Isolation and identification of an aerobic denitrifying phosphorus removing bacteria and analysis of the factors influencing denitrification and phosphorus removal

Hongying Xu, Ru Jin, Chan Zhang, Yupeng Wu and Xiaohui Wang

ABSTRACT

Excessive emission of plant nutrients (such as nitrogen and phosphorus) into the water body can induce eutrophication. Therefore, how to control eutrophic water efficiently and economically is very important. In the paper, highly efficient aerobic denitrifying phosphorus removing J16 bacteria was isolated from the activated sludge of an aerobic bioreactor in Taiyuan municipal wastewater treatment plant by using the blue–white spot screening method, an aerobic phosphorus absorption test, nitrate reduction test, nitrogen removal experiments, and plate coating and streaking methods. Through 16S rDNA gene homology comparison and physiological and biochemical identification, the J16 strain was preliminarily identified as *Escherichia coli*, with a sequence similarity of 99%. The 16S rDNA sequence of strain J16 was submitted to GenBank (accession number: MF667015). The effect of temperature, pH, percentage of inoculum and phosphate-P (PO_4^{3-}-P) concentration on denitrification and phosphorus removal efficiency was investigated through a single-factor experiment. The optimum conditions of the J16 strain for denitrification and phosphorus removal were as follows: 30 °C, neutral or weak alkaline (pH: 7.2–8), and 3% of inoculum, respectively. The denitrification and phosphorus removal efficiency of strain J16 was the highest when PO_4^{3-}-P and nitrate-N(NO_3^--N) concentrations were 8.9 and 69.31 mg/L, and the removal were 96.03% and 94.55%, respectively. In addition, strain J16 could reduce phosphoric acid to phosphine (PH_3) and remove some phosphorus under hypoxia conditions. This is the first study to report the involvement of *Escherichia coli* in nitrogen and phosphorus removal under aerobic and hypoxia conditions. Based on the above results, the strain J16 can effectively remove nitrogen and phosphorus, and will be utilized in enhancing treatment of nitrogen and phosphorus-containing industrial wastewater and phosphorus reclamation.

Key words | aerobic denitrifying phosphorus removing bacteria (ADPB), activated sludge, environmental factors, identification, isolation, 16S rDNA

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INTRODUCTION

With the continual improvement in human living standards and the rapid development of industrial production, industrial wastewater and domestic sewage containing excessive amounts of nutrients are discharged into water bodies such as rivers, lakes, and reservoirs, resulting in severe water eutrophication (Zhao *et al.* 2010; Qin *et al.* 2013). At present, the management of eutrophicated water body has become one of the environmental problems to be solved urgently, and it is also the research hotspot of global experts and scholars. There are many reports on denitrification and phosphorus removal in wastewater at home and abroad (Comean 1986; Wang *et al.* 2008; Yu & Li 2015; Nie *et al.* 2017). Usually, the wastewater containing nitrogen and phosphorus is treated by activated sludge process.

In the traditional activated sludge process, denitrification is considered to occur in anoxic environments (Li *et al.* 2005), and oxygen (O_2) inhibits denitrification reductase activity. Under aerobic conditions, O_2 is generally considered to be the first choice for an electron acceptor.

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Therefore, denitrifying bacteria preferentially use O_2 when they breathe, which prevents nitrate radical (NO_3^-) and nitrite radical (NO_2^-) from becoming final electron acceptors. At the same time, the removal of phosphorus in the traditional process is mainly through the superstrong phosphorus absorption capacity of polyphosphate bacteria. Denitrification and phosphorus removal, which are two independent processes, can occur simultaneously (Kuba *et al.* 1996; Ahn *et al.* 2002; Merzouki *et al.* 2005; Tsuneda *et al.* 2006), achieving 'carbon dual use'. In traditional denitrification and phosphorus removal processes, 'carbon dual use' can solve problems arising due to carbon source competition and the difference in sludge age between denitrifying bacteria and phosphorus removal bacteria (An *et al.* 2010).

In recent years, denitrification has been confirmed to occur under aerobic conditions after isolation and discovery of aerobic denitrifying bacteria (Bell & Ferguson 1991; Ferguson 1994; Su *et al.* 2001; Chen *et al.* 2003). In aerobic denitrifying bacteria, nitrate reductase is present in the periplasm rather than the membrane (Bell *et al.* 1990). In the hypothetical respiratory pathway of aerobic denitrifying bacteria, electrons are passed either to O_2, NO_3^-, NO_2^- or nitrous oxide (N_2O) (Moir *et al.* 1995). This indicates that aerobic denitrifying bacteria can use nitrate nitrogen to replace O_2 as the electron acceptor for the oxidation of poly-β-hydroxybutyrate. Therefore, a novel method, which can enhance the efficiency of nitrogen and phosphorus removal in the traditional activated sludge process, has been opened up by the discovery of aerobic denitrifying bacteria. At present, the screening and isolation of aerobic denitrifying phosphorus removing bacteria (DPB) have been major research topics in the field of wastewater biotreatment technology. To date, the following genera of DPB have been identified: *Pseudomonas*, *Alcaligenes*, *Paracoccus*, *Bacillus*, *Rhodococcus*, and *Enterobacter* (Patureau *et al.* 2000; Joo *et al.* 2005; Yang *et al.* 2008; Guo *et al.* 2016). In 2009, Yu *et al.* isolated a short, rod-shaped *Pseudomonas* strain that could perform denitrification and phosphorus removal under aerobic conditions (Yu *et al.* 2009). In addition, Ma *et al.* (2011) and An *et al.* (2012) isolated strains of *Bacillus* and *Bacillus cereus*, respectively, both of which were aerobic DPB. In 2017, Nie *et al.* isolated two highly effective aerobic and denitrifying phosphorus removing strains of *Achromobacter* and *Brevundimonas* (Nie *et al.* 2017). In spite of this, more microbial resources with the efficient removal of nitrogen and phosphorus from wastewater under aerobic conditions must be exploited and characterized at present.

Our objectives were therefore to identify the strain isolated from aerobic activated sludge in the North suburban sewage treatment plant of Taiyuan City and to examine the capability for removal of nitrogen and phosphorus of the strain. Influence factor analysis of removal efficiency for nitrogen and phosphorus was further performed. Finally, the efficiency of the strain in removing total phosphorus (TP) and producing phosphine (PH_3) under hypoxic conditions was studied.

MATERIALS AND METHODS

Culture media used

The following media were used for isolation of the strains: beef extract peptone medium (3 g/L beef extract, 10 g/L peptone and 5 g/L NaCl) yeast extract glucose (YG) medium (1 g/L yeast extract, 1 g/L glucose, 0.3 g/L K_2HPO_4, 0.25 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, pH7.2) and Luria–Bertani (LB) medium (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, pH7.2).

A phosphorous-limited medium and a phosphorus-rich medium were used for the blue–white bacteria selecting experiment. The phosphorous-limited medium contained 17.4 mg/L K_2HPO_4, 100 mL/L glucose-MOPS solution, 0.2 mg/L x-pi, 0.2 mg/L VB_1 and 5 g/L agar, and the phosphorus-rich medium contained 346.4 mg/L K_2HPO_4, 100 mL/L Glucose-MOPS solution, 0.2 mg/L x-pi and 5 g/L agar.

A glucose-3-(*N*-morpholino)propanesulfonic acid(MOPS) solution contained 0.1 g/L glucose and 100 mL/L 10× MOPS mixture. The 10× MOPS mixture was prepared by adding 8.372 g of MOPS, 0.717 g of Tricine, and 30 mL of deionized water. Then, 10 M KOH was added to obtain a pH of 7.4, and the total volume was adjusted to 44 mL. Then, 1 mL of freshly prepared 0.01% $FeSO_4$ solution was added. Finally, the following components were then added in turn: 5 mL of 1.9 M NH_4Cl, 1 mL of 0.276 M K_2SO_4, 0.025 mL of 0.02 M $CaCl_2 \cdot 2\,H_2O$, 0.21 mL of 2.5 M $MgCl_2 \cdot 6\,H_2O$, 10 mL of 5 M NaCl, 0.02 mL of a mixture of trace elements (0.09 g/L $(NH_4)_6Mo_7O_{24}$, 0.62 g/L H_3BO_3, 0.18 g/L $CaCl_2$, 0.06 g/L $CuSO_4$, 0.40 g/L $MnCl_2$, 0.07 g/L $ZnSO_4$) and 38.7 mL of deionized water.

The nitrogen- and phosphorus-rich medium consisted 3.32 g/L $CH_3COONa \cdot 3H_2O$, 152.8 mg/L NH_4Cl, 50 mg/L K_2HPO_4, 91.26 mg/L $MgSO_4 \cdot 7\,H_2O$, 25.68 mg/L $CaCl_2 \cdot 2\,H_2O$, 500 mg/L KNO_3, 8.5 g/L PIPES buffer, 2 mL/L mixture of trace elements (63.7 g/L Na_2EDTA, 5.06 g/L $MnCl_2 \cdot 4\,H_2O$, 2.2 g/L $ZnSO_4$, 5.0 g/L $FeSO_4 \cdot 7H_2O$, 5.5 g/L $CaCl_2$, 1.1 g/L $Na_2MoO_4 \cdot 4\,H_2O$, 1.57 g/L $CuSO_4 \cdot 5\,H_2O$, 1.61 g/L $CoCl_2 \cdot 6\,H_2O$) and 10 mg/L NaCl; the pH of this medium was 7.2.

The nitrate reduction reaction medium consisted of 3 g/L beef paste, 5 g/L peptone and 1 g/L KNO_3, pH 7.2.

The synthetic wastewater contained 0.4 g/L glucose, 0.1 g/L peptone, 0.01 g/L yeast extract, 0.25 g/L CH_3COONa, 0.05 g/L NaCl, 0.07 g/L K_2HPO_4, 0.15 g/L $MgSO_4 \cdot 7H_2O$ and 0.18 g/L NH_4Cl.

Isolation, purification and screening of the strains

For the purpose of microbial isolation, activated sludge samples collected from the aerobic bioreactor of the North suburban sewage treatment plant of Taiyuan City were used as the sample. The activated sludge samples (after gravity settling) were mashed in a magnetic mixer (X85-2S, MeiYingPu, China), and were allowed to rest for 2 h. Then, 10 mL of the supernatant liquid was added to a 250 mL triangular flask containing 100 mL of beef extract peptone medium. The flask was sealed with a nine-layer gauze (to ensure adequate oxygen supply) and maintained in an oscillation incubator (HZQ-X100, ShengBlue, China) (30 °C, 150 r/min) for 24 h. The culture liquid was diluted and plated using YG medium, and incubated at 30 °C for 48 h. Bacterial colonies were isolated from the culture plates of each dilution gradient and purified on the YG solid medium. The purified bacterial colonies were transferred to LB medium slants and cultured for 3 days at 30 °C. After culturing, the slant tubes were removed from the incubator and stored at 4 °C for further use.

The strains grown after isolation and purification were inoculated in the phosphorus-limited and phosphorus-rich glucose-MOPS culture media, respectively, and cultured at 30 °C for 1–2 days until the appearance of the visible blue–white spots. Bacterial colonies that produced blue spots on both the culture media were selected as initial strains. The initial strains were inoculated in the synthetic wastewater culture medium, cultured in an incubator (30 °C, 150 r/min) for 24 h, then the bacterial culture was centrifuged for 15 min at 5,000*g*. The phosphorus content of the supernatant liquid was measured using the TP determination method. Then, the nitrate reduction experiments were conducted with the strains in which the phosphorus uptake rate was higher than 50%. The strains that produced nitrogen gas were inoculated in the phosphorus and nitrogen-rich culture medium (30 °C, 150 r/min) for 24 h. Then, the bacterial culture was centrifuged and the concentrations of PO_4^{3-}-P and NO_3^--N in the supernatant fluid were determined. The strains that showed highly efficient denitrification and phosphorus removal were selected as target strains.

Physiological and biochemical identification of the strain

The physiological and biochemical characteristics of the strain were determined by references (Dong & Cai 2002).

Cloning and sequence analyses of 16S rDNA

The strain screened in this study was identified by the analysis of the sequence of the gene encoding 16S rDNA. The fragment amplifications were performed using the universal PCR primers: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-AAGGAGGTGATCCAGCC-3′). The PCR reaction system (50 μL): $MgCl_2$ (1.5 mM), genomic DNA (10 ng), dNTP (200 μM), primer (0.4 μM) and Taq DNA polymerase (1.25 U). The reaction conditions: denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and chain extension at 72 °C for 90 s, for a total of 30 cycles. The 16S rDNA sequence of the strain was submitted to GenBank for BLASTN comparison, and the sequences with similarity of more than 97% were selected for phylogenetic analysis.

Denitrification and phosphorus removal experiment of the strain

All denitrification and phosphorus removal experiments were performed in 150 mL conical flasks filled with 100 mL of nitrogen- and phosphorus-rich medium (69.31 mg/L NO_3^--N and 8.9 mg/L PO_4^{3-}-P). To prepare the inoculum, 150 mL of the bacterial culture was centrifuged, washed twice in Tris-HCl buffer (40 mM, pH 7), and then a certain amount of bacteria (wet weight) was added to the above conical flasks (with the pH adjusted to 7.2 or so). The flasks were sealed with Mininert valves under aerobic conditions and incubated for 24 h at 30 °C and at a constant oscillation of 150 r/min.

In order to optimize the efficiency of denitrification and phosphorus removal, tests under different conditions of temperature (20 °C, 25 °C, 30 °C, 35 °C and 40 °C), pH (5.5, 6.5, 7.2, 8.0 and 9.0), percentage of inoculum (0.5%, 1%, 1.5%, 2%, 3%, 4% and 5%), PO_4^{3-}-P concentrations (4.45 mg/L, 8.90 mg/L, 13.35 mg/L, 17.80 mg/L and 22.25 mg/L) were performed for 24 h, and the residual contents of PO_4^{3-}-P and NO_3^--N were determined at the end of the experiment. All removal assays were performed in triplicate with CK (a treatment with sterile inoculum) to eliminate nitrogen and phosphorus removal caused by non-biological factors.

The PH_3 producing experiment of the strain

The experiment was carried out by a static reaction device. The effective volume of the liquid phase of the reactor was 0.7 L (experimental strain 0.3 L, experimental water 0.4 L), and the effective volume of the gas phase was 0.3 L, using water bath heating, magnetic agitator stirring, a reactor and a receiving bottle for light shading treatment. Nitrogen was introduced into the reactor to achieve anoxia, and the generated PH_3 gas was introduced into the brown reagent bottle. The experiment water was simulated organic wastewater containing phosphorus and nitrogen (10 mg/L TP, 50 mg/L TN, a small amount of trace element). The temperature in the reactor was adjusted to 30 °C, the magnetic stirring speed was 1,000 r/min, the pH was 8, and the operation was continued for 72 h; 100 mL of the absorption liquid (10 M H_2SO_4) was added to a 250 mL brown reagent bottle and the PH_3 gas generated in the reactor was introduced into the bottle for timed sampling and measurement. After the reaction, the TP content and PH_3 production in the reactor were measured, and the efficiency of phosphorus removal by the strain was investigated. The experiment was provided with CK (a treatment with sterile inoculum) in triplicate.

Analytical methods

The concentrations of TP, PO_4^{3-}-P and NO_3^--N were measured by acid persulfate digestion followed by vanado molybdo phosphoric acid method, ammonium molybdate spectrophotometry and thymol spectrophotometry respectively (Yu *et al.* 2009). Removal rates (*RR*) was calculated using the following equations:

$$RR(\%) = \frac{C_0 - C_t}{C_0} \times 100$$

where, C_0 and C_t (mg/L) are the initial concentration and concentration of PO_4^{3-}-P and NO_3^--N at time t and t is the treatment time of the microbial strain.

Measuring methods of PH_3: 5 mL of the absorption solution was added to a 50 mL colorimetric tube, and high-purity nitrogen injected for 5 min (removing hydrogen sulfide gas to prevent its influence on the determination of PH_3 gas). Then, 8 mol/L NaOH solution was added to the tube slowly, using dinitrophenol as an indicator, and the pH was adjusted to 3 or so. The concentration of TP in the absorption liquid and the amount of PH_3 gas collected was measured and calculated. Then the mass of PH_3 entering the absorption liquid at this time could be calculated as follows:

$$m = CV \times 10^{-3} \times \frac{M}{M_P}$$

where, m is the mass (mg) of PH_3 absorbed in the absorption liquid, V is the volume of the absorption liquid (mL), C is the concentration of total phosphorus in the absorption liquid measured by molybdenum anti-spectrophotometry (mg/L), M is the molecular weight of PH_3, and Mp is the molecular weight of phosphorus.

RESULTS AND DISCUSSION

Isolation, purification, and screening of strain J16

In this study, 45 different colony morphologies of pure strains (the same strains were not excluded) were obtained after five repetitions of culturing through streak isolation on the YG purification medium. In the blue–white spot screening test, 23 strains exhibited blue spots on both the phosphorus-rich and phosphorus-limited media. The 23 screened strains were inoculated to the synthetic wastewater medium after aerobic culturing for 24 h and then centrifuged. Of the 23 strains, two strains (J13 and J16) had a phosphorous removal rate of more than 50%. In the nitrate reduction test, strain J16 produced gas and was thus selected as the target strain.

The target J16 strain was inoculated in the nitrogen- and phosphorus-rich medium. After aerobic culturing for 24 h, the rates of denitrification and phosphorus removal were more than 90%. According to traditional denitrification theory (Ahn 2006; Wang *et al.* 2008), O_2 can inhibit the denitrification process because the dissolved oxygen competes with NO_3^--N for electron receptors, hindering denitrification. However, according to aerobic denitrification theory (Yao *et al.* 2013; Choi *et al.* 2017; Rout *et al.* 2017), due to the presence of periplasmic nitrate reductase in bacteria, the strain can use nitrate and oxygen together as electron receptors for respiration. In summary, strain J16 is a highly efficient aerobic DPB that can use NO_3^--N as an electron receptor.

Physiological and biochemical identification of strain J16

The isolated J16 strain was cultured in the YG solid medium for 2 days, and its physiological and biochemical

characteristics are listed in Table 1. Strain J16 was cultured for 24 h, and its morphology was observed using a scanning electron microscope (JSM-7610F, Shimadzu, Japan) (Figure 1). Strain J16 is a short bacillus, with a size of approximately (0.5–0.8) × (1.2–2.6) μm. According to the above description, the strain J16 could be identified as *E. coli* preliminarily.

Phylogeny and 16S rDNA sequence analyses of strain J16

The genomic DNA of strain J16 was used as the template for PCR amplification and 16S rDNA sequencing. The sequence of the tested J16 strain was compared with the nucleotide sequences of 16S rDNA in GenBank by using the BLAST program. Strain J16 exhibited 99% sequence similarity with *Escherichia coli*, *Shigella*, and *Cronobacter sakazakii*. The physiological and biochemical identification results of strain J16 and the homologous sequence of its 16S rDNA confirmed that strain J16 was *E. coli*. The phylogenetic tree showed that strain J16 is most similar to the bacterium endosymbiont of *Onthophagus taurus* (Figure 2).

Effects of different factors on the denitrifying phosphorus removal

Effects of temperature on the denitrifying phosphorus removal

As shown in Figure 3(a), the percentage of PO_4^{3-}-P and NO_3^--N removal tended to increase and then decrease with increasing temperatures, and the highest phosphorus removal percentage was obtained between 30 °C and 35 °C. High or low temperatures could affect the growth and enzymatic activity of strain J16. When the temperature was higher than 30 °C, the rates of denitrification and phosphorus removal of strain J16 declined slightly. At temperatures between 30 °C and 35 °C, the rates of PO_4^{3-}-P and NO_3^--N removal reached more than 95% and more than 93%, respectively. At temperatures lower than 30 °C, PO_4^{3-}-P and NO_3^--N removal rates increased rapidly with an increase in the temperature. At 25 °C, the PO_4^{3-}-P and NO_3^--N removal rates were only 83.52% and 15.67%, respectively. In summary, low temperatures had a stronger effect on the denitrification activity of strain J16 than high temperatures. The reason for this finding may be that low temperatures have a higher effect on the growth and enzymatic activity of strain J16 than high temperatures. Zhang & Wang (2006) and Ma *et al.* (2007) have indicated that the most suitable temperature for denitrification and phosphorus removal by bacteria is between 25 °C and 35 °C; this result is slightly different from the finding of the present study. The most suitable temperature was between 30 °C and 40 °C for strain J16 in this study, and strain J16 may be resistant to temperatures higher than 40 °C. The NO_3^--N and PO_4^{3-}-P removal rates could reach 91% and 93%, respectively. This result indicated that the denitrification and phosphorus removal systems are tolerant to high temperatures, which may be related to the bacterial characteristics of strain J16. This result is also inconsistent with the conclusion of Yang *et al.* (2009), who reported a wider suitable temperature range and indicated that the denitrification and phosphorus removal of DPB are basically unaffected by temperature. Approximately similar optimum temperature for *Bacillus methylotrophicus* strain L7 has already been reported as 37 °C by other researchers (Zhang *et al.* 2012). Therefore, a medium temperature should

Table 1 | Physiological and biochemical identification of strain J16

Items	Strain J16
Bacteria shapes	rhabditiform
Colony morphology	round, 1–1.2 μm, milky white, smooth surface, neatness of the edge, micro uplift, luster
Gram staining	–
Indole test	+
Methyl red test	+
V-P experiment	–
Citrate test	–

+ positive; – negative.

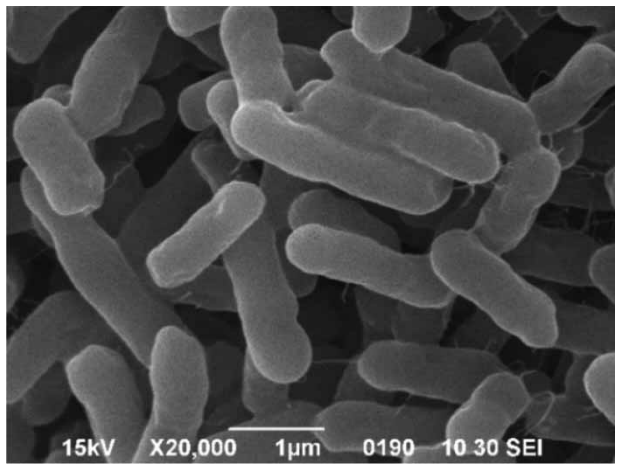

Figure 1 | The scanning electronic microscope (SEM) micrograph of strain J16.

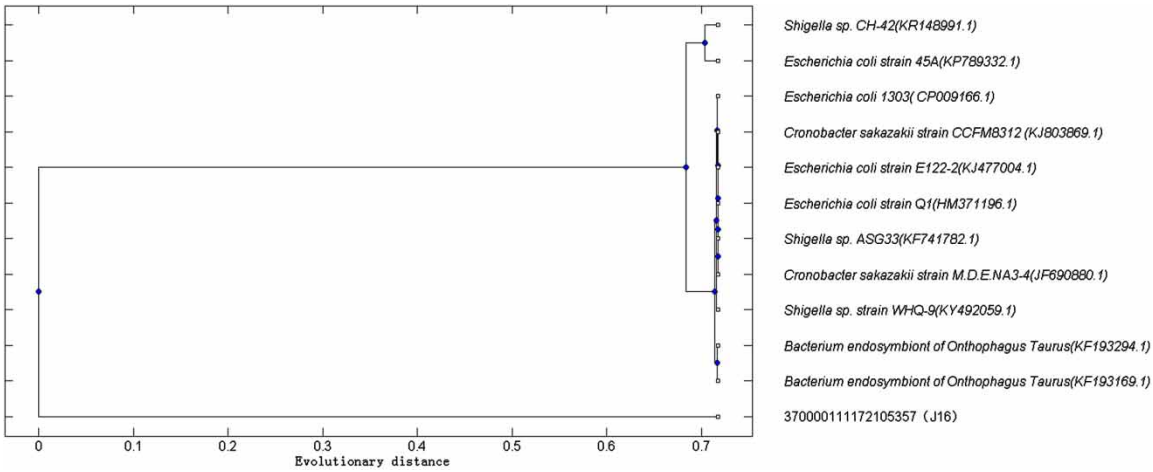

Figure 2 | Neighbor-joining phylogenetic tree based on the comparison of partial 16S rDNA gene sequences showing the phylogenetic position of strain J16, and representatives of some other reference sequences.

be employed for the growth of strains to achieve high-efficiency denitrification and phosphorus removal in practical engineering.

Effects of pH on the denitrifying phosphorus removal

The denitrification and phosphorus removal rates of strain J16 were higher than 90% when the pH of the medium was between 7.2 and 8 (Figure 3(b)). In this pH interval, when the initial total phosphorus concentration was 8.9 mg/L, the phosphorus removal rate was higher than 95%; in particular, when the pH was 7.2, the removal rate of PO_4^{3-}-P reached the maximum of 96.03%, almost eliminated completely. When the initial total nitrogen concentration was 69.31 mg/L, the denitrifying rate was higher than 90%, and when the pH was 7.2, the denitrifying rate reached 94.56%. When the pH value was less than 7.2 or more than 8, the denitrifying and phosphorus removal rates of strain J16 decreased sharply. The effect of acidic conditions on the denitrification and phosphorus removal rates of strain J16 was stronger than that of the alkaline conditions. This may be because the acidic condition exerts a higher inhibitory effect on the growth of J16 bacteria. This result differs from that of *B. cerulobacillus* LY-1, which was screened by An *et al.* (2012). For pH of 5–9, the nitrate nitrogen and PO_4^{3-}-P removal rates of strain LY-1 remained close to 85%. However, when the pH value was 6.5 or 9, the denitrifying rate of strain J16 decreased to less than 50%, but the removal rate of phosphorus was slightly lower, indicating that the effect of acid or peralkaline environments on the denitrification rate of strain J16 was stronger than that on the phosphorus removal rate. A thermophilic strain, *Bacillus* MS 30, was testified by other researchers to demonstrate remarkable nutrient removal at pH 7.5–8 (Mével & Prieur 2000). In summary, the optimum pH conditions of strain J16 for denitrifying and phosphorus removal are basically identical. A strong acidic or peralkaline environment can inhibit the enzymatic activity of J16, which hampers the efficiency of its simultaneous denitrification and phosphorus removal. Therefore, in practical engineering applications, a neutral or weak alkaline environment should be maintained for the growth of the bacteria.

Effects of percentage of inoculum on the denitrifying phosphorus removal

The removal rates of PO_4^{3-}-P and NO_3^--N increased firstly and then decreased with an increasing percentage of inoculum (Figure 3(c)). Under the optimum pH, temperature and PO_4^{3-}-P concentration conditions, when the percentage of inoculum was 3%, the denitrification and phosphorus removal rates of strain J16 were highest (the denitrification rate was 92.75% and the phosphorus removal rate 96.81%). When the percentage of inoculum of strain J16 was less than 3%, the denitrification and phosphorus removal rates increased with the percentage of inoculum, and when the percentage of inoculum was higher than 3%, the denitrification and phosphorus removal rates were slightly lower and tended to be stable. This may be because of the deficiency of nutrients in the culture medium when the percentage of inoculum is high, which leads to a slight decrease in the denitrification and phosphorus removal rates. Yu *et al.* (2009) reported that the optimum percentage

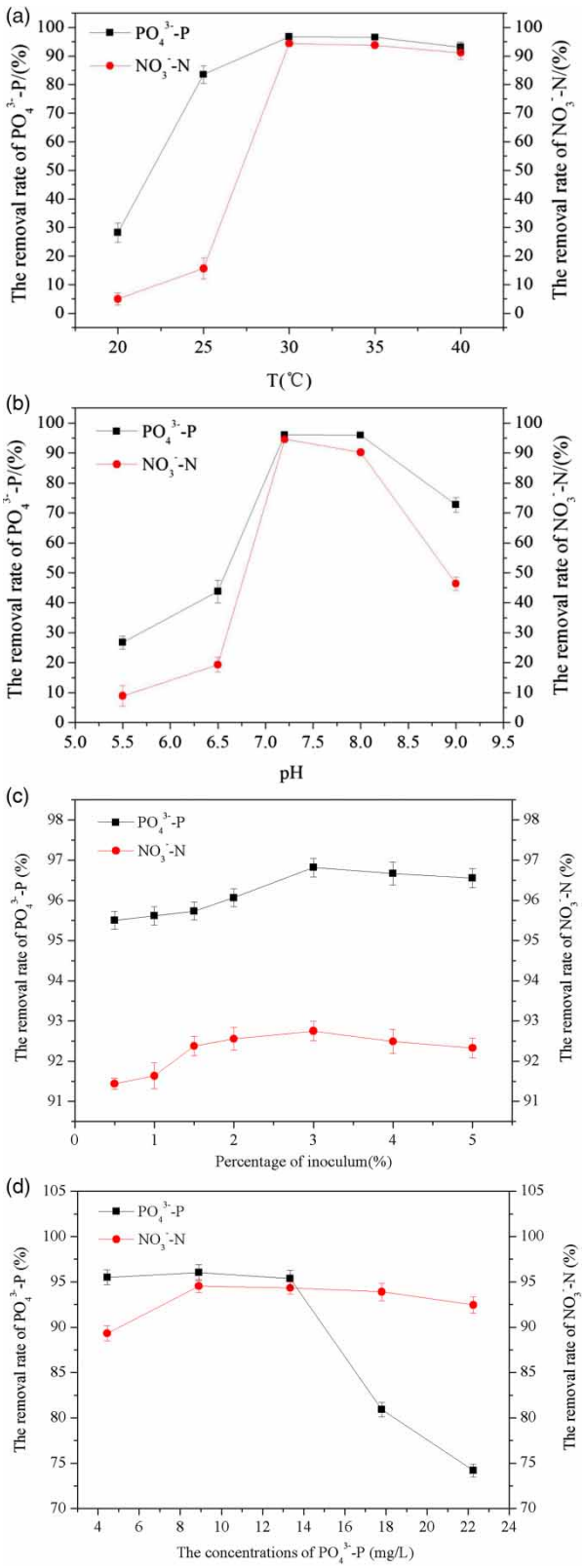

Figure 3 | Effect of temperature (a), pH (b), percentage of inoculum (c) and PO_4^{3-}-P concentrations (d) on denitrifying phosphorus removal. Values are means ± SD (error bars) for three replicates.

of inoculum of aerobic denitrifying bacteria for treating wastewater is 10%. This value is slightly higher than the percentage of inoculum of strain J16. In that study, the highest denitrifying and phosphorus removal rates were only 80% and approximately 30%, respectively, which are significantly lower than the rates obtained in this study. When the percentage of inoculum was less than 3%, the denitrification and phosphorus removal rates were lower, and when the percentage of inoculum was higher than 3%, the removal rates tended to be stable. The optimal percentage of inoculum was 3% in 100 mL of medium for strain J16.

Effects of PO_4^{3-}-P concentrations on the denitrifying phosphorus removal

The effect of initial PO_4^{3-}-P concentrations on the denitrifying phosphorus removal by strain J16 was evaluated. As shown in Figure 3(d), the removal rate of NO_3^--N by strain J16 increased firstly and then tended to be stable with the increasing PO_4^{3-}-P concentration, and when the initial PO_4^{3-}-P concentration was 8.9 mg/L, the removal rate of NO_3^--N was 94.55% (the highest). The results showed that when the NO_3^--N concentration was 69.31 mg/L in the nitrogen- and phosphorus-rich culture medium, different PO_4^{3-}-P concentrations had little influence on denitrification. When the PO_4^{3-}-P concentration was less than 13.35 mg/L, the removal rate of PO_4^{3-}-P was up to 96.03% (>95%), but the phosphorus removal rate of strain J16 declined rapidly when the PO_4^{3-}-P concentration was higher than 13.35 mg/L. The reason for this finding may be that the phosphorus content is higher than the optimum phosphorus uptake of strain J16, which leads to the rapid decrease of the phosphorus removal rate. In summary, in the nitrogen- and phosphorus-rich culture medium, when the content of NO_3^--N is constant, different PO_4^{3-}-P concentrations have no influence on denitrification, but have a considerable effect on phosphorus removal. This result is consistent with the reports of Yu *et al.* (2009).

The efficiency of removing TP and producing PH_3 under hypoxic condition

Figure 4 shows the efficiency of reduction and removal of TP and production of PH_3 under the condition of hypoxia by strain J16. The results showed that the strain J16 could also remove phosphorus under the condition of hypoxia. With the progress of the reaction time, the percentage of phosphorus removal and phosphine production gradually increased, and there was an adaptation period in the early

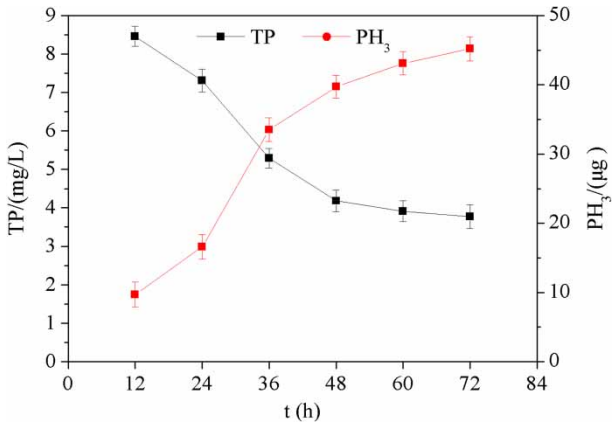

Figure 4 | Changes in PH_3 and TP over time in hypoxic conditions.

stage of the reaction, after which TP removal and PH_3 yield showed a rapidly increasing trend, and after 60 h, the trend was stable. After 72 h of reaction, the residual TP of 3.77 mg/L, the removal rate of TP was 62.3%, and the yield of PH_3 was 45.2 μg. This result is consistent with the reports of Luo *et al.* (2014) and Xu *et al.* (2018). The distinction is that there is a difference in the low PH_3 yield compared to the J16 single and mixed bacteria systems.

The traditional phosphorus removal process of wastewater is to remove phosphorus by phosphorus accumulating bacteria under aerobic conditions and then remove excess sludge. However, strain J16 can remove phosphorus from sewage either under aerobic conditions or under anoxic conditions by converting phosphate into PH_3. If the strain J16 can be applied in the actual sewage treatment process, it can not only strengthen the efficiency of phosphorus removal, but also collect the PH_3 for recycling.

CONCLUSION

In the study, *Escherichia coli* J16, a denitrification and phosphorus removal bacteria, was originally isolated from aerobic sludge in a sewage treatment plant. The data presented in this study demonstrated that *Escherichia coli* J16 could not only remove nitrogen and phosphorus by denitrification and phosphorus absorption respectively under aerobic conditions, but also remove phosphorus by forming PH_3 under anaerobic conditions. Therefore, these results strongly suggest that J16 has the potential to remove nitrogen and phosphorus from wastewater efficiently and will be useful in biological treatment processes of wastewater containing high concentrations of nitrogen and phosphorus.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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