Journal of Applied Phycology (2022) 34:423–434
https://doi.org/10.1007/s10811-021-02659-x

Establishment of high-cell-density heterotrophic cultivation of *Poterioochromonas malhamensis* contributes to achieving biological control of *Microcystis*

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Received: 28 May 2021 / Revised and accepted: 22 November 2021 / Published online: 13 January 2022

Abstract

The chrysophyte *Poterioochromonas malhamensis* has potential for controlling algal blooms through rapid grazing of toxic *Microcystis* cells and efficient degradation of microcystin. However, this method has not been used in practice because a high-cell-density method for cultivating *P. malhamensis* has not yet been established and the actual effect of the chrysophyte in controlling *Microcystis* blooms in the field is still unknown. To achieve the application of this method, high-cell-density heterotrophic cultivation of *P. malhamensis* was established through optimizing the carbon/glucose concentration, C:N ratio, temperature, pH, and dissolved oxygen concentration. Under optimized conditions, the cell concentration of *P. malhamensis* reached more than 3×10^8 cells mL^{-1}, which exceeds that reported in other studies by more than an order of magnitude. The ability of the chemoheterotrophic *P. malhamensis* to graze unicellular *Microcystis* cells was comparable to that of autotrophic and phagotrophic *P. malhamensis*. A controlled field experiment showed that chemoheterotrophic *P. malhamensis* could live in the aquatic environment with a *Microcystis* bloom and decrease the *Microcystis* biomass on the surface of the water by promoting the sedimentation of colonial *Microcystis* cells. This study offers an opportunity to drive the development of methods to control *Microcystis* blooms using predatory *P. malhamensis*.

Keywords Mixotrophic chrysophyte · *Poterioochromonas malhamensis* · Fermentation · Feeding behavior · Toxic *Microcystis*

Introduction

The chrysophyte *Poterioochromonas* is a mixotrophic flagellate microalga that can either live by autotrophy (i.e., by photosynthesis through chloroplasts) or heterotrophy (including directly assimilating dissolved organics and utilizing ingested food particles). It can graze on a diverse range of prey (e.g., other microalgae, bacteria, and organic particles) with the help of its two unequal flagella (Zhang & Watanabe, 1996). This grazing ability poses a great threat to commercial, mass culture of other microalgal species (Touloupakis et al., 2016; Ma et al., 2018), although fortunately the early detection of *Poterioochromonas* by real-time PCR (Wang et al., 2021b) and diverse methods of controlling *Poterioochromonas* in microalgal culture have been successively established (Ma et al., 2017; Wang et al., 2018; He et al., 2021; Toda et al., 2021). In contrast to these negative impacts, *Poterioochromonas* also has potential for many beneficial applications. For example, the potential effects of

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chemical compounds on the cell reproduction and viability of *Poterioochromonas malhamensis* can be easily examined, making it a good test system in ecotoxicology, toxicology, and pharmacology (Roderer, 1986). Furthermore, *P. malhamensis* cells can produce many active substances, such as chrysolaminarin (i.e., a β-glucan) serving as an immunopotentiator (Kauss & Kriebitzsch, 1969; Zeković et al., 2005) and malhamensilipin A exhibiting antiviral and antimicrobial activity (Chen et al., 1994).

To realize the potential application of *Poterioochromonas*, the acquisition of enough *Poterioochromonas* cells is a critical first step. The growth rate of *Poterioochromonas* under autotrophic conditions is extremely low, which may be due to the lack of CO_2 concentrating mechanisms (Sanders et al., 1990; Giordano et al., 2005). Therefore, *Poterioochromonas* has been described as a predominantly heterotrophic mixotroph (Holen & Boraas, 1995). The addition of prey microorganisms, such as microalgae and bacteria, can markedly improve the growth rate of *Poterioochromonas* (Zhang & Watanabe, 2001). However, this method is not suitable for large-scale production of *Poterioochromonas* because of the difficulty in providing a persistent supply of stable-quality prey. An alternative is chemoheterotrophic cultivation. Up to now, many species of microalgae, such as species of *Chlorella*, *Euglena*, and *Scenedesmus*, have been cultivated on an industrial scale to achieve high cell densities using fermenters (Jin et al., 2020). Generally, the biomass of these microalgae achieved by chemoheterotrophic cultivation is 10 times more than that achieved by autotrophic cultivation (Liu et al., 2014). For instance, a maximum biomass of 32.5 g L^{-1} for *Ochromonas danica* has been achieved by heterotrophic fermentation (Lin et al., 2014). Consequently, chemoheterotrophy should also be the most efficient way to cultivate *Poterioochromonas* on a large scale. However, although *Poterioochromonas* has been successfully cultivated using chemoheterotrophic medium containing glucose (Lewitus & Caron, 1991; Rottberger et al., 2013), the fermentation technology for cultivation of *Poterioochromonas* has not been systematically studied.

In recent decades, the promising prospect of using *Poterioochromonas* to control algal blooms has also been widely studied. Considerable laboratory data have suggested that *Poterioochromonas* can rapidly graze on toxic *Microcystis* cells (Kim & Han, 2007; Zhang et al., 2009). More importantly, *Poterioochromonas* cells can also efficiently degrade microcystins (Ou et al., 2005; Zhang et al., 2008). Therefore, *Poterioochromonas* is one of the few microorganisms that can simultaneously reduce *Microcystis* cells and microcystins. It is notable that although the feeding characteristics and scavenging activity of *Poterioochromonas* on toxic *Microcystis* cells in the laboratory have been well studied, its viability and feeding behavior in *Microcystis* blooms in the field remain unknown. Furthermore, autotrophic *Poterioochromonas* has usually been used in these studies of *Poterioochromonas* feeding on *Microcystis*, while the grazing ability of chemoheterotrophic *Poterioochromonas* on *Microcystis* remains to be investigated.

This study therefore had two main aims: to establish a method for the high-cell-density chemoheterotrophic cultivation of *P. malhamensis* by optimizing heterotrophic culture conditions; and to evaluate the effects of chemoheterotrophic *P. malhamensis* in controlling unicellular *Microcystis* under laboratory conditions and on outdoor *Microcystis* blooms. The feasibility of controlling *Microcystis* blooms using *P. malhamensis* is also discussed.

Materials and methods

Organisms and culture conditions

The chrysophyte *Poterioochromonas malhamensis* was isolated from contaminated *Chlorella sorokiniana* GT-1 culture, purified using a combination of antibiotics according to the methods of Corno and Jurgens (2006) and Destain et al. (2014), and then maintained in axenic culture. The axenic *P. malhamensis* was cultivated with a "specific flask medium" at a temperature of 25 °C under dark conditions. The "specific flask medium", adapted from Blom and Pernthaler (2010), contained 10 g L^{-1} glucose, 3 g L^{-1} yeast extract, 1.0 g L^{-1} beef liver infusion, 0.5 g L^{-1} KH_2PO_4, and 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$. Toxic *Microcystis aeruginosa* FACHB 942 was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences, China. *Microcystis aeruginosa* was cultivated using BG-11 medium (Rippka et al., 1979) at a temperature of 30 ± 1 °C under continuous illumination at 25 ± 10 μmol photons m^{-2} s^{-1}.

Optimizing the chemoheterotrophic culture conditions for *P. malhamensis*

The inoculant for large-scale microalgal fermentation is taken from cultures grown at the bench scale in shake flasks. A stable chemoheterotrophic cultivation method at the flask scale is essential for the large-scale fermentation of *P. malhamensis*. In this study, optimal levels for most of the *P. malhamensis* culture conditions were determined using 250-mL shake flasks with a working volume of 100 mL (Table 1). A total of seven culture parameters, including the concentration of glucose, ratio of carbon to nitrogen, temperature, pH of the medium, light intensity, initial cell concentration, and shaker speed, were studied. All experiments were performed in triplicate using shaking incubators (HYL-C3, Qiang Le, China). The basal culture medium for chemoheterotrophic *P. malhamensis* was the "specific flask medium" as described in the "Organisms and culture conditions" section. The *P.*

Table 1 Culture conditions for *P. malhamensis* investigated in this study

Culture system	Parameter	Levels	Other key culture conditions
Shake flask (250 mL)	Initial glucose concentration (g L^{-1})	0, 5, 10, 20, 30	CN = 10:1; T = 28 °C; P = 6.0; SS = 160 rpm
	Initial C:N ratio	2:1, 5:1, 10:1, 20:1, 30:1	CG = 10 g L^{-1}; T = 28 °C; P = 6.0; SS = 180 rpm
	Temperature (°C)	24, 28, 32, 36	CG = 10 g L^{-1}; CN = 10:1; P = 6.0; SS = 180 rpm
	Initial pH	5.0, 6.0, 7.0, 8.0, 9.0	CG = 10 g L^{-1}; CN = 10:1; T = 28 °C; SS = 180 rpm
	Light intensity (μmol photons m^{-2} s^{-1})	0 (dark), 30 ± 10 (light)	CG = 10 g L^{-1}; CN = 10:1; T = 28 °C; P = 6.0; SS = 180 rpm
	Initial cell concentration (cells mL^{-1})	10^3, 10^4, 10^5, 10^6	CG = 10 g L^{-1}; CN = 10:1; T = 28 °C; P = 6.0; SS = 180 rpm
	Shaker speed (rpm)	0, 90, 180	CG = 10 g L^{-1}; CN = 10:1; T = 28 °C; P = 6.0
Fermenter (1 L)	Concentration of dissolved oxygen (%)	0, 20, 40	T = 28 °C; P = 6.0

CG, concentration of glucose; *CN*, C:N ratio; *P*, pH value of medium; *T*, temperature; *SS*, shaker speed. Different ratios of carbon to nitrogen were realized by adjusting the concentration of yeast extract and liver extract powder (these two nitrogen sources were always added in a proportion of 3:1 calculated by weight) under a fixed glucose concentration of 10 g L^{-1}. All shake flask experiments were conducted in the dark except for the light intensity experiment. The initial cell concentration of *P. malhamensis* in the shake flask experiments (except for the initial cell concentration experiment) was 3–5 × 10^5 cells mL^{-1}

malhamensis cells were pre-stained with 1% Lugol's iodine (Leakey et al., 1994) before counting. The cell concentration of *P. malhamensis* was counted every day using a hemocytometer (Improved Neubauer, USA) under a light microscope (BX53, Olympus, Japan). The semi-quantitative analysis of glucose concentration was conducted using a Safe-AccuUG Blood Glucose Monitoring System (Model BGMS-1; Sinocare Inc., Changsha, China), with a lower limit of detection of 0.4 g L^{-1}. Generally, the glucose concentration would decrease to zero within 5 days, and therefore, the growth data of *P. malhamensis* were only collected for 4–5 days.

In addition, the effect of dissolved oxygen (DO) concentration (0, 20, and 40%) on the growth of *P. malhamensis* was also studied in 1.0-L fermenters (My-control, Applikon Biotechnology, Netherlands). For the 20% and 40% treatments, the DO was controlled automatically by additional delivery of pure oxygen. For the 0% treatment, which received no delivery of pure oxygen, the DO was only monitored by a probe and decreased from 100% to zero within 24 h. The other culture conditions for *P. malhamensis* in the fermenters were based on the optimal conditions identified in the aforementioned flask experiments. The pH of the medium was set at 6.0 (±0.2) as determined by the pH probe (Z001018510, Applisens) and was automatically maintained by the addition of 1 M NaOH or 1 M HCl solution. The temperature was set at 28 °C with a fluctuation range of ±0.5 °C. The stirring speed was 400 rpm. Aeration was maintained at an airflow rate of 1 L min^{-1}. The basal medium used during the fermentation process was adapted from a combination of the "specific flask medium" and Endo growth medium (Jin et al., 2020). It contained 10 g L^{-1} glucose, 6 g L^{-1} yeast extract, 2.0 g L^{-1} liver extract powder, 0.3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 73.5 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 4.4 mg L^{-1} $Na_2{\cdot}EDTA$, 3.15 mg L^{-1} $FeCl_3{\cdot}6H_2O$, 0.97 mg L^{-1} H_3BO_3, 0.02 mg L^{-1} $ZnSO_4{\cdot}7H_20$, 0.18 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 0.006 mg L^{-1} $NaMoO_4{\cdot}2H_2O$, and 0.012 mg L^{-1} $Co(NO_3)_2{\cdot}6H_2O$, with a ratio of carbon to nitrogen source of 5:1. The feeding medium was the 20-fold concentrated basal medium, except for the nitrogen sources (yeast extract (76 g L^{-1}) and liver extract powder (24 g L^{-1})). The glucose concentration of the medium was monitored using the Safe-AccuUG Blood Glucose Monitoring System every 6–8 h and then adjusted to 5–10 g L^{-1} by adjusting the flow velocity of the feeding medium. The initial cell concentration of *P. malhamensis* was 2.8 × 10^6 cells mL^{-1}, and the cell concentration of *P. malhamensis* was determined daily. Each treatment was performed in duplicate. The experiment was repeated three times and the same results were obtained.

Grazing ability of chemoheterotrophic *Poterioochromonas malhamensis* on unicellular *Microcystis aeruginosa*

To estimate the grazing ability of chemoheterotrophic *P. malhamensis* cells on unicellular *M. aeruginosa*, *P. malhamensis* cells cultivated with three different modes of nutrition (i.e., autotrophy, chemoheterotrophy, and phagotrophy) were used as the predator of *M. aeruginosa*. Chemoheterotrophic *P. malhamensis* was grown in the "specific flask medium" as described in the "Organisms and culture conditions" section, while autotrophic *P. malhamensis* was cultivated in BG-11 medium. For phagotrophy, the *P. malhamensis* was cultivated in BG-11 medium and fed with *M. aeruginosa* in advance, and then used in this experiment when the *M. aeruginosa* was almost cleared out. The feeding experiment was carried out in 250-mL flasks with a working volume of 100 mL BG-11 medium. Each treatment was performed in triplicate.

The initial cell concentrations of *P. malhamensis* and *M. aeruginosa* were 1.0×10^5 cells mL^{-1} and 1.0×10^7 cells mL^{-1}, respectively, in all treatments. For the control, the medium was inoculated with only *M. aeruginosa*, to give a concentration of 1.0×10^7 cells mL^{-1}. Cell concentrations of *P. malhamensis* and *M. aeruginosa* were counted every 12 h using a hemocytometer (Improved Neubauer, USA) under a light microscope. The *P. malhamensis* cells were pre-stained with 1% Lugol's iodine before counting. The micro-morphology and feeding behavior of *P. malhamensis* were recorded using a light microscope (BX53, Olympus, Japan). The culture flasks were incubated in a culture room at 25 ± 1 °C under continuous illumination at 25 ± 10 µmol photons m^{-2} s^{-1}.

Control of *Microcystis* outdoors using chemoheterotrophic *Poterioochromonas malhamensis*

The chemoheterotrophic *P. malhamensis* was further trialed for the control of outdoor colonial *Microcystis* in the field. To obtain enough *P. malhamensis*, it was cultivated using 7.5-L bioreactors (BioFlo & CelliGen 310, New Brunswick) with an initial working volume of 3 L. The basal medium and feeding medium were the same as those media used in the 1.0-L bioreactors as described in the "Optimizing the chemoheterotrophic culture conditions for *P. malhamensis*" section. The DO concentration was automatically maintained at 20% by gradually increasing the stirring speed from 50 to 400 rpm and the aeration rate from 4 to 7 L min^{-1} as the cultivation progressed. The temperature and pH value were set at 28 °C and 6.0, respectively. After a cultivation phase of 3 days, the cell concentration of *P. malhamensis* reached 3×10^8 cells mL^{-1}. To prepare the inoculum, the *P. malhamensis* cells were harvested by centrifugation at $1500\times g$ for 5 min and washed with double-distilled water (ddH_2O) three times to remove the residual organics.

Otdoor *Microcystis* control experiments were conducted around a fish pond (30° 31′ N, 114° 23′ E; Wuhan, China) with a *Microcystis* bloom (Fig. 1A–C) from October 16 to October 22 in 2018. The *Microcystis* sp. suspension (more than 1200 L) was first pumped from the pond into a 1400-L white plastic bucket (Fig. 1D, bucket 0). After homogeneous mixing, a volume of 200 L *Microcystis* suspension was then successively transferred into six further buckets, comprising three control repetitions and three experiment repetitions. This was repeated four times, giving a final working volume of *Microcystis* suspension of 800 L in each of the six buckets. For the experiment group, *P. malhamensis* was added to give a final cell concentration of 3.0×10^5 cells mL^{-1}. To avoid *P. malhamensis* sinking, a circular aerated conduit with a diameter of 65 cm was placed in the bottom of each of the six buckets (Fig. 1E). Samples were collected daily from a depth of 10–20 cm below the surface of the liquid. On the first 3 days, the *Microcystis* suspensions were not agitated before sampling; from days 4 to 6, the suspensions were evenly stirred by club before sampling. Collected samples were used to determine cell concentrations of *P. malhamensis* and *Microcystis* sp., as well as the chlorophyll-*a* concentration of the suspension. To determine the cell concentration of *Microcystis*, the collected sample was pre-heated at 60 °C for 10 min to disperse the colonial *Microcystis* (Humphries & Widjaja, 1979). The concentration of chlorophyll-*a* was determined according to the method of Ma et al. (2017). A YSI Professional Plus meter (USA) was used to determine the DO and pH value of the *Microcystis* suspension. The micro-morphology of *Microcystis* and *P. malhamensis* was observed and recorded daily by light microscopy (BX53, Olympus, Japan). The color change of the *Microcystis* suspension was also recorded by camera (Canon

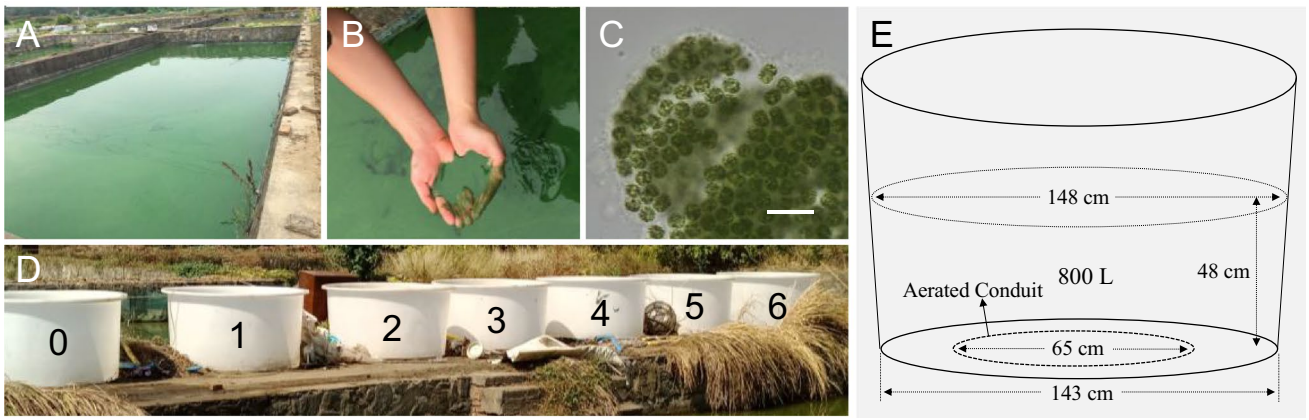

Fig. 1 The equipment and *Microcystis* sources for the outdoor experiment using *P. malhamensis*. **A**, Distant view of the fish pond with outbreak of *Microcystis*; **B**, close view of the *Microcystis* bloom; **C**, microscopic observation of *Microcystis* (scale bar = 10 µm); **D**, plastic buckets used in the study (the bucket labeled No. 0 was used for pre-mixing and the other six buckets were used in the control group (Nos. 1–3) and the experiment group (Nos. 4–6)); **E**, specification of the plastic bucket

EOS 60D, Japan). Light intensity and temperature (14–24 °C) changed with local weather conditions.

Statistical analyses

All data are presented as mean values ± standard deviations. The statistical significance of differences in different sample groups was analyzed using one-way ANOVA (analysis of variance). Tukey's multiple comparison tests were used when more than two sample groups were present. The results were considered to be significantly different at $P < 0.05$. All statistical analyses were performed using the software SPSS 16.0.

Results

Optimization of culturing conditions for chemoheterotrophic *Poterioochromonas malhamensis* in the shake flask system

In the shake flask system, the optimal levels of the four main culture parameters—glucose concentration, C:N ratio, temperature, and pH of the medium—were determined. The absence of glucose (0 g L^{-1}) led to stagnation in the growth of *P. malhamensis* (Fig. 2A). The highest biomass (3.2×10^7 cells mL^{-1}) was obtained when *P. malhamensis* was cultivated with 10 g L^{-1} glucose after 4 days of cultivation. However, in the first 2 days during which glucose was abundant, *P. malhamensis* at the glucose concentration of 5 g L^{-1} grew faster than that at 10 g L^{-1} ($P > 0.05$). Once the glucose had been consumed in the 5 g L^{-1} treatment, the cell concentration of *P. malhamensis* stopped increasing. The two highest glucose concentrations, 20 g L^{-1} and 30 g L^{-1}, were found to inhibit the growth of *P. malhamensis*. The growth of *P. malhamensis* was also greatly affected by the C:N ratio (i.e., the nitrogen concentration). The growth rate of *P. malhamensis* increased with a decrease in the C:N ratio within the range from 30:1 to 5:1 (Fig. 2B). A lower C:N ratio of 2:1 contributed little to improving the growth of *P. malhamensis*. The cell concentrations of *P. malhamensis* cultivated with a C:N ratio of 5:1 and 2:1 also stopped increasing after the glucose had been consumed. *Poterioochromonas malhamensis* at 28 °C and 32 °C grew faster than at 24 °C and 36 °C ($P < 0.05$), while the growth rates of *P. malhamensis* at 28 and 32 °C were similar ($P > 0.05$) (Fig. 2C). The optimal initial pH value for the growth of *P. malhamensis* was 6.0, followed by pH 5.0 and 7.0 in the first 3 days. However, the biomass of *P. malhamensis* after 4 days of cultivation was similar for pH 5.0, 6.0, and 7.0 ($P > 0.05$). Growth of *P. malhamensis* was considerably inhibited when the initial pH value increased to 8.0 (Fig. 2D). In the shake flask system, the maximum cell concentration of *P. malhamensis* could achieve 4.8×10^7 cells mL^{-1} (Fig. 2B).

To ensure the stable quality of inoculants for subsequent fermentation, the effects of shaker speed, illumination, and initial inoculation concentration on the growth of *P. malhamensis* in the flasks were also studied. *Poterioochromonas malhamensis* grew at an extremely low rate under stationary culture conditions,

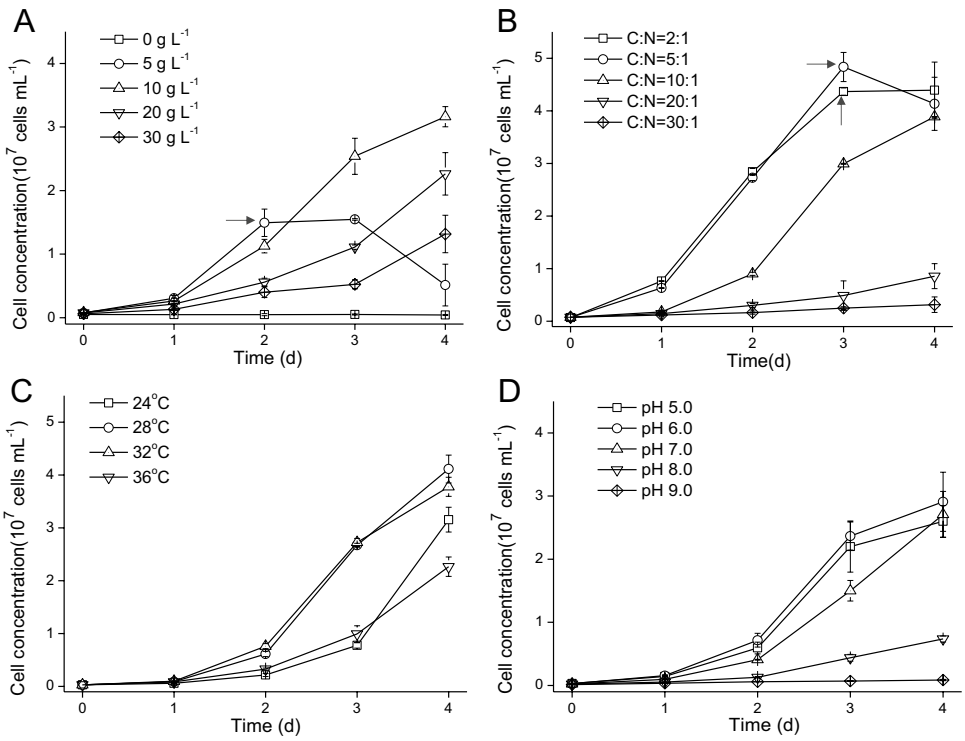

Fig. 2 Growth of chemoheterotrophic *P. malhamensis* in 250-mL flasks under different culture conditions. **A**, Glucose concentration; **B**, ratio of carbon to nitrogen source; **C**, temperature; **D**, initial pH value of medium. Arrows mean that the glucose source at this point is running out and the glucose concentration is too low to be detected. Error bars represent the standard deviation ($n = 3$)

whereas a shake significantly promoted its growth ($P<0.05$) (Fig. S1-A). *Poterioochromonas malhamensis* grew much better when shaken at 180 rpm than at 90 rpm ($P<0.05$), and exhibited a similar growth rate in dark or dim light ($P>0.05$) (Fig. S1-B). The time for *P. malhamensis* to reach the maximum cell concentration decreased with an increase in inoculation concentration (Fig. S1-C). For *P. malhamensis* inoculated to give an initial concentration of 10^6 cells mL^{-1}, the maximum cell concentration was obtained in 2 days, whereas the time for reaching maximum biomass was longer when the inoculation concentration decreased to 10^5 cells mL^{-1} or lower.

Optimization of culturing conditions for chemoheterotrophic *Poterioochromonas malhamensis* in the fermenter system

Based on the optimized culture conditions of *P. malhamensis* in shake flasks (i.e., 10 g L^{-1} glucose, C:N ratio of 5:1, temperature 28 °C, and pH 6.0), the effect of DO concentration on the growth of *P. malhamensis* was investigated in the fermenter system using a feeding medium to periodically replenish the nutrient (mainly glucose) supply. The glucose concentrations in different treatments were maintained in the range 0 to 10 g L^{-1} during the whole cultivation period, with the exception of one instance of 12 g L^{-1} at 40% DO (Fig. 3A). The cell concentration of *P. malhamensis* cultivated with 0% DO increased from 2.8×10^6 cells mL^{-1} to 6.6×10^7 cells mL^{-1} after a cultivation time of 72 h. However, the cell concentrations of *P. malhamensis* cultivated with 20 and 40% DO increased to 3.0×10^8 cells mL^{-1} and 3.6×10^8 cells mL^{-1}, respectively (Fig. 3B). It is notable that the cell concentration of *P. malhamensis* after 72 h of cultivation stopped increasing in all three treatments, with a dramatic reduction in the 40% DO treatment.

Grazing ability of chemoheterotrophic *Poterioochromonas malhamensis* cells on unicellular *Microcystis aeruginosa*

The grazing ability of chemoheterotrophic *P. malhamensis* cells on unicellular *M. aeruginosa* was compared with those of autotrophic and phagotrophic *P. malhamensis*. *Poterioochromonas malhamensis* using different modes of nutrition varied greatly in cell morphology (Fig. 4). Chemoheterotrophic *P. malhamensis* had the largest cell size and had an obvious chrysolaminarin vacuole, but the chloroplast was inconspicuous. The cell size of autotrophic *P. malhamensis* was smallest, but its chloroplast was obvious and regular. The cell size of phagotrophic *P. malhamensis* fell between that of chemoheterotrophic and autotrophic *P. malhamensis*. The chloroplast in phagotrophic *P. malhamensis* cell was also obvious. After being grazed for 84 h, cell concentrations of *M. aeruginosa* co-cultured with *P. malhamensis* had decreased to extremely low levels ($<10^4$ cells mL^{-1}) for all three modes of nutrition, while the cell concentrations of *M. aeruginosa* in the control had increased to 1.5×10^7 cells mL^{-1} (Fig. 5A). However, chemoheterotrophic and phagotrophic *P. malhamensis* showed greater grazing ability than that of autotrophic *P. malhamensis* in the first 24 h ($P<0.05$). Growth of *P. malhamensis* showed similar trends for all three modes of nutrition in the first 24 h, but the final cell concentration of chemoheterotrophic *P. malhamensis*

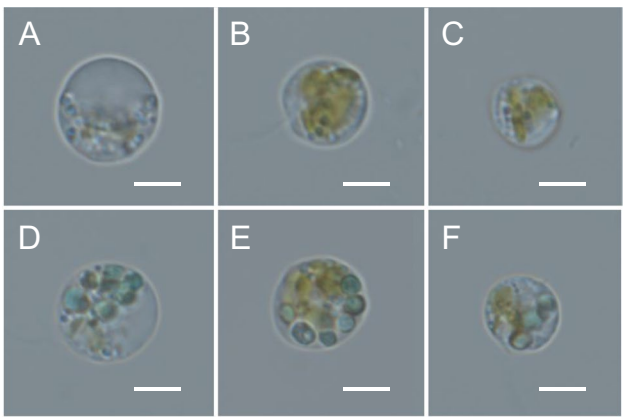

Fig. 4 Cell morphology of *P. malhamensis* with different modes of nutrition before (**A**–**C**) and after (**D**–**F**) engulfing *M. aeruginosa*. **A** and **D**, Chemoheterotrophic *P. malhamensis*; **B** and **E**, phagotrophic *P. malhamensis*; **C** and **F**, autotrophic *P. malhamensis*. Scale bars = 5 μm

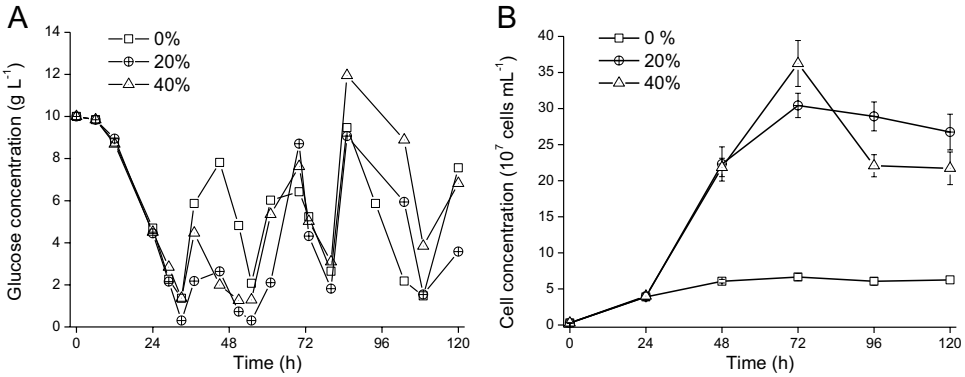

Fig. 3 Glucose concentration of the medium (**A**) and cell concentration of *P. malhamensis* (**B**) during the chemoheterotrophic cultivation of *P. malhamensis* in 1-L fermenters under different dissolved oxygen concentrations

(7×10^5 cells mL^{-1}) was higher than that of phagotrophic and autotrophic *P. malhamensis* (both around 5×10^5 cells mL^{-1}) ($P<0.05$) (Fig. 5B).

Outdoor control of *Microcystis* using chemoheterotrophic *Poterioochromonas malhamensis*

From a macro-observational perspective, the color of the *Microcystis* suspension in the experiment group (inoculated with *P. malhamensis*) turned from blue-green to slightly yellow–brown by the third day, while the water color of the *Microcystis* suspension in the control group maintained a blue-green color (Fig. 6). The chlorophyll-*a* concentration of the experiment group correspondingly decreased and reached 0.6 mg L^{-1} on the third day, while the chlorophyll-*a* concentration of the control group stayed at around the initial level of 1.1 mg L^{-1}. According to our observations, a large proportion of the *Microcystis* cells sank to the bottom of the buckets in the experiment groups. Therefore, the chlorophyll-*a* concentration of the experiment group increased substantially after an additional agitation before sampling on the fourth day (Fig. 7A). The cell concentration of *Microcystis* in the experiment group was significantly lower than that in the control group after 3 days ($P<0.05$) (Fig. 7B). The cell concentration of *P. malhamensis* was substantially unchanged in the first 4 days, but decreased to an extremely low value by the sixth day (Fig. 7B). Microscopic observation showed that *P. malhamensis* in the experiment group was incapable of grazing on colonial *Microcystis* and could only ingest a few unicellular *Microcystis* at any one time (Fig. S2-A & D). Furthermore, the cell size of *P. malhamensis* had decreased by the end of the experiment (Fig. S2-B & C). The DO concentration in the control group showed an increase in the first 2 days followed by a gradual decrease, while the DO concentration in the experiment group dramatically decreased within the first day and then remained stable after a short time of increase (Fig. 7C). The pH value of the experiment group fluctuated in the range 8.0–9.0, while the pH of the control group increased gradually to 10.8 (Fig. 7D).

Discussion

Effects of nutrients and environmental factors on the chemoheterotrophic growth of *Poterioochromonas malhamensis*

The present study provides a promising fermentation technology for acquiring abundant *P. malhamensis* cells, and the

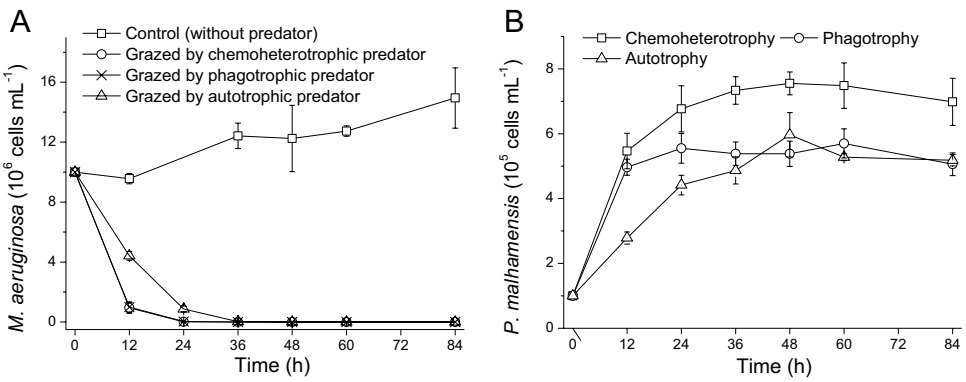

Fig. 5 Population dynamics of prey (**A**) and predator (**B**) when *M. aeruginosa* was grazed by *P. malhamensis* cultivated with different modes of nutrition: chemoheterotrophy, phagotrophy, and autotrophy. The curves represented by the cross (×) and circle (○) symbols in "**A**" are coincident. Error bars represent the standard deviation ($n=3$)

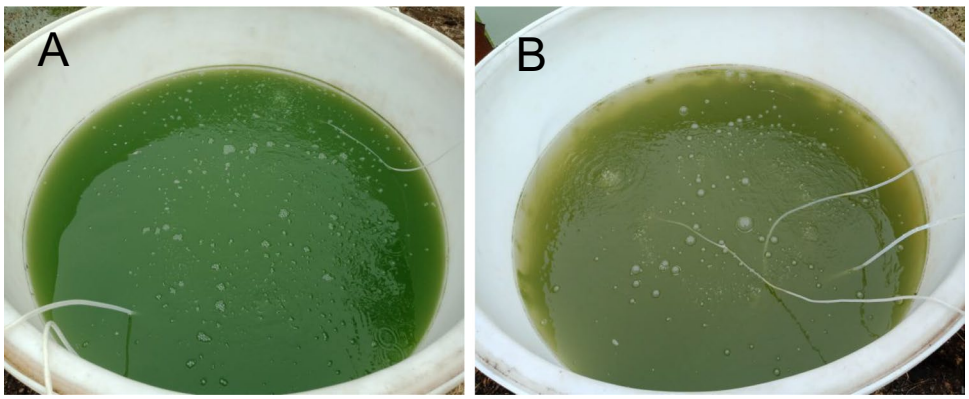

Fig. 6 Difference in the color of the *Microcystis* suspension in the control group (**A**) and the experiment group (**B**) on the third day

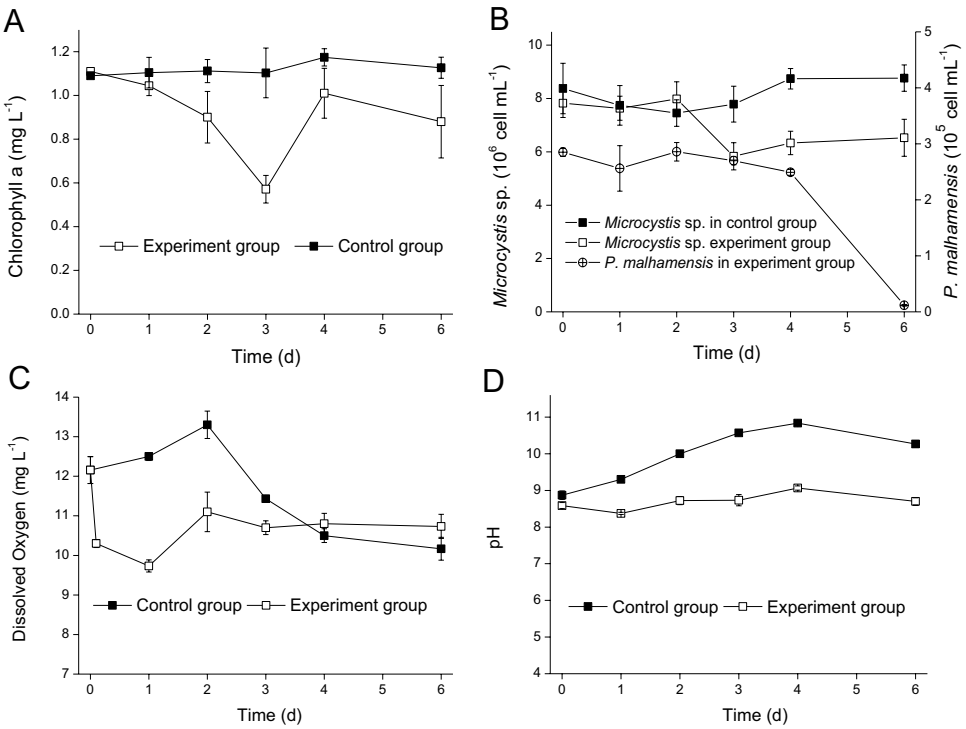

Fig. 7 Changes in chlorophyll-*a* concentration (**A**), cell concentrations of *Microcystis* and *P. malhamensis* (**B**), dissolved oxygen (**C**), and pH value (**D**) in the control group and the experiment group. In **A** and **B**, the samples on the first 3 days were collected from the suspensions without agitation, while the samples collected on days 4 and 6 were from evenly stirred suspensions

maximum biomass achieved (more than 3×10^8 cells mL^{-1}) in this study exceeds that reported in other studies (Lewitus & Caron, 1991; Rottberger et al., 2013) by more than an order of magnitude. In recent years, fermentation of microalgae has attracted increasing interest due to the ultrahigh cell densities produced, fast growth, and low occurrence of contaminants (Liu et al., 2014). Our results provide useful insights into the optimal culture conditions, in terms of nutrients and environmental factors, for high-cell-density fermentation of *P. malhamensis*.

Although other carbon sources (e.g., glycerol and ethanol) have been used to cultivate *P. malhamensis* (Lewitus & Caron, 1991), glucose is the most common carbon source in microalgal heterotrophic cultivation. The concentration of glucose and the strategy adopted for adding it to the culture have a great impact on the growth of microalgae. For example, the growth rate of the diatom *Nitschia laevis* has been found to decrease with an increase of glucose concentration from 1 to 40 g L^{-1} (Wen & Chen, 2000), which is consistent with the trends for *P. malhamensis* in this study. For *Chlorella saccharophila*, an optimum glucose concentration of 2.5 g L^{-1} has been reported, with growth inhibition occurring once the glucose concentration exceeds 25 g L^{-1} (Tan & Johns, 1991). To ensure a high growth rate of *P. malhamensis* in this study, the glucose concentration in the fermenter was maintained at a relatively low level (< 10 g L^{-1}) by periodic addition of a feeding medium (Fig. 5). The same strategy has also been used in the fermentation of unicellular *Scenedesmus acuminatus*, yielding the highest levels of biomass (286 g L^{-1}) achieved in microbial heterotrophic cultivation to date (Jin et al., 2020).

The type of nitrogen source and ratio of C:N are also critical nutritional factors affecting microalgal fermentation (Huang et al., 2010; Zheng et al., 2013). Yeast extract and liver powder are commonly used as nitrogen sources in the chemoheterotrophic cultivation of *Poterioochromonas* and its "sister genus" *Ochromonas* (Blom & Pernthaler, 2010). In fact, an inorganic nitrogen source (e.g., NH_4Cl) could also be used to cultivate *Poterioochromonas*, but additional vitamin B_1 and B_{12} would be required (data not shown). Based on our shake flask experiment, *P. malhamensis* grows faster at low ratios of C:N (i.e., 2:1 and 5:1) than at high ratios (i.e., 10:1, 20:1, and 30:1) (Fig. 2B). However, the ratio of C:N has no effect on the maximum biomass of *P. malhamensis* ($P > 0.05$). To reduce the cost of the nitrogen sources, a C:N ratio of 10:1 is therefore recommended for further large-scale fermentation of *P. malhamensis*.

Our results also provide useful comparative data on the growth of *P. malhamensis* utilizing different modes of nutrition. Heterotrophy in *Poterioochromonas* comprises phagotrophy (grazing on other particle organics/organisms) and chemoheterotrophy (utilizing dissolved organics). The effects of environmental factors (e.g., temperature, pH value, and light intensity) on the feeding behavior of *Poterioochromonas* have been well studied (Ma et al., 2018). On the whole, phagotrophic and chemoheterotrophic *Poterioochromonas* have similar requirements regarding optimal environmental conditions, including temperature, pH, and illumination. The optimal

temperature and pH for *Poterioochromonas* grazing on microalgae have been found to be 25–30 °C and 5.0–6.0, respectively (Ma et al., 2018), which is consistent with the results of this study. Furthermore, illumination has been found to have little effect on the growth rate of *Poterioochromonas* feeding on other microalgae (Zhang & Watanabe, 2001), which is similar to our results regarding the chemoheterotrophic growth of *P. malhamensis* (Fig S1-B). These results could be explained by previous findings that photosynthesis only contributes ca. 7% of the total carbon budget of *P. malhamensis* in mixotrophic conditions (Sanders et al., 1990). However, it is noteworthy that previous studies have revealed that *Poterioochromonas* could not live for more than 4 days in continuous dark conditions and is presumed to be a light-dependent protist (i.e., some factor(s) supplied by photosynthesis in *Poterioochromonas* are required for heterotrophy) (Zhang & Watanabe, 2001; Zhang et al., 2009). In our study, we found that the cell concentration of *P. malhamensis* in dark conditions decreased dramatically with the depletion of added glucose (Fig. 2A–B and S1-C). However, during the long-term subculturing of *P. malhamensis* in continuous dark conditions, *P. malhamensis* grew well provided that abundant glucose was continuously supplied. This finding overturns the previous hypothesis.

In addition to needing sufficient glucose, the rapid growth and metabolism of *P. malhamensis* at high cell concentrations will also require a large amount of oxygen. Although mixotrophic protists (e.g., *Poterioochromonas*) can tolerate very low DO concentrations as their internal supply of pure oxygen through photosynthesis in chloroplasts (Fenchel, 2014), the photosynthetic capacity of *Poterioochromonas* decreases dramatically with increasing environmental organic concentration (Lewitus & Caron, 1991). Any increase in DO (either in flasks by shaking or in fermenters by addition of purified oxygen) would therefore be expected to enhance the biomass of *P. malhamensis*, and this is borne out by our results (Fig. 3 and S1-A).

Feasibility of controlling *Microcystis* blooms using *Poterioochromonas malhamensis*

Frequent outbreaks of cyanobacterial blooms have caused great damage to global biodiversity and the equilibrium of aquatic ecosystems (Zhang et al., 2009; Rigosi et al., 2014). In classical biological manipulation to control a cyanobacterial population, large zooplanktons (e.g., *Daphnia*) are presumed to be one of the main predators (Sarnelle, 1992; Boon et al., 1994). However, the practical application of large-sized zooplankton for biological control is difficult because of their selective grazing on high-food-quality eukaryotic algae (Ger et al., 2019), lower tolerance to toxic cyanobacteria (Lyu et al., 2019), and inability to degrade cyanotoxins (Shams et al., 2014). On the other hand, small-sized protists are generally considered the major predators of phytoplankton in natural plankton ecology (Sherr & Sherr, 2002), typically accounting for 60–70% of daily phytoplankton consumption (Calbet & Landry, 2004). Therefore, it is theoretically feasible to control *Microcystis* blooms using the mixotrophic flagellate *P. malhamensis*. Moreover, compared to other zooplankton, *P. malhamensis* has many other advantages for controlling *Microcystis* blooms, as well as the high-cell-density cultivation method developed in this study and its ability to degrade microcystins (Ou et al., 2005).

First, the grazing ability of *P. malhamensis* on unicellular *Microcystis* is comparatively strong. We found that the number of *Microcystis* cells that could be consumed by *P. malhamensis* in 24 h (10^7 cells mL^{-1}) was 100 times the initial number of *P. malhamensis* cells (10^5 cells mL^{-1}), and the cell concentration of *P. malhamensis* increased up to sevenfold during the same 24-h grazing phase (Fig. 5). Second, recent studies have shown that the chrysophytes *Poterioochromonas* and *Ochromonas* favor toxic *Microcystis* over chlorophytes, which should help the efficient recovery of chlorophytes upon a reduction in the *Microcystis* population either in the laboratory or in in situ microcosms (Zhang et al., 2009, 2018, 2020). Furthermore, *Poterioochromonas* is very adaptable to environmental conditions. In fact, *Poterioochromonas* can survive under conditions of temperature from 10 to 36.7 °C, light intensity from 0 to 1100 μmol photons m^{-2} s^{-1}, and a pH from 2.5 to 10.0 (Ma et al., 2018). Under extreme conditions, such as low temperature and lack of nutrients, *Poterioochromonas* can form siliceous cysts (stomatocysts) and germinate again when the environmental conditions are suitable (Andersen et al., 2017). In this study, *P. malhamensis* was found to be capable of living in the aquatic environment with *Microcystis* blooms, but a rapid decrease of *P. malhamensis* cell concentration was observed on the sixth day, which might have been due to the lack of available prey (Fig. 7). So, in this respect, it seems unlikely that *P. malhamensis* would form another algal bloom after grazing on a large number of prey *Microcystis* cells. Last but not least, the addition of *P. malhamensis* appears to promote the sedimentation of *Microcystis* cells, which could reduce the dominance of *Microcystis* in surface waters, thereby facilitating the growth of other microalgal species. Considering that pH fluctuation is generally considered one of the vital reasons causing the flocculation of microalgal cells (Nguyen et al., 2019; Tan et al., 2020), this beneficial effect may be due to the addition of *P. malhamensis* being able to inhibit the increase in the pH value of *Microcystis* suspensions (Fig. 7).

However, we have to admit that there are still critical issues that remain to be resolved before successful control of *Microcystis* blooms using *P. malhamensis* can be achieved. First and foremost, *P. malhamensis* can only graze on microalgal prey with a cell size less than 10 μm (Ma et al., 2018), while *Microcystis* in the field is mostly colonial with a colony size larger than 100 μm (Xiao et al., 2018). Therefore, *P. malhamensis* has been presumed to be incapable of grazing

on colonial *Microcystis* (Van Wichelen et al., 2016), which was also verified by our observations (Fig. S2). To solve this problem, the key point is to effectively disaggregate the colonial *Microcystis* into unicellular or small-sized colonies using physical methods such as high-turbulent mixing and ultrasonic radiation (Ahn et al., 2003; Li et al., 2018; Wang et al., 2021a), chemical methods such as chlorination (He & Wert, 2016), or biological methods such as bacterial degradation (Wang et al., 2015). These colony disaggregation technologies should allow *P. malhamensis* to achieve its greatest potential in controlling *Microcystis* blooms. Furthermore, the timing of *P. malhamensis* addition might also influence how well it can control *Microcystis* growth. *Poterioochromonas malhamensis* should best be able to inhibit *Microcystis* growth during the early stage of a *Microcystis* bloom when *Microcystis* is dominated by unicellular or small-sized colonies (Liu et al., 2017). Finally, the ecological security of releasing cultured *P. malhamensis* into aquatic ecosystems should be carefully evaluated. Previous studies (Boxhorn et al., 1998; Boenigk & Stadler, 2004; Zhang et al., 2011) have revealed that many large-sized zooplankton (e.g., *Daphnia magna* and *Brachionus angularis*) would die after feeding on *P. malhamensis*, which shows that *P. malhamensis* might be harmful for certain higher trophic zooplankton. Consequently, more field experiments should be carried out to explore the effect of *P. malhamensis* on the community structures of zooplankton and phytoplankton. The high-cell-density cultivation method of *P. malhamensis* established in this study will, at the very least, offer the opportunity to research these issues and to investigate further the potential for controlling *Microcystis* blooms using predatory *P. malhamensis*.

Conclusions

A high-cell-density chemoheterotrophic method of cultivating *P. malhamensis* was established. Under the optimized conditions of 10 g L^{-1} glucose, a C:N ratio of 5:1, temperature of 28 °C, pH of 6.0, and 20% DO, the cell concentration of *P. malhamensis* reached more than 3×10^8 cells mL^{-1}, which exceeds that reported in other studies by more than an order of magnitude. Furthermore, the chemoheterotrophic *P. malhamensis* proved to be effective in grazing unicellular *Microcystis* cells and decreasing the *Microcystis* biomass on the surface of the water by promoting the sedimentation of colonial *Microcystis* cells. This study will play an important role in driving the development of methods to control *Microcystis* blooms using predatory *P. malhamensis*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10811-021-02659-x.

Acknowledgements Thanks are due to Dr. Binliang Wang and Qingyang Song for helping with the outdoor control experiment. The authors also thank the National Aquatic Biological Resource Center (NABRC) at the Institute of Hydrobiology, Chinese Academy of Sciences, for providing support.

Author contribution Mingyang Ma and Fuchen Wang performed the experiments, analyzed the data, and wrote the paper. Chaojun Wei and Hongxia Wang participated in the outdoor experiment. Jianping Chen and Hu Jin participated in the fermentation of *P. malhamensis* in the 7.5-L bioreactors. Lirong Song provided algal cultures and offered crucial suggestions on the analysis of the results. Qiang Hu and Yingchun Gong contributed to the design of the experiments, the drafting of the paper, and revising it critically. All authors gave approval for publication.

Funding This work was funded by the National Key Research and Development Program of China (No. 2019YFD0900302), the National Natural Science Foundation of China (No. 31772419, No. 31872201, and No. 32002413), the National Key Research and Development Project (No. 2017YFE0125700), the China Postdoctoral Science Foundation (No. 2019M662749), and the Agricultural Science and Technology Innovation Action Project of Hubei Province of China (2018).

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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