR analysis is conducted to investigate the functional groups. The FTIR spectra of fraction A2 are illustrated in Fig. 5. It shows that the FTIR absorption spectra are at 3,379; 2,960; 1,654; 1,409; 1,339; 1,115; 749 and 702 cm^{-1}. The absorption peaks could be divided into two distinct regions: peaks at >1,700 and <1,700 cm^{-1}. For the first region, the absorption at 3,379 cm^{-1} is attributed to the stretching of the O–H bond in hydroxyl functional groups, indicating the presence of several –OH groups; For the second region, the adsorption around the peak at 2,960 cm^{-1} reveals that the aliphatic chains with a small shoulder (C–H asymmetric stretching in CH_2 and CH_3) are existed in fraction A2. In addition to the distinctive adsorption band at 1,654 cm^{-1} which results in the absorption of the protein secondary structure as namely amides I, fraction A2 has an absorbance band representative of the functional group of the protein secondary structure amides II (1,409 cm^{-1}). Moreover, other absorbance bands from 700 to 1,400 cm^{-1} may indicate the presence of the –C=C–, C–C, C–O and O-H bonds.

3.4. Purification and identification of fraction A2 with HPLC-MS

The anti-cyanobacterial fraction A2 from silica gel CC is further analyzed and identified using HPLC-MS. HPLC-MS results suggest that there are four major components in this fraction, which are consistent with earlier results of TLC analysis (Fig. 4). These four peaks are represented with retention times of 1.87 min (P1), 2.32 min (P2), 3.80 min (P3) and 5.43 min (P4), respectively (Fig. 6a), nevertheless, P1 shows no anti-cyanobacterial activity (date not shown). Furthermore, the EI mass spectrums for P2, P3 and P4 compound are illustrated in Fig. 6b–d. The results of $[M+H]^+$ ion indicate that the molecular mass of P2, P3 and P4 compound could be determined at m/z 147, 166 and 205. Compared to the spectral data in the database, these EI mass spectrums are very similar to that of L-lysine (molecular weight (MW), 146), L-phenylalanine (MW, 165) and L-tryptophan (MW, 204).

To further confirm the hypothesis that the major anti-cyanobacterial components secreted by *B. amyloliquefaciens* T1 are L-lysine (Lys), L-phenylalanine (Phe) and L-tryptophan (Trp), pure amino acids such as Lys, Phe and Trp are analyzed with HPLC. HPLC results suggest that the three amino acids, which is consisted

with the active fractions separated by size-gel exclusion chromatography (Figs. S3–S5), are eluted at the following retention times: 2.0 to 3.0 min, 5.5 to 6.5 min, and 8.5 to 9.5 min, respectively; Moreover, these distinctions are consistent with the HPLC-MS results, indicating the three amino acids of Lys, Try and Phe are present in A2 fraction.

3.5. Anti-cyanobacterial effect of amino acids on M. aeruginosa

In order to confirm whether the purified amino acids are good algicides against *M. aeruginosa*, anti-cyanobacterial activity assay is applied to determine their anti-cyanobacterial efficiencies. The anti-cyanobacterial activities of fraction A2 and commercial amino acids are illustrated in Fig. 7. Results demonstrate that the inhibition efficiency of fraction A2, Lys and Phe on *M. aeruginosa* is 98.7% ± 2.1%, 94.1% ± 3.5% and 80.8% ± 4.3%, respectively,

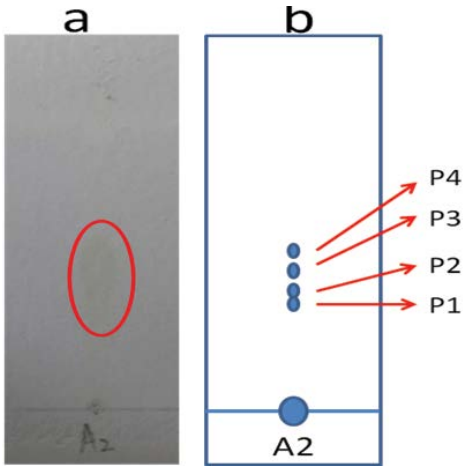

Fig. 4. TLC analysis of anti-cyanobacterial fraction A2.

while the inhibition efficiency of Trp is only 1.2% ± 0.2%, indicating Trp has no biological toxicity against *M. aeruginosa*. Meanwhile, the anti-cyanobacterial activity of amino acids mixtures (Lys+Phe, Lys+Trp, Phe+Trp and Lys+Phe+Trp) are also investigated. It is obvious that the anti-cyanobacterial activity of Lys+Phe and Lys+Phe+Trp treatments are as effective as that of fraction A2, however, the anti-cyanobacterial activities of Lys+Trp and Phe+Trp treatments are lower than that of other treatments, and the inhibition efficiency for the Lys+Trp and Phe+Trp is only 72.3% ± 3.1% and 59.8% ± 3.8%, respectively.

4. Discussion

Researches on feasible and environmental approaches to control HCBs have important theoretical and practical application [24,29]. Microbiological technology has been widely concerned because of its safety, high efficiency and without secondary pollution [4,13,16]. As one of the most widespread microorganisms in water, soil and air environment, *Bacillus* species have been widely used in the fields of food processing, agricultural production and environmental protection [23,29], in addition, they are developed as biocontrol agents due to the ability to form heat- and desiccation-resistant spores [23]. Their positive roles in improving water quality makes them widely used as probiotics in aquaculture. On the part of eutrophic water treatment and remediation, evidence of *Bacillus* sp. that inhibits the growth and causes lysis of cyanobacteria is firstly provided in 1985 [30]; in recent years, the *Bacillus* species, such as *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Bacillus tequilensis*, have been found to possess stronger anti-cyanobacterial activity against *M. aeruginosa* than other microorganisms isolated from freshwater [20,24,29,31,32]. Among these microorganisms, *B. amyloliquefaciens* T1 exhibits the strongest anti-cyanobacterial activity with an inhibition efficiency of 100% on the 4th d. Furthermore, our previous study shows cyanobacteria such as *M. aeruginosa* 905, *M. aeruginosa* 907, *M. aeruginosa* 908,

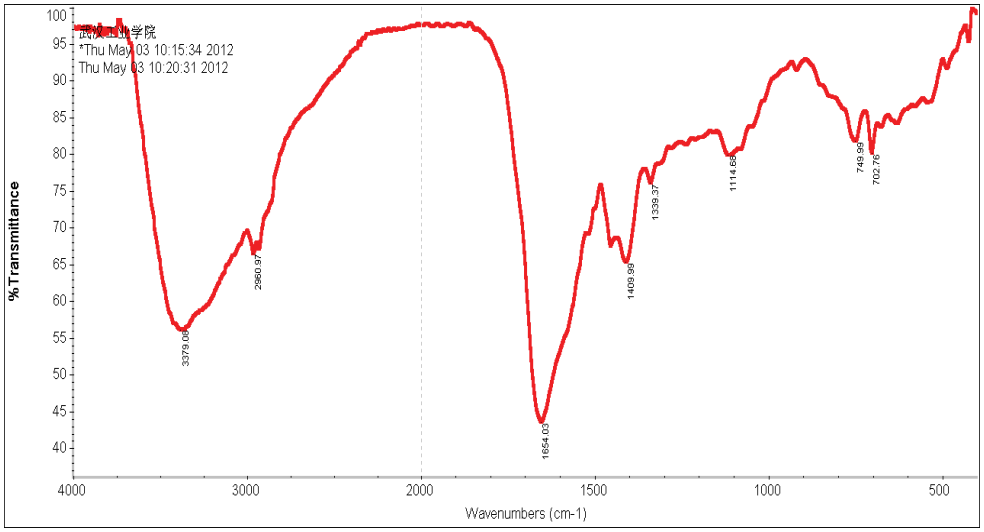

Fig. 5. FTIR spectra of fraction A2.

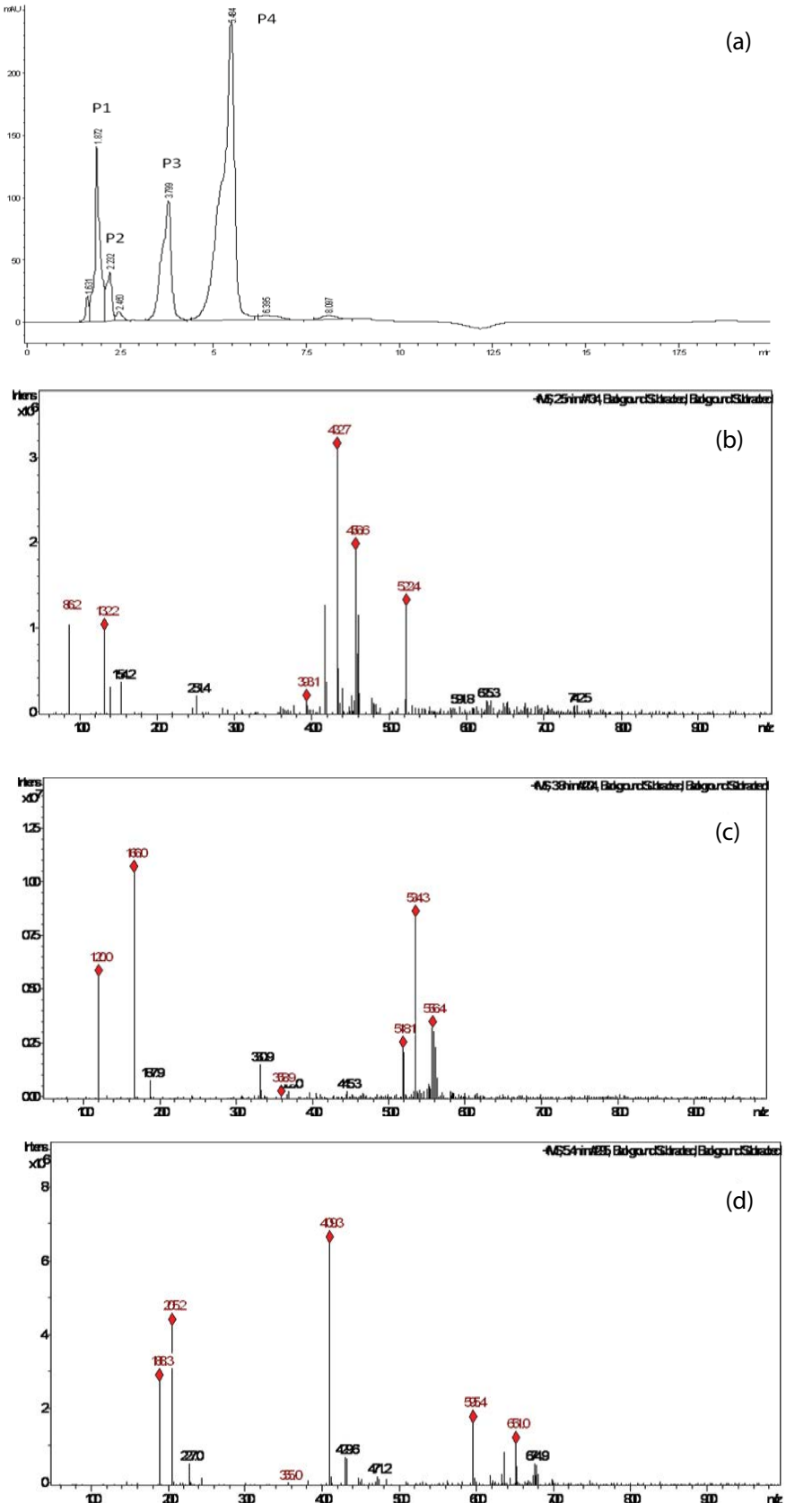

Fig. 6. The results of HPLC-MS analysis of fraction A2 (a) liquid chromatogram (P1 = 1.872 min; P2 = 2.232 min; P3 = 3.799 min; P4 = 5.484 min), (b) mass spectrum of a compound with the retention time of 2.23 min, (c) mass spectrum of a compound with the retention time of 3.80 min and (d) mass spectrum of a compound with the retention time of 5.43 min.

M. aeruginosa 912 and *M. aeruginosa* 7806 are significantly inhibited by T1. Therefore, the anti-cyanobacterial action mode and anti-cyanobacterial active products secreted by *Bacillus* sp. are worthy of further study.

The anti-cyanobacterial action mode is particularly important for studying the interaction between bacteria and algae/cyanobacteria. It is generally recognized that the anti-cyanobacterial action modes could be defined as direct interaction and indirect interaction [10–12]. In the present study, the cell-free filtrate of *B. amyloliquefaciens* T1 exhibits high anti-cyanobacterial effect against *M. aeruginosa* as nearly the same as that of the bacterial suspension, demonstrating the anti-cyanobacterial active compounds are secreted by *B. amyloliquefaciens* T1 and presented in the cell-free filtrate. Furthermore, the anti-cyanobacterial action mode of *B. amyloliquefaciens* T1 is achieved by indirect interaction. Numerous studies on the anti-cyanobacterial action modes have demonstrated that the anti-cyanobacterial activity depends on the extracellular anti-cyanobacterial active substances, which are released by of *Bacillus* sp. [21–23], and these results are in accordance with our study.

In view of silica gel CC and TLC are used for pre-fractionation of active substances [26,27], the anti-cyanobacterial compounds from *B. amyloliquefaciens* T1 are isolated and purified by silica gel CC and TLC, and the compounds are further investigated by FTIR and HPLC-MS. Chromatographical experiments reveal that the anti-cyanobacterial compounds in fraction G2 is able to be directly separated by TLC while they are difficult to be separated from fraction G1 and G3, although these two fractions have good anti-cyanobacterial activity on *M. aeruginosa*. Based on the FTIR spectrum and HPLC-MS analysis, the anti-cyanobacterial compounds are identified as Lys, Phe and Trp, moreover, the peak shapes and the retention times of pure amino acids such as Lys, Phe and Trp are in accordance with the HPLC-MS results. However, inspection of the anti-cyanobacterial effects reveals that Trp doesn't have excellent anti-cyanobacterial activity (Fig. 7). The possible reason of such phenomenon maybe that different amino acids have different effects on the metabolism process of *M. aeruginosa* [33].

The isolation and identification of anti-cyanobacterial compounds from *Bacillus* sp. is also investigated in recent studies. For example, two anti-cyanobacterial compounds, S-5A (cyclo[Gly-Pro]) and S-5B (cyclo[ProVal]), secreted by *Bacillus* sp. Lzh-5 are identified [8]; in addition, another two low MW anti-cyanobacterial compounds [indole-3-carboxaldehyde and cyclo(Pro-Phe)] produced by strain *Bacillus* sp. S51107 are also reported [34]. Although *Bacillus* sp. has anti-cyanobacterial effect on *M. aeruginosa*, its anti-cyanobacterial active substances are quite different, and the amino acids that secreted by *Bacillus* sp. can significantly inhibited the growth of *M. aeruginosa* has not been reported previously. In the present study, we demonstrate that Lys and Phe inhibits the growth of *M. aeruginosa* with an inhibition efficiency of 94.1% ± 3.5% and 80.8% ± 4.3%, respectively, and the anti-cyanobacterial effect of Lys is much higher than that of the other anti-cyanobacterial compounds produced by other *Bacillus* sp. [8,34].

Anti-cyanobacterial compounds are not only secreted by *Bacillus* sp., but also produced by other anti-cyanobacterial bacteria such as *Aquimarina* sp., *Streptomyces* sp. and *Shewanella* sp. [9,10,12,13]. Luo et al. [9] reports that the anti-cyanobacterial active compound produced by *Streptomyces* sp. L74 (with the anti-cyanobacterial effect against *M. aeruginosa*) could directly isolated and purified with HPLC, and further identified as triterpenoid saponin with a molecular formula of $C_{42}H_{70}O_{13}$; another anti-cyanobacterial compound named NIG355, produced by *Streptomyces malaysiensis* O4-6, is also identified based on the quadrupole time-of-flight mass spectrometry and nuclear magnetic resonance spectra analysis [12]. While the anti-cyanobacterial compounds from *B. amyloliquefaciens* T1 are identified as Lys and Phe. These results demonstrate that different microorganisms secrete different anti-cyanobacterial active substances.

Consistent with our study, *Streptomyces* strains have been reported to secrete L-lysine, which exhibits anti-cyanobacterial activity by destroying the cell wall of cyanobacteria *M. aeruginosa* NIES 112, *M. aeruginosa* NIES 298 and *Anabaena ucrainica* [34]. It is also demonstrated that L-lysine inhibits the growth of *M. novacekii* TAC20-1 and *M. viridis* NIES102 [17,33], and cells of cyanobacteria are completely destroyed in 48 h with the addition of lysine at concentrations up to 10 mg L^{-1} [35]. A similar phenomenon is observed that anti-cyanobacterial antibiotics extracted from *Streptomyces* sp. KY-34 cause inhibition effects on *M. aeruginosa* due to the destruction the cellular structure,

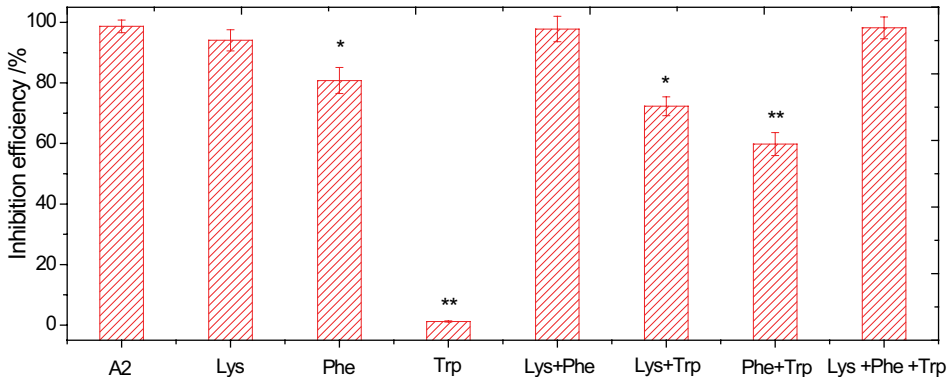

Fig. 7. The anti-cyanobacterial activity of fraction A2 and commercial amino acids after 4 d incubation (* and ** represent a statistically significant difference of $p < 0.05$ and $p < 0.01$).

induction of oxidative damage and reduced photosynthesis [6], and this may be the same inhibition mechanism of T1. By reason of the foregoing, the anti-cyanobacterial effects by Lys and Phe may serve as another evidence for the fact that biological process is especially efficient for the elimination of *M. aeruginosa*.

Amino acids and peptides are the constitutional components that are abundant in natural water [33], they could be naturally utilized by cyanobacteria in aquatic environments [15,16], and they also could inhibit the growth of cyanobacteria [17,35]. With respect to our experiments, as shown in Fig. 7, it is apparently that *M. aeruginosa* could be inhibited by both Lys and Phe, in addition, our previous study shows that the anti-cyanobacterial effect is stable at a wide range of temperature (–20°C~70°C) and pH (7.0~10.0) [24], indicating that the strain T1 has a potential application for the eutrophication bioremediation in situ. Hence, there are a number of reasonable advantages to use these amino acids isolated from *B. amyloliquefaciens* T1 for controlling HCBs.

In conclusion, in order to develop a biological agent for eutrophication control, the anti-cyanobacterial action mode and the anti-cyanobacterial compounds secreted by *B. amyloliquefaciens* T1 are investigated. The anti-cyanobacterial active components secreted by T1 are existed in the cell-free filtrate and the anti-cyanobacterial action mode of T1 on cyanobacterium *M. aeruginosa* is achieved by indirect interactions. The purified anti-cyanobacterial active compounds are identified as Lys and Phe with HPLC-MS. The anti-cyanobacterial activity assay indicates the purified amino acids have good anti-cyanobacterial effects against *M. aeruginosa* with the inhibition efficiency of 94.1% ± 3.5% and 80.8% ± 4.3% for Lys and Phe, respectively. Regardless of the isolation and identification of anti-cyanobacterial compounds secreted by *B. amyloliquefaciens* T1, anti-cyanobacterial mechanism and potential application of these amino acids should also be further investigated.

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Supplementary information

Fig. S1. Inhibition effects of different fractions separated from *Bacillus amyloliquefaciens* T1 on the growth of *M. aeruginosa* (The treatment from left to right was CK, CH_3OH, fraction A1, G1, G2, G3 and G4, respectively).

Fig. S2. Inhibition effects of different fractions separated from *Bacillus amyloliquefaciens* T1 on the growth of *M. aeruginosa* (The treatment from left to right was CK, CH_3OH, the cell-free filtrate, fraction A1 and A2, respectively).

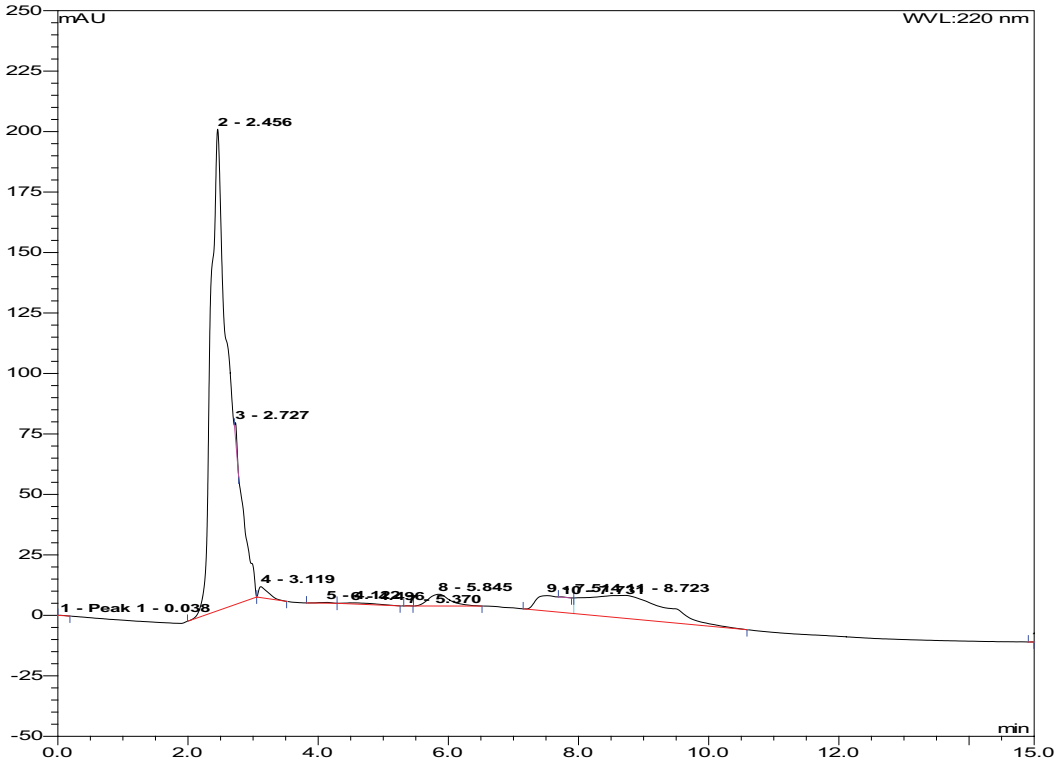

Fig. S3. HPLC chromatogram of the L-lysine.

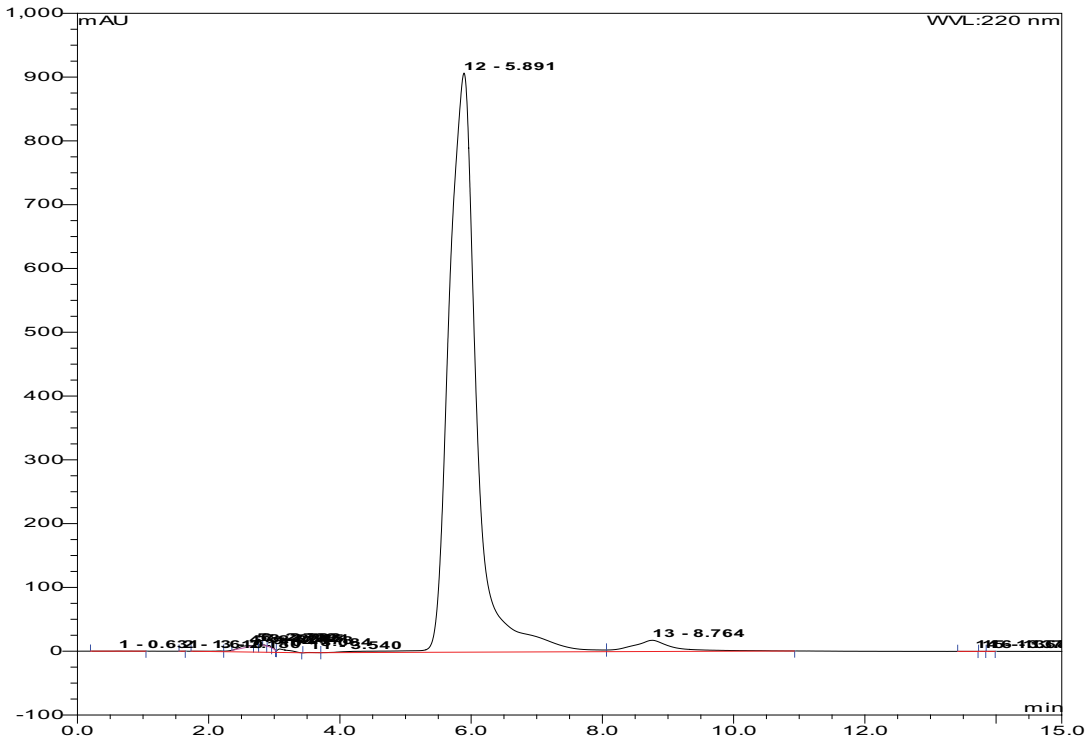

Fig. S4. HPLC chromatogram of the L-phenylalanine.

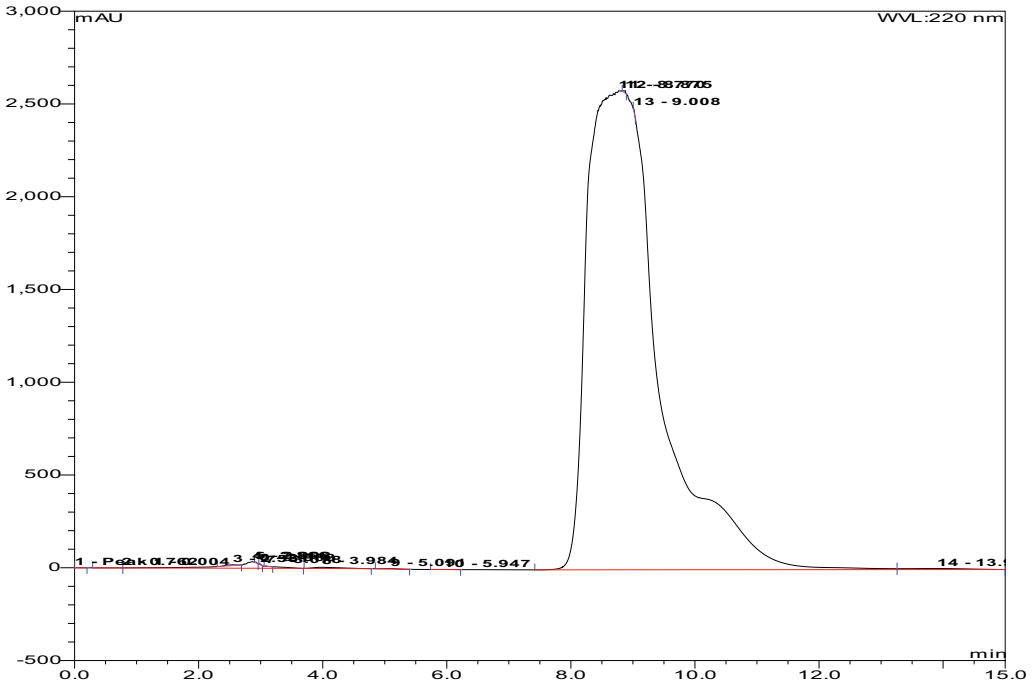

Fig. S5. HPLC chromatogram of the L-phenylalanine.