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Original Research

Physiological Responses of *Microcystis aeruginosa* to Extracellular Degradative Enzymes and Algicidal Substance from Heterotrophic Bacteria

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Abstract

It is a promising way to control *Microcystis* bloom by the algicidal substances from some special heterotrophic bacteria. However, the algicidal mechanism of the common known 1-hydroxyphenazine and the potential impact of extracellular degradative enzymes from total accompanying heterotrophic bacteria on its algicidal characteristics remains unknown. Here, the physiological changes of *Microcystis aeruginosa* were investigated under the stress of 1-hydroxyphenazine and extracellular degradative enzymes individually or together. The results showed that the extracellular degradative enzymes from heterotrophic bacteria had a weak inhibitory effect on the growth of *M. aeruginosa* and made *M. aeruginosa* suffered oxidative damage. 1-hydroxyphenazine promoted the cells death of *M. aeruginosa* with a manner independent of reactive oxygen species (ROS) level. 1-hydroxyphenazine might play a role in promoting the cyclic electron transport to reduce ROS in *M. aeruginosa*. The reduction of total anti-oxidative capacity and the depletion of glutathione might induce the death of *M. aeruginosa* under stress of 1-hydroxyphenazine. The addition of extracellular degradative enzymes eventually delayed the algae death and alleviated the inhibitory effect of 1-hydroxyphenazine on algal ATPase hydrolytic activity and total antioxidant capacity. The heterotrophic partnership seemed to be helpful to increase the stress resistance of *M. aeruginosa*.

Keywords: *Microcystis aeruginosa*, heterotrophic bacteria, extracellular enzymes, algicidal substance, 1-hydroxyphenazine, heterotrophic partnership

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Introduction

As global warming and eutrophication of water bodies, cyanobacteria blooms are boosted [1]. A series of environmental problems have been aroused worldwide, such as water hypoxia, release of toxic secondary metabolites [2]. Toxigenic *Microcystis aeruginosa* is a primary bloom-forming cyanobacteria in the world [3]. Because of the hepatotoxicity and potential hepatocarcinogenicity of microcystins, much attention has been focused on *M. aeruginosa*.

Heterotrophic bacteria are important members accompanying cyanobacteria in aquatic ecosystem. About 14% of organic carbon required by heterotrophic bacteria are provided by extracellular organic carbon from phytoplankton [4]. *M. aeruginosa* can be considered as incubator for heterotrophic bacteria when it becomes dominant in phytoplankton in eutrophic water [5]. Colonial *M. aeruginosa* cells are surrounded by thick surface layer which is mainly composed of polysaccharides and proteins. Materials in surface layer can be absorbed by algae itself and also be used by heterotrophic bacteria [6]. Furthermore, many members of heterotrophic bacteria are active in the decomposition of *Microcystis* [7].

Some *Microcystis*-lying bacteria are found among heterotrophic bacteria associated with *Microcystis* bloom [8]. Their number is directly related to the density of algae [9]. However, the control of cyanobacteria is dependent on a density threshold at which algicidal bacteria can release lytic substances and induce algal cell disruption [10]. It is a promising way to control *Microcystis* bloom by the lytic substances because of the breakthrough of density limitation. Nowadays, many lytic substances against cyanobacteria have been identified from bacterial exudate [8]. Although some algicidal substances have been revealed to damage the algae cell wall and elevate the reactive oxygen species (ROS) and malondialdehyde in algae cell [11-12], it is not enough. The lytic mechanism of many known algicidal subtances with different structures against cyanobacteria has not been thoroughly explored. *Pseudomonas aeruginosa* is a common known algicidal bacterium in the colonies of *M. aeruginosa*. 1-hydroxyphenazine as a relatively stable and algicidal substance was identified from *P. aeruginosa* [13-14]. But little is known about how its algicidal subtances such as 1-hydroxyphenazine inhibit the growth of *M. aeruginosa*.

Colonial *M. aeruginosa* cells are found to be less sensitive to algicidal bacteria than unicellular cells in lab [15]. Thick surface layer may act as buffers against some damages [16]. However, the concentration of total heterotrophic bacteria are on average 23 times as much as cyanobacteria in eutrophic waters [17]. The activities of extracellular degradative enzymes from heterotrophic bacteria can rapidly increase with the activity of algal photosynthesis [18]. Moreover, the role of heterotrophic bacteria can be further enhanced because of higher dissolved organic carbon concentrations resulted from mixing events or high flows [19]. Whether extracellular degradative enzymes of total heterotrophic bacteria can interfere with the buffering effect of algal surface layer and promote the algicidal effect remains unknown.

The aims of this study were to explore the physiological changes of *M. aeruginosa* under stress of 1-hydroxyphenazine and the influence of extracellular degradative enzymes from heterotrophic bacteria on these algicidal characteristics. For this, the cells density, oxidative stress, energy status, intracellular iron-binding capacity and fructose 1, 6-diphosphatase activity of *M. aeruginosa* were investigated under the stress of 1-hydroxyphenazine and extracellular degradative enzymes from heterotrophic bacteria individually or together. We hope it is helpful to make better use of algicidal substances to control cyanobacteria bloom.

Materials and Methods

Strain and Culturing Conditions

The microcystin-producing strain *Microcystis aeruginosa* (FACHB-912) was obtained from Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). This strain was cultured in 10% modified Hoagland's medium (pH 7.0) [20] supplemented with Ca^{2+} at a final concentration of 36 mg L^{-1} in incubator to maintain colonial status. Medium was autoclaved at 121ºC for 20 min before used. The incubator was maintained at 28ºC with a constant relative humidity of 75% and illuminated with the density of 200 μmol photons m^{-2} s^{-1} in 12-hour diurnal cycles.

Preparation of Extracellular Degradative Enzymes

The heterotrophic bacteria was obtained from surface sediment-water mixture of Taihu lakeside wetland in Yixing (China) where was suffered from *Microcystis* bloom. Heterotrophic bacteria was enriched in the same 10% modified Hoagland's medium (pH 7.0) which was supplemented with tryptone (2 g L^{-1}), yeast extract (2 g L^{-1}) and NaCl (1 g L^{-1}). The community structure of these culturable bacteria was shown in Fig. 1 by analysis of high-throughput sequencing at Shanghai Personal Biotechnology Co., Ltd. (China). The main culturable bacteria belonged to proteobacteria. After cultured at 28ºC for 18 h with shaking at 100 rpm, cells were discarded from culture fluids by centrifugation at 4ºC, 8000 *g* for 15 min. Then, extracellular enzymes was precipitated from non-cell culture fluids by ammonium sulfate (60% saturation). Enzymes precipitate was dissolved in 10 mM HEPES buffer (pH 7.0), dialyzed in the same HEPES buffer for 6 hours at 4ºC and dialyzed again in 10% modified Hoagland's medium (pH 7.0) for overnight at 4ºC. These buffer and medium used for dialysis were autoclaved at 121ºC for 20 min. Enzymes

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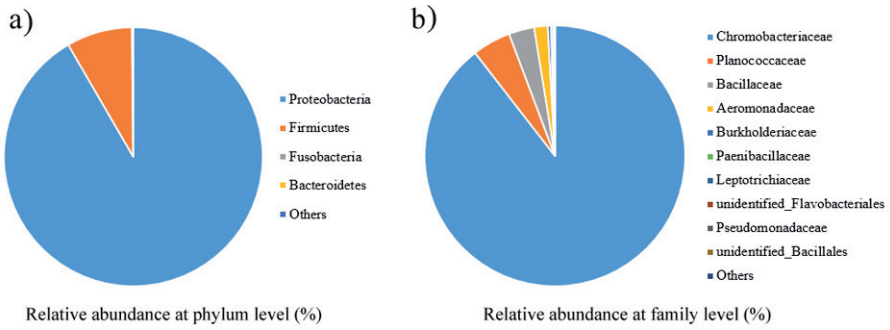

Fig. 1. The community structure of the culturable bacteria.

concentration was determined by the Bradford method using BSA as a standard.

Experimental Design

M. aeruginosa was amplified in 10% modified Hoagland's medium supplemented with Ca^{2+} for 7 days and then transferred into aseptic conical flasks (2 L) when it was at exponential growth phase (5.37×10^6 cells ml^{-1}). Next then, 1-hydroxyphenazine (purity >99%, Tokyo Chemical Industry Co., Ltd., Japan) and extracellular enzymes were then respectively added into *M. aeruginosa* cultures in flasks at final concentration of 5.00 mg L^{-1} and 1.75 mg L^{-1} with the exposure time from 0 to 4 days, while the *M. aeruginosa* cultures without 1-hydroxyphenazine and extracellular enzymes were used as controls (Fig. 2). The dose of 1-hydroxyphenazine was selected based on its lower concentration for effective growth inhibition of *M. aeruginosa* in our preliminary experiments. The amount of extracellular enzymes was applied according to the secretion of heterotrophic bacteria at the concentration of 1.07×10^7 cells ml^{-1} which was close to the bacterial density in cyanobacteria-blooming Taihu Lake in summer. Samples were taken for analysis of algal density and physiological parameters at 1-day interval. All experiments were conducted with three independent replicates. The culture fluids were stirred at 300 rpm for 5 min twice a day by magnetic force. All treatments were cultivated under the culturing conditions described above.

Measurement of Algae Density

Algae fluids were used for analysis. Algae cells were counted on a hemocytometer using a microscope (XS-213; Jiangnan Optical Instrument Factory, Nanjing, China), after stained with Lugol's iodine solution.

Determination of Physiological Parameters

Algae cells were harvested by centrifugation at 4ºC, 5000 *g* for 5 min, washed with 10 mM HEPES buffer (pH 7.0) and resuspended in same HEPES buffer. The ultrasonic cell lysate was cleared of cell debris using centrifugation at 4ºC, 8000 *g* for

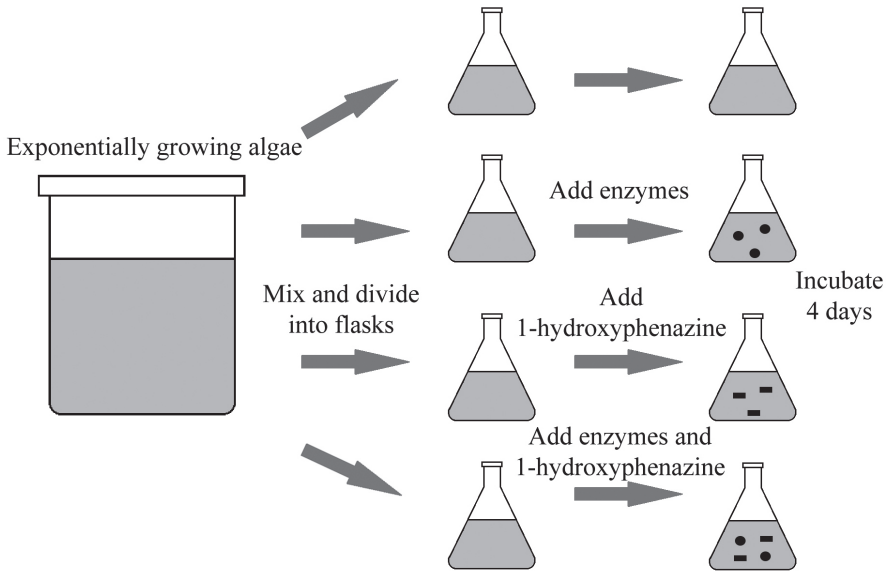

Fig. 2. Schematic diagram of the experimental procedure.

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10 min and then used for analysis of physiological parameters. Protein concentration was quantified by the Bradford method using BSA as a standard. Total anti-oxidative capability (T-AOC) was measured by ferric ion reduction method using the T-AOC detection kit (Jiancheng Bioengineering Institute, China). The total glutathione (T-GSH) content was assayed by 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) method using a commercial kit (Jiancheng Bioengineering Institute, China). Malondialdehyde (MDA) content was detected by thibabituric acid (TBA) method using a commercial kit (Jiancheng Bioengineering Institute, China). The content of nitrotyrosine-containing proteins was determined by competitive enzyme immunoassay method using OxiSelectTM nitrotyrosine ELISA kit (Cell Biolabs Inc., USA). ATP content was determined by phosphomolybdic acid clolrimetric method using a commercial kit (Jiancheng Bioengineering Institute, China). Total ATPase hydrolytic activity was detected by the method of inorganic phosphate determination using ultramicro total ATPase assay kit (Jiancheng Bioengineering Institute, China). Fructose 1, 6-diphosphatase (FBPase) activity was assayed by double antibody sandwich method using a commercial ELISA kit (Jiangsu Feiya Biotechnology Co., Ltd, China). Total iron-binding capacity (TIBC) was measured by bipyridine colorimetric method using a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China). All procedures were performed in accordance with manufacturer's instructions. All assays were carried out in triplicate.

Statistical Analysis

Data were presented as mean±standard deviation (SD). Differences between controls and treatments were determined by one-way ANOVA. Pearson correlation analysis was used to analyze the correlation among parameters. Linear regression analysis was used to test the relationship. Statistical significances were conformed at $p<0.05$. All statistical analyses were performed using IBM SPSS Statistics 20.0 (IBM SPSS Inc., USA).

Results

Effect of Extracellular Degradative Enzymes and Algicidal Substance from Bacteria on the Growth of *M. aeruginosa*

When extracellular enzymes and 1-hydroxyphenazine from heterotrophic bacteria were exposed to *M. aeruginosa* separately or together, the algal growth was found inhibited to different extents compared to the controls (Fig. 3). Algae density was significantly lower in enzymes treatments than that in controls after 3 days. Algae cell death was triggered both in 1-hydroxyphenazine treatments and in combined treatments of enzymes and 1-hydroxyphenazine (hereafter called combined treatments). However, the remaining algae density was notably higher in combined treatments than that in 1-hydroxyphenazine treatments after 3 days.

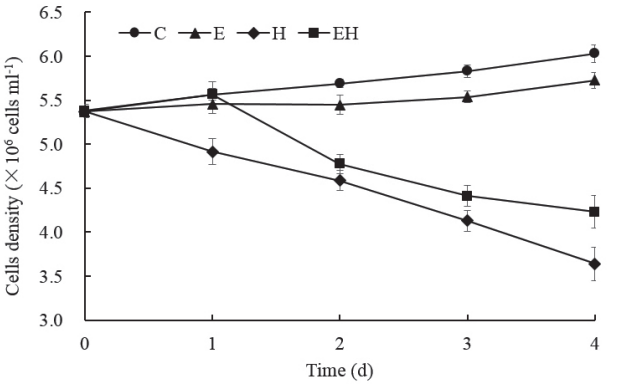

Fig. 3. Effect of extracellular degradative enzymes and 1-hydroxyphenazine from bacteria on the growth of *M. aeruginosa*. C: control; E: enzymes treatments; H: 1-hydroxyphenazine treatments; EH: combined treatments of enzymes and 1-hydroxyphenazine.

Effect of Extracellular Degradative Enzymes and Algicidal Substance from Bacteria on Oxidative Stress of *M. aeruginosa*

Compared with controls, algal MDA content was apparently elevated by a maximum of 136% in enzymes treatments (Fig. 4a). But algal MDA content in 1-hydroxyphenazine treatments and combined treatments were both close to controls. There was not significant differences between 1-hydroxyphenazine treatments and combined treatments. Correlation analysis revealed that algal MDA content had notably negative linear correlation with algae density in controls (r= -0.678, $p<0.05$), while it had notably positive linear correlation with algae density in 1-hydroxyphenazine treatments (r = 0.912, $p<0.05$) and combined treatments (r = 0.822, $p<0.05$). But this correlation was not significant in enzymes treatments.

Algal nitrotyrosine-containing proteins level in enzymes treatments was significantly higher than that in controls after 2 days, and then dropped to controls level at day 4 (Fig. 4b). Algal nitrotyrosine-containing proteins level in combined treatments was similar to that of controls. Algal nitrotyrosine-containing proteins level in 1-hydroxyphenazine treatments was not significantly different from controls level until 4 days later. Correlation analysis showed that algal nitrotyrosine-containing proteins level had significantly negative linear correlation with algae density in controls (r = -0.645, $p<0.05$), while that had significantly positive linear correlation with algae density in combined treatments (r = 0.630, $p<0.05$). But this

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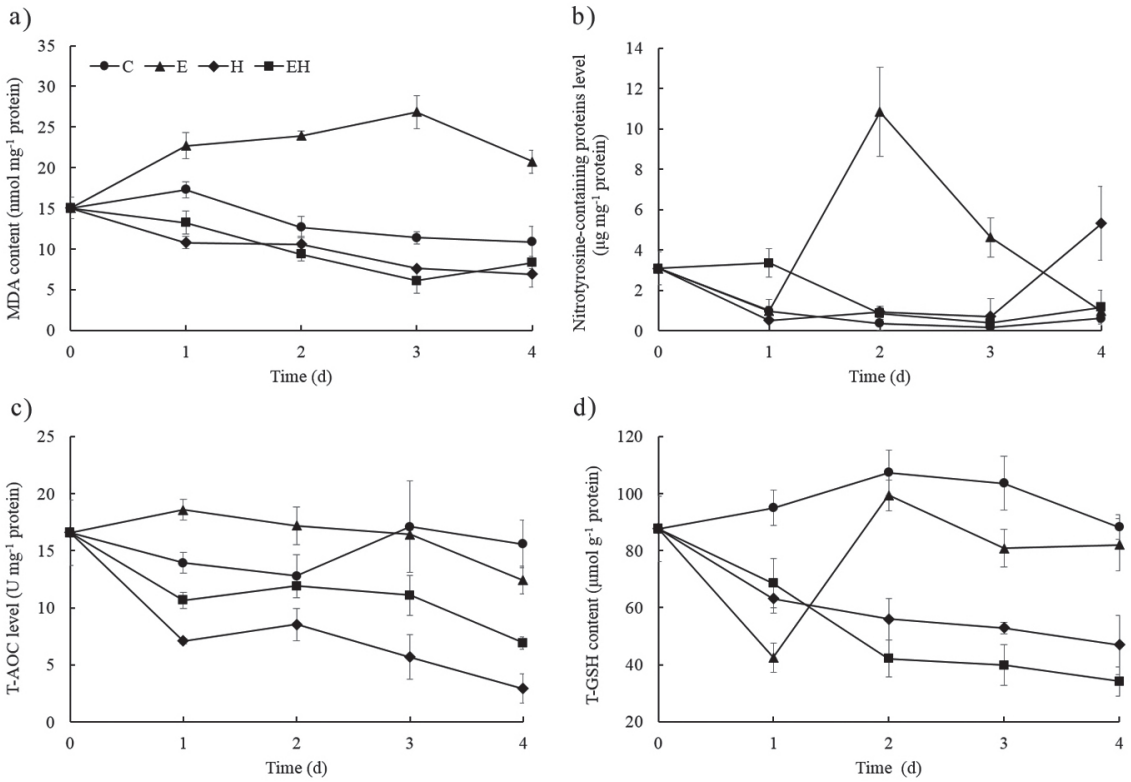

Fig. 4. Effect of extracellular degradative enzymes and 1-hydroxyphenazine from bacteria on oxidative stress of *M. aeruginosa*. a) MDA content; b) Nitrotyrosine-containing proteins level; c) T-AOC level; d) T-GSH content. C: control; E: enzymes treatments; H: 1-hydroxyphenazine treatments; EH: combined treatments of enzymes and 1-hydroxyphenazine.

correlation was not significant in enzymes treatments and 1-hydroxyphenazine treatments.

Algal T-AOC in controls was kept in a dynamic balance. Algal T-AOC in enzymes treatments was only notably higher than that in controls at day 2 and then dropped to controls level (Fig. 4c). Algal T-AOC in 1-hydroxyphenazine treatments was significantly lower than that in controls at day 1, 3 and 4. Although Algal T-AOC in combined treatments was significantly lower than that in controls at last, it was still significantly higher than that in 1-hydroxyphenazine treatments. Correlation analysis indicated that algal T-AOC had significantly positive linear correlation with algae density in1-hydroxyphenazine treatments ($r = 0.828$, $p<0.05$) and combined treatments ($r= 0.598$, $p<0.05$). But this correlation was not significant in controls and enzymes treatments.

Algal T-GSH content in controls was also maintained at a dynamic balance. Algal T-GSH content in enzymes treatments was cut down fleetly and then recovered to controls level (Fig. 4d). Algal T-GSH content no matter in 1-hydroxyphenazine treatments or in combined treatments was significantly lower than that in controls. But there was not significant differences between 1-hydroxyphenazine treatments and combined treatments. Correlation analysis revealed that algal T-GSH content had significantly positive linear correlation with algae density in1-hydroxyphenazine treatments ($r = 0.763$, $p<0.05$) and combined treatments ($r = 0.843$, $p<0.05$). But this correlation was not significant in controls and enzymes treatments.

Effect of Extracellular Degradative Enzymes and Algicidal Substance from Bacteria on Intracellular Iron-Binding Capacity of *M. aeruginosa*

As shown in Fig. 5, algal TIBC was significantly reduced by a maximum of 55% in enzymes treatments compared to controls. Algal TIBC in 1-hydroxyphenazine treatments was significantly less than that in enzymes treatments after 2 days. Algal TIBC was notably reduced by a maximum of 85% in 1-hydroxyphenazine treatments compared to control. But there was not significant differences between 1-hydroxyphenazine treatments and combined treatments. Correlation analysis showed that algal TIBC had significantly positive linear correlation with algae density in 1-hydroxyphenazine treatments ($r = 0.836$, $p<0.05$) and combined treatments ($r = 0.652$, $p<0.05$), while that had significantly negative linear correlation with algae density in enzymes treatments ($r = -0.643$, $p<0.05$). But this correlation was not significant in controls.

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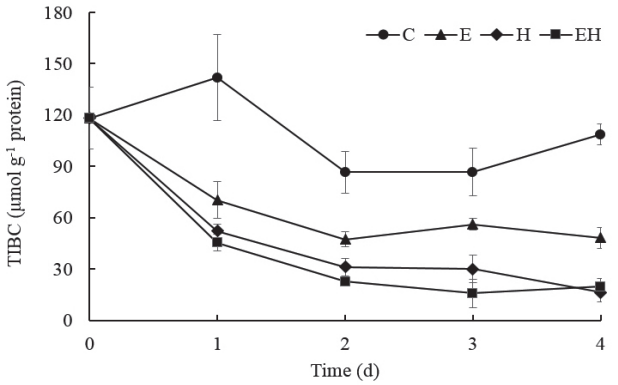

Fig. 5. Effect of extracellular degradative enzymes and 1-hydroxyphenazine from bacteria on intracellular iron-binding capacity of *M. aeruginosa*. C: control; E: enzymes treatments; H: 1-hydroxyphenazine treatments; EH: combined treatments of enzymes and 1-hydroxyphenazine.

Effect of Extracellular Degradative Enzymes and Algicidal Substance from Bacteria on FBPase Activity of *M. aeruginosa*

As shown in Fig. 6, algal FBPase activity in enzymes treatments was close to that in controls with no significant difference. Algal FBPase activity was apparently promoted in 1-hydroxyphenazine treatments compared to controls. But algal FBPase activity in combined treatments was increased one day later than that in 1-hydroxyphenazine treatments. And finally, algal FBPase activity in combined treatments was significantly lower than that in 1-hydroxyphenazine treatments. Correlation analysis indicated that algal FBPase activity had significantly negative linear correlation with algae density in 1-hydroxyphenazine treatments ($r = -0.975$, $p<0.05$) and combined treatments ($r = -0.921$, $p<0.05$). But this correlation was not significant in controls and enzymes treatments.

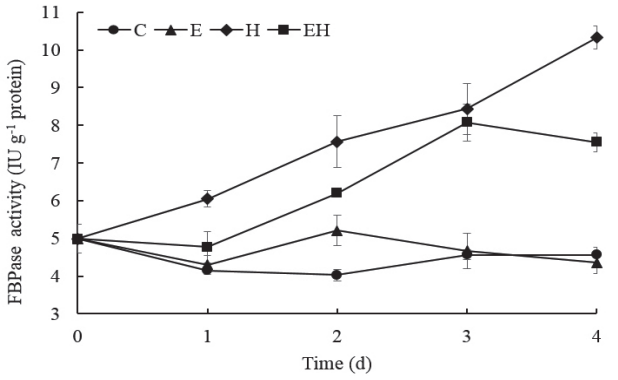

Fig. 6. Effect of extracellular degradative enzymes and 1-hydroxyphenazine from bacteria on FBPase activity of *M. aeruginosa*. C: control; E: enzymes treatments; H: 1-hydroxyphenazine treatments; EH: combined treatments of enzymes and 1-hydroxyphenazine.

Effect of Extracellular Degradative Enzymes and Algicidal Substance from Bacteria on Energy Balance of *M. aeruginosa*

Algal ATP level in enzymes treatments and in combined treatments were both not significantly different from that of controls (Fig. 7a). Algal ATP level in 1-hydroxyphenazine treatments was fleetly increased and then recovered to controls level. Correlation analysis indicated that algal ATP level had significantly positive linear correlation with algae density in controls ($r = 0.594$, $p<0.05$). But this correlation was not significant in any other treatments.

Algal ATPase hydrolytic activity in enzymes treatments was kept at the same level of controls after a short promotion (Fig. 7b). Algal ATPase hydrolytic activity in 1-hydroxyphenazine treatments was gradually inhibited and was significantly lower than that in controls after 2 days. Algal ATPase hydrolytic activity in combined treatments was finally significantly inhibited after a short promotion. But algal ATPase hydrolytic activity in combined treatments was still apparently higher than that in 1-hydroxyphenazine treatments over time. Correlation analysis showed that algal ATPase hydrolytic activity had significantly positive linear correlation with algae density in 1-hydroxyphenazine treatments ($r = 0.882$, $p<0.05$) and combined treatments ($r = 0.742$, $p<0.05$). But this correlation was not significant in controls and enzymes treatments.

Discussion

1-hydroxyphenazine showed a strong algicidal activity. Cells death in *M. aeruginosa* was apparently promoted under the stress of 1-hydroxyphenazine. Many algicidal substances were known to mainly promote the production of ROS, lead to oxidative damage of cellular components, interfere with the photosynthesis and result in the lack of energy [21-22]. But the algicidal mode of 1-hydroxyphenazine was a little different. When algal cell death occurred under the stress of 1-hydroxyphenazine, the MDA content of *M. aeruginosa* did not increase and the nitrotyrosine-containing proteins level of *M. aeruginosa* was not elevated until day 4. MDA and nitrotyrosine-containing proteins were usually used as biomarkers to reflect oxidative damage of cells by ROS [23-24]. Furthermore, when algal cell death occurred under the stress of 1-hydroxyphenazine, algal ATP content was still maintained at a dynamic balance and eventually closed to controls. FBPase was a key regulatory enzyme in the Calvin cycle of cyanobacteria for carbon fixation. FBPase gene was reported essential to sustain growth for cyanobacteria [25]. Overexpression of FBPase was proved to increase the growth of cyanobacteria [26]. FBPase was also considered as the target for some algicides to control algal blooms. But

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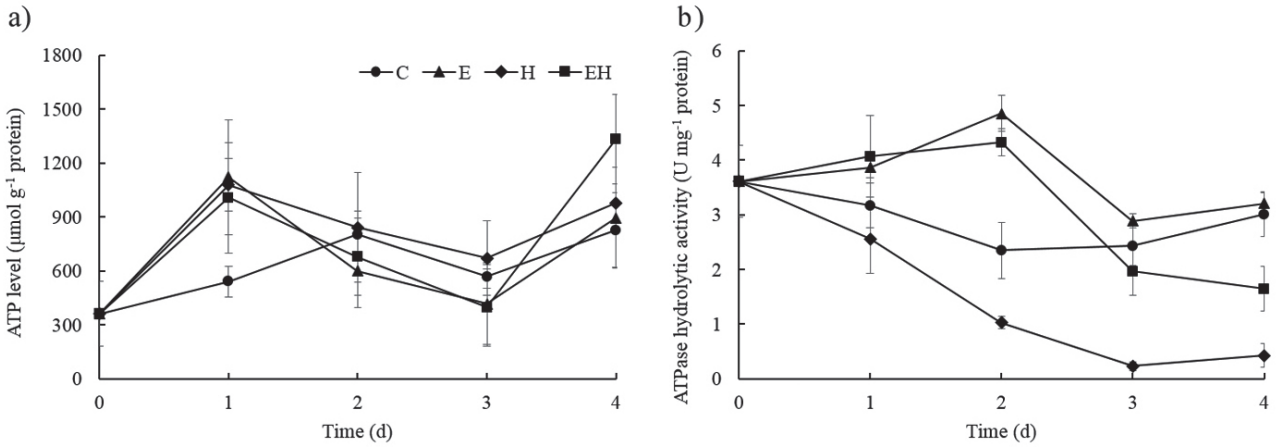

Fig. 7. Effect of extracellular degradative enzymes and 1-hydroxyphenazine from bacteria on energy balance of *M. aeruginosa*. a) ATP level; b) ATPase hydrolytic activity. C: control; E: enzymes treatments; H: 1-hydroxyphenazine treatments; EH: combined treatments of enzymes and 1-hydroxyphenazine.

our results indicated that FBPase was not the target of 1-hydroxyphenazine. FBPase has been found to be sensitive to the AMP∶ATP ratio [27]. Our data showed that ATPase hydrolytic activity of *M. aeruginosa* was severely inhibited by 1-hydroxyphenazine. That was likely to reduce the production of AMP and result in the stimulation of FBPase. Previous research found that *Scenedesmus obliquus* (green algae) was able to undergo inhibition in a ROS-independent manner [28]. HeLa cells and *Escherichia coli* were also reported to undergo apoptosis with a manner independent of ROS level, but dependent on GSH depletion [29-30]. Based on our data, it was assumed that the reduction of total anti-oxidative capacity and the depletion of GSH might induce the death of *M. aeruginosa* under the short-term stress of 1-hydroxyphenazine.

The extracellular degradative enzymes from heterotrophic bacteria showed an inhibitory effect on the growth of *M. aeruginosa* and made *M. aeruginosa* suffered oxidative damage. These results were consistent with that of some heterotrophic bacteria acting on cyanobacteria [31-32]. The accumulation of ROS was found to be closely related to the rate of photosynthetic electron transport [33]. But flavodoxin level in cyanobacteria was found to be elevated under iron deficiency and was then conducive to scavenging ROS [34]. *Synechococcus* sp. (cyanobacteria) was reported to downregulate the genes that encoded iron-rich proteins when co-cultured with *Vibrio parahaemolyticus* [35]. According to our data, lower TIBC of *M. aeruginosa* might reflect a physiological iron-deficiency in 1-hydroxyphenazine treatments and combined treatments. However, the TIBC of *M. aeruginosa* in enzymes treatments was also reduced a little compared to controls. Therefore, flavodoxin might not be the main factor which led to ROS reduction in 1-hydroxyphenazine treatments and combined treatments. In addition, there existed photosystem I-NAD(P)H dehydrogenase cyclic electron flow in cyanobacteria which only produced ATP [36]. The oxidative stress could be alleviated by the enhancement of cyclic electron transport [37]. Based on the status of algal cellular ATP pool in our experiments, it was likely that 1-hydroxyphenazine might enhance the cyclic electron transport in *M. aeruginosa* and extracellular degradative enzymes could not prevent the effect of 1-hydroxyphenazine on the cyclic electron transport. This assumption needed to be further tested. Moreover, it was interesting that cells death of *M. aeruginosa* was delayed and alleviated even when they were oppressed by the double role of 1-hydroxyphenazine and extracellular degradative enzymes, compared to 1-hydroxyphenazine treatments. The addition of extracellular degradative enzymes eventually alleviated the inhibitory effect of 1-hydroxyphenazine on ATPase hydrolytic activity and total antioxidant capacity. Contrary to our original assumption, these data seemed to support that heterotrophic partnership was helpful to increase the stress resistance of *M. aeruginosa*. In order to get the same inhibitory effect on *Microcystis*, more algicidal substances might be needed in the environment with accompanying bacteria. Although there were some differences between culturable bacteria and natural bacteria communities, it was a meaningful attempt to distinguish the different roles of extracellular degradative enzymes and algicidal substance from heterotrophic bacteria to cope with *M. aeruginosa*.

Conclusions

1-hydroxyphenazine had a lytic effect on *M. aeruginosa* cells with a manner independent of ROS level. The reduction of total anti-oxidative capacity and the depletion of GSH might be the important modes by which 1-hydroxyphenazine acted on *M. aeruginosa*. The extracellular degradative enzymes from heterotrophic bacteria had a weak inhibitory effect on the growth of

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M. aeruginosa and made *M. aeruginosa* suffered oxidative damage. But the addition of extracellular degradative enzymes eventually alleviated the inhibitory effect of 1-hydroxyphenazine on *M. aeruginosa*. More algicidal substances might be needed to deal with *M. aeruginosa* in natural environment with accompanying bacteria. Further investigation of comparative transcriptomics is warranted to make it clearer how the heterotrophic partnership increases the stress resistance of *M. aeruginosa*. It will help us make better use of algicidal substances to eliminate harmful algal blooms.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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