

Original Research

Ammonium Inhibits Performance of *Rhodopseudomonas palustris* in Cyanobacterial Substrate

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Abstract

Though it is feasible of *Rhodopseudomonas palustris* (*R. palustris*) stimulation in the cyanobacterial substrate, less is known about its performance under the high ammonium-nitrogen ($\text{NH}_4\text{-N}$) circumstance. In the present study, the performance of grown *R. palustris* Strain PUF1 under an $\text{NH}_4\text{-N}$ gradient were investigated. Results showed that both the bacterial density and the pigment synthesis were severely inhibited at an $\text{NH}_4\text{-N}$ concentration of 6.0 g/L, while the ultrathin structure of survived PUF1 wasn't obviously changed in comparison to $\text{NH}_4\text{-N}$ concentration ≤ 3.0 g/L. Noticeably, at an $\text{NH}_4\text{-N}$ concentration of 3.0 g/L PUF1s recovered its biosynthesis of pigments in a six-day acclimation period. Importantly, the PUF1s thrived in algal substrate under the $\text{NH}_4\text{-N}$ concentration ≤ 1.0 g/L with per mL algal substrate 8.96 to 9.88×10^8 colony formation unit (CFU) on day six. Moreover, it was more diverse of the bacterial consortia in the low $\text{NH}_4\text{-N}$ treatments (≤ 1.0 g/L) than that of $\text{NH}_4\text{-N}$ concentration 3.0-6.0 g/L. Additionally, the excess $\text{NH}_4\text{-N}$ reduced the sequestration of phosphorus by PUF1s from the algal substrate. Based on the above findings, an $\text{NH}_4\text{-N}$ threshold up to 1.0 g/L was recommended, it herein produces substantial *R. palustris* biomass and achieves efficient nutrient sequestration from the protein-rich cyanobacterial feedstock.

Keywords: *Rhodopseudomonas palustris*, cyanobacteria, ammonium inhibition

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Introduction

In recent decades the incidents of Harmful Algal Blooms (HABs), especially the blooms of cyanobacteria, have been reported in most eutrophic waters, i.e. ponds, reservoirs, lakes and estuaries throughout the world [1, 2]. The mechanical collection is the physical method using well equipped algae-water separation station and a mobile boat to effectively remove the dense algal cells in HAB periods. This kind of method is commonly used in the emergency management of HABs and can prevent the nutrients and cyanotoxins in cyanobacteria from being released into the controlled aquatic ecosystems. In China the Taihu Lake, Chaohu Lake and Dianchi Lake are among the three most polluted lakes. In these districts, the Chinese government organizes salvage operations of cyanobacteria every year. As in eutrophic Taihu Lake, it reports a production of cyanobacterial sludge – 1.20, 1.64, 1.60 and 2.37 million tons in 2014, 2015, 2016 and 2017, respectively (Jiangsu Almanac, Nanjing University Press, 2014-2017) [3]. Presently it still lacks proper handling of the algal sludge which might become the pollution source in making the stinking odors and even degrading the nearby aquatic ecosystems due to the surface runoff. So that it matters of the researches conducted to make good use of the salvaged cyanobacteria.

Ubiquitously distributed, the bacterium *Rhodopseudomonas palustris* (*R. palustris*) enjoys extraordinary flexibility within the four modes of metabolisms to support its life [4]. This behavior makes it one of the most versatile microorganisms among the known purple non-sulfur bacteria (PNSB). PNSB play roles in the biochemical cycle of carbon (C), nitrogen (N), phosphorus (P), sulfur (S) and iron (Fe) and are good candidates in the bioremediation and wastes or wastewater treatments [5-8]. As reported the mixed culture purple phototrophic bacteria are an effective fishmeal replacement in aquaculture [9]. The protein content in *R. palustris* strains comprise of 60% or more of their cellular weight showing the possibility of single cell protein production from *R. palustris* biomass [10]. *R. palustris* TIE-1's capacity to produce polyhydroxybutyrate (PHB) varied fairly under different conditions, photoelectroautotrophy and photoferroautotrophy showed the highest PHB electron yield and the highest specific PHB productivity [11]. It also revealed the Plant Growth-Promoting Rhizobacteria inoculant of *R. palustris* in sustainable agriculture [12]. In general, the *R. palustris* biomass have broad market prospect.

Cyanobacterial populations are the potential nutrients via the anaerobic digestion through their carbohydrate and protein contents in supporting other microbes, such as *Scenedesmus obliquus* [3]. In theory the feasibility of *R. palustris* production from cyanobacterial waste yields valuable cells that are important in promoting the related industry of biomaterial extraction, fish feed supplement and

probiotics production. It further reutilized the nutrients in cyanobacterial microorganisms that alleviates the nutrient release of algal erosion. One of our previously published work demonstrated the vigorously grown *R. palustris* PUF1 on using dried cyanobacterial materials as prepared from Dianchi Lake [13], but little is known of the potential factors in limiting the yield and performance of PUF1 in cyanobacterial substrate. Since cyanobacterial cells are rich in the protein content, it easily cause the accumulation of $\text{NH}_4\text{-N}$ in the anaerobic digestion of cyanobacteria which might compromise the performance of PUF1 especially when high concentration cyanobacterial inputs and/or long retention time are applied. So far, it is still little information on the performance of *R. palustris* in cyanobacterial substrate. The present study focused on the response of cell morphology, *in vivo* spectrum, microbial community and phosphate sequestration to gradient ammonium aiming at a cost-effective biomass production of *R. palustris* from cyanobacterial wastes.

Materials and Methods

Materials

The bacterium *Rhodopseudomonas palustris* strain PUF1 was isolated from the Donghu Lake in Wuhan, China. This isolate is straight or slightly curved rod-shaped, 3.05 to 10.06 μm in length, 0.32 to 0.68 μm in diameter, with laminar membranes. Additionally, this bacterial culture appears dark purple-red with major pigments being bacteriochlorophyll a and carotenoids [13]. The pure culture was daily preserved in the double-layer solid ATYP medium (agar content, 1.5%). The composition of the ATYP medium is referred to Lu et al. (2018) [14]. Cells in the exponential growth phase were used for the experiment. Before inoculation, bacterial suspensions were rinsed twice with 0.85% sodium chloride to remove the ATYP medium.

The cyanobacteria used in the present study was collected in August, 2015 from eutrophic Lake Dianchi in Kunming, China. The wet algal sludge was air-dried to a final ten percent moisture content. Before use, algal solids were mechanically ground to a particle size of under 0.4 mm. The major elements in per gram dry algal solids was on average 429.6 mg C, 82.5 mg N and 6.40 mg P [13].

Experimental Design

In the present work, the photo-bioreactors were serum bottles with a working volume of 300 mL. The algal substrate 3 g/L was used by suspending certain algal solids in the aseptically distilled water. The PUF1 inoculated was initially 2.00×10^7 CFU/mL by the colony enumeration. Before being exposed to light, the inoculated bottles were two-hour dark adapted to consume the residual dissolved oxygen and then

incubated at 28°C with a light intensity of 3000 lux on the bottle surface. Ammonium chloride ($\geq 99.8\%$, GR, Sinopharm Chemical Reagent Co., LTD) was supplemented to final concentrations of 0.5, 1.0, 3.0 and 6.0 g N/L. The only algal substrate was designated as 0 g N/L. The initial pH in the culture broth was adjusted to 6.8 with 1 mol/L HCl (or NaOH).

Analytical Methods

Cell Growth

For the growth determination, three milliliter bacterial suspension in each treatment was retrieved and then centrifuged at 6000 g for 10 min to obtain the cell pellet. The cell pellets obtained were immediately pictured as shown Fig. 1a). Moreover, the viable cells at 24 h and 144 h were further checked by enumerating the pigmented colonies preserved on the solid ATYP medium (1.5% agar), results are shown in Fig. 1b).

Transmission Electron Microscopy

The Transmission Electron Microscope (TEM) was used to examine the cell ultrathin structure. The cell pellets at 144 h were washed by 0.1 mol/L phosphate buffer solution (pH 7.2), then fixed by 2.5% (v/v) glutaraldehyde solution for 2 hours at room temperature and post fixed with 1.0% osmium tetroxide for 1 hour at room temperature after removal of glutaraldehyde. Afterwards, cells free from osmium tetroxide were dehydrated through a series of ethanol treatment (75-100%) and acetone (75-100%) and further infiltrated with Spurs resin at concentrations of 5%, 33%, 66% for 2 hours each. The embedding was performed in 100% resin. Samples were polymerized for 20 hours at 70°C, sectioned with UC7 (Leica, Austria), stained with uranyl acetate and lead citrate and viewed using HT-7700 (Hitachi, Japan).

In vivo Spectrum

Pigments of bacteriochlorophylls and carotenoids in *R. palustris* give its *in vivo* spectrum the characteristic absorption peaks. Bacterial cells at 72 h and 144 h were collected to check the changes of the character absorption spectrum to up-regulated $\text{NH}_4\text{-N}$. For the *in vivo* spectrum measurement, the intact cell suspension was resuspended with 60% sucrose and scanned by the Cary WinUV spectrophotometer (Agilent Technologies, America) in a scanning range of 300 to 1000 nm.

DNA Extraction and High-Throughput Sequencing

The microbial consortia resided in the algal substrate at 144 h were determined by high-throughput sequencing. The environmental DNA from each

sample was isolated using the E.Z.N.A.[®] Bacterial DNA Kit (Omega Bio-tek, America) according to the manufacturer's protocol. The V3-V4 region of 16S rRNA gene was amplified from the genomic DNA using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') [15]. The 20 μL PCR reaction system contained 0.5 μL of DNA template, 0.5 μL of each primer (10 mM), 10 μL of 2 \times Es Taq MasterMix (CWBIO, China) and 8.5 μL distilled water. PCR conditions were as follow: 10 min at 94°C, followed by 45 cycles of 10 s at 94°C and 30 s at 60°C, and final extension 5 min at 72°C. The amplified products were purified with AxyPrep[™] DNA Gel Extraction Kit (Axygen Biosciences, USA). The purified products were sent to Shanghai Majorbio Technology Co., Ltd. (Shanghai, China) to perform using Illumina high-throughput sequencing on the Miseq platform. Raw fastq files were quality-filtered and assembled using QIIME. Bioinformatic analysis of the sequencing data was performed on the free online platform of Majorbio I-Sanger Cloud Platform (www.i-sanger.com).

Phosphate Phosphorus Determination

The phosphate-phosphorus ($\text{PO}_4^{3-}\text{-P}$) in the algal substrate potentially correlates with the bacteria activity indicating the inhibitive effect of $\text{NH}_4\text{-N}$. To obtain the supernatant, PUF1s and other solids were separated from the liquid by centrifugation (10,000 g, 10 min). The phosphate-phosphorus ($\text{PO}_4^{3-}\text{-P}$) in supernatants was determined by Molybdenum blue complex formation method.

Data Presentation and Statistical Analysis

All experiments were conducted in triplicate. Means and standard deviations are presented. One-way analysis of variance was used to analyze statistical difference between means at $P < 0.05$.

Results

As shown in Fig. 1a), it visually verified the growth performance of PUF1. On the sixth day the pigmented colonies in treatment e continuously decreased to $1.20 (\pm 0.46) \times 10^5$ CFU/mL with the initial $\text{NH}_4\text{-N}$ 6.0 g/L and in treatment d with the initial $\text{NH}_4\text{-N}$ 3.0 g/L it indicated a cell increase to $9.29 (\pm 1.07) \times 10^7$ CFU/mL which improved by 4.6-fold compared with the initial PUF1s, while in treatments a, b and c cell numbers substantially augmented to $8.96\text{-}9.88 \times 10^8$ CFU/mL with no significant difference of $\text{NH}_4\text{-N}$ concentration ≤ 1.0 g/L (Fig. 1b). Based on the above results, a $\text{NH}_4\text{-N}$ threshold of up to 1.0 g/L was recommended at the algal concentration of 3.0 g/L.

As shown in Fig. 2, cell suspensions under an

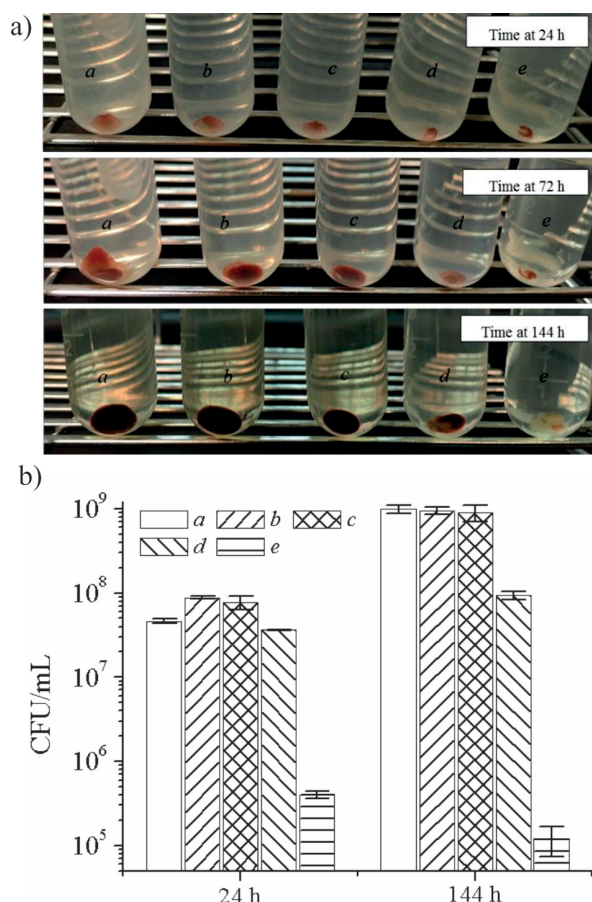


Fig. 1. Growth potential of PUF1. a) Cell pellets at a culture age of 24 h, 72 h and 144 h, respectively. b) Cell numbers measured at 24 h and 144 h by colony enumeration. Letters of a, b, c, d and e separately represent $\text{NH}_4\text{-N}$ concentrations of 0, 0.5, 1.0, 3.0 and 6.0 g/L.

$\text{NH}_4\text{-N}$ concentration ≤ 1.0 g/L always involved the characteristic absorption peaks at 806 and 866 nm indicating the presence of pigment bacteriochlorophyll a [16], while PUF1s of an $\text{NH}_4\text{-N}$ concentration of 6.0 g/L lost all the designated peaks from bacteriochlorophylls and carotenoids over the whole experimental period. Noticeably, suffering a middle $\text{NH}_4\text{-N}$ concentration of 3.0 g/L PUF1s restored its pigment synthesis ability after a six-day acclimation period. Therefore the fabricated photopigments in PUF1 associated with the incubation time and $\text{NH}_4\text{-N}$ concentration.

Representative TEM images of PUF1 under different $\text{NH}_4\text{-N}$ concentrations are presented in Fig. 3. PUF1 was a Gram-negative, rod, vibrioid or peanut shaped cells. It reported that *R. palustris* formed rosette-like clusters in older cultures and reproduced as budding [16]. The cell division occurred by budding was also observed (Fig. 3b). By the TEM examination it also revealed the internal membranes of the lamellar type and cellular inclusions of PHBs and polyphosphate (Poly-P), same with previous researches [6, 11, 16]. Overall, the ultrathin structure of PUF1 seemed no obvious damage even to an $\text{NH}_4\text{-N}$ concentration of up to 6.0 g/L in comparison to the $\text{NH}_4\text{-N}$ concentration ≤ 3.0 g/L.

The white part in Fig. 3c) shows the PHB accumulation. Bar, 1 μm . Letters of a, b, c, d and e separately represent $\text{NH}_4\text{-N}$ concentrations of 0, 0.5, 1.0, 3.0 and 6.0 g/L.

As shown in Fig. 4, genus *Aneurinibacillus* at a value of 0.61 in the control treatment (CK, the only algal substrate without PUF1 inocula) dropped to 0.12 in T1 (the treatment a) and in T5 genus *Bacillus* and *Rhodospseudomonas* were the most dominant subgroups

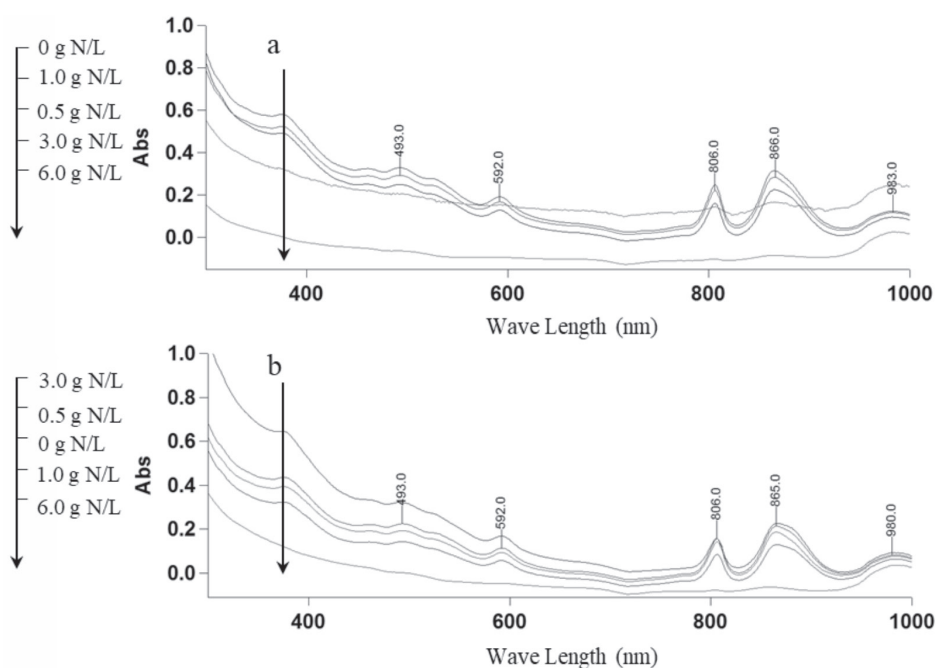


Fig. 2. Spectra of intact cells at 72 h a) and 144 h b). The left-down arrow marked with nitrogen concentration corresponded to the orders of stacked lines.

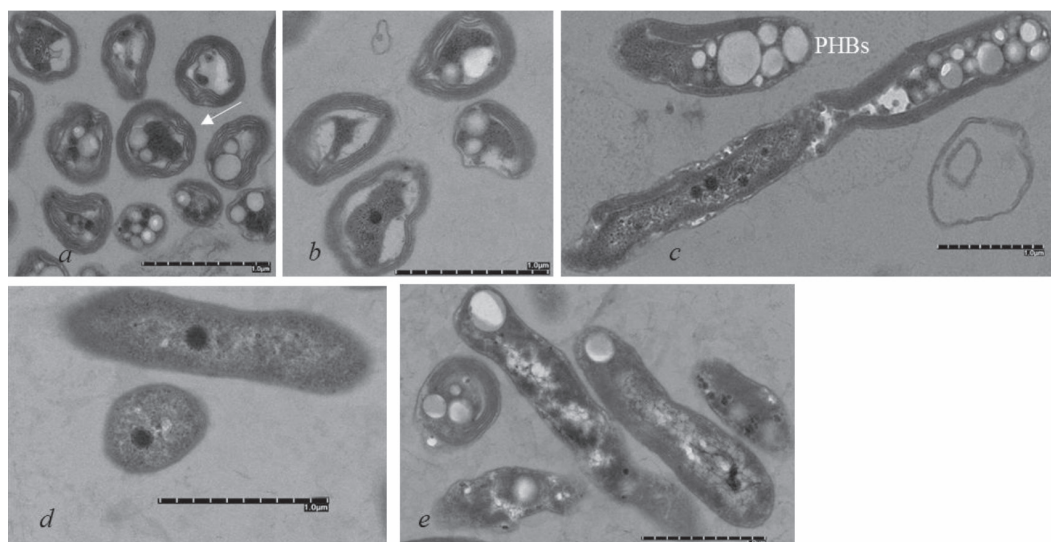


Fig. 3 The ultrathin structure of PUF1. The arrow shows the lamellar membranes. The white part in Fig. 3c shows the PHB accumulation. Bar, 1 mm. Letters of a, b, c, d and e separately represent $\text{NH}_4\text{-N}$ concentrations of 0, 0.5, 1.0, 3.0 and 6.0 g/L.

having a respective ratio of 0.20 and 0.795 (the treatment e). The genus *Aneurinivacillus*, *Clostridium_sensu_stricto_10* and *Clostridium_sensu_stricto_12* were relatively abundant of $\text{NH}_4\text{-N}$ concentration ≤ 0.5 g/L and strains from genus *Bacillus*, *Paenibacillus* and *Clostridium_sensu_stricto_13* were among the most tolerant microbes of $\text{NH}_4\text{-N}$ 3.0-6.0 g/L. The Chao index in T1, T2 and T3 (the treatments a, b and c) significantly differed ($P = 0.02$) from that of in T4 and T5 (the treatment d and e).

The microbial metabolism severely associates with the nutrient digestion and assimilation. Hence the phosphate in algal substrates well confirmed the $\text{NH}_4\text{-N}$ inhibition effects. As shown in Fig. 5, the dissolved $\text{PO}_4^{3-}\text{-P}$ in 0-12 h was likely to be ascribed to the free diffusion rather than the bacterial metabolism.

0-48 h the $\text{PO}_4^{3-}\text{-P}$ accumulated indicating the greater dissolution than ingestion by PUF1s, whereas it observed a fast depletion of $\text{PO}_4^{3-}\text{-P}$ from 72 h to 144 h corresponding to the mid-log phase to stationary phase with considerable cells then. Additionally, the $\text{PO}_4^{3-}\text{-P}$ in algal substrate with an $\text{NH}_4\text{-N}$ concentration of 3 g/L set to be significantly higher than that of with 6 g/L $\text{NH}_4\text{-N}$ after an incubation time of 48 h showing the continuously severe inhibitory effect at 6 g/L $\text{NH}_4\text{-N}$.

Discussion

Nowadays waters eutrophication and HABs are one of the most serious pollutions in the whole world [1, 2]. In China the HABs, especially in the Taihu Lake,

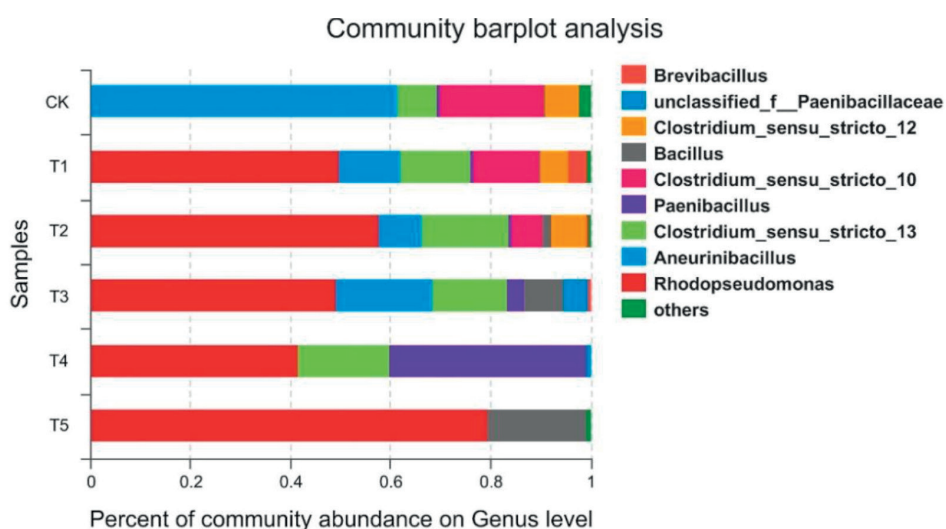


Fig. 4. Percent of community abundance on Genus level. CK, algal substrate only. T1, T2, T3, T4 and T5, same algal substrate with *R. palustris* inocula having respective $\text{NH}_4\text{-N}$ concentrations of 0, 0.5, 1.0, 3.0 and 6.0 g/L.

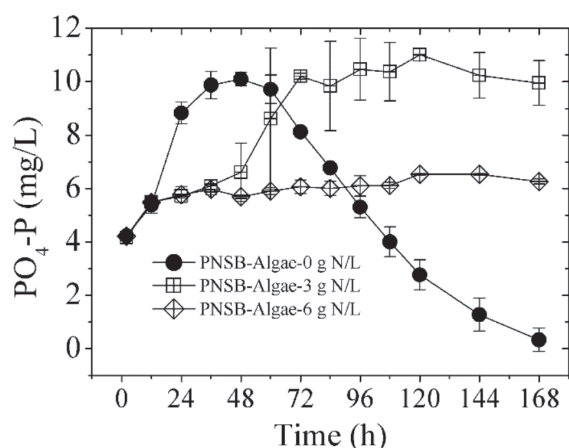


Fig. 5 Dynamics of $\text{PO}_4^{3-}\text{-P}$ in algal substrate with varied $\text{NH}_4\text{-N}$ concentrations.

Chaohu Lake and Dianchi lake has attracted much more attentions for their severe damage on human health and aquatic ecosystems. The thick algae mat on the surface water prevents the penetration of solar irradiation into the lake bottom that inhibits the photosynthesis of submerged plants [17]. Also algae erosion in lake rapidly depletes the limited dissolved oxygen that results in the anoxia and harms to the animals [17]. In HAB occurring days, the intensive salvage operation is performed producing huge amount of cyanobacterial wastes [3]. To date a high proportion of salvaged algae needs to be properly treated. Like plants, the cyanobacteria can also perform photosynthesis. The carbohydrate synthesized can be good nutrients in cultivating the probiotics in environments [3]. We previously demonstrated the stimulation of *R. palustris* PUF1 in the only substrate of dried cyanobacteria, however, much more informations are awaited to be discovered for a fruitful scale production of this process.

On account of the high protein content in cyanobacteria, the $\text{NH}_4\text{-N}$ accumulation might heavily inhibits the cell activities even to a toxic level in anaerobic digestion, and this condition may become worse with more cyanobacterial inputs or under a longer retention time. Therefore, the grown *R. palustris* PUF1 to gradient $\text{NH}_4\text{-N}$ in cyanobacterial substrate was probed in the present study aiming at a cost-effective biomass production and efficiently reutilized the cyanobacterial wastes. Results showed a considerable cell biomass under a $\text{NH}_4\text{-N}$ concentration ≤ 1.0 g/L with normal pigment synthesis and cell ultrathin structure. It is well acknowledged that bacteria have the ability to adapt to changing conditions to ensure their reproduction and maintain growth under inferior conditions. The study proved that the trehalose was accumulated in protection of the enzymatic functionality against salt stress to allow hydrogen production [18]. Based on the above findings, we deemed it more susceptible of the pigment synthesis to changed $\text{NH}_4\text{-N}$ than the cell morphology by TEM observation. It documented that the depression

of photopigment synthesis resulted in reduced ability of photoheterotrophic growth in photosynthetic biofilm under anaerobic conditions with the increase in illumination intensity beyond the light saturation [19]. Thus changes of *in vivo* spectrum of intact *R. palustris* might be an ease indicator to evaluate the inhibitory effect of high ammonium on PUF1's survival.

The ammonium control is not only key to the cell growth, but it also greatly impacts on the nutrient sequestration from the extracellular environment. It's well known that the microorganisms grew rapidly under growth-sufficient conditions degrading a large amount of bioavailable substrates [6]. Polyphosphate is a linear biopolymer composed of three to hundreds of phosphate residues that are linked by high-energy phosphoanhydride bonds [20]. Several isolated PNSB were superior in poly-P accumulation through bacterial assimilation and accumulation. In the present study, the $\text{PO}_4^{3-}\text{-P}$ depleted in cultures was mainly sequestered by PUF1 in the exponential and stationary phase. It was in consistence with Puyol et al. (2015) [21], showing the more poly-P accumulation in static mode rather than in growth mode of PNSB. The study showed that the phosphorus content during illuminated anaerobic incubation ranged from 13% to 15% of the bacterial cell dry weight in the isolated *R. palustris* G11 [22]. It also reported that when the carbon and/or energy source were/was removed, *R. palustris* G11 released intracellular poly-P or PHB to obtain energy to grow or maintain its growth [6]. Our results also found a release of phosphorus from PUF1 into algal substrate with the retention time of 14 day when the bioavailable organic acids was depleted (data not shown). Therefore, the study on a careful evaluation of compositions of cyanobacterial substrate to balance the contents of bioavailable C, N, P, minerals and growth factors is needed and will further enhance the performance of PUF1 growth and nutrient recovery.

As far as we can see, the ammonium changed the microbial community in algal substrate. The genus *Bacillus* stood out as one of the most $\text{NH}_4\text{-N}$ tolerant microorganisms except the PUF1. It is widely accepted of the beneficial probiotic bacteria under the genus *Bacillus*. Researches showed the *Bacillus* strains effective in promoting growth, suppressing diseases, and inducing systemic resistance in fish and shrimp hosts, and improving overall environment for sustainable aquaculture system [23]. A genetically engineered *Bacillus subtilis* served as a new bio-absorbent that acted in recovering organic matters and ions from wastewaters [24]. The biofloculants have drawn an increasing interest because they are biologically active, biodegradable, nonpolluting and harmless to the environment. Cost-effective biofloculants produced improved the cell harvest ratio which is critically important in commercialization of cell biomass-based industrial processes [14]. A study by Bukhari et al. (2016) [25] indicated the biofloculant production of *Bacillus marisflavi* NA8 using valuable

palm oil mill effluent. Of large scale production of *R. palustris* biomass, it confronts a low cell harvest ratio, since PNSB are small in size and maintain stable suspension state in the culture medium that are hard to be separated from water and sequentially, they flow out of the bioreactors with effluents [26]. Thus combining the probiotic bioflocculant produced *Bacillus* with *R. palustris* might enhance the cell harvest promoting their application in large scale. On the other hand it is also desirable of a combined production of *R. palustris* and *Bacillus* from the cyanobacterial substrate since they both prefer an anaerobic circumstance, degrade biowastes, and are functionally used in aquaculture.

Conclusions

Growth performance of *R. palustris* PUF1 in cyanobacterial substrate highly depends on the concentration of ammonium. A threshold of $\text{NH}_4\text{-N}$ concentration up to 1.0 g/L is recommended for the scale production of *R. palustris* PUF1 from cyanobacterial substrate considering the aspects on cell biomass, morphology, pigment synthesis and nutrient sequestration. Moreover, a quick examination on the pigment synthesis is helpful in judging the cell performance in daily incubation periods. A clear $\text{NH}_4\text{-N}$ resistant mechanism of grown *R. palustris* PUF1 in cyanobacterial substrate needs to be explored in depth, especially the molecular mechanism responsible for the repair of pigment synthesis.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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