Desalination and Water Treatment
www.deswater.com

doi: 10.1080/19443994.2016.1144531

57 (2016) 25028–25035
November

Taylor & Francis
Taylor & Francis Group

Optimal growth conditions and nutrient removal characteristic of a denitrifying phosphorus-accumulating organism

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Received 31 May 2015; Accepted 8 January 2016

ABSTRACT

A highly efficient, denitrifying and phosphorus-accumulating bacterial strain was isolated from the Xuzhou Water Operation Co., Ltd, sewage treatment plant. The strain contains ploy-P and PHB granules. It was identified as a *Klebsiella* sp. using 16S rDNA sequencing and physiological and biochemical analyses, and named as *Klebsiella* sp. N14. The growth of the strain and its capability of nutrient removal were affected by pH, temperature, and the carbon source. The optimal pH for growth and nutrient removal was 8, and the optimal temperature was 30 °C. Sodium acetate was identified as the best carbon source. The phosphorus (PO_4^{3-}-P) concentration in the culture supernatant resulting from aerobic training of N14 in synthetic wastewater for 24 h decreased from 81 to 12.4 mg/L. The phosphorus removal rate was 84.69%. The nitrate (NO_3^--N) concentration decreased from 180 to 15 mg/L. The nitrite (NO_2^--N) concentration decreased from 44 to 6.7 mg/L. The denitrification rate was 87.94%. This study describes a novel denitrifying, phosphorus-accumulating bacterial strain, and the optimal conditions for nitrogen and phosphorus removal by this strain.

Keywords: Growth characterization; Denitrification rate; Denitrifying phosphorus-accumulating organism (DPAO); Identification; Phosphorus removal rate

1. Introduction

Excessive emission of nitrogen and phosphorus is one of the main causes of the eutrophication of water bodies [1]; therefore, economical methods to reduce this eutrophication have received increasing attention by the urban sewage disposal sector. In the past 20 years, rapid advances have been made in wastewater denitrification and biological phosphorus removal technologies. Many researchers have developed a series of denitrification and phosphorus removal processes, and have practically applied these concepts in wastewater treatment [2–4], thus extending the knowledge of wastewater nutrient removal processes. With progress in research on phosphorus removal techniques in the field of microbiology, the discovery of denitrifying phosphorus-accumulating organisms (DPAOs) has provided new ideas and

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perspectives for the simultaneous nitrogen and phosphorus removal from sewage. Thus, biological denitrifying phosphorus removal technology has become a prime focus and hotspot in the field of sewage treatment research.

DPAOs are facultative anaerobic organism that utilize O_2, NO_3^--N, or NO_2^--N as electron acceptors; they release phosphorus under anaerobic conditions and utilize phosphorus under aerobic conditions. The rate of phosphorus uptake by DPAOs is considerably higher than that of phosphorus release; thus, a sludge with high phosphorus content is discharged from the system [2], so that nitrogen and phosphorus removal can be carried out simultaneously. The presence of DPAOs alleviates the variation in sludge retention time between nitrifying and phosphorus-accumulating organisms (PAOs), and resolves the conflict in carbon source utilization between denitrifying organisms and PAOs [5]. To date, some DPAOs have been individually screened, including *Comamonas*, *Acinetobacter*, *Pseudomomas*, *Bacillus*, *Rhodocyclus*, and *Paracoccus* spp. [6]. However, these organism and their characteristics were studied at an early stage, and there was no systematic approach toward identification of their species, which requires further development [7,8].

In the present study, we screened a DPAO strain, *Klebsiella* sp. N14, from activated sludge, and we evaluated the effects of growth factors such as carbon source, temperature, and pH on the bacterial growth and their ability to degrade nitrogen and phosphorus pollutants. We thus aimed to enrich the biological denitrifying phosphorus removal theory. The findings of this study may help to improve the efficiency of the wastewater biological denitrification and phosphorus removal processes.

2. Materials and methods

2.1. Source of strain

Activated sludge from the biochemical tank of a wastewater treatment plant was enriched and domesticated under anaerobic/anoxic conditions. The wastewater treatment process was "A_2/O" (Anaerobic–Anoxic–Oxic), and the population equivalent (PE) was 749.6 thousand. Thereafter, we screened for denitrifying organisms; subjected them to staining for poly-P and PHB granules; tested for nitrate reduction, nitrogen gas production, and phosphorus uptake abilities [9]; and a DPAO was thus qualitatively isolated and named as strain N14.

2.2. Culture media

Lysogeny Broth (LB) medium was used to culture the organisms and activate the strains. The medium was composed of 5 g yeast extract, 1 g peptone, 5 g NaCl, and 1 L ddH_2O (pH 7.0–7.2).

The denitrifying phosphorus-rich medium contained 2 g/L KNO_3, 5 g/L $CH_3COONa{\cdot}3H_2O$, 0.05 g/L K_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L $CaCl_2$, and 0.2% trace elements (pH 7.2–7.6).

The trace element solution contained 1.5 g/L $FeCl_3{\cdot}6H_2O$, 0.15 g/L H_3BO_3, 0.03 g/L $CuSO_4{\cdot}5H_2O$, 0.03 g/L KI, 0.06 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.12 g/L $MnCl_2{\cdot}4H_2O$, 0.12 g/L $ZnSO_4{\cdot}7H_2O$, and 0.12 g/L $CoCl_2{\cdot}2H_2O$.

2.3. Morphological, physiological, and biochemical properties of strains

Strain characterization was conducted according to previously described methods [10].

2.4. 16S rDNA PCR amplification and phylogenetic analysis

PCR was conducted according to previously described methods [9]. Bacterial genomic DNA was used as a template to amplify 16S rDNA with a pair of universal primers: upstream primer (7F), 5′-CAGAGTTTGATCCTGGCT-3′; downstream primer (1540R), 5′-AGGAGGTGATCCAGCCGCA-3′. The PCR system contained: 0.5 μL template (50 ng/μL), 2.5 μL 5 × buffer (with Mg^{2+}), 1 μL dNTPs (2.5 mmol/L, respectively), 1 μL dream Taq-TM DNA polymerase (1U/μL), 0.5 μL upstream and downstream primers (10 μmol/L, respectively); ddH_2O was added to bring the volume to 25 μL. The PCR program was as follows: denaturation at 98℃ for 3 min; 30 cycles of denaturation at 98℃ for 25 s, annealing at 55℃ for 25 s, extension at 72℃ for 1 min; final extension at 72℃ for 10 min; and termination at 4℃. PCR products were analyzed by agarose gel electrophoresis and sent to China Shanghai Biological Technology Co., for sequencing.

N14 strain 16S rDNA gene sequences were submitted to NCBI, were compared to similar sequences through the Basic Local Alignment Search Tool (BLAST), and then were aligned using Clustal X1.83 software. The phylogenetic tree was built with the neighbor-joining method using Mega5.0 software, and the stability of the tree branch was analyzed with bootstrap analysis repeated 1,000 times.

2.5. Effect of culture conditions on bacterial characteristics

2.5.1. pH

The phosphorus-rich denitrification medium was prepared at pH values of 5, 6, 7, 8, and 9 (100 mL). Cultures were inoculated at an optical density at 600 nm (OD_{600}) of 0.1 and cultured at 26℃ on a 140 rpm shaker for 24 h. After culture, the OD_{600} and concentration of PO_4^{3-}-P, NO_3^--N, and NO_2^--N in the supernatant were measured. The denitrification rate and phosphorus removal rate were calculated.

2.5.2. Temperature

The phosphorus-rich denitrification medium was prepared at the same pH values described above. Cultures were inoculated at an OD_{600} of 0.1 and cultured on a 140 rpm shaker for 24 h. Incubation temperatures were set to 20, 25, 30, 35, or 40℃. After culture, the OD_{600} and concentration of PO_4^{3-}-P, NO_3^--N, and NO_2^--N in the supernatant were measured. The denitrification rate and phosphorus removal rate were calculated.

2.5.3. Carbon source

The inoculum was cultured as mentioned above with a mass: volume ratio of 1% and sodium acetate, propionic acid, sodium citrate, or sodium tartrate as the sole carbon source. After culture, the OD_{600} and concentration of PO_4^{3-}-P, NO_3^--N, and NO_2^--N in the supernatant were measured. The denitrification rate and phosphorus removal rate were calculated.

2.5.4. Nitrogen and phosphorus removal performance

The inoculum was seeded into 200 mL of synthetic wastewater (0.6 g/L glucose; 0.1 g/L peptone; 0.01 g/L yeast extract; 0.5 g/L sodium acetate; 0.05 g/L NaCl; 0.09 g/L dipotassium hydrogen phosphate trihydrate; 0.4 g/L magnesium sulfate heptahydrate; 0.2 g/L potassium nitrate). The initial concentration of nitrate and phosphorus was 180 and 81 mg/L, respectively. The OD_{600} was adjusted to 0.1 and the culture was incubated with a suitable pH, temperature, and shaking at 140 rpm. The concentration of PO_4^{3-}-P, NO_3^--N, and NO_2^--N in the supernatant was measured at 0, 4, 8, 12, 16, 20, and 24 h.

2.5.5. Analysis method [11]

PO_4^{3-}-P was detected using Mo-Sb spectrophotometry. Five milliliters of inoculum were filtered, trickled into a colorimetric tube (50 mL), and then diluted to the standard calibration line with double-distilled water. Following this 1 mL of ascorbic acid solution (10%) was blended. After 30 min, 2 mL of molybdate salt solution was blended and allowed to react for 15 min. The mixture was then transferred to a cuvette (3 mL), and the absorbance was measured at 700 nm with a spectrophotometer. NO_3^--N was detected using the phenol disulfonic acid method as previously described. Five milliliters of inoculum were filtered, trickled into an evaporating dish, placed in a water bath, and allowed to evaporate until it was dry. Disulfonic acid phenol (1 mL) was added, and the mixture was fully ground. Water (10 mL) and ammonia (3 mL) were added slowly. The mixture was transferred to a colorimetric tube (50 mL), diluted to the standard calibration line with double-distilled water and blended. The mixture was then transferred to a cuvette (3 mL), and the absorbance was measured at 410 nm with a spectrophotometer. NO_2^--N was detected using α-naphthylamine spectrophotometry. Five milliliter of inoculum were filtered, trickled into a colorimetric tube (50 mL), and then diluted to the standard calibration line with double-distilled water. A color-developing agent (1 mL) was blended and allowed to react for 20 min. The mixture was then transferred to a cuvette (3 mL), and the absorbance was measured at 540 nm with a spectrophotometer.

The denitrification and phosphorus removal rates were calculated in accordance with the absorbance results. On the basis of these results and the standard curve, three types of nutrient concentration were calculated:

$$\text{Denitrification rate: } \eta_1 = (A - A_t - B_t)/A \times 100\% \quad (1)$$

$$\text{Phosphorus removal rate: } \eta_2 = (C - C_t)/C \times 100\% \quad (2)$$

$$\text{Concentration of nutrients: } \rho = m/v \quad (3)$$

where η_1: denitrification rate, %; A: NO_3^--N initial concentration, mg/L; A_t: NO_3^--N concentration of the solution at time t, mg/L; B_t: NO_2^--N concentration of the solution at time t, mg/L; η_2: phosphorus removal rate, %; C: PO_4^{3-}-P initial concentration, mg/L; C_t: concentration of the PO_4^{3-}-P in the solution at time t, mg/L; ρ: concentration of the nutrients, mg/L; m: quality of the nutrients from the standard curve, μg; v: volume of the water sample, mL.

All experiments were repeated in triplicate.

3. Results and discussion

3.1. N14 strain characteristics

3.1.1. Morphological characteristics

N14 formed milk-white colonies with neat edges. The colonies had a smooth, moist surface and raised centers, and they were translucent. The organisms were 0.5 × 1.0-μm short rods with occasional short chains. Staining demonstrated the accumulation of poly-P and PHB particles within the organisms (Fig. 1).

3.1.2. Physiological–biochemical characteristics

N14 is a non-spore-forming Gram-negative bacterium with a thick capsule. N14 was positive for nitrate reduction, nitrate reduction producing N_2, oxidization of H_2S, catalase, Voges–Proskauer, and indole. The strain was negative for producing ammonia, oxidase, and methyl red. It could utilize citrate and glucose to grow, and could not hydrolyze starch or liquefy gelatin (Table 1). Based on biochemical analysis, the strain was identified as belonging to the genus *Klebsiella*.

3.1.3. 16S rDNA sequence and phylogenetic analysis

The 1485-bp 16S rDNA sequence of N14 was submitted to NCBI (Accession number: KP284569) and aligned against known gene sequences using BLAST to determine homology. The results showed that N14 was closely related to some known *Klebsiella* strains, and exhibited 99% similarity. The phylogenic tree (Fig. 2) showed greater than 50% branch support of all branches. Therefore, strain N14 was determined to be *Klebsiella* sp. based on its morphological and physiological–biochemical characteristics and 16S rDNA sequence analysis.

3.1.4. Effect of growth conditions on nitrogen and phosphorus removal performance

3.1.4.1. pH. The pH of the culture medium can affect the charge state and redox potential, thus affecting microbial absorption of nutrients and enzymatic reactions. Therefore, pH change can have a large impact on nitrogen and phosphorus removal [12]. Fig. 3(a) shows that the denitrification rate and phosphorus removal rate gradually increased as the pH increased from 5 to 8. The highest growth was achieved at a pH of 8, where the OD_{600} reached 1.16. The highest rates of nitrogen and phosphorus removal (85.9 and 78.6%, respectively) were achieved under these conditions. At a pH of 5, the OD_{600} only reached 0.31. This acidity resulted in the lowest rates of nitrogen and phosphorus removal (32.5 and 29.1%, respectively). A pH of 9 resulted in better growth than that in acidic conditions (OD_{600} = 0.95), and supported nitrogen and phosphorus removal rates of 71.8 and 64.6%, respectively. These results indicate that the optimum pH for denitrification and phosphorus removal by N14 is neutral to slightly alkaline. This is consistent with studies conducted by Filipe et al. [13] and Ma et al. [14]. Fig. 3(a) also shows that the absorbance values of the strains have a simple normal distribution with nitrogen and phosphorus removal rates in the pH range of 6–9; it indicates that denitrification and phosphorus removal rates have a positive correlation with bacterial growth. Ammonia was not detectable (data not shown), which is consistent with nitrate reduction producing nitrogen gas.

3.1.4.2. Temperature. Temperature typically has the following effects on bacterial growth and metabolism. At low temperatures, the bacterial cell membrane is gel-like, transmembrane transport of nutrients is blocked, and growth is inhibited. As the temperature rises, intracellular biochemical reactions and growth are accelerated. When the temperature exceeds the upper

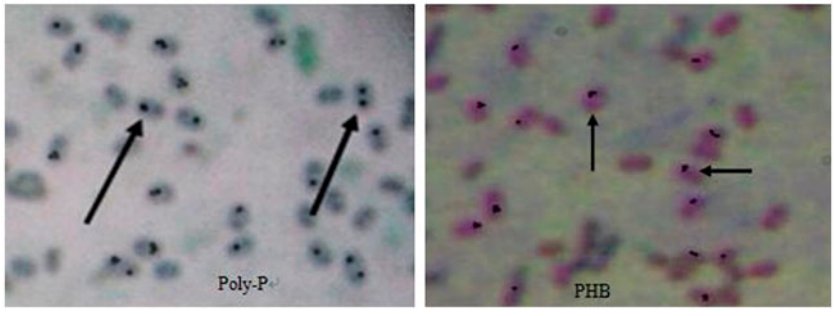

Fig. 1. Poly-P and PHB particles staining of strain N14.

Table 1
N14 main physiological–biochemical characteristics

Objects	Results	Objects	Results
Gram stain	–	Oxidization of H_2S	–
Spore	–	Utilization of citrate	+
Capsule	+, Thick	Utilization of glucose	+
Nitrate reduction	+	Hydrolyzing starch	–
Nitrate reduction producing N_2	+	Liquefying gelatin	–
Producing ammonia	–	Voges–Proskauer	+
Catalase	+	Indole	–
Oxidase	–	Methyl red	–

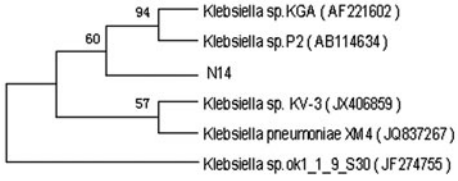

Fig. 2. Phylogenic tree of strain N14 based on 16S rDNA sequence analysis.

limit, temperature-sensitive protein and nucleic acid denaturation intensifies, bacterial growth stops, and the cells can be killed [15]. Therefore, an optimum growth temperature is defined for different bacterial species. After aerobic training for 24 h, the OD_{600}, denitrification rate and phosphorus removal rate gradually increased as the incubation temperature increased from 20˚C (0.89, 70.5, and 50.2%, respectively) to 30˚C (1.15, 86.2, and 80.7%, respectively) (Fig. 3(b)). These were the optimal values, except for that for phosphorus removal, which peaked at 78.9% at 25˚C. These results suggest that N14 may tolerate low temperatures for growth. At 35˚C, the OD_{600}, denitrification, and phosphorus removal rate declined slightly (1.06, 71.4, and 68.7%, respectively). At 40˚C, the OD_{600}, denitrification, and phosphorus removal rate declined obviously (0.71, 40.8, and 31.6%, respectively). The decreases were more robust at higher temperatures. The optimum temperature range for the growth of this strain is 20–35˚C.

3.1.4.3. Carbon source. Nitrogen and phosphorus removal by DPAOs are affected by the type of carbon source and the rate of carbon utilization. Studies have shown that some DPAOs can rapidly absorb short-chain volatile fatty acids during the anaerobic phase and store them as poly-P-hydroxyalkanoates (PHAs) [16]. The external carbon source can affect the absorption of phosphorus in the presence of nitrate and oxygen. N14 growth is optimal when sodium acetate is used as the carbon source (Fig. 3(c)). Using this substrate, the OD_{600}, denitrification, and phosphorus removal were 1.25, 88.5, and 81.3%, respectively. The next best performance resulted from the use of propionic acid as a carbon source with an OD_{600}, denitrification rate, and phosphorus removal rate of 1.11, 84.4, and 79.2%, respectively. The third best performance resulted from the use of sodium potassium tartrate as a carbon source with an OD_{600}, denitrification rate, and phosphorus removal rate of 0.57, 61.5, and 57.1%, respectively. When sodium citrate was used as the sole carbon source, the OD_{600}, nitrogen and phosphorus removal rate were the lowest (0.30, 25.6, and 44.7%, respectively), possibly because of the slow absorption of this substrate by N14, resulting in less storage of glycogen and conversion into PHA. With sodium citrate, the phosphorus removal rate was higher than the denitrification rate, potentially indicating that dissolved oxygen (DO) in the medium was used as a final electron acceptor rather than NO_3^--N.

3.1.4.4. N14 nitrogen and phosphorus removal. When the inoculum containing *Klebsiella* sp. N14 was seeded into 200 mL of synthetic wastewater at 30˚C and pH value of 8 for 24 h, the concentration of PO_4^{3-}-P was reduced from 81 to 12.4 mg/L, and the concentration of NO_3^--N was reduced from 180 to 15 mg/L. The NO_2^--N concentration increased within the first 8 h from 0 to 44 mg/L, and it was gradually reduced after 16 h to 6.7 mg/L. The nitrogen and phosphorus removal rates reached 87.94 and 84.69%, respectively; The largest degradation ratios of NO_3^--N and PO_4^{3-}-P were 16.25 mg/(L h), 2.75 mg/(L h), respectively, within 24 h (Fig. 4).

Robertson and Kuenen (1988) put forward the earliest theory of aerobic denitrification. During their research, they identified denitrification phenomena under aerobic conditions, and for the first time isolated three aerobic denitrifying bacteria, namely, *Thiosphaera pantotropha*, *Pseudmonas* sp., and *Alcaligenes*

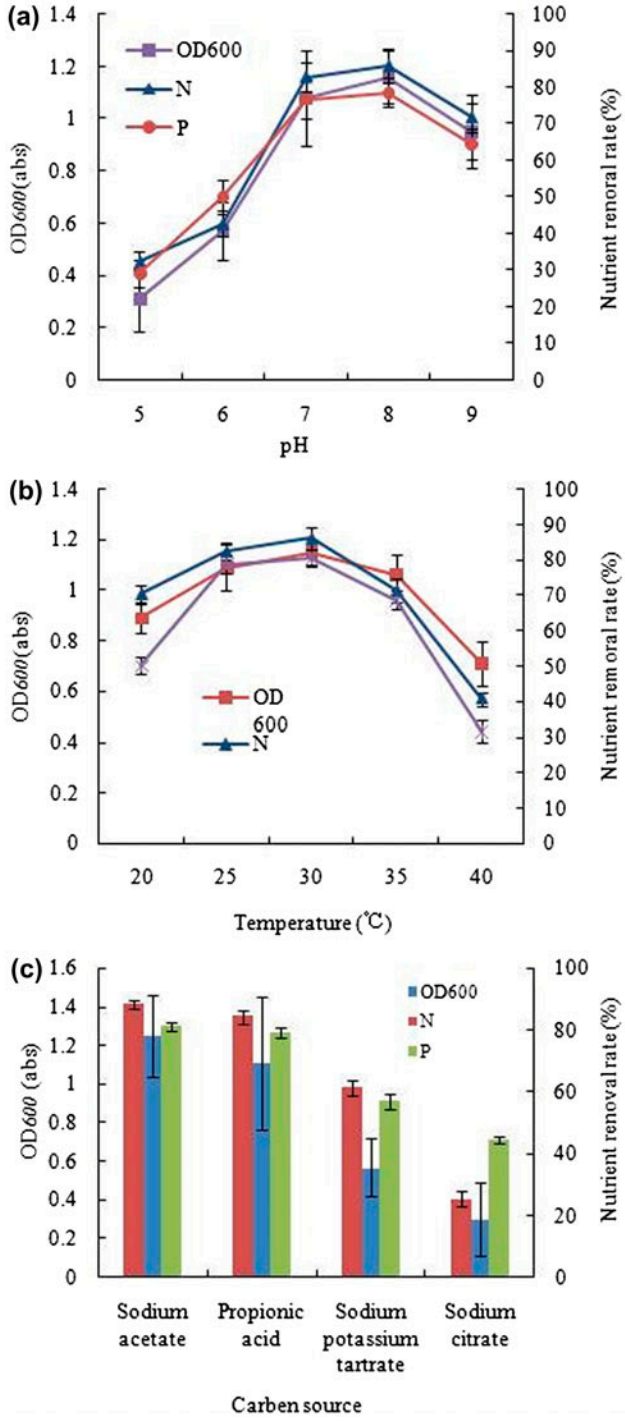

Fig. 3. Effect of conditions on nitrogen removal.

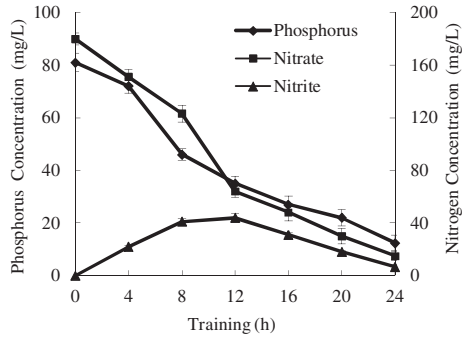

Fig. 4. N14 nitrogen and phosphorus removal.

faecalis [17]. The mechanism of aerobic denitrification can be explained from the perspective of micro-environment and biology theories. Micro-environment theory [18]: because of the limitation of oxygen diffusion in the microbial flocculant, a DO gradient was produced, leading to a suitable aerobic environment for flocs, and an internal micro-environment for anaerobic denitrification. DO is higher on the surface of the vitro flocculant, and in this micro-environment, aerobic heterotrophic bacteria and aerobic nitrifying bacteria multiply. Deep inside the flocs, oxygen transfer is blocked; the oxidation and nitrification of organics consumes a large amount of oxygen producing an anoxic zone inside the flocs; denitrifying bacteria predominate and this leads to aerobic denitrification. Biology theory: Jetten et al. argued that there are aerobic denitrifying enzymes within some bacteria, and that electron flow can simultaneously transmit to denitrifying enzymes in the system and oxygen in the process of collaborative respiration. Therefore, denitrification can occur in an aerobic environment. Some heterotrophic nitrification bacteria are aerobically denitrifying and can also perform nitrification and denitrification; therefore, they can directly convert ammonia to NO_x, and then let out the system. This also makes it possible for nitrification and denitrification to be completed simultaneously in the same reactor [19].

In recent years, researchers have found that denitrification occurs under aerobic conditions during practical projects. Sixteen strains of denitrifying bacteria were isolated from activated sludge an intermittent anaerobic/aerobic treatment tank by Frette et al. They carried out denitrification under both aerobic and anaerobic conditions [20]. A strain of aerobic denitrification bacteria named *Pseudomonas putida* was screened out during the operation of a continuously running biological filtration tower; this strain could reduce NO_3^--N under aerobic conditions, and the nitrogen removal rate was 94.84% in 24 h [21]. Three types of aerobic denitrifying bacteria were added to the biological filtration system; after 26 d a biofilm with aerobic denitrifying bacterial strains was obtained and the nitrogen removal rate was found to be 93.6% [22]. These results fully confirm the existence

of aerobic denitrification bacteria, and these can be used in practical processes. In this study, *Klebsiella* sp. N14 could use organic carbon sources to grow and reduced nitrate to nitrite, 0and then to N_2. Upon aerobic training in synthetic wastewater for 24 h, the nitrogen removal rate was 87.94%. Therefore, strain N14 enabled denitrification under aerobic conditions; it was a kind of aerobic denitrifier.

A strain with denitrifying and phosphorus-uptake capabilities was isolated from printing and dyeing wastewater. The strain was identified as *Klebsiella* and anaerobically cultured for 24 h, the denitrification rate was 86.67% and the phosphorus removal rate was 77.01% [23]. A denitrifying phosphorus-accumulating bacterial strain was screened from the stable operation of the continuous flow sludge system. This strain was identified as *Klebsiella*, hypoxia cultured, and the unit bacterial phosphorus uptake was found to be 1.13×10^{-11} mg/CFU [24]. In the study, *Klebsiella* sp. N14 also formed poly-P and PHB particles and showed phosphate uptake, which further confirmed that it had the ability to accumulate phosphorus. Therefore, N14 is a type of DPAOs with functions of simultaneous denitrification and uptake of phosphorus in aerobic conditions.

4. Conclusions

In this study, a high-efficiency synchronous nitrogen and phosphorus-removing DPAO was isolated from the activated sludge, it contains ploy-P and PHB granules. It was identified as *Klebsiella* sp. N14 by 16S gene sequence analysis and physiological–biochemical experiments. Strain N14 exhibits better growth and higher nitrogen and phosphorus removal in sodium acetate or propionate as carbon sources, a pH of 7–9, and a temperature of 25–35˚C. Nutrient removal is optimum at a pH of 8, a temperature of 30˚C, and a carbon source of sodium acetate. Upon aerobic training in synthetic wastewater for 24 h, the phosphorus concentration in culture supernatants decreased from 81 to 12.4 mg/L. The phosphorus removal rate was 84.69%. Nitrate concentration decreased from 180 to 15 mg/L. Nitrite concentration decreased from 44 to 6.7 mg/L. The denitrification rate was 87.94%. Therefore, the strain N14 can efficiently synchronize nitrogen and phosphorus removal in wastewater for the efficient denitrifying phosphorus organisms.

Acknowledgements

The study was supported by grants from the Six Talent Summit of Jiangsu Province under Grant No. JNHB-005; the Science and Technology Plan Projects of Ministry of Housing and Urban-Rural Development of the People's Republic of China under Grant Nos. 2015-K6-014 and R22016061; the Natural Science Foundation of College of Jiangsu Province under Grant No. 13KJD610002; and the Young Project of Xuzhou Institute of Technology under Grant No. XKY2015309.

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