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Original Research

Nutrient Recovery from Cyanobacteria Biomasses Using Purple Nonsulfur Bacterium *Rhodopseudomonas palustris*

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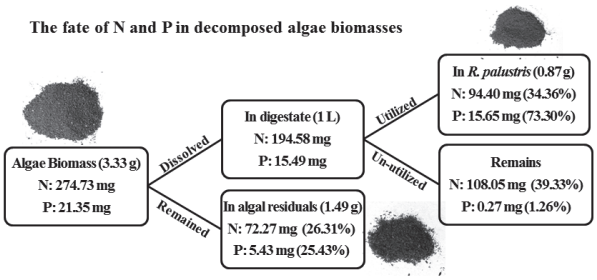
Abstract

Occurrences of harmful cyanobacterial blooms are a worldwide environmental problem in most eutrophic lake ecosystems. But what should be noticed is that cyanobacteria can be used as a useful resource due to the wide range of metabolites they produce. Nutrient partitioning using purple nonsulfur bacteria (PNSB) has the potential to biologically concentrate nutrients. The present study evaluated the kinetics of nutrients released from decomposed field blue green algae (BGA) biomasses. The potential of nutrient acquisition from decomposed BGA biomasses for culturing *Rhodopseudomonas palustris* (*R. palustris*) was investigated via fed-batch experiments. Results indicated that *R. palustris* stimulated in algae substrates with algae biomasses ranging from 3.33 to 10 g/L. Removal efficiencies of N and P in the stationary phase of growth were at least 40% and 95%, respectively, of all the nitrogen (N) and phosphorus (P) released. Additionally, the cellular contents like total lipid and poly-β-hydroxybutyrate (PHB), as well as the fatty acids produced by *R. palustris*, were consistent. Hence, practice based on the bacterial production for the nutrient recovery from BGA biomasses provides a new insight in field algae disposal. It will lower the chances of secondary pollution due to algae decay and produce giant cells of *R. palustris* and surely will prosper the industries applying *R. palustris*.

Graphic Abstract



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Keywords: purple nonsulfur bacteria, *Rhodopseudomonas palustris*, biomass production, blue green algae, nutrient recovery

Introduction

Though difficult to verify, it is widely held that harmful algal bloom outbreaks have aggravated over the past few decades at both global and regional scales [1]. Blooms of blue green algae (BGA) are more frequent and widespread, causing global public health and environmental concern. In 2007, 40% of the total area of Lake Taihu was incredibly covered by cyanobacterial blooms challenging more than two million residents with a week-long drinking water crisis [2-3]. Physical and chemical methods can remove algae from water being the algae sludge. Dealing with this algae sludge will be an issue point for preventing secondary pollution from algae decay [4]. Therefore, management of these huge biomasses is an ongoing challenge for lake managers and policymakers.

Algae biomasses take in huge quantities of carbon (C), nitrogen (N), and phosphorus (P) from water bodies. Besides the essential elements of C, N, P, and the minerals of microalgae met the nutrient requirements of the anaerobic microflora [5]. As mentioned, algae are not only the product of eutrophication in natural lakes but also can be used as potent substrate due to the abundant nutrients they release by anaerobic digestion [6].

As numerously reported, purple nonsulfur bacteria (PNSB) are an important subgroup of anoxygenic phototrophs used for waste biodegradation and hydrogen production [7-8]. *Rhodopseudomonas palustris* (*R. palustris*) is among the most metabolically versatile microorganisms known and reserves a much larger inventory of degradation genes than current knowledge by genome sequencing [9]. Phototrophic bacteria are apt to store organic carbon as poly-hydroxybutyrate (PHB) [10] and help with nutrient removal, e.g., by inorganic poly-phosphate formation [11]. Additionally, *R. palustris* TN110 showed great potential as a biofertilizer in that it provides ammonia and plant growth-promoting substances (of indole-3-acetic acid and 5-aminolevulinic acid), and reduces heavy metals and methane greenhouse gasses [12]. Though PNSB have significant potential in bioremediation, few researchers have focused on the selection of a natural feedstock for *R. palustris* biomass production.

On most occasions, raw substrates used for sustaining PNSB are wastewaters having combined effects of waste removal and energy production (i.e., hydrogen) [13]. The common characteristic of the wastewater steams is the abundance of bioavailable organic materials (lipids, carbohydrates, proteins). These substances are also important compositions in the algae cell [14], which are a potential nutrient source [6]. It was reported that microalgal biomasses or extracts coupled with anaerobic bacteria (e.g., *Lactobacillus amylovorus* or *Clostridium butyricum*) sustained *Rhodobium marinum* strain A-501 [15] and *Rhodobacter sphaeroides* strain KD131 [16]. However, the strains of algae utilized are exclusively green algae either from freshwater species of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* or the marine species of *Dunaliella tertiolecta* [15-17]. Few researchers have investigated the potential of field BGA as substitutive substrate for *R. palustris*. On the contrary, it is more meaningful using field BGA as eco-feedstock to the mass production of *R. palustris*.

It is urgent to deal with the increasing amount of BGA biomass for water quality improvement and secondary pollution prevention. PNSBs are known as fast-growing microorganisms that can be used as an alternative mediator for combined biological carbon, nitrogen, and phosphorous removal [18]. In the present study, three aspects on the feasibility of nutrient recovery form decomposed algae using *R. palustris* are to be involved: 1) Kinetics of nutrients released from the decomposed BGA biomasses, 2) Determining the growth performance of *R. palustris* in algae substrates, and 3) Identifying nutrient removal efficiency and quantify the cellular contents of *R. palustris*. In this paper, we present the first description of using field algae as the potent feedstock for *R. palustris* production achieving the goals of nutrient recovery. These results provide new insights into the management of BGA and concomitantly produce *R. palustris* cells that are biological water purifiers.

Material and Methods

Bacteria

The pure strain of PNSB was maintained in ATYP liquid medium under weak illumination (1,000 lux) at 28°C. This strain was isolated in a sediment sample from Lake Donghu (Wuhan, China; for the compositions of ATYP, see [19]). The online blast result discriminates the isolate to species of *Rhodopseudomonas palustris* under accession number KU886140 and it was designated *R. palustris* strain PUF1. Similar sequences of its relatives were downloaded from the database of Genbank (*ncbi.nlm.nih.gov/nucleotide*) for a phylogenetic analysis shown in Fig. 1. The phylogenetic tree was constructed with MEGA 5.1 software by the neighbor-joining method and the topology of the tree was evaluated using a bootstrap with 1,000 replications.

Field Algae Collection

Fresh BGA were collected in August 2015 at the downwind area in Lake Dianchi (Kunming, China). The harvested fresh algae were concentrated to form algae sludge (~5% total solids). Next, smooth the thickened BGA biomasses onto the nonwoven cloth to dry in shade. After drying, the algae biomasses have a moisture

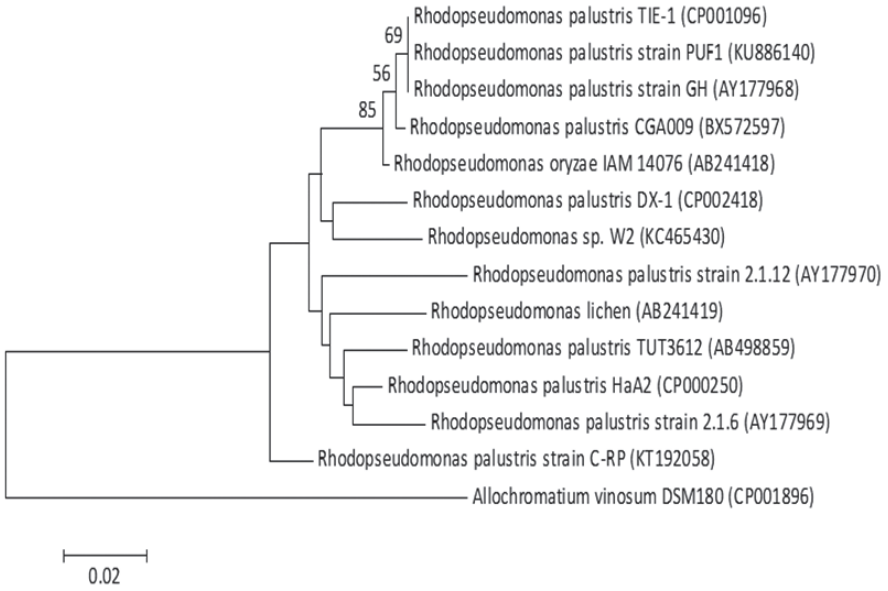

Fig. 1. A phylogenetic tree based on puf M sequences of R. palustris strain PUF1 and other closely related species. The numbers at the nodes represent the level of bootstrap support (%) based on the neighbor-joining analyses of 1,000 resampled datasets. The scale bar represents the branch length.

content of approximately 10%. Finally, they were ground mechanically into small granules within 0.43 mm forming the homogenous feedstock.

Experimental Design

Kinetics of Nutrients Released from Decomposed BGA Biomasses

In this section, experiments were performed for kinetics determination on nutrient releasing derived from the decomposed algae biomasses. Suspend the pre-weighed algae biomasses with aseptic double-distilled water to a final algal concentration of 5, 10, 15, and 20 g/L. Total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) in the liquid were periodically examined.

Growth Performance of R. palustris at Different Algae Substrates

In this study, serum bottles (diameter 6.5 cm, height 14 cm) with a working volume of 300 mL were used as the photo-bioreactors. *R. palustris* stain PUF1 was used as the initial inoculant after having it rinsed three times to remove the ATYP medium. Before experiments, certain amounts of BGA together with the bioreactors were sterilized at 121ºC for 30 min. After cooling to room temperature, approximately 300 mL aseptic distilled water was added to the bioreactors followed by *R. palustris* inoculation. An initial cell concentration of ~0.04 (OD_{650nm}, optical density at 650 nm) was recommended and the experimental sets were incubated at 28ºC with an observed light intensity of ~2,000 lux on the surface.

Analytical Methods

Analysis of Cell Growth

The bacterial biomass of *R. palustris* was determined by measuring OD650 nm [20]. A logistic (sigmoid) model was adopted to fit the whole growth curve. OD can be described as a function of time using Equation 1:

$$OD\ (t) = a\ /\ (1 + \exp\ (-k\ (x - xc))) \quad (1)$$

…where a represents the maximum OD (OD_{max}) and k indicates the specific growth rate, and xc is the time to half OD_{max}, which can be normalized to compare growth rates with diverse starting ODs and different start-up rates. The cell cultures (2 mL) were centrifuged at 10,000 g for 10 min to obtain the cell pellets. The bacteriochlorophyll a (Bchl a) of *R. palustris* cells were extracted with acetone and methanol (7 : 2 (v/v)). The supernatants from extracts were measured at 775 nm [21] for the determination of Bchl a concentration.

Total Lipid, PHB, and Fatty Acids Determination

We examined cells of *R. palustris* in stationary phase growth. The amounts of total lipid and PHB were calculated as contents to the dry cell weights. A certain amount of lyophilized cells (generally 0.20 g) were directly extracted with methanol: chloroform (1:2, V:V) for total lipid measurement. The extracts were dried under nitrogen [22]. PHB is a biodegradable material that produces and accumulates in the late exponential and stationary

phases of PNSB. The extraction of PHB was performed according to a protocol previously described [23]. Thawed cell pellets were used for the profile of cellular fatty acids according to the indicated reference [24].

Water Quality Determination

Approximately 8 mL of cell suspension was periodically collected. Supernatant was used for water quality determination according to the standard methods (APHA, 1998) [25]. Parameters of TN (total nitrogen), TDN (total dissolved nitrogen), NH4-N (nitrogen in ammonia), TP (total phosphorus), TDP (total dissolved phosphorus), T-COD (total chemical oxygen demand), and D-COD (dissolved chemical oxygen demand) were determined.

Statistical Analysis

Three replicates were performed for each experiment. Mean values and the standard deviations were presented. Significant differences among means were calculated at a p-value <0.05. Student's t-test was used to compare the total lipid and PHB yields from algae substrates and ATYP medium and nutrient removal efficiencies from different algae substrates using IBM SPSS Statistics software (Release ver. 18.0.0; SPSS Inc.).

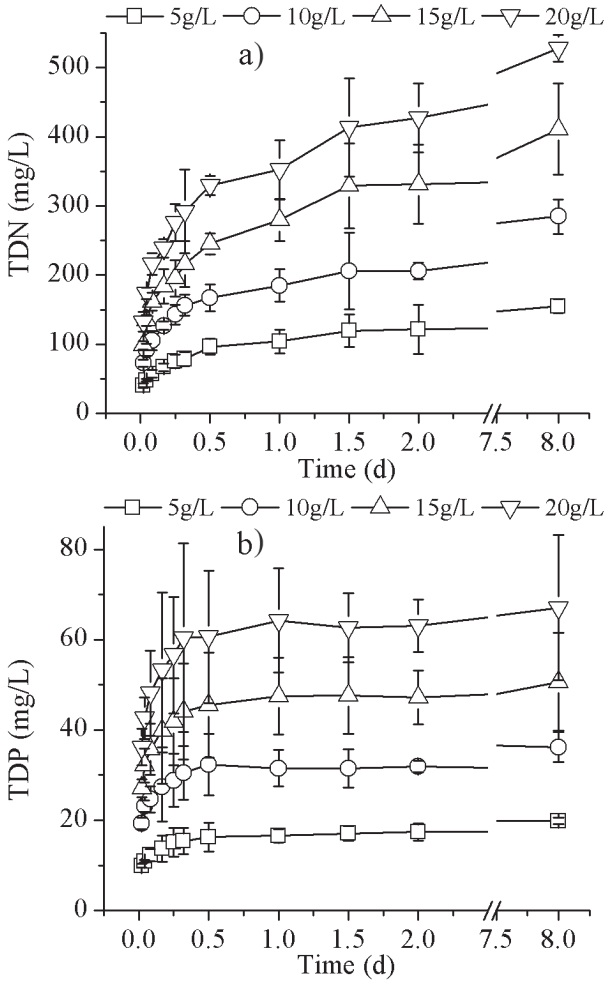

Fig. 2. Kinetics of nutrient releasing from algae biomasses calculated on the concentration of TDN a) and TDP b) in the digestates. Values are means of three replications ± standard deviation.

Results and Discussion

Kinetics of Nutrient Releasing from Decomposed BGA Biomasses

Determining the kinetics of nutrient releasing from algae biomasses is a way to apprehend their digestion potential. As shown in Fig. 2, this indicated a fast nutrient excretion calculated by TDP and TDN from decomposed cyanobacteria. More than half phosphorus (P) and nitrogen (N) were dissolved in one day (usually12 hours) of different algal amounts (5, 10, 15, and 20 g/L). Noticeably, the amount of TDN was progressively evolved due to the conversion of nitrogenous substances, e.g., protein.

Being alternative substrates, algae concentrates with intact cell walls require pretreatment (either by ultrasonic disruption, enzymatic hydrolysis, and/or thermal decomposition), especially for the concentrate like *Chlorella vulgaris* that has a rigid cell wall [5, 26]. The dehydration procedure damages the cell wall of the BGA. This step makes the organic matters more accessible to the anaerobic microflora and thus were more easily being degraded. The success of *R. palustris* selection from algae biomasses was the bioavailability to organic and inorganic materials from the cellular inclusions apart from light conditions.

Nutrient Comparison between Algae Substrate and ATYP Medium

The incubation system of algae substrates automatically converted to anoxia within 12 to 20 hours either with or without *R. palustris* inoculants (data not shown). In the present work, overall nutrients (also the dissolved parts at the initiation time) of algae substrates (3.33 g/L) and ATYP medium were compared. Shown in Fig. 3, the comparison indicated relative abundances of N and C contents in algae substrates. The initial NH_4-N and TDN in algae substrates (released within two hours) were less, whereas they were progressively increased due to phototrophic activity from nitrogenous substance conversions (see results of NH_4-N and TDN in Figs 4a-b). Noticeably, the amount of phosphorus of algae substrates was relatively low. We performed experiments to find out whether phosphorus was the limiting factor in the growth of PUF1. A final P-concentration of 100 mg/L was adjusted using phosphate with BGA concentration of 3.33 g/L, but no enhanced biomass production in stationary phase growth. Concomitantly, phosphorus reduced in the digestion did not differ much (data not shown). Cyanobacteria contain a vast amount of organic molecules, such as low-molecular-

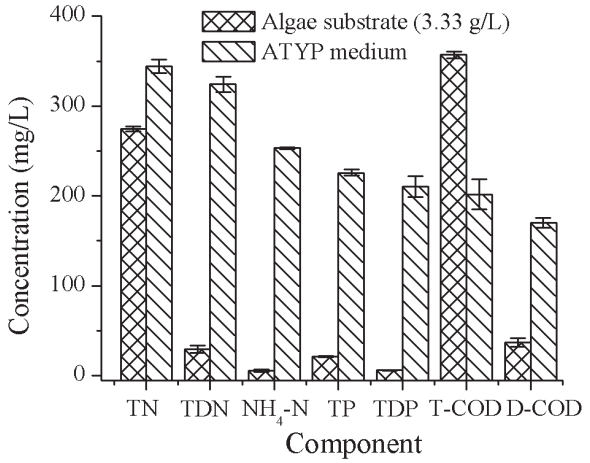

Fig. 3. Nutrient comparison between algae substrate (3.33 g/L) and ATYP medium. Values are means of three replications ± standard deviation. Concentrations of NH_4-N, TDN, TDP, and D-COD were the initial amounts released in two hours of algae substrates.

Table 1. Main characteristics of algae biomass, algal residual, and *R. palustri*

Element content	Algae biomass	Algal residual*	*R. palustris*
N (%)	8.25	4.84	10.15
C (%)	42.96	43.71	51.46
P (mg/g)	6.40	3.64	15.08
K (mg/g)	5.99	0.50	1.36
Ca (mg/g)	10.48	16.07	2.52
Mg (mg/g)	2.68	1.73	2.39
Fe (mg/g)	0.83	1.16	0.40
Zn (µg/g)	28.75	36.99	37.71
Ash (%)	6.71	7.21	5.90

*Algae biomasses after digestion were algal residuals. *R. palustris* cells loosely attached to the algal residuals were removed by dilutions with distilled water until there was no obvious red color found. The retrieval rate of algal residuals was 44.84% (mean of six replicates).

weight compounds and extrapolymeric substances composed of proteins, lipids, and nucleic acids, as well as acetate, propionate, lactate, and ethanol as fermentation products [14]. All of these molecules were beneficial in developing the growth substrate. For direct evidence, see Table 1 on the elemental analysis of algae biomasses (used for experiments), algal residuals (recollected after digestion), and *R. palustris* (in algae substrates).

Therefore, the decomposed cyanobacteria can be potential supplements for *R. palustris* generation. Hiraishi et al. (1989) explained that organic nutrient strength affected the content of PNSB and the metabolic activity of photosynthetic sludge for wastewater treatment [27].

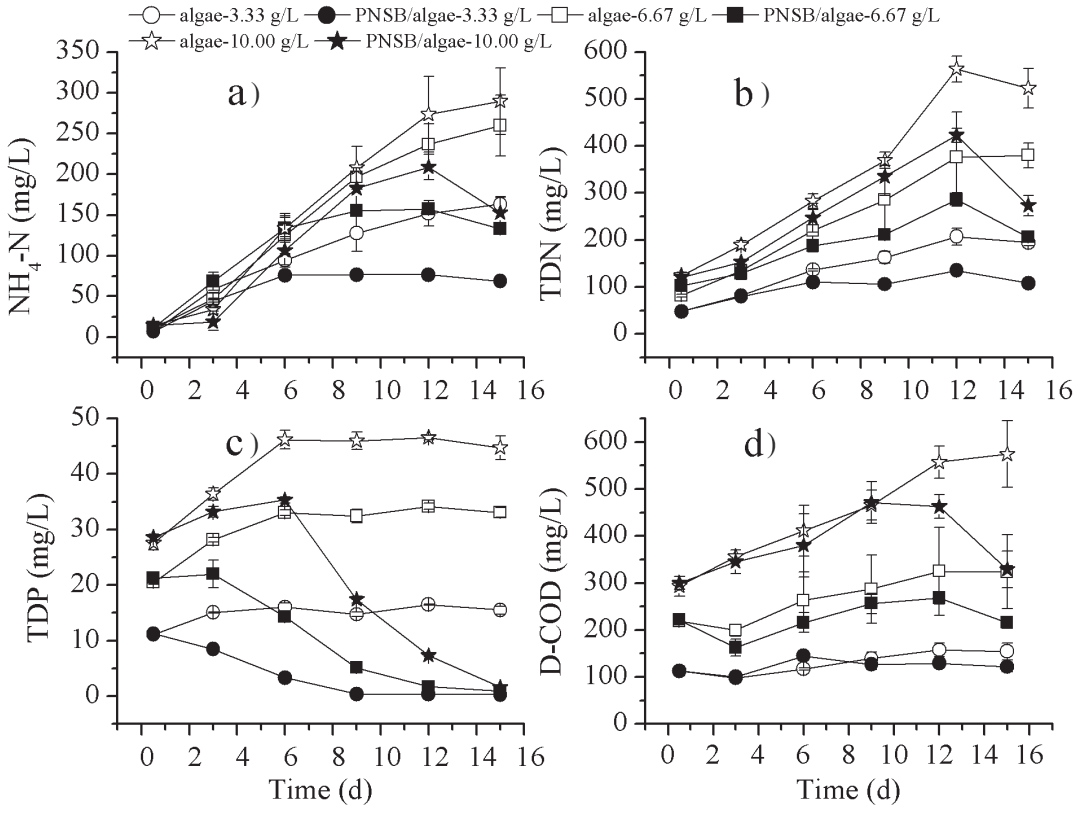

Fig. 4. Time course of NH_4-N a), TDN b), TDP c), and D-COD d) of different algae substrates. Values are means of three replications ± standard deviation. PNSB, namely *R. palustris* strain PUF1.

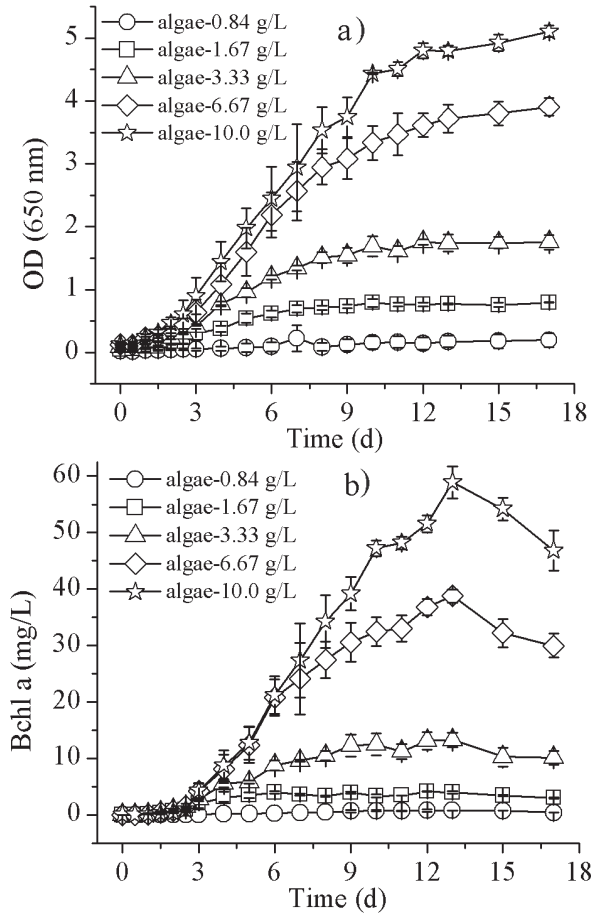

Fig. 5. Trends of $OD_{650\ nm}$ a) and Bchl a b) versus time attained under different algae substrates. Values are means of three replications ± standard deviation. OD_{650nm} was the corrected value that subtracted the absorbance from algae substrate. A dilution factor of five (one part cultures with four parts distilled water) was applied when in high density.

It observed a gradual inhibition of anaerobic ammonium oxidation activity by the high-strength organic matter, also an accumulated nitrite when the influent concentration of COD was 2,000 mg/L [28]. We speculate that the amount of algae biomasses added would affect bacterial performance, which is an essential topic to determine a rational range for growing *R. palustris* in algae substrates. Meanwhile, factors involving biomass output and waste removal efficiency were to be calculated.

Growth Performance of *R. palustris* with Different Algae Substrates

Growth performances of *R. palustris* under different algae substrates varying from 0 to 10 g/L were compared (Fig. 5). None of (or little) growth of strain PUF1 was observed in algae substrates of 0.84 g/L, while algae substrates (10 g/L) gave a maximal Bchl a concentration of ~59 mg/L, 3.69 folds of 16 mg/L in ATYP medium (Table 2). Vigorous degradation was undergone in the log phase between 72 h to 216 h of algae substrates 3.33 to 10 g/L. Both OD650 nm and Bchl a content in the stationary phase were positively correlated (R^2 >0.98) with the algae concentrations (from 0.84 to 10 g/L). The range of specific growth rate (k, h^{-1}) of *R. palustris* from algae substrates was 0.025-0.030 (see Table 2), which was 42.4-50.8% of experiments performed using ATYP medium. A report showed a growth rate of 0.021h^{-1} of *R. palustris* 42OL cells grown in an outdoors 50 L photobioreactor containing RPP medium, and a longer lag phase of 46 h existed after inoculation [29]. The specific growth rates of five high-hydrogen producers of anoxygenic photosynthetic bacteria were variable, that they were 0.0269 h^{-1} to 0.0475 h^{-1} under anaerobic-low light conditions (3,000 lux) at 30ºC [30].

Table 2. Basic growth information based on logistical kinetic model and biomass growth between ATYP medium and different field algae substrates.

Substrates	*xc* (h)*	*k* (h^{-1})	OD_{max}	DW* (g/L)	Bchl a (mg/L)
ATYP medium	59	0.059	1.8	0.91	16
Algae-1.67 g/L	124	0.030	0.8	0.40	4
Algae-3.33 g/L	136	0.028	1.7	0.86	12
Algae-6.67 g/L	157	0.026	3.8	1.92	38
Algae-10.0 g/L	177	0.025	4.9	2.47	58.92

*The dry weight (DW) of *R. palustris* strain PUF1 was calculated by the following empirical formula: *y* (OD650nm) = 1.9768 *x* + 0.002 (*x*: DW (g/L), N = 23, R^2 = 0.95). 50 mL bacterial cultures were filtrated through a 47 mm GF/F glass microfiber filter (pore size ~0.7 µm, Whatman, CAT No. 1828-047, GE Healthcare Life Sciences, Bucks, UK) and the filter was dried to a constant weight at 105ºC for 12 h. The DW was determined by weighing the filters.

As shown in Fig. 5b), Bchl a concentration, in batch experiments of algae substrates 3.33 g/L, first approached its peak value (usually in seven days). Afterward, it was significantly reduced. In early cultivation, biosynthesis of Bchl a was temporarily depressed in algae substrates of 10 g/L compared to algae substrates of 6.67 g/L. This inhibitive effect diminished seven days later and was followed by a steep rise of Bchl a in the following days. Results showed an important effect of organic loading rate on enzyme activity [31], suggesting that strength from the ions and organic nutrients regulated the photosynthesis of photosynthetic microorganisms [9, 32]. The increasing non-soluble particles and soluble pigments were another potential factors affecting growth performances by changing the refractive indexes of algae substrates, since smaller particles, regardless of shape, generally inhibited light penetration into the reactor more than larger particles [33].

Nutrient Utilization

Anaerobic digestion of algae biomasses caused large shifts in nutrient levels. As shown in Table 1, N contents of algal materials before and after digestion were 8.25

Table 3. Removal rates of NH_4-N, TDN, TDP, and COD in the digestate by *R. palustris* strain PUF1 in a 15-day experiment.

Algae substrate	Removal rate (%)			
	NH_4-N (%)	TDN (%)	TDP (%)	D-COD (%)
3.33 g/L	58.15±1.67	43.89±0.24	98.07±0.40	30.78±2.04
6.67 g/L	48.35±9.3	44.92±0.92	97.08±0.57	36.42±1.65
10.0 g/L	47.05±6.61	47.74±0.58	96.23±0.64	38.71±9.04

(±0.08)% and 4.84 (±0.02)%, while they were 6.41 (±0.18) and 3.64 (±0.13) mg/g for P calculation. Based on a retrieval rate of 44.84%, N and P released from algae biomasses were 74.54% and 73.69%. In the digestate, NH_4-N was the major nitrogen comprising TDN by the onset of log phase growth (Figs 4a-b). As shown in Fig. 4d), of the first nine-day incubation, D-COD levels in controls (without *R. palustris* inoculants) were comparable to treatments having *R. palustris* added. This indicated a balanced degradation and consumption of lower fatty acids on these days. Rapid uptake of volatile fatty acids by PNSB was reported [34]. Results demonstrated that total COD actually increased during the batch experiments due to phototrophic activity with increasing soluble COD consumption in batch experiments on settled wastewater [18].

Longer-term NH_4-N removal is directly linked to soluble COD assimilation [18]. In the present study, both D-COD and NH_4-N from operations were linearly correlated with OD_{650nm} from strain PUF1, especially NH_4-N (R^2 = 0.79, N = 18). Results indicated that cell assimilation contributed to the longer-term reduction of soluble COD, nitrogen, and phosphorous in the digestates. As shown in Table 3, the removal efficiencies of NH_4-N, TDN, TDP, and D-COD in the digestates on average were 58.15%, 43.89%, 98.07%, and 30.78% of algae substrates 3.33 g/L. Importantly, no marked difference of the removal efficiencies was found within the batch experiments of algae substrates 3.33, 6.67, and 10 g/L.

Noticeably, the increase of NH_4-N in controls indicates the existence of indigenous bacteria brought by algae biomasses, while it is reasonable to suppose the nutritional difference of controls and treatments in algae substrates to the assimilation of strain PUF1. The amounts of N and P acquired by strain PUF1 were 94.40 (±3.55) and 15.65 (±0.06) mg/L (see the reductions of TDN and TDP shown in Figs. 4b-c, whereas they were 88.31 and 13.08 mg/L on average based on the elemental results of *R. palustris* from Table 1). This case supports the idea that photo-heterotrophs using light energy gain an advantage over strict heterotrophs [35]. Reports show that versatility and flexibility with respect to the utilization of lower fatty acids provide *Rhodopseudomonas* species with a competitive advantage over *Rhodobacter* species [36-37]. Being attached to particles gives aerobic anoxygenic photosynthetic (AAP) bacteria an enhanced ability to synthesize Bchl a [35].

Though low in carbon to nitrogen (C/N) ratio of microalgae [26], it seems not to be a defect in single-stage biomass production of *R. palustris* cells provided with a rational loading rate. Regarding the bacterial growth and subsequent costs from wastewater retreatment and power supply, algae concentration of 1.67 to 10 g/L is viable, whereas algae biomasses of 3.33 to 6.67 g/L is advisable. Bioaugmentation using selected strains or mixed cultures in wastewater treatments benefit catabolizing specific compounds, e.g., refractory organics, or overall COD enhancing removal efficiency (for details see [38]). PNSB, an eco-friendly and cheap tool for bioremediation, has high efficiency in wastewater treatment in the reduction of N, P, and COD [39]. By 1:4 dilutions, reduction of 50% in COD was observed by applying *Rhodobacter sphaeroides* Z08 during pharmaceutical wastewater treatment with biomass yield of 780 mg/L and specific growth of 0.015 h^{-1} [40].

Total Lipid, PHB, and Fatty Acid Production in *R. palustris*

In the present study, total lipid, PHB, and fatty acids of strain PUF1 were determined for algae substrates and ATYP medium. Total lipid and PHB attained in *R. palustris* with algae substrates (3.33 g/L) were 37.68 (±1.17)% and 11.67 (±1.30)%. And they were comparable to the value of 37.86 (±0.97)% and 13.64 (±2.26)% observed in ATYP medium. Total lipid content of the dry biomasses of *R. palustris* PUF1 fell into the range 36-39% of the literature using the same species when the irradiance was within the 56-151 W m^{-2} range [41]. Results indicated a higher PHB production rate of 8.4% on butyrate of 28 mM (2.47 g/L) than that of 5.3% on acetate of 39 Mm (2.34 g/L) in *R. palustris* WP3-5 [10]. The type of nitrogen source does not cause any marked variation in the intracellular concentration of PHB [42]. *R. palustris* strain PUF1 contained large amounts of saturated, unsaturated, and cyclo fatty acids. Results showed that fatty acids of 18:1w7c and 16:00 of strain PUF1 grown on algae substrates (3.33 g/L) accounted for up to 60.14% and 13.66%, and were comparable to a pure culture with ATYP medium, in which 18:1w7c was 58.74% and 16:00 was 9.36%. These results indicate that algae biomasses are a potent feedstock for the biomass generation of *R. palustris*, thereby providing a new direction on the management of harmful algae.

Conclusions

Field BGA biomass has proven to be an ideal substrate for culturing *R. palustris*. Nutrients from decomposed algae biomasses were biologically concentrated through growth. More than 40% of nitrogen in the digestate was removed while it was 95% or more for the phosphorus in longer-term (15 days) experiments with algae biomasses ranging from 3.33 to 10 g/L.

Our work demonstrated that practically applying *R. palustris* for nutrient recovery from field BGA biomasses is promising. It achieves great ecological and economic benefits since the added products from *R. palustris*. For further investigation, more attention should be given to the ammonia toxicity due to the low C/N ratio of algae, and a mutualistic microbial community will make sense for advanced nutrient utilization and wastewater treatment.

Acknowledgements

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