JOURNAL OF PURE AND APPLIED MICROBIOLOGY, FEBRUARY 2014. Vol. **8**(1), p. 373-378

Study on Three Denitrifying Phosphorus Removal Strains from a Low Carbon-Source Treatment System

Zhan Jin, Fangying Ji*, Xuan Xu, Qian Guo and Qi Li

Key Laboratory of Three Gorges Reservoir Region's Eco-Environment, Ministry of Education, Chongqing University, No. 174 Shazhengjie Road, Shapingba District, 400045, Chongqing, China.

(Received: 02 August 2013; accepted: 19 September 2013)

Three strains, 13#, 15# and 17#, which had ability of denitrifying phosphorus-uptake, were isolated and identified from a low carbon-source treatment system. Through 16S rDNA sequence analysis and homology comparison, three strains were identified as *Klebsiella pneumonia* strain, *Ralstonia pickettii* strain and *Acinetobacter junii* strain, respectively. The nitrogen and phosphorus removal study showed that all three strains are denitrifying phosphorus removal bacteria, especially the 13# which is considered as the efficient DPAOs for treating low carbon source sewage. The phosphorus release and phosphorus uptake amounts of 13# were 0.72 and 0.88 mg/L, respectively. 15# and 17# did not absorb excess phosphorus because of the high NO_3^--N concentration (>50mg/L). Furthermore, 13# and 15# are capable of denitrification under aerobic condition suggesting that the LS-SDP existed simultaneous nitrification and denitrification (SND) besides denitrifying phosphorus removal.

Key words: Denitrifying Phosphorus, Low carbon source, Nitrification, denitrification.

Polyphosphate accumulation and denitrification are important biochemical processes in biological nutrient removal (BNR) system. However, carbon source competition exists in simultaneous biological nitrogen and phosphorus removal system[1], resulting in the low efficiency of nitrogen and phosphorus removal in wastewater treatment plant. This phenomenon is especially prominent when treating urban sewage with low carbon source[2]. In fact, the low carbon source problem of urban sewage is currently widespread[3]. Achieved high efficiency of nitrogen and phosphorus removal under low carbon source condition has turned to be one of the big problems in the field of the environment.

From the late 1980s, several studies on activated sludge systems and laboratory cultures had proven that phosphorus accumulating organisms (PAOs) can use nitrate as an electron acceptor in the absence of molecular oxygen[4,5]. Therefore, polyphosphate accumulation and denitrification can be combined as an alternative to conventional biological nitrogen and phosphorus removal process.

There are some advantages of simultaneous nitrogen and phosphorus removal through the denitrifying phosphorus removal pathway in wastewater treatment process, such as simultaneous removal of N and P, less sludge production. Furthermore, the major advantage is that it can reduce the requirement for substrates[6]. With the development of phosphorus and nitrogen removal techniques in microbiological field, denitrifying phosphorus removal have been investigated more and more.

* To whom all correspondence should be addressed.
E-mail: jfytou@sina.cn

Some authors have suggested that PAOs could be divided into two coexisting groups: denitrifying PAO (DPAOs) and non-DPAO[7]. DPAOs can utilize either nitrate or oxygen as an electron acceptor, whereas non- DPAOs can only use oxygen. Further, DPAOs have also shown to be able to utilize nitrite as an electron acceptor despite it can have inhibitory effect on the aerobic P-uptake. Some authors have shown results supporting the presence of two different types of PAOs in denitrifying P removal systems. It was demonstrated that P was initially taken up under anoxic conditions, but anoxic P uptake eventually ceased despite the continued presence of nitrate when anaerobic–anoxic–aerobic tests was performed[8]. Once oxygen was introduced to the system, P uptake then re-started. The proposed explanation for this observation was the co-existence of DPAOs, capable of using nitrate as electron acceptor in the anoxic phase until their PHA pools were depleted, and non-DPAOs, only able to use oxygen for phosphorus removal, which would be responsible for the subsequent aerobic P uptake. Recent microbiological studies are focused on assessing the link between the process operation conditions and their microbial community structure under different conditions such as the type of carbon source. On the other hand, influence factors of denitrifying phosphorus removal process were also studies, e.g. the threshold concentration that nitrite can be used for anoxic phosphorus uptake in the denitrifying phosphorus removal process[9]. Nevertheless, little information is available on the performance of denitrifying phosphorus removal on full-scale or pilot plants are fed with real low carbon source sewage and few references can be found where the efficient DPAOs were isolated from low carbon source influent system.

Based on the above, the denitrifying phosphorus removal in the low carbon source sewage treatment system with enhanced denitrifying phosphorus removal function (LS-SDP) was investigated. The previous study mainly concentrated on the design of LS-SDP[10] and the establishment of the denitrifying phosphorus removal performance in this system when treating the low carbon source sewage (COD/TN<4.25, COD<200mg/L)[11]. In this study, the main objective is to investigate the nitrogen and phosphorus removal characteristics of the DPAOs isolated from LS-SDP. In addition, they were identified through 16S rDNA sequence analysis.

MATERIALS AND METHODS

Collection of microbial source

Isolation of denitrifying phosphorus removal strains was done from activated sludge of stable operation LS-SDP feeding with real low carbon source sewage.

Isolation, identify and 16S rDNA sequence analysis

Separation medium (include denitrifying bacteria and polyphosphate accumulation bacteria separation medium) and composition of trace element[12,13]; Phosphorus deficient medium and Rich phosphorous and nitrogen solution[14]; COD solution: 1 L distilled water with 1 g CH_3COONa. Nitrate reduction test was done with Du-canaliculus. Metachromatic granules and Poly-^{2}-hydroxybutyrate (PHB) reagent were also stained and observed under oil lens

Target strains were isolated[15] and their DNA were extracted and amplified by PCR[16]. Sequencing was accomplished by Invitrogen Corporation Shanghai Representative Office. The homology comparison of sequencing result and known 16s rDNA sequences listed in the GenBank was done by BLAST. According to the result of sequencing, homologous sequence was found in the GenBank database. The sequences were aligned with CLUSTAL X software. A phylogenetic tree was constructed using MEGA 4.0 with the neighbor-joining and Bootstrap analysis (1000 re-samplings) was used to evaluate the tree topology.

Experiment of nitrogen and phosphorus removal of DNPAOs

The strains of above inclined medium were inoculated into beef-protein medium with inoculation loop. After being activated in the constant temperature water bath oscillator at 30°C for 24h, bacteria solution was inoculated into 200 mL phosphorus deficient medium. Inoculation amount was 1%.

Simulating the practical operation process of the wastewater treatment plant, nitrogen and phosphorus removal capability of three strains were studied by the culture method of pre-anaerobic/ anaerobic /aerobic. They were cultured

in the constant temperature water bath oscillator at 30°C for 36 h. Then 5 mL aseptic COD solution was added in for phosphorus release and cultured in the incubator at 30°C under anaerobic condition for 4 h (pre-anaerobic). Then 5 mL aseptic rich phosphorous and nitrogen solution was added in and cultured in the incubator at 30°C for the next 4 h (anaerobic). At last 5 mL aseptic COD solution and 5 mL rich phosphorous and nitrogen solution were added into bacteria solution and cultured in the constant temperature shaking table at 30°C (aerobic) for 6 h. They were sampled at the beginning and the end of each stage (pre-anaerobic, anaerobic and aerobic), respectively. Meanwhile, sampling and determination the concentration of total nitrogen, nitrate, nitrite, ammonia nitrogen and phosphate were conducted in every step. The operations all above were on the aseptic table. Each experiment consists of three parallel.

Analytical methods

COD, TN, NH_4^+-N, NO_3^--N, NO_2^--N and PO_4^{3-}-P were measured according to the Chinese State Environmental Protection Agency (SEPA) Standard Methods[17].

RESULTS AND DISCUSSION

Isolation of the DNPAOs from the low carbon source treatment system

21 strains were obtained from denitrifying bacteria separation medium and 8 strains were obtained by second selective culture in polyphosphate accumulation bacteria separation medium**.**

Among them, 13#, 15# and 17# strains can deoxidize nitrate to gas. Furthermore, only 13#, 15# and 17# strains' Metachromatic and PHB granules staining test results were positive (Table 1). Thus, it could be judged preliminarily that 13#, 15# and 17# were the object strains capable of denitrifying phosphorus removal.

Table 1. Screening result of DNPAOs

Strains	Denitrifying nitrates to gas	PHB granules	Metachromatic granules
2#	yes	no	no
4#	yes	no	no
5#	yes	no	no
9#	no	no	no
13#	yes	yes	yes
14#	no	no	no
15#	yes	yes	yes
17#	yes	yes	yes

Identification of the isolate

Comparing 16s RNA sequence of 13#, 15# and 17# strains (1429 letters, 1429 letters and 1419 letters) with GenBank database, it can be found

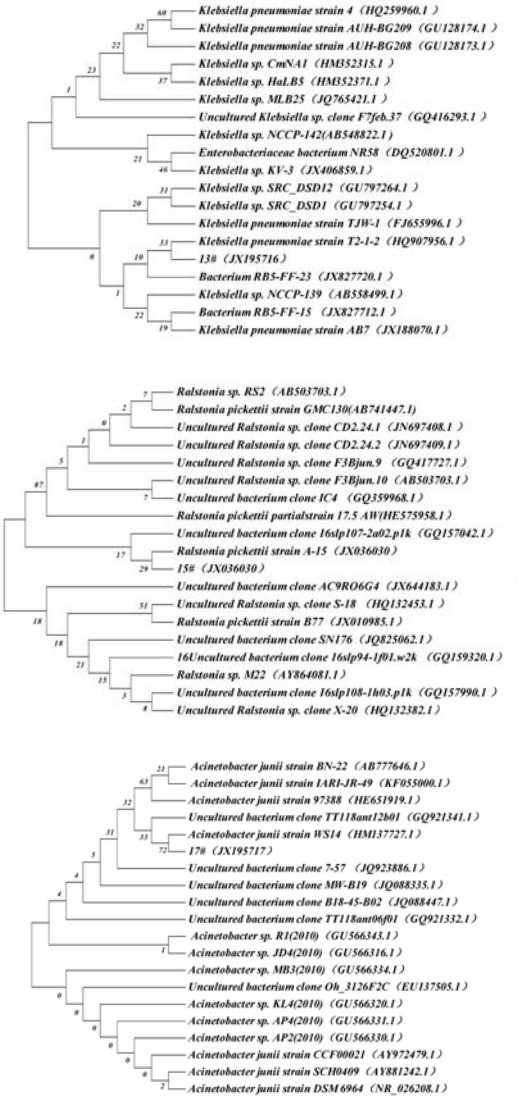

Fig.1. Aphylogram (neighbor-joining method) showing genetic relationship between Isolate strain and other related reference microorganisms based on the 16SrRNA gene sequence analysis (A: 13#; B: 15#; C: 17#). Species names are followed by the accession numbers of their 16SrRNA sequences.

that the similarity level of 16s rDNA of 13# and several *Klebsiella pneumoniae* strains reaches more than 99%. 13# is almost certain to be *Klebsiella pneumonia* strains, named as *Klebsiella pneumoniae* strain 13#(GenBank Accession number: JX195716). While the 16s rDNA similarity levels of 15# and several *Ralstonia pickettii* strains, 17# and several *Acinetobacter junii* strains reach more than 98% and 99%, respectively. So 15# is certain to be *Ralstonia pickettii* strain, named as *Ralstonia pickettii* strain 15#(GenBank Accession number: JX036030), and 17# is certain to be *Acinetobacter junii* strain, named as *Acinetobacter junii* strain 17#(GenBank Accession number: JX195717).

The nitrogen and phosphorus removal experimental of three strains

Target strains were inoculated into phosphorus deficient medium and cultured for about 36 h (DO_{600} is basically stable). The denitrifying phosphorus removal characteristic of three strains in the low carbon-source treatment system was studied.

The initial COD of Pre-Anaerobic was 42(13#), 33(15#) and 35(17#) mg/L, respectively. A obviously degraded was observed at Pre-anaerobic stage with the initial COD of next Anaerobic was only 11, 5 and 5 mg/L for 13#, 15# and 17# strains, respectively. The initial COD of 13#, 15# and 17# at Aerobic stage adjusted to 40, 38 and 34 mg/L, respectively after aseptic COD solution was added (Fig. 2). However, the higher initial TN concentration of three strains led to the low COD/TN ratio during the whole processing from Pre-anaerobic to aerobic stage which indicated that the nitrogen and phosphorus removal experiments of three strains were done under low carbon source condition.

The differential values of PO_4^{3-}-P concentration at pre-anaerobic and anaerobic stages showed that all 3 strains had the phenomena of phosphorus release in pre-anaerobic and phosphorus uptake in next anaerobic (Fig. 3). The phosphorus release amounts were 0.72(13#), 0.58(15#) and 0.52(17#) mg/L, respectively and the phosphorus uptake amounts in next anaerobic were 0.88(13#), 0.21(15#) and 0.47(17#) mg/L, respectively. Meanwhile, Fig. 3 also shows that the NO_3^--N and TN concentration differential

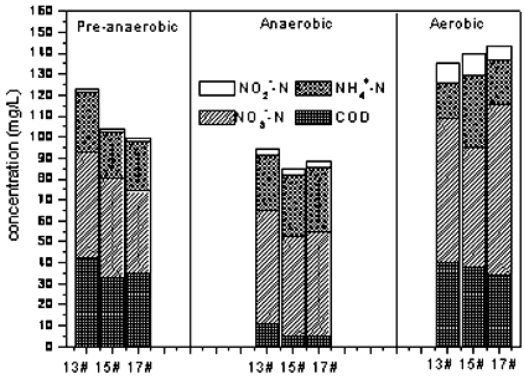

Fig 2. The initial COD and TN concentration (include NH_4^+-N, NO_2^--N, NO_3^--N) of each stage

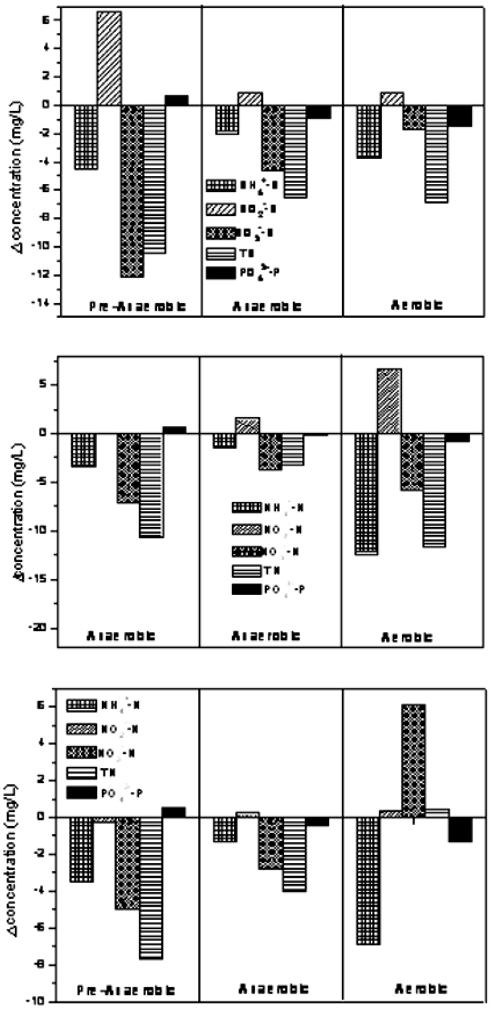

Fig. 3. The differential value of NH_4^+-N, NO_2^--N, NO_3^--N, TN and PO_4^{3-}-P concentration at each stage (A: 13#; B: 15#; C: 17#)

values of three strains at anaerobic stage were obviously decreased compared with pre-anaerobic stage showing that the denitrification rate of anaerobic stage was lower than pre-anaerobic stage. It was noteworthy that 15# and 17# did not appear denitrification excess phosphorus uptake. The possible reason was that the high NO_3^--N concentration (about 50 mg/L) had a negative effect on phosphorus uptake and this explanation is supported by other report [18]. Fig. 2 shows that the NO_3^--N concentration of 15# and 17# at anaerobic stage was 47.5 and 50.1 mg/L respectively while 13# was 50.7 mg/L. The results demonstrated that 13# could be considered as the efficient DPAOs under low carbon source condition.

Combined with fig 2, the different nitrogen and phosphorus removal performance between pre-anaerobic and anaerobic stages might be attributed to the presence of carbon source available at pre-anaerobic. It was well-known that the carbon source, especially the Sodium acetate [19], was benefited to denitrification and phosphorus uptake. At anaerobic stage, the external carbon source was almost exhausted and the internal carbon source was used to denitrifying phosphorus removal which led to the decrease of denitrification rate.

The NH_4^+-N of three strains was decreased throughout all the stages even under anaerobic condition and the results indicated that three strains might be heterotrophic denitrifying bacteria. It has been report that *Klebsiella pneumoniae strain* is aerobic denitrifying bacteria [20] *Ralstonia pickettii strain* is high oxygen-resistant denitrifying bacteria [21] and *Acinetobacter junii strain* is aerobic heterotrophic denitrifying bacteria [22]. Many aerobic denitrifying bacteria were also capable of heterotrophic nitrification which could convert ammonia into nitrogen gas without intermediary accumulation of nitrite. However, 13# got high NO_2^--N accumulation and reached 8.16 mg/L at pre-anaerobic stage. This might be due to the denitrification process which was coinciding with previous studies [20].

The NH_4^+-N removal amounts of 3 strains were 3.68, 12.38 and 6.93 mg/L, respectively under aerobic condition when the TN removal amounts of 13# and 15# were 6.91 and 11.65, respectively. The TN concentration of 17# remained essentially constant throughout the whole aerobic stage which reveals that 17# do not have denitrification ability under aerobic condition.

Therefore it can be concluded that all three strains are denitrifying phosphorus removal bacteria. In particular, 13# is the efficient DPAOs for treating low carbon source sewage. Furthermore, 13# and 15# are capable of denitrification under aerobic condition suggesting that the LS-SDP existed simultaneous nitrification and denitrification (SND) besides denitrifying phosphorus removal.

ACKNOWLEDGMENTS

The authors acknowledge the support by National Natural Science Foundation of China (NO.51108483) and Natural Science Foundation Project of CQ CSTC (NO.cstcjjA20002)

REFERENCES

1. REN Nan-qi, WANG Xiu-heng, DONG Jing-hao, Simultaneous Phosphorus and Nitrogen Removal in Novel HITNP System. *Chinese J Environ Sci;* 2007: **28**(1):108-112.
2. Katarzyna Bernat, Irena Wojnowska-Barylaa, Adriana DobrzyDska. Denitrification with endogenous carbon source at low C/N and its effect on P(3HB) accumulation. *Bioresour Technol*; 2008**; 99**: 2410-2418.
3. WANG Tao, ZHAO Na, DENG Rongseng, NIE Zhuona, MAI Songbing. Research on the Strengthening Efect of the Nitrogen and Phosphorus Removal by the Multi-loop Reactor in Low Carbon-resource Condition. *J Chongqing Jianzhu Univer,* 2005: **27**(6): 62-66.
4. Comeau Y, Hall K J, Handcock R E W, Oldham W K . Biochemical model for enhanced biological phosphorus removal. *Water Res;* 1986: **20**(12):1511-1521.
5. Barker P S, Dold, P L . Denitrification behaviour in biological phosphorus removal activated sludge systems. *Water Res;* 1996 **30**(4): 769-780.
6. Kuba T, van Loosdrecht M C M and Heijnen J J .Phosphorus and nitrogen removal with minimal COD requirement by integration of denitrifying dephosphatation and nitrification in a two-sludge system. *Water Res;* 1996: **30**(7):1701-1710.
7. Freitas F, Temudo M, Reis M A M . Microbial population response to changes of the operating

conditions in a dynamic nutrient-removal sequencing batch reactor. *Biopro Biosystems Engg;* 2005: **28**(3):199-209.

8. Sin G, Niville K, Bachis G, Jiang T, Nopens I, van Hulle S, Vanrolleghem P A .Nitrite effect on the phosphorus uptake activity of phosphate accumulating organisms (PAOs) in pilot-scale SBR and MBR reactors. *Water SA;* 2008: **34**(2), 249-260.
9. Zhou Y, Pijuan M and Yuan Z . Free nitrous acid inhibition on anoxic phosphorus uptake and denitrification by poly-phosphate accumulating organisms. *Biotechnol. Bioeng.*; 2007: **98**(4):903-912.
10. Ji Fangying, Jin Zhan, Guo Qian, Li Qi, Liu Na, Chen Si . A method of low carbon source sewage treatment system with enhanced denitrifying phosphorus removal. China, 2012: CN102659288A.
11. Ji Fangying, Guo Qian, Jin Zhan, ChenQingkong, XiongLi . Study on enhanced denitrifying phosphorus removal from domestic sewage with low carbon source. *China Water & Waste water*; 2012: **28**(13):1-4.
12. MA Fang, WANG Chun-li, WANG Li-li . Screening of denitrifying polyphosphate-accumulating organisms and their biological characteristics. *J Harbin Engg Univer,* 2007: **28**(6): 631-635.
13. BAO Lin-lin, LI Dong, LI Xiang-kun, HUANG Rong-xin, ZHANG Jie, LV Yang, XIA Guang-qing .Phosphorus accumulation by bacteria isolated from a continuous-flow two-sludge system. *J Environ Sci,* 2007 ; **19**(4):391-395.
14. MerzouKi M, DELGENEs J P, BERNET N . Polyphosphate-accumulating and denitrifying bacteria isolated from anaerobic-anoxic and anaerobic-aerobic sequencing batch reactors. *Curr Microbio;* 1999: **38**: 9-17.
15. Tirsten S, Jorgensen and Anneli S L Pauli .Polyphosphate Accumulation among Denitrifying Bacteria in Activated Sludge. *Environ Microbio:* 1995: **1**(3):161-168
16. Johwan Ahn, Tomotaka Daidou, Satoshi Tsuneda, Akira Hirata. Characterization of denitrifying phosphate-accumulating organisms cultivated under different electron acceptor conditions using polymerase chain reaction-denaturing gradient gel electrophoresis assay. *Wat. Res.* 2002: **36**(2): 403-412.
17. SEPA. Water and wastewater monitoring methods, 4th Edn. SEPA, BeiJing, China, 2002.
18. Zhao Qing, Yuan Linjiang, Wang Zhiying., Study on characteristics of denitrifying dephosphorization. *Techniques and Equipment for Environmental Pollution Control* 2005; **6**(9): 20-24.
19. Zhen Hu, Jian Zhang, Shanping Li, Huijun Xie., Impact of carbon source on nitrous oxide emission from anoxic/oxic biological nitrogen removal process and identification of its emission sources. *Environ Sci. Pollut. Res.* 2013; **20**(2): 1059-1069.
20. Li Xiaokun, Qiu Yexian, Yang Zhiyuan, Xu Zhenhua, Liu Yurong., Screening of Denitrifying Phosphate Accumulating Organisms from Printing and Dyeing Wastewater and Characteristics of Nitrogen and Phosphorus Removal. *Environental science and management* 2009; **34**(8):90-92.
21. HE Fan, KANG Yi-Jun, SHAN Jun, HU Jian, YIN Shi-Xue., An Oxygen-tolerant Denitrifying Strain and Its Denitrifying Processes. *Microbiology* 2008; **35**(1):35-39.
22. Jin Min,Wang Jingfeng, Kong Qingxin, Zhao Zuguo, Wang Xinwei, Shen Zhiqiang, Chen Zhaoli, QIU Zhigang and LI Junwen., Isolation and Denitrification Mechanism of an Aerobic Heterotrophic Bacterium *Acinetobacter sp. YY-5*. Chin J Appl. Environ Biol.* 2009; **15**(5):692-697.