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Effects and mechanisms of L-phenylalanine on growth of *Microcystis aeruginosa*

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Abstract: The effects and possible mechanisms of action of L-phenylalanine on the growth of *Microcystis aeruginosa* cells were explored by cell counting and flow cytometry assays. L-phenylalanine promoted the growth of *Microcystis aeruginosa* at concentrations between 0.078 and 0.312 μg/mL, but inhibited growth at concentrations between 0.625 and 20 μg/mL in 24 h exposure. The dose-effect and time-course relationships between exposure to L-phenylalanine and growth inhibition of *Microcystis aeruginosa* were observed. The IC_{50} value of L-phenylalanine for growth inhibition of *Microcystis aeruginosa* was 6.2 μg/mL (95% confidence interval was 0.005 to 16.76 μg/mL). The membrane integrity of the cells showed significant variations after 24 h exposure to L-phenylalanine. Meanwhile, no effects on esterase activity of the cells were observed until after 48 h exposure to L-phenylalanine. In conclusion, L-phenylalanine has hormesis effects and algae control effects on *Microcystis aeruginosa*. The latter is closely related to alterations or disorders in the cell membrane and with variation of esterase activity in the cells.

Key words: algae control; L-phenylalanine; mechanisms; flow cytometry; membranes; growth

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Blooms of cyanobacteria (blue-green algae) are widespread in freshwater lakes and reservoirs throughout the world, especially during the summer. Algal blooms produce many toxic substances[1]. Toxic blooms are known to cause major problems in water supplies, resulting in growing public health concerns[2]. A number of restoration technologies have been applied to control blooms of *Microcystis* species and other cyanobacteria[3]. Algicidal bacteria are an important part of the structure and function of an aquatic biological ecosystem and play a very important role in maintaining the balance of the algal biomass[4]. In recent years, it has been suggested that the sudden disappearance of water blooms may be related to bacterial infection[5]. Therefore, algicidal bacteria and their algae-lysing bioactive substances have attracted increasing attention as a biological means to prevent and treat algal blooms[6-7].

A native algicidal bacterium from Taihu Lake with activity against *Microcystis aeruginosa* was found in our previous study. The strain was tentatively identified as *Aeromonas punctata* based on its physiology, biochemical reactions, and 16S rDNA sequence. The bacterium lyses algal cells by producing extracellular bioactive substances, one of which was determined to be L-phenylalanine by LC/MS-IT-TOF[8]. The purpose of the present work is to explore the characteristics and possible mechanisms of L-phenylalanine-mediated inhibition of the growth of *Microcystis aeruginosa*.

1 Materials and Methods

1.1 Algal cultures

Microcystis aeruginosa was purchased from the Freshwater Algal Culture Collection of the Institute of Hydrobiology, China. *Microcystis aeruginosa* was cultured in the BG-11 medium[9]. The algal cells were maintained as unialgal axenic cultures at 28 ℃ and a pH of 7.2, with illumination at 150 μmol photons per m^2 per s under a 12-h light/12-h dark (12L/12D) regimen.

1.2 Effect of L-phenylalanine on the growth of *Microcystis aeruginosa*

For the detection of growth inhibition by L-phenylalanine, algal cells in the logarithmic growth phase (3×10^6 cells/mL) were inoculated in the BG-11 medium. The stock solution of L-phenylalanine at 400 μg/mL was used to make serial twofold dilutions in the BG-11 medium. The final concentrations of L-phenylalanine in the algal cultures were 0.078 to 20 μg/mL. After 24, 48, and 72 h, the number of algal cells was counted using a hemacytometer at a magnification of 400×. Three replicate cultures were used for every L-phenylalanine concentration.

1.3 Influence factors to the growth of *Microcystis aeruginosa*

Algal cells in the logarithmic growth phase (3×10^6 cells/mL) were inoculated in the BG-11 medium and were exposed to L-phenylalanine at the concentration of 10 μg/mL. The experimental variables included culture temperatures (4, 28, or 37 ℃), pH values of the medium (pH was adjusted to 5, 7, or 9 by 0.1 mol/L HCl or 0.1 mol/L NaOH) and culture illumination conditions (light-only, 12L/12D, or dark-only).

1.4 Membrane integrity and esterase activity of *Microcystis aeruginosa*

Algal cells in the logarithmic growth phase (3×10^6 cells/mL) were inoculated in the BG-11 medium and were exposed to different concentrations of L-phenylalanine (0, 0.625, 1.25, 2.5, 5, 10, or 20 μg/mL). After 2, 24, and 48 h, membrane integrity and esterase activity were determined simultaneously using a FACSCalibur Flow Cytom-

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eter (Becton Dickinson, USA) equipped with a xenon-ion excitation lamp (488 nm). The fluorescence of cells stained with propidium iodide (PI) was measured to assess changes in the membrane integrity of cells exposed to L-phenylalanine. Esterase activity was interpreted as the mean rate of fluorescein diacetate (FDA) conversion to fluorescein, expressed as the mean fluorescein fluorescence. The flow cytometer assay provided fluorescence data for individual cells, so frequency histograms of these data were used to define esterase activity states. Stock solutions of PI (dissolved in double distilled water) and FDA (dissolved in acetone) were prepared at concentrations of 100 μmol/L and 1 mmol/L, respectively, and were stored at 4 and −20 ℃, respectively. For each algal culture sample, 600 μL of algal culture was placed in a polystyrene tube with either 10 μL of PI stock solution or 20 μL of FDA stock solution. The samples were incubated at room temperature for 8 min for the PI assay and 15 min for the FDA assay.

1.5 Statistical analysis

L-phenylalanine inhibition effects were expressed as an IC_{50} value. The IC_{50} value and 95% confidence interval were calculated using SPSS 13.0. After testing the data for normality and homogeneity of variance, single-factor analysis of variance (one-way ANOVA) was used to determine which treatments (PI or FDA) differed significantly from the control at a level of significance of 0.05.

2 Results

2.1 Effect of L-phenylalanine on growth of *Microcystis aeruginosa*

The growth of *Microcystis aeruginosa* was promoted by L-phenylalanine at concentrations between 0.078 and 0.312 μg/mL, whereas L-phenylalanine inhibited the growth of *Microcystis aeruginosa* at concentrations between 0.625 and 20 μg/mL in 24 h cultures (see Fig. 1). The lowest effective concentration to inhibit the growth of *Microcystis aeruginosa* was 0.625 μg/mL. At 20 μg/mL, L-phenylalanine inhibited almost 70% of the cell growth of *Microcystis aeruginosa* during a 24 h exposure. Therefore, *Microcystis aeruginosa* responded to L-phenylalanine in a dose-dependent manner ($r = 0.99$, $P < 0.01$). The IC_{50} value of L-phenylalanine for growth inhibition of *Microcystis aeruginosa* in 24 h cultures was 6.2 μg/mL (95% confidence interval was 0.005 to 16.76 μg/mL). It can therefore be assumed that L-phenylalanine had both hormesis effects and algal control effects on *Microcystis aeruginosa*.

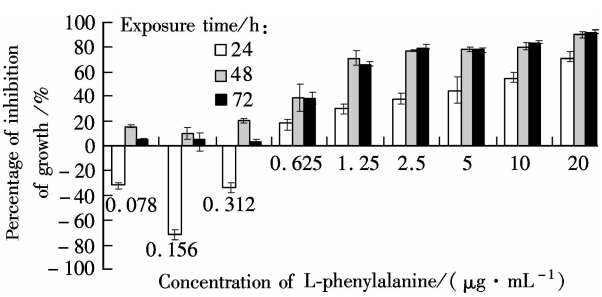

Fig. 1 Effects of various concentrations of L-phenylalanine on growth of *Microcystis aeruginosa*

2.2 Factors influencing the growth of *Microcystis aeruginosa*

The effects of culture temperatures, pH values of the medium, and culture illumination conditions on the growth of *Microcystis aeruginosa* exposed to L-phenylalanine at 10 μg/mL are shown in Fig. 2. The optimal parameters are as follows: culture temperature is at 37 ℃, the medium is at a pH of 5, and the culture illumination condition is 12-h light/12-h dark.

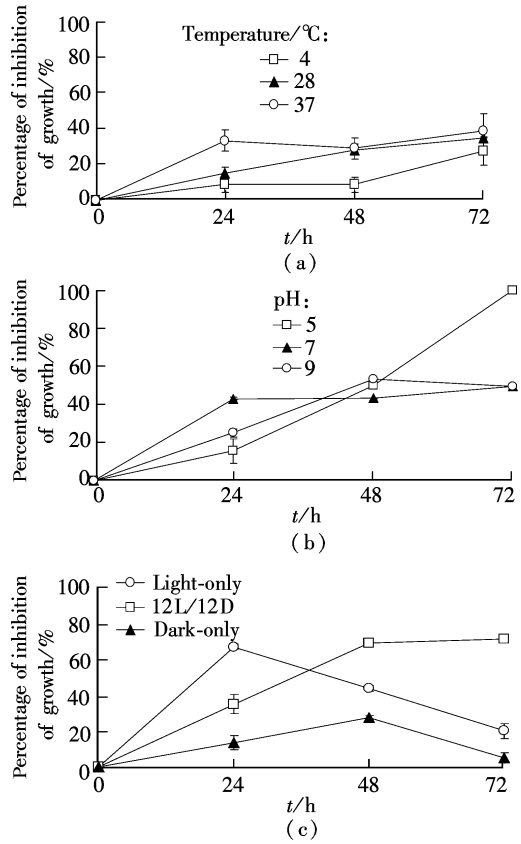

Fig. 2 Effects of various factors on the growth of *Microcystis aeruginosa* cultures containing L-phenylalanine at 10 μg/mL. (a) Culture temperature; (b) pH value of the medium; (c) Culture illumination condition

2.3 Effect of L-phenylalanine on the membrane integrity of *Microcystis aeruginosa*

The effect of L-phenylalanine on membrane integrity was measured after 2, 24, and 48 h exposure using PI fluorescence detection by flow cytometry (see Fig. 3). The "*" indicates a significant ($P < 0.05$) difference from the control. At various concentrations of L-phenylalanine, the percentage of the fluorescent cells of *Microcystis aeruginosa*, as measured by PI staining, did not show statistically significant variation ($P > 0.05$) relative to the control after 2 h exposure. In other words, short exposure to L-phenylalanine (2 h) had no effects on membrane integrity. However, after 24 h and 48 h exposure, cells exposed to L-phenylalanine at concentrations between 1.25 and 20 μg/mL showed statistically significant increases ($P < 0.05$), although the intensity of orange fluorescence decreased after 48 h exposure.

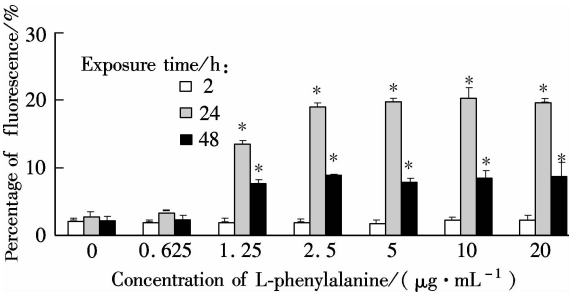

Fig. 3 Membrane integrity of *Microcystis aeruginosa* after exposure to L-phenylalanine

2.4 Effect of L-phenylalanine on esterase activity of *Microcystis aeruginosa*

The esterase activity as an acute toxicity endpoint was assessed over 2 to 48 h exposure to L-phenylalanine (see Fig. 4). At the concentrations of L-phenylalanine tested in this paper, no effects on the esterase activity of *Microcystis aeruginosa* cells were observed after 2 or 24 h exposure compared to the control ($P > 0.05$). Nevertheless, esterase activity was significantly suppressed after 48 h exposure to L-phenylalanine at five of the six concentrations tested.

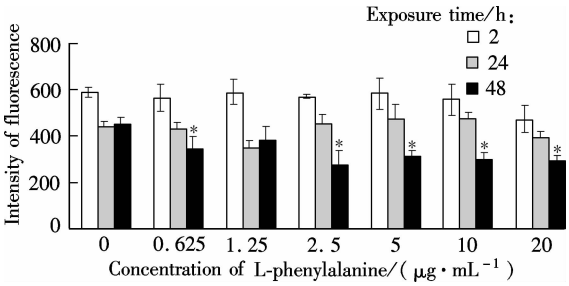

Fig. 4 Esterase activity of *Microcystis aeruginosa* after exposure to L-phenylalanine

3 Discussion and Conclusion

Algicidal bacteria, as a more economical and environment-friendly solution to controlling algal blooms, are becoming a hot topic of research. Recent studies investigated whether diverse microorganisms were vulnerable to the growth-inhibitory effects of exogenous metabolites such as amino acids, even though the endogenous synthesis of the metabolites was obligatory for growth[10]. It was reported that lysine could inhibit the growth of *Microcystis aeruginosa* significantly at a concentration of 5 μg/mL[11], and alanine, leucine, and arginine could be used as the sole nitrogen source to support the growth of *Microcystis aeruginosa*[12].

The results obtained in our research revealed that L-phenylalanine, one of the algal control compounds extracted from the native algicidal *Aeromonas punctata* from Taihu Lake, displayed a potent ability to inhibit the growth of *Microcystis aeruginosa*. The lowest effective concentration of L-phenylalanine for inhibition of *Microcystis aeruginosa* was 0.625 μg/mL, which was much lower than that for lysine[11]. Furthermore, L-phenylalanine might have hormesis effects on the growth of *Microcystis aeruginosa* because it promoted growth at lower concentrations and inhibited growth at higher concentrations. Hence, it would be interesting to know how L-phenylalanine affects the growth of the cells. This study applied flow cytometry to investigate the possible effects of L-phenylalanine on the membrane integrity and esterase activity of *Microcystis aeruginosa* cells. The algal cells exposed to L-phenylalanine showed increased injury (i.e. reduced membrane integrity) under flow cytometry observation, but no variation in cell esterase activity after 24 h exposure. However, decreased esterase activity in cells was found after 48 h exposure. It was presumed that the growth inhibition of *Microcystis aeruginosa* by L-phenylalanine was caused by dissolution of the algal cell membranes, which is the same as that for L-lysine[13].

In conclusion, L-phenylalanine has hormesis effects and algal control effects on the growth of *Microcystis aeruginosa*. It can efficiently inhibit the growth of *Microcystis aeruginosa* by disrupting the cell membrane integrity and the esterase activity in the cells. These findings are helpful in the application of algicidal bacteria and L-phenylalanine to control cyanobacterial blooms in lakes and reservoirs.

References

[1] Anderson D M. Approaches to monitoring, control and management of harmful algal blooms (HABs) [J]. *Ocean & Coastal Management*, 2009, **52**(7): 342 - 347.

[2] van Gremberghe I, Vanormelingen P, van der Gucht K, et al. Influence of *Daphnia* infochemicals on functional traits of *Microcystis* strains (Cyanobacteria) [J]. *Hydrobiologia*, 2009, **635**(1): 147 - 155.

[3] Waybright T J, Terlizzi D E, Ferrier M D. Chemical characterization of the aqueous algistatic fraction of barley straw (*Hordeum vulgare*) inhibiting *Microcystis aeruginosa* [J]. *Journal of Applied Phycology*, 2009, **21**(3): 333 - 340.

[4] Zhao Y J, Liu Y D. Possible microbial control on the adverse impacts of algae-current information about the relationship between algae and microbes [J]. *Acta Hydrobiologiac Sinica*, 1996, **20**(2): 173 - 181.

[5] Wu G, Xi Y, Zhao Y J. The lastest development of research on algae-lysing bacteria [J], *Research of Environmental Sciences*, 2002, **15**(5): 43 - 46.

[6] Zhao C P, Pu Y P, Yin L H, et al. Isolation and algicidal effect of a lytic *Microcystis* bacterium from Taihu Lake [J]. *Journal of Southeast University*: *Natural Science Edition*, 2005, **35**(4): 602 - 606.

[7] Kang Y K, Cho S Y, Kang Y H, et al. Isolation, identification and characterization of algicidal bacteria against *Stephanodiscus hantzschii* and *Peridinium bipes* for the control of freshwater winter algal blooms [J]. *Journal of Applied Phycology*, 2008, **20**(4): 375 - 386.

[8] Li X Q, Yang F, Yin L H, et al. Priliminary separation and detection of the algicidal substances of *Aeromonas Punctata sp* in Lake Taihu [C]//*Proceedings of the 5th International Academic Conference on Environmental and Occupational Medicine*. Dujiangyan, China, 2010: 12 - 14.

[9] Rippka R, Deruelles J, Waterbury J B, et al. Generic assignments, strain histories and properties of pure cultures of cyanobacteria [J]. *Journal of General Microbiology*, 1979, **111**(1): 1 - 61.

[10] Conrad R S, Sokatch J R, Jensen R A. Relationship of metabolite inhibition of growth to flow-of-carbon patterns in nature [J]. *Life Sciences*, 1976, **19**(3): 299 - 320.

[11] Lin B G, Yang L Y, Xiao L, et al. Mechanism of the inhibition effect of lysine on *Microcystis aeruginosa* [J]. *Journal of Agro-Environment Science*, 2008, **27**(4): 1561 - 1565.

[12] Dai R H, Liu H J, Qu J H, et al. Effects of amino acids on

microcystin production of the *Microcystis aeruginosa* [J]. *Journal of Hazardous Materials*, 2009, **161**(2/3): 730 - 736.
[13] Yamamoto Y, Kouchiwa T, Hodoki Y, et al. Distribution and identification of actinomycetes lysing cyanobacteria in a eutrophic lake[J]. *Journal of Applied Phycology*, 1998, **10**(4): 391 - 397.

L-苯丙氨酸对铜绿微囊藻生长的作用与机制

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摘要:应用细胞计数法结合流式细胞术探讨了 L-苯丙氨酸对铜绿微囊藻生长的作用与可能机制. 结果表明:作用 24 h 后,较低浓度 L-苯丙氨酸(0.078 ~ 0.312 μg/mL)对藻细胞生长表现为促进作用,而在较高浓度(0.625 ~ 20 μg/mL)时表现为抑制作用. L-苯丙氨酸对铜绿微囊藻生长的抑制作用存在剂量效应和时间效应关系. 24 h 生长抑制作用的 IC_{50} 为 6.2 μg/mL(95% 可信区间是 0.005 ~ 16.76 μg/mL). 用 L-苯丙氨酸染毒 24 h 后,藻细胞膜完整性受到明显影响,而在 48 h 后,细胞酯酶活性才发生改变. 研究结果提示 L-苯丙氨酸对铜绿微囊藻的生长存在 hormesis 效应并具有控藻作用,其控藻作用机制可能与 L-苯丙氨酸导致藻细胞膜完整性受损和酯酶活性改变有关.

关键词:控藻;L-苯丙氨酸;机制;流式细胞术;膜;生长
中图分类号:X506