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Efficient Ammonium Removal by Bacteria Rhodopseudomonas Isolated from Natural Landscape Water: China Case Study

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Abstract: In this study, we isolated a strain of photosynthetic bacteria from landscape water located in Southwest University, Chongqing, China, and named it Smobiisys501. Smobiisys501 was *Rhodopseudomonas* sp. according to its cell morphological properties and absorption spectrum analysis of living cells. The analysis of the 16S rDNA amplification sequence with specific primers of photosynthetic bacteria showed that the homology between Smobiisys501 and *Rhodopseudomonas* sp. was 100%, and the alignment results of protein sequences of the bacterial chlorophyll Y subunit showed that Smobiisys501 and *Rhodopseudomonas palustris* were the most similar, with a similarity of more than 92%. However, Smobiisys501 could not utilize glucose and mannitol as a carbon source and had a low fatty acid content, which were different from the related strains of the genus *Rhodopseudomonas*. Moreover, the DNA-DNA relatedness was only 42.2 ± 3.3% between Smobiisys501 and the closest strain *Rhodopseudomonas palustris*. Smobiisys501 grew optimally at 30 °C and pH 7.0 in the presence of yeast extract, and it could efficiently remove ammonium (99.67% removal efficiency) from synthetic ammonium wastewater. All the results indicated that Smobiisys501 was a novel species of *Rhodopseudomonas*, with the ability to remove ammonium.

Keywords: *Rhodopseudomonas* sp.; anaerobic; characteristics; ammonium

1. Introduction

The genus *Rhodopseudomonas* was first described by Czurda and Maresch [1] and is one of the photosynthetic bacteria (PSB), known as purple nonsulfur bacteria. Up to now, many bacterial strains of *Rhodopseudomonas* have been reported, isolated from water and mud [2–4]. According to Bergey's Manual of Systematic Bacteriology, the genus *Rhodopseudomonas* was classified into seven species: *Rhodopseudomonas palustris*, *Rhodopseudomonas marina*, *Rhodopseudomonas blastica*, *Rhodopseudomonas sulfoviridis*, *Rhodopseudomonas rutila*, *Rhodopseudomonas acidophila*, and *Rhodopseudomonas viridis* [5].

However, delineating species of the genus *Rhodopseudomonas* is taxonomically challenging due to the poor correlation between phenotypic and genotypic traits, hampering the identification and classification of newly isolated strains [6]. At present, many molecular biological methods including the sequence alignment, G+C content determination, DNA-DNA hybridization, and phospholipid fatty acid analysis have been successfully employed to identify the genus *Rhodopseudomonas* [2,7,8]. An example of this is PPF2/PPR2, a pair of specific primers designed according to the particular sequence of 16S rDNA of *Rhodopseudomonas* that could accurately identify *Rhodopseudomonas* [9]. The *bchY* gene (Y subunit of chlorophyllide oxidoreductase) of anoxygenic photosynthetic bacteria could also distinguish *Rhodopseudomonas* and anoxygenic photosynthetic bacteria [10]. Therefore, in order

to classify the new strains more accurately, the phenotypic characteristics and molecular biological characteristics should be used in combination to identify the isolated strains of *Rhodopseudomona*.

The construction of a landscape waterbody for reshaping the living environment is increasingly popular. However, most landscape water bodies are closed or semi-closed catchments, and the organic nitrogen in them is easily changed to ammonium (NH_4^+-N). It is notable that excessive ammonium in water can damage the liver and kidney of fish, and make the water stinky. Therefore, removing the undesirable ammonium in landscape water is essential and urgent. *Rhodopseudomonas* has been used as the most effective bioagent in the field of environmental protection, such as in the treatment of sewage, household, and restaurant wastewater owing to its ability to efficiently remove ammonium [11–14]. However, little research has been conducted on the application of the genus *Rhodopseudomonas*, even the PSB in the treatment of landscape water ammonium pollution. Furthermore, the pollutant concentration and composition in landscape water are different from life sewage, household, and restaurant wastewater.

In this work, a rod-shaped and red-pigmented strain, named Smobiisys501, was identified by combining the phenotypic characteristics and molecular biological characteristics (including physiological and biochemical characteristics, phospholipid fatty acid (PLFA), DNA-DNA hybridization, 16S rDNA sequence, and *bchY* protein sequence analysis). The effects of temperature, pH, and nitrogen source on its growth characteristics were investigated in PSB enrichment medium. Meanwhile, the ammonium removal efficiency of Smobiisys501 from synthetic ammonium wastewater was examined carefully. All the results illustrated that Smobiisys501 was a novel species of *Rhodopseudomonas*, and it could be used to treat ammonium wastewater because of its high ammonium removal efficiency.

2. Materials and Methods

2.1. Characterization and Identification of the Bacterial Strain

The water samples for bacterial isolation were taken from the pool of Resources and Environment College, Southwest University, Chongqing, China. The PSB enrichment medium was used to enrich the photosynthetic bacteria, which contained (L^{-1}): CH_3COONa 3.3 g, NH_4Cl 0.6 g, K_2HPO_4 0.9 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $NaHCO_3$ 0.2 g, and yeast extract 1.5 g. CH_3COONa, NH_4Cl, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, and $NaHCO_3$ are all analytically pure (AR), and yeast extract and agar are biochemical reagents (BR). All the reagents were purchased from new titanium chemical industry in Chongqing. For the preparation of solid plates for isolation and purification, 2% (*w*/*v*) agar was added to the PSB enrichment medium [15]. The collected water (1 mL) was added to the anaerobic bottle (1 L) filled with PSB enrichment medium. The bottle was incubated anaerobically in a constant illumination box (light: 2000 lx) at 30 °C for about 7 d. Then, 1 mL culture was transferred into 1 L fresh PSB enrichment medium, and the medium was incubated at the same conditions. These works were done three consecutive times until a dark red coloration was achieved [16]. Purification of single colonies was achieved with successive re-streaking in PSB enrichment medium containing 2% (*w*/*v*) agar [17].

Cell morphology of the strain was observed by optical microscopy (Olympus BH-2) and scanning electron microscopy (SEM, JEM-2100F). The physiological and biochemical characteristics of the strain (including carbon source utilization, oxidization H_2S test, starch hydrolysis test, et al.) were identified according to related literatures [18,19]. Cell pigment scans and absorption spectra of the living cells suspension of the strain were performed using cell pellets resuspended in 60% sucrose utilizing an Aminco DW-200 UV-Visible spectrometer in the split mode [16].

Genomic DNA of the strain was extracted and purified using a Qiagen genomic DNA extraction kit from Sigma. The specific primers PPF2/PPR2 for photosynthetic bacteria (fwd: 5′-CTGGAAGTCTTGAGTATGGC-3; rev: 5′-AGTAAACCCACTAACGGCTG-3′) [7] and the specific degenerate primers for anaerobic phototrophic bacteria (APB) (fwd: 5′-CCNCARACNATGTGYCCNGC NTTYGG-3′; rev: 5′-GGRTCNRCNGGRAANATYTCNCC-3′) [8] were used for the amplification of

the partial 16S rDNA gene and the partial *bchY* gene of the isolates, respectively. PCR products were separated on a 2% agarose gel and purified by a BioSpin gel extraction kit (BioFlux). The purified product was cloned into the pMD®20-T vector (Takara, Liaoning, China) and then sequenced by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). Then, the sequences were submitted to NCBI for their accession number. Sequence alignment and multiple alignment were performed using the NCBI search tool program (BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi) and CLUSTAL W. Additionally, the phylogenetic tree was constructed using MEGA 6.0 software.

DNA-DNA hybridization of the strain was detected by a Perkin Elmer Lambda 35 UV/VIS Spectrophotometer [20]. Levels of DNA–DNA relatedness were determined by triplicate measurements between Smobiisys501 and its closest strain *Rhodopseudomonas palustris*, reciprocally (mean ± SD, $n = 3$). The phospholipid fatty acid (PLFA) composition of Smobiisys501 was analyzed by an Agilent 6850 gas chromatograph (FID detector) after being extracted, saponified, and methylated according to the standard protocol given by the MIDI [21]. The chromatographic conditions were as follows: HP-5 column (25.0 m × 200 μm × 0.33 μm), sample volume was 1 μL, split ratio was 10:1, the carrier gas (H_2), tail blowing high purity N_2, and used air as auxiliary gas, the velocity was 8 mL·min^{-1}; the temperature of the vaporization chamber and detector was 250 °C and 300 °C, respectively. Pre column pressure was 10.0 psi (1 psi = 6.895 kPa), the full scan range of mass spectrometry was 30 to 600 mr·Z^{-1}, and the two-order program column temperature was 170 °C (5 min)→260 °C→310 °C, maintained for 1.5 min. The standard product was purchased from MIDI Company (New York, NY, USA), and the C19:0 was used as an internal standard to convert the absolute content of PLFA.

2.2. Effect of Culture Conditions on Bacterial Growth Characteristics

In order to obtain the optimal growth conditions of Smobiisys501, three cultivation conditions which affect the growth of Smobiisys501 including temperature (10, 20, 30, 35, 40, 45, and 50 °C), pH (4, 5, 6, 7, 8, 9, and 10), and nitrogen source (nitrate ($LiNO_3$, $NaNO_3$, KNO_3), ammonium (CH_3COONH_4, NH_4Cl, $(NH_4)_2C_2O_4$), $(NH_4)_2SO_4$), organic nitrogen (urea, peptone, and yeast extract)) were determined [22]. The pH value of raw water was adjusted to desirable values using 0.1 M NaOH or 0.1 M HCl. Smobiisys501 was activated on PSB solid plates, and then a single colony of Smobiisys501 was inoculated into 100 mL PSB enrichment medium cultured at 30 °C for 7 d. In total, 1 mL of pre-cultured Smobiisys501 was centrifuged at 4000 r·min^{-1} for 8 min and washed twice with sterilized pure water before being inoculated into a 250 mL anaerobic bottle full of PSB enrichment medium (OD_{600} = 0.3, different nitrogen sources were used to replace NH_4Cl in PSB enrichment medium when explored the effect of nitrogen source on growth of Smobiisys501). Furthermore, the anaerobic bottles were incubated in a constant illumination box (light: 2000 lx) at 30 °C for 48 h. Then, the OD_{600} values were determined using a spectrophotometer (UV1000, Techcomp Limited, Shanghai, China) at the wavelength of 600 nm. All experiments were conducted in triplicate.

2.3. Application of Smobiisys501 to Ammonium Removal

Synthetic ammonium wastewater (contained (L^{-1}): CH_3COONa 0.34 g, NH_4Cl 0.02 g, K_2HPO_4 0.09 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $NaHCO_3$ 0.34 g) was used to investigate the ammonium removal efficiency of Smobiisys501. Smobiisys501 was incubated on PSB solid plates, and then a single colony of Smobiisys501 was inoculated into 100 mL PSB enrichment medium cultured at 30 °C for 7 d. Different concentrations of pre-cultured Smobiisys501 were centrifuged at 4000 r·min^{-1} for 8 min and washed twice with sterilized pure water before being inoculated into a 250 mL anaerobic bottle (0.5 mL: 0.2% inoculums of Smobiisys501 isolate; 1 mL: 0.4% inoculums of Smobiisys501 isolate; 1.5 mL: 0.6% inoculums of Smobiisys501 isolate) which was filled with synthetic ammonium wastewater. Moreover, synthetic ammonium wastewater without inoculation was used as the control (CK). All experiments were conducted in triplicate. The cultures were incubated at the optimal pH and temperature. Additionally, the concentration of ammonium in water was measured every 3 d using the indophenol blue method at room temperature [23]. The ammonium removal efficiency was calculated by the

equation: $Rv = (T1 - T2)/T1 \times 100\%$ to assess the ammonium removal ability of Smobiisys501. Note that Rv, $T1$, and $T2$ represent the ammonium removal efficiency, the initial concentration of ammonium, and the final concentration of ammonium in water after incubation, respectively.

2.4. Statistical Analysis and Graphical Work

Statistical analysis and graphical work were carried out using Excel, SPSS Statistics, and Origin8.6. The results were presented as means ± SD (standard deviation of means).

3. Results and Discussion

3.1. Characterization of Smobiisys501

3.1.1. Morphological and Absorption Spectra Characteristics

Smobiisys501 was found to grow well under the light of the anaerobic condition, but could not to grow under a dark and anaerobic condition. Colony morphologies of Smobiisys501 were red–brown, convex, and circular, with a diameter of 1.2–1.5 mm when it grew on the solid plates of PSB enrichment medium after incubation at 30 °C for 7 d. The cells were spherical on acid medium (Figure 1a), and long rods on alkaline medium (Figure 1b) under an optical microscope. The microscopic scanning test of the Smobiisys501 utilizing SEM clearly showed that the single cell of Smobiisys501 was budding and the size of a single bacterial cell was about 0.6–0.8 mm wide and 1.2–1.5 mm long (Figure 1c). Additionally, the double staining test of the isolate showed that the isolate was a Gram-negative species. The absorption spectra of the living cells showed characteristic absorption peaks at 375, 490, 520, 805, and 870 nm, indicating the presence of bacteriochlorophyll a and carotenoids of the spheroidene series [24,25]. These are characteristic features of a photo bacterium, which illustrated that Smobiisys501 is a typical PSB, and might be *Rhodopseudomonas* sp.

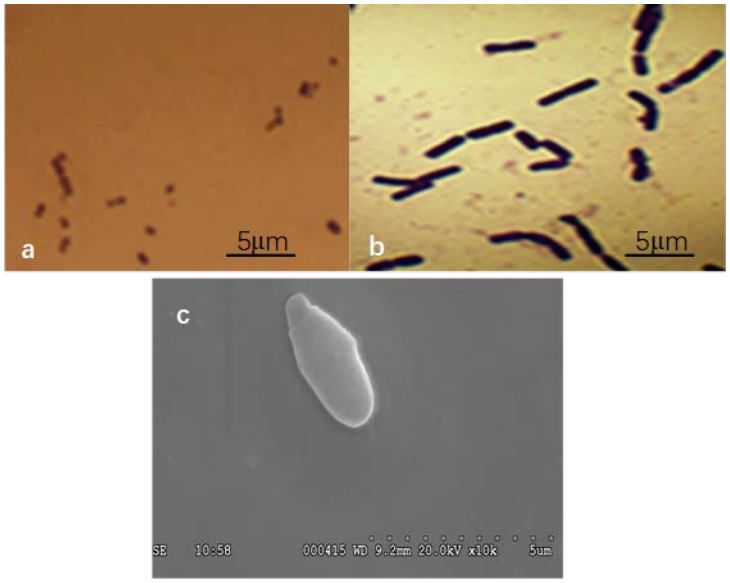

Figure 1. The morphology of Smobiisys501 under a microscope. (**a**): Cell morphology of Smobiisys501 under an optical microscope (10 × 100) in acid medium (pH = 6); (**b**): Cell morphology of Smobiisys501 under an optical microscope (10 × 100) in alkaline medium (pH = 9); (**c**): Cell morphology of Smobiisys501 under an electron microscope (2000 × 10).

3.1.2. Phylogenetic Analysis

According to sequence similarity calculations of the 16S rDNA gene, Smobiisys501 was most closely related to *Rhodopseudomonas* sp. (AB696784, 99%), *Rhodobacter sphaeroides* (HM053959,

99%), *Rhodopseudomonas faecalis* (HQ154127, 99%), *Rhodopseudomonas palustris* (KJ722766, 99%), and *Rhodopseudomonas thermotolerans* (NR108528, 99%). The 16S rDNA-based phylogenetic tree constructed using the neighbor-joining method showed the same result, where Smobiisys501 was placed in a cluster within the genera *Rhodopseudomonas* (Figure 2). The results strongly supported the species-level classification of Smobiisys501 within the genus *Rhodopseudomonas*.

The 507bp *BchY* gene was transformed into a 168 aa protein sequence. Phylogenetic analysis based on the *bchY* protein sequence indicated that Smobiisys501 was most closely related to *Rhodopseudomonas palustris* (ALB25898, 100%), *Rhodopseudomonas palustris* (WP027279534, 95%), *Rhodopseudomonas palustris* (WP011662766, 92%), *Rhodopseudomonas* sp. AAP120 (WP054165526, 89%), and *Rhodopseudomonas* sp. B29 (WP022723271, 88%). The *bchY*-based phylogenetic tree depicting the phylogenetic relationships of Smobiisys501 with the related strains using the neighbor-joining method is shown in Figure 3, where Smobiisys501 was most adjacent to *Rhodopseudomonas palustris*. The phylogenetic result based on the *bchY* protein was consistent with those based on the 16S rDNA gene, suggesting that Smobiisys501 belonged to the genus *Rhodopseudomonas*, and might be *Rhodopseudomonas palustris*.

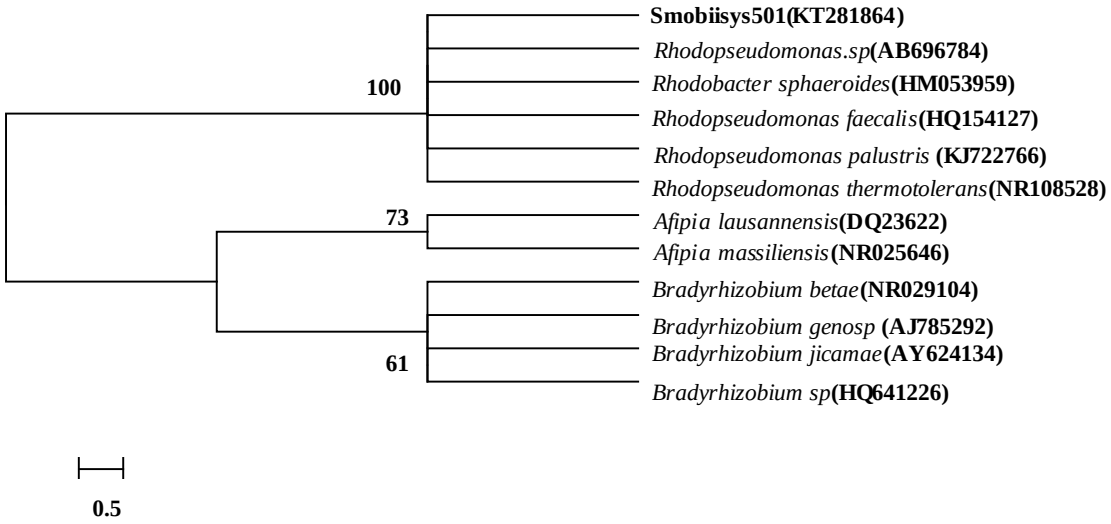

Figure 2. Phylogenetic tree of Smobiisys501 based on the 16S rDNA gene homology.

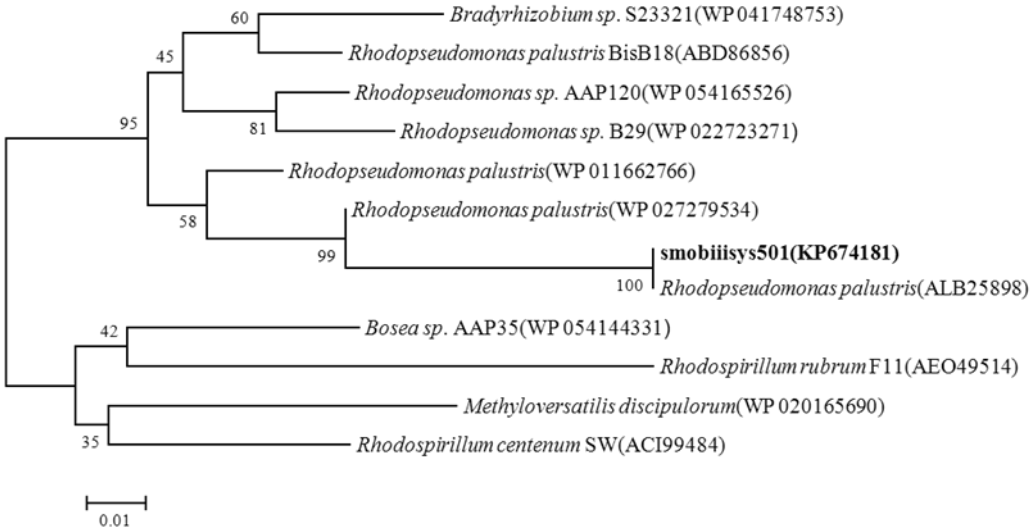

Figure 3. Phylogenetic tree of the strain Smobiisys501 based on the *BchY* protein homology.

3.1.3. Physiological-Biochemical Characteristics

Smobiisys501 was a Gram-negative bacterium, which contained oxidase and catalase and was positive for the production of indole, and it could also utilize sodium acetate. However, it was negative for the oxidization of H_2S and starch hydrolysis, and it did not have the ability to utilize methanol, glucose, sodium thiosulfate, and mannitol (Table 1). Previous research found that *Rhodopseudomonas faecalis*, *Rhodopseudomonas palustris*, and *Rhodobacter sphaeroides* (which were most adjacent to Smobiisys501) had the ability to utilize glucose and mannitol, demonstrating that there were some differences in the utilization of carbon sources between Smobiisys501 and the three type species of *Rhodopseudomonas* sp. [26,27].

Table 1. The physiological and biochemical characters of Smobiisys501.

Physiological Characteristics		Biochemical Characteristics	
Carbon source utilization	+	Oxidase	+
Sodium acetate	+	Catalase	+
methanol	−	Indole	+
glucose	−	Oxidization H_2S	−
Sodium thiosulfate	−	Starch hydrolysis	−
Mannitol	−	Gram stain	−

3.1.4. DNA–DNA Hybridization

Normally, when strains share a similarity of more than 97% in the 16S rDNA gene sequence identity, the DNA–DNA hybridization technique has been especially successful in resolving taxonomic relationships at the species level and below [28]. In this study, the closest strain *Rhodopseudomonas palustris* was selected for DNA–DNA hybridization with Smobiisys501. The value of genomic DNA relatedness as shown by the DNA-DNA hybridization was only 42.2%, which was well below the 70% threshold proposed for species delineation [29].

3.1.5. Cellular Fatty Acid Analysis

A total of more than ten kinds of fatty acids of Smobiisys501 were detected, and the proportion of $C_{18:1\omega7c}$ was the highest, which could reach 38.43%. Smobiisys501 showed a similar major fatty acid composition to the related type strains of the genus *Rhodopseudomonas* (Table 2), but there were significant quantitative differences when cultivated under the same conditions by comparing it with the three adjacent strains in the phylogenetic trees [26,27], which indicated that Smobiisys501 was different from the other three strains.

Table 2. Cellular fatty acid profiles (%) of Smobiisys501 and closely related species of the genus *Rhodopseudomonas*.

Fatty Acid Composition (%)	1	2	3	4
$C_{12:0}$	3.05	1.6	-	1.7
$C_{16:0}$	19.13	20.8	5.1	14.9
$C_{16:1\omega7c/16:1\omega6c}$	19.29	10.2	1.9	6.2
$C_{18:1\omega7c}$	38.43	51	77.2	63

1 represents Smobiisys501; 2 represents *Rhodopseudomonas palustris* ATCC 17001T; 3 represents *Rhodobacter sphaeroides* DSM158T; 4 represents *Rhodopseudomonas faecalis* JCM 11668T.

Morphological and absorption spectra analysis showed that the main characteristics of Smobiisys501 were consistent with the characteristics of the genus *Rhodopseudomonas*. The alignment result of the 16S rDNA amplification sequence with specific primers of photosynthetic bacteria (PPF2/PPR2) showed that the homology between Smobiisys501 and *Rhodopseudomonas* sp. was 100%,

and the alignment results of protein sequences of the bacterial chlorophyll Y subunit showed that Smobiisys501 and *Rhodopseudomonas palustris* were the most similar, with a similarity of more than 92%. However, the physiological and biochemical characteristics, the main fatty acid analysis, and the DNA-DNA hybridization found that there were obvious differences between Smobiisys501 and the related strains of the genus *Rhodopseudomonas*. For instance, Smobiisys501 could not utilize glucose and mannitol as a carbon source and had a lower fatty acid content. Furthermore, the DNA-DNA relatedness was only 42.2 ± 3.3% between Smobiisys501 and the closest strain *Rhodopseudomonas palustris*. These differences indicated that Smobiisys501 might be a new member of the genus *Rhodopseudomonas*.

3.2. Effects of Culture Conditions on the Growth of Smobiisys501

The effect of temperature, pH, and nitrogen sources on the growth of Smobiisys501 is shown in Figure 4. Within the temperature range of 10–50 °C, Smobiisys501 grew better at higher temperatures, but when the temperature reached 35 °C, bacterial growth decreased. The optimal growth temperature of Smobiisys501 was about 30 °C (Figure 4a), which was consistent with the reported strain of the genus *Rhodopseudomonas* [30]. In this study, the growth of Smobiisys501 was affected by the pH, like in previous studies [31,32]. The optimal pH was 7 with OD_{600} values of 0.53 after anaerobic incubation for 48h (Figure 4b). At pH 6.0–9.0, Smobiisys501 grew better. However, it could not grow at pH 4 (OD_{600} = 0.01) and pH 10 (OD_{600} = 0.01). These findings also indicated that Smobiisys501 has a wide adaptability to the pH in water, and it could survive in weak acidic and weak alkaline environments. The result was consistent with the reported strain of the genus *Rhodopseudomonas* [33]. As shown in Figure 4c, $(NH_4)_2SO_4$, $NaNO_3$, and yeast extract were the most suitable ammonium nitrogen, nitrate nitrogen, and organic nitrogen for the growth of Smobiisys501, respectively. Interestingly, yeast extract could dramatically stimulate the growth of the strain compared to other nitrogen sources. This might because the yeast extract contains organic carbon sources which will accelerate the biomass growth. However, CH_3COONH_4, $(NH_4)_2C_2O_4$, urea, and peptone could not noticeably sustain the growth of strain, although they also contain organic carbon sources. The results implied that containing organic carbon was not the main factor which meant that the yeast extract could significantly promote the growth of the strain. Further analysis is needed for specific reasons. On the basis of the study above, yeast extract was found to be the optimal nitrogen source for strain growth.

3.3. Assessment of Ammonium Removal by Smobiisys501

Research has shown that the excess of ammonium is one of the main causes of eutrophication. Therefore, reducing the content of ammonium will be an effective means to control the eutrophication of a water body [34]. The purpose of this experiment was to investigate the effect of inoculation on NH_4^+-N removal efficiency while the water initial pH value and temperature were controlled at 7 and 30 °C, respectively. The concentration of NH_4^+-N was significantly decreased in 3 d after being treated by Smobiisys501 in comparison with the control (Figure 5). While there was a different ammonium removal efficiency among three inoculation quantity treatments, the higher the inoculation quantity, the better the ammonium removal efficiency. The optimum inoculation quantity of Smobiisys501 was 0.4% when taking into account the medicament cost and ammonium removal efficiency, and under this condition, the ammonium removal efficiency reached 99.67%. These results showed that Smobiisys501 has the ability to effectively remove ammonium in wastewater, and could be used for the governance of ammonium polluted water. Moreover, NO_2-N and NO_3-N were nearly undetectable during the experiments, which indicated that Smobiisys501 could reduce the ammonium in water under an anaerobic condition owing to assimilation [35] rather than dissimilation.

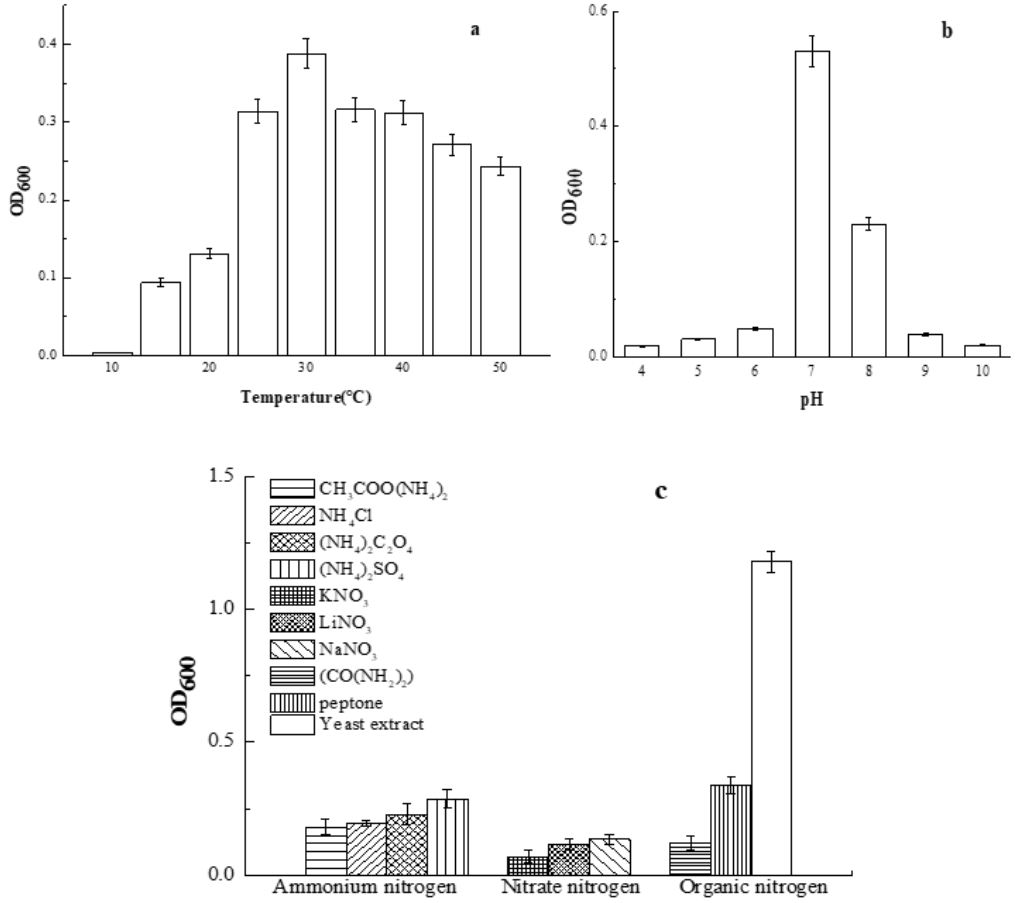

Figure 4. Effect of culture conditions on the growth of Smobiisys501. (**a**) represents the effect of temperature; (**b**) represents the effect of pH; (**c**) represents the effect of nitrogen sources.

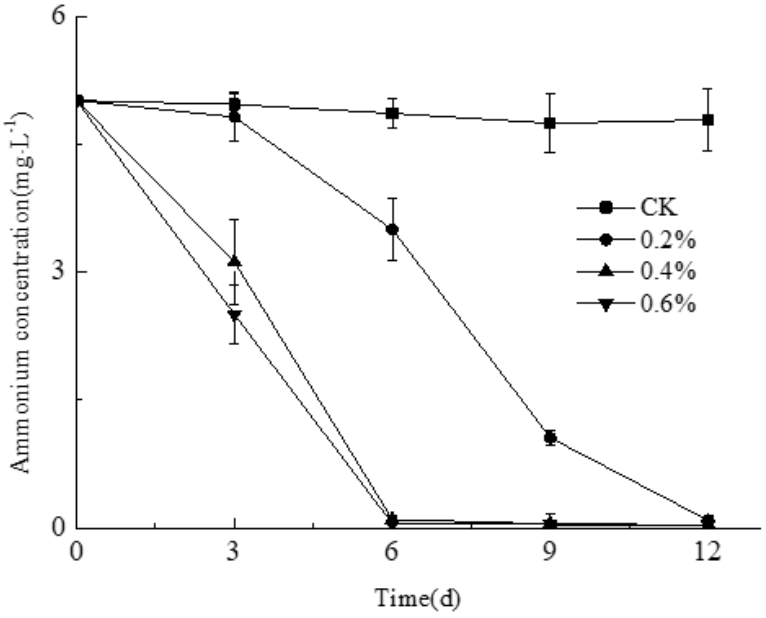

Figure 5. Variation of NH_4^+ with time in synthetic ammonium wastewater treated by Smobiisys501.

4. Conclusions

This study reports a new member of the genus *Rhodopseudomonas*, named Smobiisys501, as an anaerobic bacterium. Smobiisys501 belongs to the genus *Rhodopseudomonas* according to the morphological and absorption spectra analysis, as well as the alignment result of the 16S rDNA amplification sequence with specific primers of photosynthetic bacteria. The alignment result of protein sequences of the bacterial chlorophyll Y subunit showed that Smobiisys501 might be *Rhodopseudomonas palustris* because they exhibited the highest similarity (similarity of more than 92%). But there was a great difference in the biological properties of Smobiisys501 and *Rhodopseudomonas palustris* according to physiological biochemical characteristics, the main fatty acid analysis, and the DNA-DNA hybridization. These differences indicated that Smobiisys501 was a novel species of *Rhodopseudomonas*. Smobiisys501 grew optimally at 30 °C and pH 7.0 in the presence of yeast extract. In addition, Smobiisys501 exhibits the highest ammonium removal ability at 30 °C and pH 7.0, where the ammonium removal efficiency was up to 99.67%. Accordingly, Smobiisys501 can be potentially used for removing ammonium in landscape water.

Author Contributions: X.H. and C.Y. conceived and designed the experiments; X.H., C.Y., and M.F. carried out the experiments; X.H., C.Y., M.F., and Z.L. analyzed the data; X.H., Z.L., J.N., and D.X. wrote the main manuscript text and all authors reviewed the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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