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Enhancement of denitrifying phosphorus removal and DPAOs identification in the anaerobic/anoxic sequencing batch reactor system

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ABSTRACT

This study proposed an anaerobic/anoxic sequencing batch reactor system to enrich denitrifying polyphosphorus accumulating organisms (DPAOs) for sewage treatment and explored the microbial community structure and phosphorus removal mechanism. The experimental results illustrated that DPAOs contributed to metabolic mechanism, nitrogen and phosphorus removal, bacterial diversity and communities. The treatment effect was the best under suitable conditions of C/P20, sodium acetate as carbon source, pH7.5, and the removal rates of chemical oxygen demand, TP and NO_3^-–N was 88.21%, 92.23% and 92.46% severally. Moreover, the successful enrichment of DPAOs was verified with biodiversity and microbial community structure. Highly efficient DPAO NG2 was isolated, which was preliminarily identified as *Gordonia terrae* with a sequence similarity of 100% through physiological and biochemical identification and homology comparison of 16S rDNA gene. Eventually, the metabolic activities of DPAOs was studied to clarify the relationship between energy conversion and organic matter degradation.

Keywords: Denitrifying polyphosphorus accumulating organisms; *Gordonia terrae*; Metabolic mechanism; Microbial community

1. Introduction

Due to population pressure and the increase of corresponding eutrophication activities, water quality deterioration has became one of the world's major environmental problems [1]. In the process of chemical and agricultural production, a large number of nitrogen and phosphorus would be entered into the environment and lead to eutrophication of water [2]. Therefore, developing and improving sustainable and economical technologies for nitrogen and phosphorus emission reduction is an important priority for researchers in this field [3–6]. Microorganisms have been used for nitrogen and phosphorus removal in wastewater treatment plants for more than 30 y [7–9]. Denitrifying phosphorus removal technology has the advantages of low carbon source consumption, wide application range and less sludge production. It is a more convenient and environment-friendly phosphorus removal technology [10–11]. Denitrifying phosphorus accumulating organism (DPAO) could use nitrite (NO_2^-) or nitrate (NO_3^-) instead of oxygen (O_2) as electron acceptors to accumulate excess phosphorus under anoxic conditions [12]. Moreover, DPAO has benefits in that it provides a solution to the contradiction between carbon source utilization and sludge age, so that nitrogen and phosphorus can be removed at the same time [13].

With the deeply researched on denitrifying phosphorus removal process, scholars have carried out the relevant research on the species composition of DPAO. Fuhs and Chen [14] were the first to isolate the bacteria from the activated sludge, after identification, they confirmed that

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the strains were *Acinetobacter* of γ-Proteobacteria and had high phosphorus removal capacity. Moreover, Lötter [15] found most of the bacteria that could absorb phosphorus belonged to *Acinetobacter*, *Aeromonas*, and *Pseudomonas*. As the technical conditions become more and more mature, more other microbial communities have been found in biological phosphorus removal sludge by using 16S rRNA cloning library, fluorescence in situ hybridization (FISH) and high-throughput sequencing (HTS) [16]. The results of fish probe showed that Acinetobacter was not superior to other flora, such as Actinobacteria and Actinobacteria. HTS technology could be used to explore the relationship between bacterial community and metabolism [17], the most abundant bacteria could be considered to hold sway in the culture system [18].

This study precisely evaluated the efficiency of pollutant treatment, the microbial diversity, and microbial community structure of anaerobic/anoxic sequencing batch reactor (A^2SBR) activated sludge system. Moreover, a DPAO, NG2 strain was screened and identified by 16S rDNA, the characteristics and metabolic mechanism of NG2 was analyzed to characterize the microorganisms responsible for phosphate removal. The above research results provided theoretical basis and technical support for the application of denitrifying phosphorus accumulating bacteria in practical engineering practice of wastewater biological treatment.

2. Material and methods

2.1. Process setup and operation

Fig. 1 shows a schematic representation of the sequencing batch reactor (SBR) used in this study. The SBR was made from double-layer organic glass with a tapered bottom, and the outer layer was connected with the water bath pot for water bath circulation heating, so as to ensure that the system was in the optimal constant temperature state. The working volumes of the SBR was 12 L with the inner diameter of 140 mm and a height of 850 mm. The system was connected with PHS-10 portable acidity meter to monitor the pH value online. When the pH value was not within the set range, the peristaltic pump would automatically add acid-base buffer solution to the reactor until the pH reached the set range (7.50–7.80).

The SBR, operating for nitrogen and phosphorus removal. The sludge of Sanbaotun Sewage Treatment Plant in Fushun City, Liaoning Province was inoculated, which adopted A^2/O process. The concentration of MLSS between 2,800 and 3,300 mg/L and the sludge retention time of the SBR was 15 d. The reactor ran three cycles a day for 5–5.5 h, a typical cycle as described in Table 1. The concentrations of chemical oxygen demand (COD), TP and NO_3^-–N in the influent and effluent were monitored every day.

2.2. SBR static test device

The effective volume of the static test device for denitrifying phosphorus removal was 3L. N_2 was injected into the reactor to maintain an anaerobic environment, and nitrate nitrogen with a mass concentration of 35 mg/L was added through a peristaltic pump as an electron acceptor to control the anoxic reaction conditions. The operation mode was the same as in the reactor to investigate the influence of different influencing factors on denitrifying phosphorus and nitrogen removal.

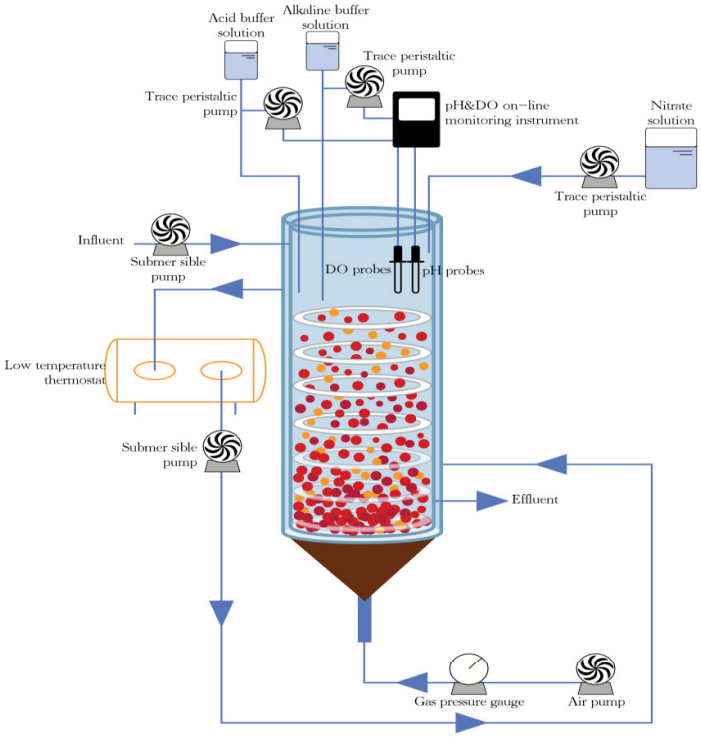

Fig. 1. SBR experimental setup.

2.3. Domestic wastewater

Artificial sewage was used in the experiment. The average concentrations of CH_3COONa, NH_4Cl and KH_2PO_4 were 212 ± 48, 7 ± 4, 10 ± 3 mg/L, respectively. In addition, $CaCl_2$, $MgSO_4·7H_2O$ and trace elements (1.00 mg/L) were added. The pH was controlled between 7.5 and 7.8 by $NaHCO_3$.

2.4. Media

Denitrification medium (/L): 5 g of sodium citrate, 1 g of potassium nitrate, 1 g of potassium dihydrogen phosphate, 1 g of potassium hydrogen phosphate, 15 g of agar, pH 7.2–7.4.

Polyphosphate medium (/L): 3.68 g of sodium acetate trihydrate, 28.73 mg of mgdisodium hydrogen phosphate, 57.27 mg of ammonium chloride, 131.82 mg of magnesium sulfate heptahydrate, 26.71 mg of potassium sulfate, 2 mL of trace elements, 17.20 mg of calcium chloride dihydrate, 15 g of agar, pH 7.0.

Phosphorus limited medium (/L): 82.14 mg of $MgSO_4·7H_2O$, 3.32 g of $CH_3COONa·3H_2O$, 152.76 mg of NH_4Cl, 10.98 mg of $CaCl_2·2H_2O$, 22.98 mg of $Na_2HPO_4·2H_2O$, 16.92 mg of K_2SO_4, 2.76 mL of trace elements, pH 7.0.

Nitrogen and phosphorus rich medium (/L): 3.32 g of $CH_3COONa·3H_2O$, 305.52 mg of NH_4Cl, 35.11 mg of $KH_2PO_4·2H_2O$, 91.26 mg of $MgSO_4·7H_2O$, 25.68 mg of $CaCl_2·2H_2O$, 300 mg of KNO_3, 2 mL of trace elements.

Nitrate reduction medium (/L): 1 g of KNO_3, 2.42 g of $KH_2PO_4·2H_2O$, 1 g of glucose, 1 g of agar, 20 g of peptone.

2.5. Analytical methods

COD, PO_4^{3-}–P, NO_3^-–N were measured according the 723C visible spectrophotometer, using rapid closed catalytic digestion, molybdenum antimony spectrophotometry and ultraviolet spectrophotometry respectively. MLSS was detected using Filter paper weighing method, pH value was measured by PHS-25 pH meter, HQ40d portable digital display oxygen meter and constant temperature water bath were used to detect dissolved oxygen and temperature. The method of Oehmen et al. [19] was used for the analysis of PHB, and the glycogen was measured by HPLC according to the requirements of Bond et al. [20].

2.6. Phosphorus release/absorption efficiency

The strains to be tested were put into denitrifying liquid medium or polyphosphate liquid medium respectively and cultured at 30°C, and the shaking speed was 140 rpm. Centrifugation (4,000 rpm, 10 min) of the enriched bacterial liquid, discarding the supernatant, washing with sterile distilled water, centrifuging, and repeating the cleaning operation twice. The strain was inoculated into a phosphate limited medium and shaken for 24 h in a shaker at 30°C to ensure sufficient phosphorus release. The supernatant was then washed with distilled water and centrifuged for 10 min. After that, it was put into the nitrogen and phosphorus rich culture medium for 24 h. The concentrations of NO_3^-–N and PO_4^{3-}–P were detected for every 30 min.

2.7. Determination of growth curve

The selected strains were inoculated in denitrifying/phosphorus accumulating medium under aseptic conditions, and cultured in constant temperature oscillation incubator at 30°C and 140 rpm. The blank culture medium was taken as blank sample, and the absorbance value of the bacterial liquid to be tested was determined by spectrophotometer at the wavelength of 600 nm. At this time, the absorbance value of bacterial liquid represented bacterial turbidity (OD_{600}), that is, bacterial concentration. The physiological and biochemical properties were determined by chemical methods.

2.8. Sequence analysis of 16S rDNA coding gene

The gene DNA of the strain was purified and extracted with TaKaRa kit. Then, total DNA was used as template and 27F and 1492R as primers for PCR amplification. The PCR system was 50 μL. The reaction conditions were as follows: pre-denaturation at 94°C for 4 min; then denaturation at 94°C for 30 s; annealing at 60°C for 30 s; extending for 30 s at 72°C for 30 cycles; extending at 74°C for 5 min; and terminating preservation at 5°C. The PCR products were analyzed by 16S rDNA and 1% agarose gel electrophoresis. The results were compared in GenBank database by blast software, and phylogenetic tree was constructed by using 16S rDNA sequence of related species.

2.9. Flora diversity and community structure

Three sludge samples (sun1: sludge samples from water plant; sun3: sludge samples from the 12th day of sludge acclimation; sun5: sludge samples from the 42th day of sludge acclimation) came from the water outlet respectively. PCR amplification was performed by Geneamp 9700 thermal circulator and analyzed by Illumina miseq

Table 1
Operating procedure of SBR process

Stage	Procedure	Running steps
First	(i) Anaerobic phase for 120 min (ii) Aerobic phase for 120 min (iii) Settling for 30 min	Anaerobic/aerobic alternative operation mode was used to make the traditional phosphorus accumulating organism became the dominant bacteria in the system.
Second	(i) Anaerobic phase for 120 min (ii) Anoxic phase for 150 min (iii) Settling for 30 min	Anaerobic/anoxic mode was adopted to continue domestication, and nitrate was continuously added as electron acceptor to make the system enter into anoxic mode. At this stage, DPAO became the dominant bacteria.

platform and mothur 1.30.2. Bacterial diversity (Shannon and Simpson) and richness (Chao1) indices were calculated using the mothur package 1.10.1.

3. Results and discussions

3.1. Enrichment of denitrifying phosphorus accumulating organism

As illustrated in Fig. 2, the concentration of COD and PO_4^{3-}–P descended gradually in first 11 d, while they remained stable to reach the levels of 47.19 ± 3.19 mg/L and 0.35 ± 0.21 mg/L, respectively in the next 3 d. COD was mainly used in the anaerobic stage, this process consumed the proton dynamic potential (PMF) in the phosphate accumulating bacteria, it was necessary to decompose poly-P in the form of orthophosphate to release extracellular, so as to reconstruct PMF and produce ATP, that is, organic substance consumption and phosphorus releasing in active sludge were interrelated. In the aerobic phase, PAOs consumed PHB to generate ATP, part of which was used for supplying enough nutrients to assure its own synthesis and life sustaining activities, and the other part was used for excessive phosphorus absorption, which was manifested as aerobic phosphorus absorption [21]. Compared with previous studies, the removal efficiency of PO_4^{3-}–P in A/O stage has been significantly improved [22], which might be due to the use of acetic acid instead of other carbon sources in this study. Acetic acid was took to be the first-rank carbon source for PAOs and DPAOs, and showing the best performance in the process of phosphorus removal and denitrification. Previous studies have shown that the high removal rate of PO_4^{3-}–P was closely related to the dominant flora PAOs in the sludge system [23]. In this study, TP presented a 96.44% ± 4.78% removal rate, which was consistent with the research results. At this moment, the system maintained the main environment of PAOs and was ready for the enrichment of DPAOs in the next stage.

After enrichment of PAOs in the first stage, changed the cultivation conditions, stopped aerobic aeration, and added KNO_3 as electronic acceptor of DPAO after the anaerobic stage, which lasted 15 d. In anaerobic phase, the external carbon source was absorbed by DPAO and stored in cells in the form of PHB. The macroscopic appearance of this phase was phosphorus releasing. During anoxic phase, DPAO used PHB as carbon source and NO_3^-–N as electron acceptor to absorb excessive phosphorus. At the beginning of the reaction, the traditional phosphorus accumulating bacteria, as the dominant bacteria in the reactor, could not use NO_3^-–N as electron acceptor for phosphorus absorption, and the removal rates of TP and NO_3^-–N were low. With the operation of the reactor, DPAOs has became the dominant bacteria. After 15 d of enrichment culture, the effluent concentrations of COD, NO_3^-–N and TP were 20.16 ± 3.45 mg/L, 2.57 ± 1.35 mg/L and 0.75 ± 0.29 mg/L respectively, which met Class A standard of GB 1899-2003, and the removal rate of TP and NO_3^-–N increased to 92.23% ± 4.45% and 92.46% ± 4.63% severally. At this time, the enrichment of DPAO was completed. It was worth noting that the concentration of NO_3^-–N in the effluent was very low, which indirectly ensured that phosphorus will not be released again in the anoxic stage [24,25]. It was prominent from Fig. 2 that the removal rate of COD in anoxic stage only decreased slightly, which proved that the growth of DPAO consumed less COD

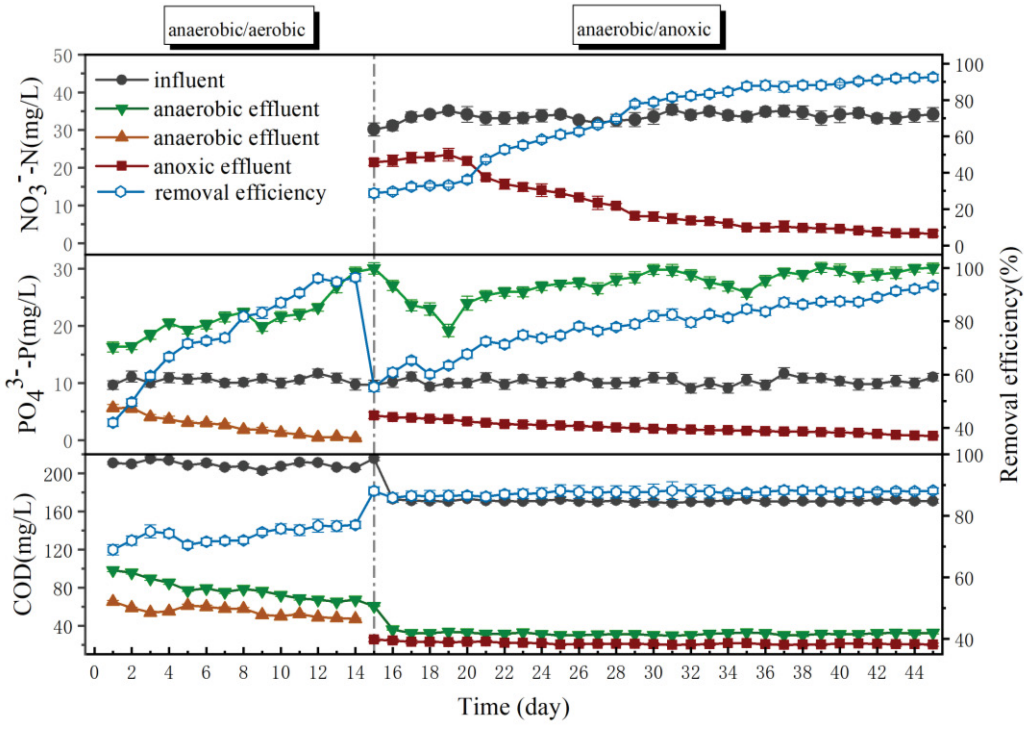

Fig. 2. Concentration and removal efficiency of COD, PO_4^{3-}–P and NO_3^-–N in SBR.

[26], PHAs formed in anaerobic stage were utilized to improve denitrification and phosphorus removal [27].

3.2. Effects of different C/P on the denitrifying phosphorus removal

The dynamic variation of COD, TP and NO_3^-–N in the effluent are shown in Figs. 3–5. The organic substance consumption was similar in different C/P ratios under the C/P ratio of 10, 20, 30 and 40. The rapid utilization of external carbon source by DPAOs decreased COD concentration at a great lick in the first 30 min of anaerobic reaction. As the reaction continued, the utilization rate of external carbon source descended slowly, when the influent C/P ratio were 10 and 20, the COD concentration were 11.72 ± 3.38 mg/L and 31.46 ± 4.32 mg/L respectively in the end of anaerobic process. At this time, there was less residual carbon source in the system, which would not cause denitrifying bacteria or other heterotrophic bacteria to interfere with the anoxic phosphorus uptake of DPAO. At the end of anoxic, the carbon sources were almost exhausted, and the removal rate reached 100% and 88.06% ± 4.45%, respectively. When the influent C/P was 30 and 40, the COD concentration of the system were 87.46 ± 4.67 mg/L and 141.42 ± 5.12 mg/L respectively in the end of the anaerobic stage. Consequently, there were still many external carbon sources remaining in the system to enter the anoxic reaction, and the removal rates were low, only 81.88% ± 4.65% and 75.44% ± 3.96%, respectively.

Different C/P ratio also had great influence on the change of TP concentration in the system. When the influent C/P were 10, 20 and 30, the TP concentration of anaerobic effluent were 21.54 ± 3.45 mg/L, 35.71 ± 4.17 mg/L and 38.82 ± 3.16 mg/L respectively. However, when the influent C/P reached 40, the TP concentration of anaerobic effluent was 36.16 ± 4.45 mg/L, and the phosphorus release was similar to that of influent C/P 20. In anoxic stage, TP effluent concentrations were 4.41 ± 2.43 mg/L, 0.74 ± 0.36 mg/L, 2.54 ± 1.56 mg/L and 2.96 ± 1.08 mg/L respectively, and TP removal rates reached 61.18%, 93.19%, 75.24% and 73.31%, respectively.

The removal efficiency of NO_3^-–N showed the same trend, as the influent C/P were 20, 30 and 40. The effluent NO_3^-–N concentrations were 2.86 ± 2.01 mg/L, 1.29 ± 0.98 mg/L and 0.21 ± 0.11 mg/L, and the removal rates reached 91.83% ± 4.12%, 96.31% ± 4.17% and 99.40% ± 3.45%, respectively. Only in the late stage of C/P ratio of 10, the removal rate of NO_3^-–N gradually slowed down, and a large amount of NO_3^-–N remained at the end of anoxic stage. In conclusion, to achieve higher phosphorus and nitrogen removal efficiency, C/P could not only be able to synthesize sufficient PHB in the anaerobic stage and store it in the cells, but also control the concentration of the remaining external carbon source in the anoxic stage. Therefore, the optimum C/P of the system was 20.

In investigating the influencing factors of C/P ratio, during the rise of C/P ratio, the phosphorus release capacity showed a trend of first increasing and then decreasing, the reasons were as followed that the PHB synthesized by DPAO would increase, that is, the more sufficient phosphorus release reaction was. Due to the limited intracellular polymerized phosphate in DPAO, when the C/P ratio rose

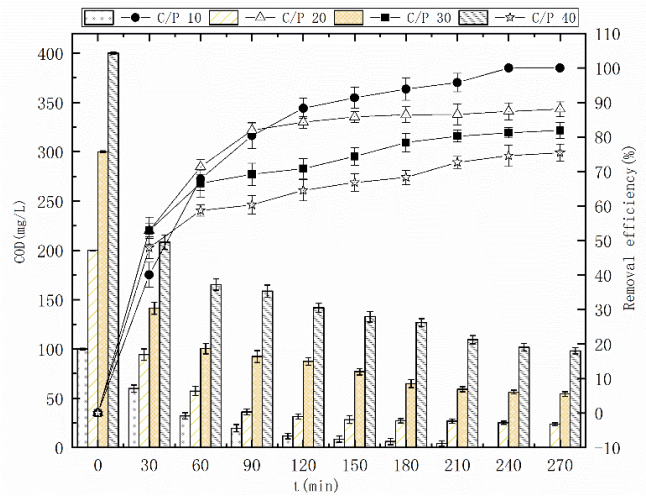

Fig. 3. Effect of different C/P ratio on COD removal efficiency.

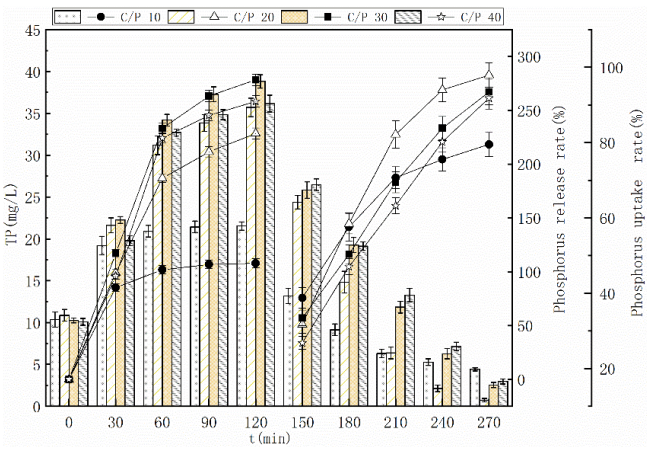

Fig. 4. Effect of different C/P ratio on TP removal efficiency.

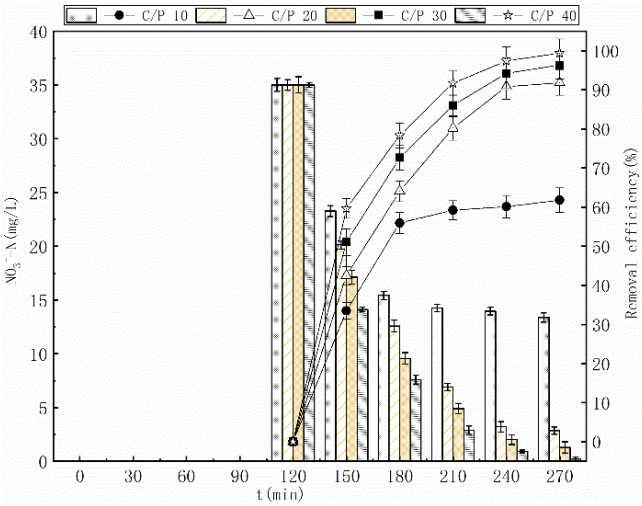

Fig. 5. Effect of different C/P ratio on NO_3^-–N removal efficiency.

again, the absorption of external carbon sources through the energy generated by phosphorus release was restricted [28]. While higher C/P ratio made a large amount of residual carbon source were stranded outside the anoxic phase, which made the conventional denitrifying bacteria compete with DPAO for the electron acceptor, and nitrite utilization by DPAO was lower than conventional denitrifying bacteria, at a competitive disadvantage, the denitrifying phosphorus uptake was also suppressed. When the influent C/P ratio was 10, remaining a large amount of NO_3^-–N at the end of the anoxic stage, the low COD influent concentration led insufficient synthesis of PHB by DPAO, and then the energy generated by oxidation PHB was not enough for the full denitrifying phosphorus removal reaction [29]. Moreover, due to the low residual COD concentration, the conventional denitrifying bacteria in the system could not use NO_3^-–N for denitrification. When the influent C/P ratio was 40, the highest removal rate of nitrate nitrogen was close to 100%, which was because the remaining external carbon source after the anaerobic stage was used by conventional denitrifying bacteria for denitrification [30].

3.3. Effects of different carbon sources on the denitrifying phosphorus removal

It can be seen from Figs. 6 and 7 that sodium acetate, sodium propionate and glucose were used as carbon sources respectively, and the initial COD concentration was controlled to be 200 mg/L. Under anaerobic conditions, the three different carbon sources showed obvious degradation of carbon source matrix and release of phosphorus in different degrees. The COD removal rates with sodium acetate, sodium propionate and glucose as carbon sources were 82.22% ± 5.45%, 81.82% ± 4.13% and 61.17% ± 5.09% respectively, and the TP release rates were 248.44% ± 6.05%, 213.91% ± 4.47%, 111.28% ± 5.12% severally. The degradation effect of sodium acetate was the first, followed by sodium propionate, and glucose was the last. In the anoxic stage, DPAO with sodium acetate and sodium propionate as carbon sources showed the high metabolic activity, sodium acetate was slightly better than sodium propionate, and the glucose as external carbon source was the worst. The removal rates of TP reached 84.05% ± 5.98%, 64.20% ± 5.21% and 33.46% ± 4.78% respectively. Therefore, sodium acetate was the most suitable carbon source in this experiment.

The effect of different carbon sources on denitrifying phosphorus removal could be analyzed by carbon source utilization rate ($P_{release}/C_{uptake}$). $P_{release}/C_{uptake}$ could represent the activity of phosphorus accumulating bacteria (PAOs) in the system. The previous study found that there was a good correlation between $P_{release}/C_{uptake}$ and the relative number of PAOs and GAOs in the biological phosphorus removal system [31]. The carbon source utilization efficiencies of sodium acetate, sodium propionate and glucose were 0.16 mg PO_4^{3-}–P/mg COD, 0.14 mg PO_4^{3-}–P/mg COD and 0.10 mg PO_4^{3-}–P/mg COD, respectively. Based on the analysis of the test results, the difference of carbon source utilization efficiency between sodium acetate and sodium propionate was not much, which indicated that the denitrifying phosphorus removal system had no selectivity for sodium acetate and sodium propionate. Compared with the former two, the utilization efficiency of carbon source of glucose was lower, this was because glucose, as a carbon source, could replace glycogen in cells to directly provide energy for microbial metabolism, selectively promoted the growth of GAOs and inhibited the growth of DPAOs [32].

3.4. Effects of different pH on the denitrifying phosphorus removal

To study the operation of denitrifying phosphorus and nitrogen removal system under different pH, the pH were adjusted to 6.5, 7.5, 8.0 and 8.5 by using 0.5 mol/L HCl and NaOH solution, then the phosphorus removal situation was analyzed. Fig. 8 showed the dynamic variation of TP in a cycle under different pH. In anaerobic stage, when the pH increased from 6.5 to 8.0, the phosphorus release increased gradually, from 23.74 ± 4.78 mg/L to 41.26 ± 4.12 mg/L. However, when the pH reached 8.5, the phosphorus release was only 19.58 ± 3.65 mg/L. In the anoxic stage, as the increase of pH, the amount and rate of phosphorus uptake ascended synchronously. When the pH

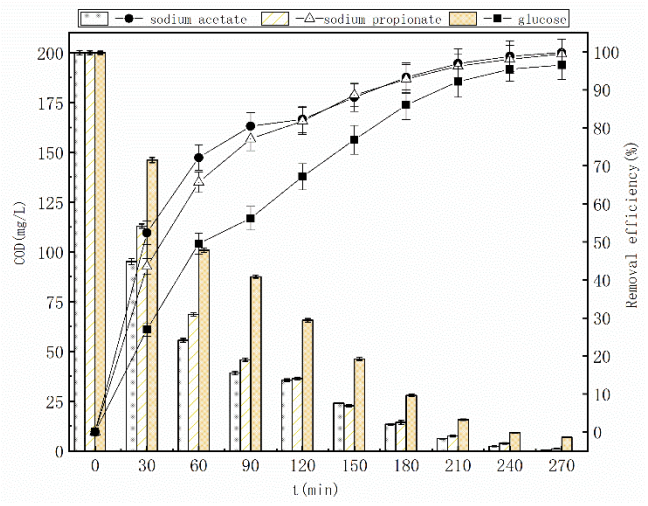

Fig. 6. Effect of different carbon sources on COD removal efficiency.

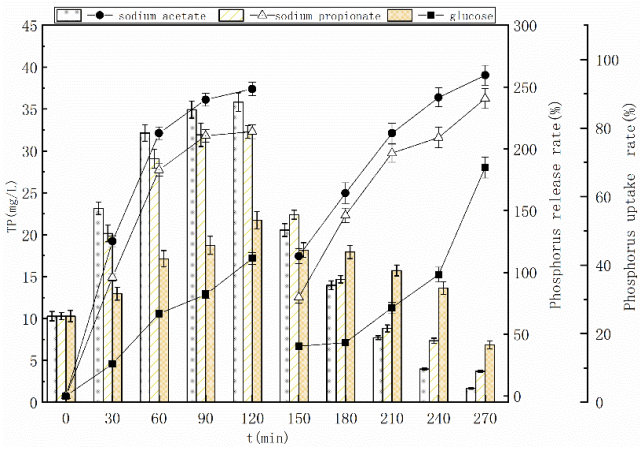

Fig. 7. Effect of different carbon sources on TP removal efficiency.

of the system were 7.5 and 8.0, the phosphorus uptake was 36.65 ± 5.43 mg/L and 40.44 ± 5.55 mg/L, respectively, and the TP removal rates were 95.3% ± 4.34% and 91.8% ± 5.45%, respectively. When the pH value was further increased to 8.5, the phosphorus uptake was only 22.76 ± 4.25 mg/L. It could be seen from the above that when the pH was 7.5, the effect of phosphorus removal was the best.

The permeability and surface charge of microbial cell membrane were affected by pH, so the growth and reproduction of microorganisms were closely related to the pH of the system [33]. In anaerobic stage, acetic acid entering denitrifying phosphorus accumulating bacteria consumed bacterial proton mobility (PMF). In order to reconstruct PMF, DPAOs decomposed poly-P to maintain the constant PMF. While the increase of pH leaded to the decrease of PMF, denitrifying phosphorus accumulating bacteria would decompose more intracellular poly-P to maintain the constant PMF, thus increasing the release of phosphorus. At pH 8.5, the phosphorus release did not increase but decreased, which was due to the phosphate precipitation caused by too high pH, resulting in the decrease of soluble phosphorus [34]. In the anoxic stage, too high or too low pH would affect the phosphorus absorption reaction. When pH was low, the release of phosphorus in DPAOs was inhibited, resulting in insufficient PHB synthesis. While the ineffective release of phosphorus caused by too high pH had a negative effect on the synthesis of PHB by DPAO, thus further affected the subsequent phosphorus absorption [32].

3.5. Bacterial diversity and communities of sludge samples

In order to further explore the microbial metabolic mechanism for removing nitrogen and phosphorus, the microbial flora community structures at three different stage were obtained by high throughput sequencing technology. As can be seen from Table 2, the effective sequence of each sample was more than 39,000 and the OTU counts obtained were 4,663; 3,140 and 3,636 respectively. Obviously, compared with other samples, sample sun1 had the highest richness and diversity in terms of maximum Shannon and Simpson estimators, which indirectly proved that subsequent stages of domestication made some progress.

The abundance was accurate to 0.01%, and the gene classification of the three samples was analyzed in Fig. 9a. The dominant phyla mainly contained Proteobacteria, Bacteroidetes, Chloroflexi. The total abundance of Proteobacteria in the sample reached 40%, which contributed to most of the PAOs discovered at present belong to Proteobacteria [35].

Researchers found similar phenomena in sewage treatment plant [36,37], which indirectly showed that Proteobacteria played a main role in nitrogen and phosphorus removal.

Then, Bacteroidetes ranked second about 20%, followed by Chloroflexi (3.95%–7.12%), Planctomycetes (1.97%–6.9%) and Ignavibacteriae (2.61%–6.99%). The third and fourth ranked Chloroflexi and Planctomycetes had the ability to degrade a wide range of organic pollutants, which improved the ability of denitrification [38]. There was no significant difference in bacterial community between sun2 and sun3 samples, but the relative abundance of some phyla changed significantly.

For example, the relative abundance of Proteobacteria, Bacteroidetes, Chloroflexi and Firmicutes decreased at A/O stage, while increased at A/A stage. In contrast, Planctomycetes and *Candidatus Saccharibacteria* dropped, while Chloroflexi and Acidobacteria accumulated in the process of acclimation.

The classification of functional communities at the genus level was shown in Fig. 9b. Through comparison, it was found that the flora composition at the genus level was more different than that at the phylum level. The three stages corresponded to three dominant genera. In sun1, *Phaeodactylibacter*, as a predominant genus, was the facultative anaerobic denitrifying bacteria, it obtained nitrate or nitrite in anoxic environment and had a competitive relationship with DPAOs. In the later stage of the system process, the content of *Phaeodactylibacter* decreased from 4.69% to 1.06%, which indirectly promoted the process of denitrifying phosphorus removal. In sun3, the dominant genus became *Parcubacteria* accounted for 9.44%, and in Sun5, the ratio of *Ignavibacterium* was the highest, reaching 10.53%. Through experiments, researchers found that *Ignavibacterium* had a variety of functions. For example, it could cooperate with DPAOs to enhance the ability of

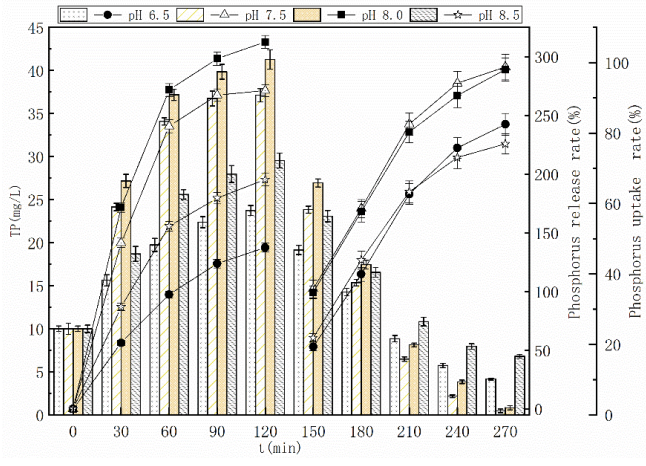

Fig. 8. Effect of different pH values on TP removal efficiency.

Table 2
Indicators of microbial community in the sample

Samples	Reads	OTUs	Shannon	Ace	Chao1	Simpson	Coverage
Sun1	40,774	4,663	6.73	18,941.47	12,521.37	0.0034	0.9326
Sun2	42,516	3,140	5.79	15,728.61	9,902.56	0.0110	0.9550
Sun3	39,692	3,636	5.95	20,321.44	12,497.27	0.0083	0.9404

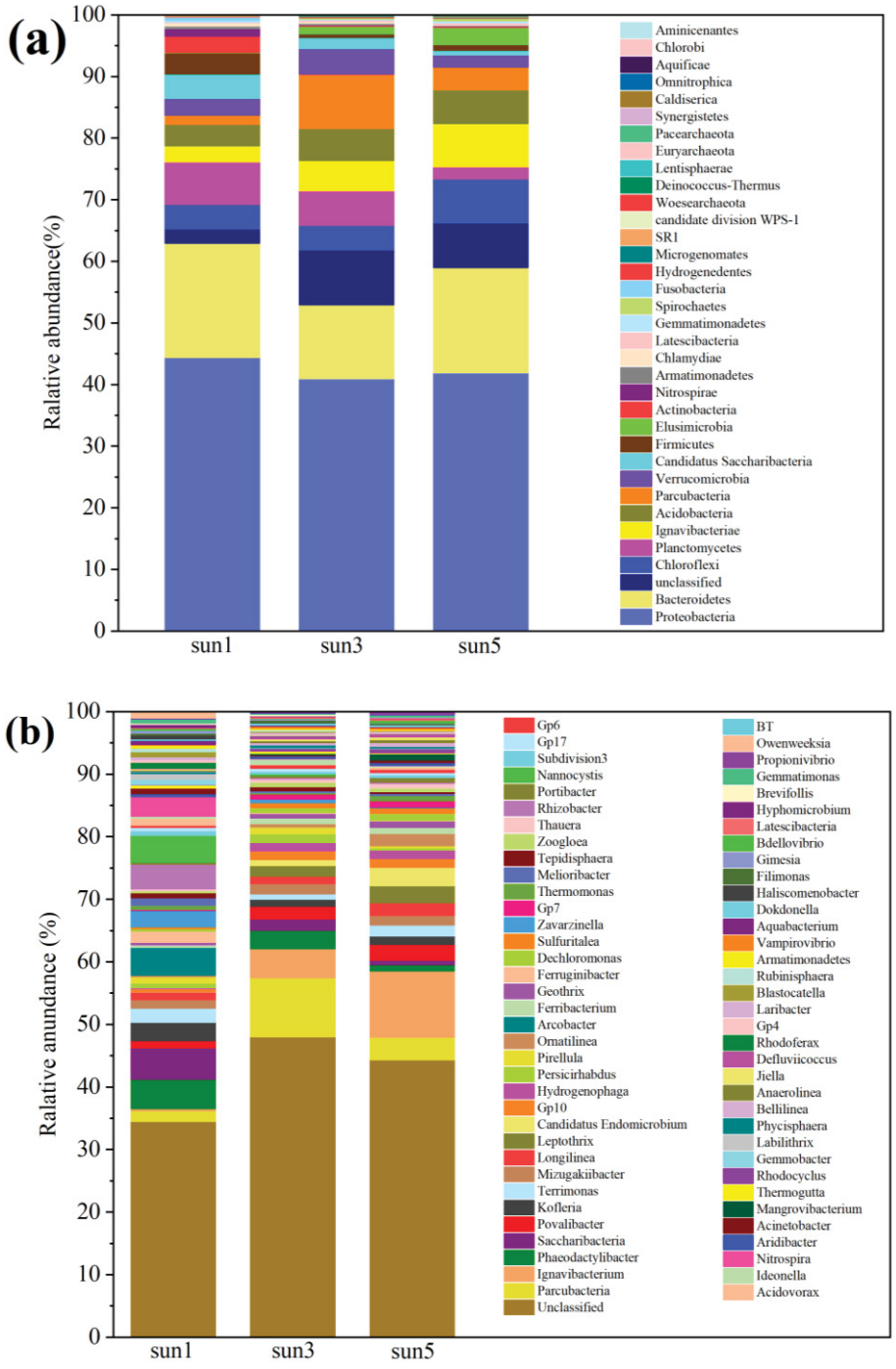

Fig. 9. Microbial community structures at (a) phylum and (b) genus levels.

nutrient removal and accelerate the process of endogenous denitrification [25]. In addition, *Ignavibacterium* was also a kind of DPAOs, which had the ability to convert organic matter into CO_2 [39,40]. In the process of denitrifying phosphorus removal, some new flora began to enrich and gradually increase, while its content in seed sludge was very small (less than 1%), so it can be inferred that a generic microbial community beneficial to nitrogen and phosphorus removal was formed in the process of domestication [41,42].

3.6. Physiological and biochemical characteristics of NG2

High efficient DPAO was screened from A²SBR reactor and named NG2, which had high ability of nitrogen and phosphorus removal. The TP removal rate was as high as 89.46%, and the removal effect of NO_3^-–N also reached 91.68%. Subsequently, the growth curve of strain NG2 was determined (Fig. 10). The results showed that the generation cycle of strain NG2 was short, which had the strong

adaptation environment ability. It entered the logarithmic growth period from 3 h to 16 h, the doubling time was about 13 h, and the maximum growth rate was 0.087. NG2 entered the stable stage after 18 h, and the total amount of bacteria hold steady. To sum up, NG2 had more obvious growth advantages. Then, NG2 was cultured on the specific medium plate for 48 h, the surface of cultured colonies was orange red, opaque, soft texture and smooth. As shown in Fig. 11, the microscopic examination of the strains demonstrated that the bacterial strain was a short rod-shaped bacterium without spores, flagella and motility, and was a Gram-positive bacillus. These characteristics were also confirmed in the literature of Shigematsu et al. [43]. In the end, the main physiological-biochemical of NG2 was identified. The results of glucose oxidation fermentation showed that NG2 was a fermentative strain. Both nitrate reduction and nitrite reduction were positive, that is to say, the strain could take nitrate or nitrite as electron acceptor to reduce it to N_2 under anoxic condition, thus showing the function of denitrification. Methyl red test, utilization of sodium citrate and indole test were positive, V-P test and gelatin liquefaction were negative.

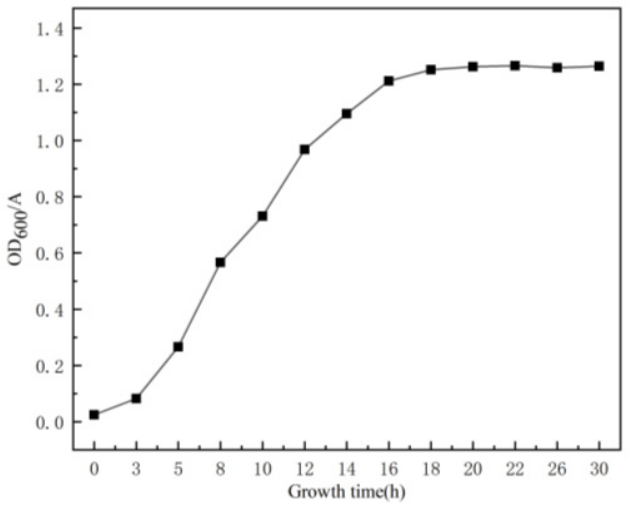

Fig. 10. Growth curve of bacterial strain.

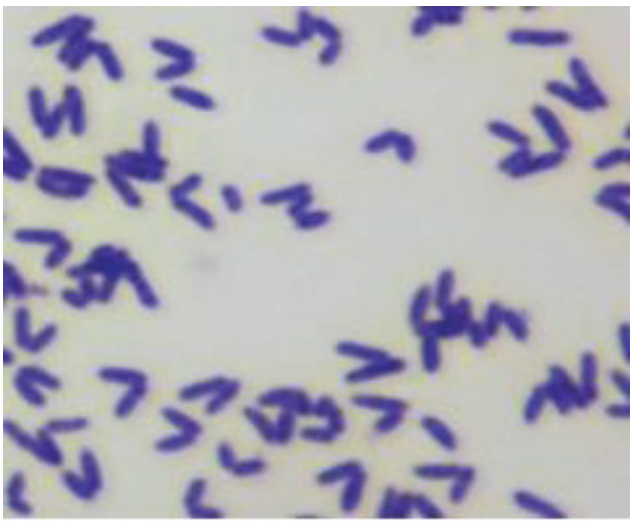

Fig. 11. Gram-Färbung of bacterial strain.

3.7. 16S rDNA gene sequencing of NG2

The strain NG2 was used as the template for 16S rDNA sequencing. The length of the sequenced fragment was 1389bp, which met the sequencing requirements. Then the phylogenetic tree was constructed by MEGA5.1 software using the related species with more than 95% homology (as shown in Fig. 12). Strain NG2 exhibited 100% sequence similarity with *Gordonia terrae* AY771329, *Gordonia terrae* AY771333 and *Gordonia terrae* AY771330. By comparing its 16S rDNA homologous sequence, it was confirmed that strain NG2 was *Gordon terrae*.

3.8. Metabolic characteristics of NG2

The metabolic characteristic of NG2 was shown as Fig. 13. The change values of PHB and TP were 80.842 and 13.3 mg/L respectively, under anaerobic conditions. The ratio of phosphate released to PHB synthesized was 0.16 mg P per mg PHB. According to the following equation:

$$CH_{1.5}O_{0.5}(PHB) + 1.125O_2 \rightarrow CO_2 + 0.75H_2O \quad (1)$$

$$C_6H_{10}O_5(\text{glycogen}) + 6O_2 \rightarrow 6CO_2 + 5H_2O \quad (2)$$

Calculated from the molecular formula: 1.67 g COD per g PHB and 1.08 g COD per g glycogen. Simultaneously, ΔPHB = 135.00 mg COD/L, ΔCOD = 108.03 mg/L, so the rate of carbon absorption to PHB synthesis (ΔCOD × 100%/ΔPHB) was 80.02%. Meanwhile, the concentration of glycogen fell to 70.69 mg/L, Δglycogen = 48.27 mg COD/L. Thus, the percentage of degraded glycogen to synthesized PHB (Δglycogen × 100%/ΔPHB) was 35.76%. Therefore, we can infer that the synthesis of PHB mainly came from the

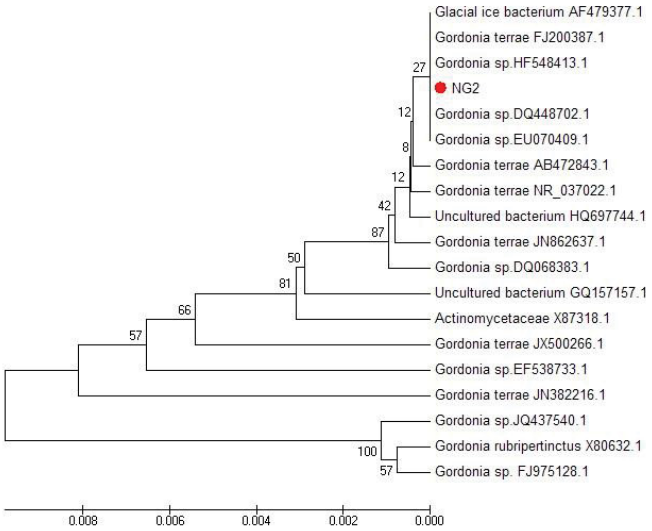

Fig. 12. Species phylogenetic tree.

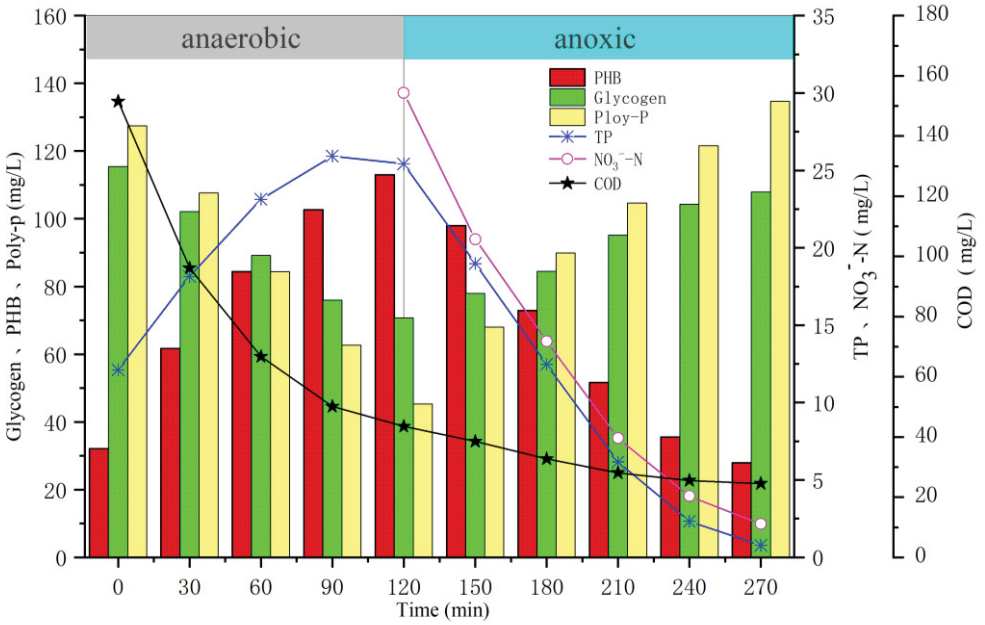

Fig. 13. Substrate transformation of anaerobic/anoxic reaction.

absorption of carbon and a small amount from the transformation of stored glycogen. At present, it was generally accepted that the energy of DPAOs came from two ways: phosphate release and stored glycogen degradation.

Strain NG2 gained amount of energy by the way of releasing phosphate, under anaerobic conditions. In addition, NG2, as a kind of fermentation bacteria, which could produce part of energy through fermentation, this process was described as the degradation of glycogen. The ratio of phosphate absorption to PHB degraded (ΔP/PHB) was 0.30 mg P per mg PHB under anoxic conditions, which had a higher level than that under anaerobic conditions. Thus, strain NG2 took up and accumulated a large amount of phosphate. DPAOs used NO_3^-–N to to generate energy, 43.78% of which was used for synthesis of glycogen, and 56.22% for phosphorus absorption. As NG2 was a fermentative bacterium, the energy generated by PHB degradation was used for phosphate absorption and glycogen synthesis in an average way, and its metabolic mechanism was the same as that of traditional DPAOs.

4. Conclusions

In order to achieve the effect of simultaneous removal of nitrogen and phosphorus in wastewater, SBR system was used, which had the operation mode of A/O and A/A cycles. After 30 d of acclimation, the DPAOs were successfully cultivated. The optimal nutrient removal efficiencies reached to 88.21% (COD), 92.23% (TP) and 92.46% (NO_3^-–N) severally under the conditions of C/P20, sodium acetate as carbon source and pH 7.5. Then the bacterial diversity and community structure of sludge samples were analyzed, as well as the changes and causes of flora at the phylum and genus level in the three stages of domestication. Furthermore, a strain named NG2 was separated from sludge, which was identified as *Gordonia terrae* with a sequence similarity of 100%. Eventually, the metabolic characteristics of NG2 described the transformation process of glycogen, carbon source and PHB, which indirectly proved that its metabolic mechanism was the same as that of traditional DPAOs. This experiment realized the synchronous and efficient removal of nitrogen and phosphorus, and deeply analyzed its internal mechanism, so as to provide a strong theoretical basis for the practical application of denitrifying phosphorus accumulating bacteria in sewage.

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Data availability statement

All relevant data are included in the paper or its Supplementary Information.

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