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ORIGINAL PAPER

Catalyzing denitrification of *Paracoccus versutus* by immobilized 1,5-dichloroanthraquinone

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Abstract The accelerating effect of non-dissolved redox mediator (1,5-dichloroanthraquinone) on the biological denitrification was investigated in this paper using 1,5-dichloroanthraquinone immobilized by calcium alginate (CA) and a heterotrophic denitrification bacterium of *Paracoccus versutus* (GU111570). The results suggested that the denitrification rate was enhanced 2.1 fold by 25 mmol l^{-1} 1,5-dichloroanthraquinone of this study, and a positive correlation was found for the denitrification rate and 1,5-dichloroanthraquinone concentrations from 0 to 25 mmol l^{-1}. According to the change characteristic of NO_3^- and NO_2^- during the denitrification process, the tentative accelerating mechanism of the denitrification by redox mediators was put forward, and redox mediator might play the role of reduced cofactors like NADH, N(A)DH and SDH, or the similar ubiquinol/ubiquinone (Q/QH_2) role during the denitrification process.

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Introduction

With the fast development of modern industry and agriculture, inorganic nitrogen compounds have become common contaminates in surface water and groundwater in recent years. Ammonium, nitrate and nitrite, discharged into the environment, can cause serious problems such as the eutrophication of rivers and deterioration of water sources, as well as hazards to human health (Wang et al. 2009; Wan et al. 2009; Chen et al. 2009).

Biological denitrification is the most common technology employed for nitrogen removal in wastewater treatment. This technology is an anoxic process in which nitrate is reduced to nitrite and subsequently to nitrogen gas by facultative denitrifying bacteria. Denitrifying bacteria are generally heterotrophic and utilize simple organic compounds as electron donors to achieve denitrification. Carbon source and waste management costs are usually responsible for more than 50% of the total costs of wastewater treatment, which creates a bottle-neck problem in these wastewater treatment technologies (Fernández-Nava et al. 2010). More and more new biological technologies have been developed to overcome shortcomings of previous technology in recent years, for example, the

sulfur-limestone autotrophic denitrification (SLAD) process (Feleke and Sakaibara 2002; Prosnansky et al. 2002) and the combined bioelectrochemical and sulfur autotrophic denitrification system (CBSAD) (Wan et al. 2009). However, the low removal rate and efficiency is still a key problem in this field. Therefore, it is very important to find a novel and economical biological nitrogen removal processes.

As we know, quinones can accelerate the anaerobic conversion of perchlorate, arsenate, nitrate, nitrite, nitrous oxide and azo dyes as electron donors (Aranda-Tamaura et al. 2007; Lovley et al. 1999; Guo et al. 2007, 2010a, b; Van der Zee and Cervantes 2009). Recently, the simultaneous conversion of sulphide and nitrate in a denitrifying culture was reported to be enhanced with different dissolved redox mediators (Aranda-Tamaura et al. 2007), but continuous dosing of the dissolved redox mediators implies continuous expenses and continuous discharge of this biologically recalcitrant compound. And the catalyzing effect of novel non-dissolved redox mediator catalyzing biological denitrification (RMBDN) was first explored simplify (Guo et al. 2010a, b), and the activity retained over 90% of their original value after four repeated experiment (Guo et al. 2007). However, the RMBDN technology has to be conducted in detail for the accelerating mechanism and effects.

The aim of this study was to explore the accelerating mechanism and the effect of 1,5-dichloroanthraquinone on the denitrification processes of *Paracoccus versutus* (strain GW1), which was a new isolated denitrification bacterium and has the high denitrification efficiency.

Methods and materials

Chemicals

All chemical reagents were analytical grade and were purchased from Xiandai Ltd. (Shijiazhuang, China). The chemical structure of 1,5-dichloroanthraquinone was shown in Fig. 1.

Isolation and characterization of the heterotrophic denitrification bacterium

The heterotrophic denitrification bacterium (GW1) was isolated from the denitrification reactor in ''The key laboratory of Environmental Biotechnology of Hebei''. The sludge (5 ml) and buffer solution (45 ml) were mixed by the blender for 1 min, and then deposited for 10 min. About 10 ml suspended liquid was added into the enrichment and isolation medium, which contained 100 ml mineral medium with 1 ml trace element solution. The mixed culture was cultivated in a shaker (150 rev min^{-1}) at 30°C. The plates containing bromothymol blue as indicator of the denitrification bacteria were incubated at 30°C, and colonies were carefully observed for their uniformity and differences. The morphologically distinct colonies were selected and transferred to new plates to check for purity. Then the isolated bacterium was identified by the 16 S ribosomal genomic DNA according to previous paper (Guo et al. 2008). The optimum denitrification condition of the isolated bacterium was also conducted.

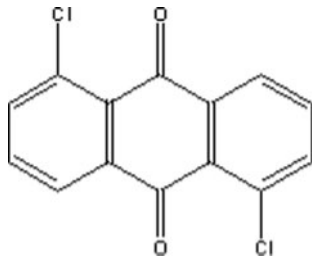

Fig. 1 The chemical structure of 1,5-dichloroanthraquinone

The enrichment and isolation medium contained the following (l^{-1}): 0.1–0.3 g NO_3^-–N, 1.25 g $KHCO_3$, 0.025 g KH_2PO_4, 0.3 g $CaCl_2{\cdot}2H_2O$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.00625 g $FeSO_4$, 0.00625 g EDTA and 1 ml trace elements solution. The trace element solution consisted of (l^{-1}): 15 g EDTA, 0.43 g $ZnSO_4{\cdot}7H_2O$, 0.24 g $CoCl_2{\cdot}6H_2O$, 0.99 g $MnCl_2{\cdot}4H_2O$, 0.25 g $CuSO_4{\cdot}5H_2O$, 0.22 g $NaMoO_4{\cdot}2H_2O$, 0.19 g $NiCl_2{\cdot}6H_2O$, 0.21 g $NaSeO_4{\cdot}10H_2O$, 0.014 g H_3BO_4, 0.05 g $NaWO_4{\cdot}2H_2O$ per liter. NO_3^-–N was added into the influent synthetic wastewater in the form of $NaNO_3$.

Biomass concentration was determined by optical density (OD) at 660 nm, and the concentration of Strain GW1 was 5.8 mg dry cell weight l^{-1} in all experiments.

1,5-dichloroanthraquinone immobilization by Calcium alginate

Calcium alginate was selected as the anthraquinone immobilization support according to the previous

paper (Guo et al. 2007). The mixed immobilization solution with different 1,5-dichloroanthraquinone concentration (g l^{-1}) and sodium alginate (0.05 g l^{-1}) was introduced into a syringe, and was then pressured to drop into a 5 g $CaCl_2$ l^{-1} solution to form beads of 3.0–4.0 mm in diameter. The beads were suspended in the $CaCl_2$ solution for 4 h to enhance their mechanical stability at 4°C. The 1,5-dichloroanthraquinone concentration was calculated by the mass ratio of added anthraquinone and the volume of the mixed solution for immobilization. The 1,5-dichloroanthraquinone weight of every bead was calculated using the ratio of the added 1,5-dichloroanthraquinone mass and the numbers of the bead.

The effects of 1,5-dichloroanthraquinone on the denitrification process

To evaluate the effect of 1,5-dichloroanthraquinone on the denitrification process, 1,5-dichloroanthraquinone immobilization beads were placed in 350 ml growth medium containing 600 mg NO_3^-–N l^{-1}. Denitrification with an identical amount of 1,5-dichloroanthraquinone-free immobilization beads was also performed as the control. The residual nitrate and nitrite concentration in the solution was detected as a function of time until equilibrium.

The pH value is an important parameter for the denitrification processes. And the pH change was measured during the denitrification process with redox mediator.

The control experiment with 1,5-dichloroanthraquinone-free immobilization beads were similar to the procedures of immobilized 1,5-dichloroanthraquinone beads.

Analytical methods

The samples were centrifuged for 15 min at 8,000×g to remove the insoluble particles from the supernatant.

Nitrite concentration was measured by using the colorimetric methods and nitrate concentration was determined through UV-spectrophotometry (Tianmei, UV2600, UV/VS spectrophotometer, China). The pH was determined with a digital pH Meter (Delta-320, China).

Results and discussion

The optimum denitrification conditions of *Paracoccus versutus*

Strain GW1 was identified to be a member of *Paracoccus versutus* by 16S rDNA sequence (1,416 bp, GU111570) analysis and physic-biochemical tests, respectively. It was a Gram-positive, non-motile, facultative, round-shaped bacterium measuring 0.7–1.0 μm in diameter. No flagellum was found in their electron microscopy. Colonies were smooth, glistening, circular, low-convex and white. It was oxidase-positive and catalase-positive. Sucrose, lactose, maltose, cellulose, mannitol and glycerol were not used by strain GW1 to produce acid compounds, and fructose was hydrolyzed.

The optimum temperature range of denitrification was 35–40°C. The appropriate pH range was 7.0–8.5. At the same time, sodium acetate was selected as the carbon source in this study, and the optimum ratio of C/N was 6.0.

The effect of 1,5-dichloroanthraquinone on the denitrification process

The adsorption results of 1,5-dichloroanthraquinone-free bead served as the blank control for the experiment. The 200 CA beads caused little change (0.2%) nitrate in 36 h (data not shown). Therefore, the adsorption of CA beads for nitrate could be neglected in the experiment in this study. With a fixed initial nitrate concentration (300 mg l^{-1}) because of many industrial wastewater containing high nitrate concentration (Shen et al. 2009; Rodriguez et al. 2007) and the 200 CA beads with different 1,5-dichloroanthraquinone concentrations, Fig. 2 showed that the denitrification rates by strain GW1 increased with the addition of 1,5-dichloroanthraquinone. Figure 3 also suggested that the pH value increased with the decrease of nitrate concentration for all experiments, but the pH value with 1,5-dichloroanthraquinone was lower than that of the control. The catalyzing denitrification process by 1,5-dichloroanthraquinone followed a modified pseudo-zero order model, which was $k = dC_{Nitrate}/dt$. The reaction constant of k (mg NO_3^-–N g^{-1} l^{-1} h^{-1}) were 18.90, 24.68, 33.86, 37.96 and 42.61 at the 1,5-dichloroanthraquinone of 0, 0.5, 5.0, 10.0 and 25.0 mmol l^{-1} by multiple

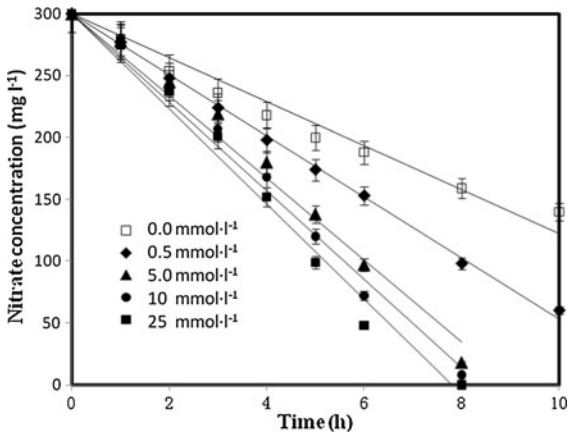

Fig. 2 The nitrate change curve of denitrification system with different concentration of 1,5-dichloroanthraquinone (Initial nitrate concentration: 300 mgNO_3–N l^{-1}; 35°C; pH: 7.0; carbon source: sodium acetate; C/N: 6; 5.8 g dry cell weight l^{-1})

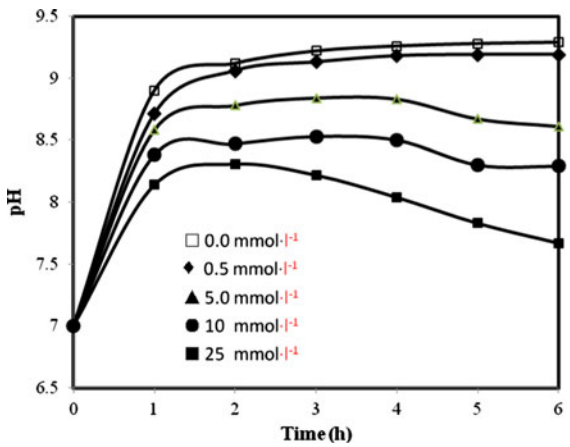

Fig. 3 The pH change curve of denitrification system with different concentration of 1,5-dichloroanthraquinone (Initial nitrate concentration: 300 mgNO_3–N l^{-1}; 35°C; pH: 7.0; carbon source: sodium acetate; C/N: 6; 5.8 g dry cell weight l^{-1})

regression analysis, respectively. A positive correlation was found for 1,5-dichloroanthraquinone concentrations from 0 to 25 mmol l^{-1} (Fig. 4), and the specific denitrification rate was enhanced 2.1 fold by 25 mmol l^{-1} 1,5-dichloroanthraquinone. When the 1,5-dichloroanthraquinone concentration was over 25 mmol l^{-1}, the accelerating effect of 1,5-dichloroanthraquinone on the denitrification process was similar that of 25 mmol l^{-1} 1,5-dichloroanthraquinone in this experiment, which might be related to the amount of nitrate reductase present. The accelerating effect by redox mediator on the denitrification was

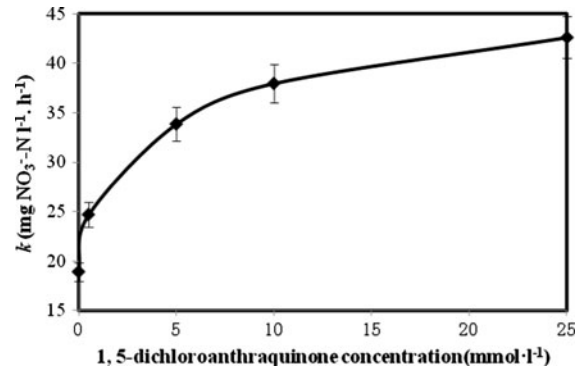

Fig. 4 Denitrification rate by strain GW1 with different concentration of 1,5-dichloroanthraquinone (Initial nitrate concentration: 300 mgNO_3–N l^{-1}; 35°C; pH: 7.0; carbon source: sodium acetate; C/N: 6; 5.8 g dry cell weight l^{-1})

similar to the results during the decolorization of azo dye (Guo et al. 2008). Different initial nitrate concentration (100–600 mg l^{-1}) was conducted to study the effect of nitrate concentration on the denitrification rate with 25 mmol l^{-1} 1,5-dichloroanthraquinone. Fig. 5 showed that the denitrification rate was not significantly affected by the initial concentration in the tested range of nitrate concentration and the average denitrification rate was 44.03 ± 10.63 mg NO_3^-–N l^{-1} h^{-1} or 182.19 ± 43.99 g NO_3^-–N g^{-1} dry cell weight d^{-1}, which was enhanced 3–10 times compared to the reported data (Shen et al. 2009; Rodriguez et al. 2007).

During the denitrification process, the nitrite concentration was first accumulated up to the highest concentration and was then converted into nitrous

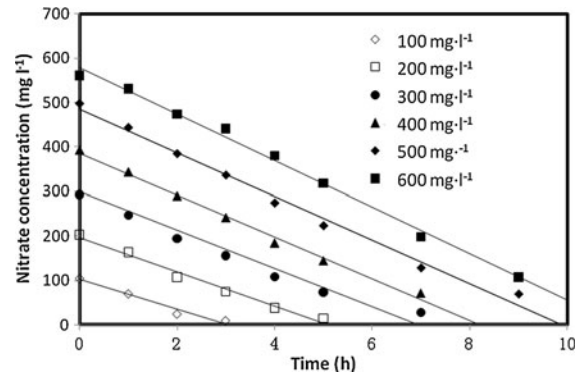

Fig. 5 The effect of different initial nitrate concentration on the denitrification efficiency with 25 mmol l^{-1} 1,5-dichloroanthraquinone (35°C; pH: 7.0; 25.0 mmol l^{-1} 1,5-dichloroanthraquinone; carbon source: sodium acetate; C/N: 6; 5.8 g dry cell weight l^{-1})

oxide, nitric oxide and nitrogen gas. The highest concentration of nitrite was about 132 mg l^{-1} with 25 mmol·l^{-1} 1,5-dichloroanthraquinone at 6 h of reaction (Fig. 6). The reduction rate of nitrate to nitrite increased with the 1,5-dichloroanthraquinone concentration, and the reduction rate of nitrite was also enhanced by 1,5-dichloroanthraquinone. Compared to the control (0 mmol l^{-1} 1,5-dichloroanthraquinone), the nitrate concentration was reduced completely and the removal rate was 80% after 8 h reaction. As we knew, the denitrification steps were sequential and the product of one enzyme is a preceding step for the next one. Fine-tuned regulation of the concentration and activity of the denitrification enzymes was, therefore, required in order to keep the free concentrations of NO_2^- and NO below cytotoxic levels. NO_2^--reductase was induced in response to NO_2^- availability. The activity of NO_2^--reductase gave rise to an initial increase of NO_2^- concentration.

The accelerating mechanism of redox mediator during the denitrification processes

The effect of redox mediator on the denitrification should be taken into consideration that two nitrate reductases are present in some strains: a periplasmic and a membrane-bound one (Alefounder and Ferguson. 1980; Bedzyk et al. 1999; van Spanning et al. 2006). In all bacteria, the enzymes of denitrification

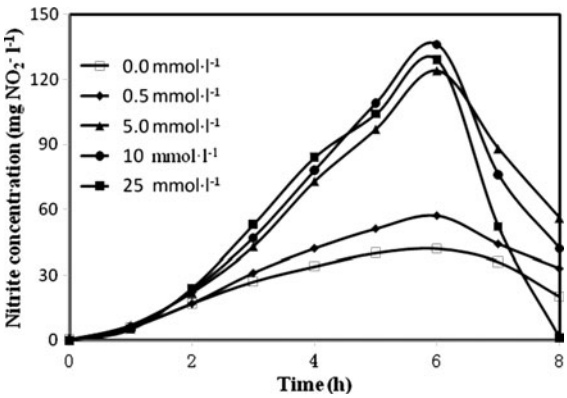

Fig. 6 The nitrite change during the denitrification processes with different concentration of 1,5-dichloroanthraquinone (Initial nitrate concentration: 300 mgNO_3–N l^{-1}; 35°C; pH: 7.0; carbon source: sodium acetate; C/N: 6; 5.8 g dry cell weight l^{-1})

received e^- from the respiratory chain system that was part of the cytoplasmic membrane. Denitrification-specific components need to start at the ubiquinol/ubiquinone component of the chains. Reduction of ubiquinone to ubiquinol occured using e^- originating from reductants (such as NADH, succinate, etc.) during the denitrification process and ubiquinol can be directly oxidized by a membrane-bound respiratory NO_3^--reductase (Fig. 7).

Figures 2 and 6 showed that redox mediator accelerated the denitrification of NO_3^- to NO_2^- and NO_2^- to N_2, which needed the cofactor ubiquinol/ubiquinone (Q/QH_2). A co-metabolic reaction could be the main mechanism of the denitrification, in which the reducing equivalents or reduced cofactors like N(A)DH and succinate acting as secondary electron donors, channel electrons to NO_3^- and NO_2^-. There were three ways for the tentative accelerating mechanism of the denitrification by redox mediator (Fig. 7): (1) A represent that redox mediator severed as reduced cofactors like N(A)DH and succinate acting as a secondary electron donor, channel electrons to NO_3^- and NO_2^-. The accelerating effect of redox mediator would be due to an electron shuttle between reduced enzyme cofactor (Q/QH_2) and redox mediator; (2) B represent that redox mediator severed as the reducing equivalents shuttling electron between reduced enzyme cofactor (Q/QH_2) and NO_3^- -reductase; (3) C represent that redox mediator served a similar role as the reduced enzyme cofactor (Q/QH_2).

As discussed above, redox mediator might play the role of the reducing equivalents, reduced cofactors like N(A)DH, and succinate, or the similar ubiquinol/ubiquinone role during the denitrification process. Redox mediator might affect the cell behavior in different ways, i.e. reducing the redox potential and shifting the cytoplasmic ubiquinol/ubiquinone balance in favor of the reduced form. That was why an explanation for the accelerating effect on the denitrification could be sought in terms of an alteration of the enzyme properties and of cofactor regeneration. This also explained the similar role of NADH and redox mediator for the accelerating effect during the decolorization (Russ et al. 2000; Keck et al. 1997). In our research group, the catalyzing mechanism of redox mediator on the denitrification is also studying by using cell extracts and purified enzymes, which would allow a better understanding on the nature of the process.

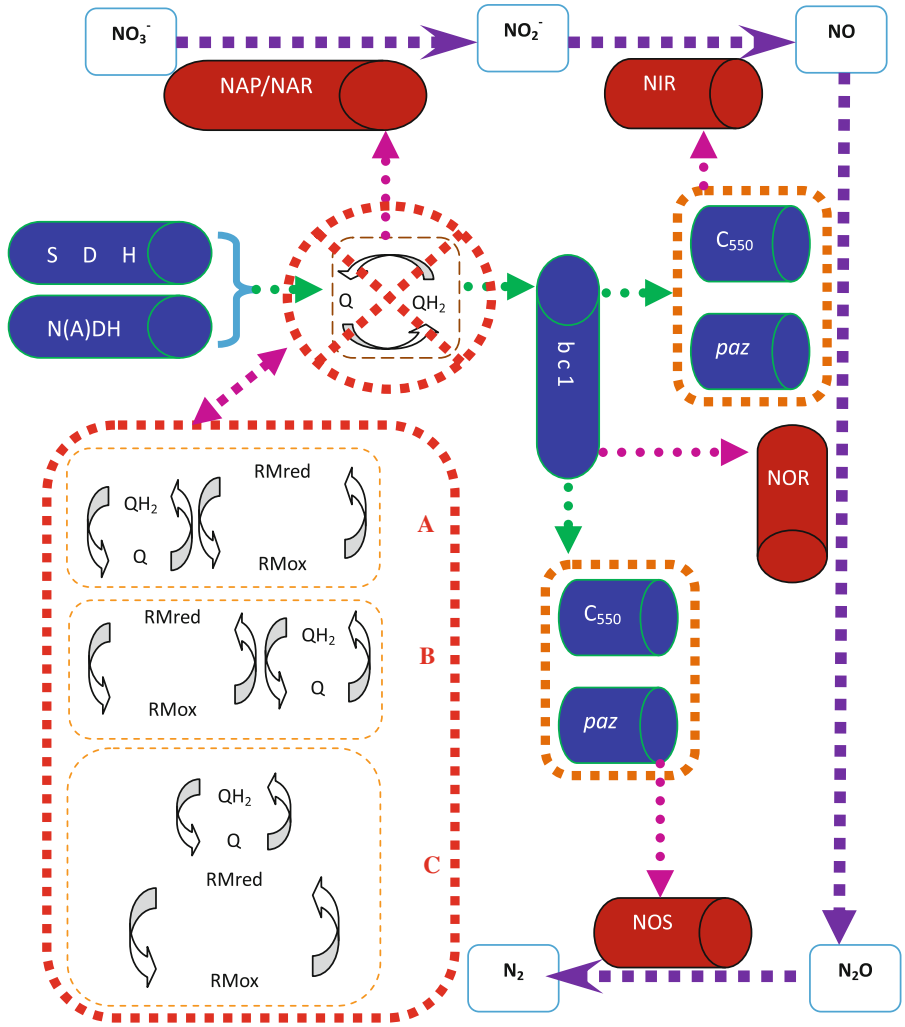

Fig. 7 The denitrification pathway with redox mediators and involved enzyme. *SDH* succinate dehydrogenase, *N(A)DH* dehydrogenase, bc_1 cytochrome bc_1 complex, c_{550} cytochrome c, *paz* pseudoazurin, *NAR* membrane-bound NO_3^--reductase, *NAP* periplasmic NO_3^- -reductase, *NIR* cd_1-type NO_2^- -reductase, *NOR* bc-type NO-reductase; NOS, N_2O-reductase, *Q*, QH_2 ubiquinol/ubiquinone, *RM* redox mediator, *red* reduced, *ox* oxidized

Conclusion

(1) It was observed that the artificial redox mediator 1,5-dichloroanthraquinone was capable of raising the denitrification rate 2.1 fold with 25 mmol l^{-1} concentration in this study, and a positive correlation was found for the denitrification rate and 1,5-dichloroanthraquinone concentrations from 0 to 25 mmol l^{-1}.

(2) According to the change characteristic of NO_3^- and NO_2^- during the denitrification process, the accelerating mechanism of redox mediator was first presumed to act as a reducing equivalent, such as reduced cofactors like NADH, NAD(P)H and succinate, or the similar ubiquinol/ubiquinone role during the denitrification processes.

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