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The Impact of Silver Nanoparticles on Bacterial Aerobic Nitrate Reduction Process

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Abstract

Due to the effective antimicrobial properties of silver nanoparticles (Ag NPs), these particles are receiving an extensive interest for applying in wide range of consumer products and water purification systems. Entering the Ag-based material in wastewater system can influence the biological cycle such as nitrogen. Denitrification as a part of nitrogen cycle is an effective biological process in wastewater systems which can be affected by Ag NPs. The objective of this research was to study the impact of Ag NPs on aerobic nitrate reduction. We showed that *Rhizobium sp* and *Azotobacter sp* isolates were able to reduce nitrate aerobically. Adding 0.2 ppm of Ag NPs in culture medium of *Azotobacter* PHB$^+$ enhanced the of the nitrate reduction activity about 20% and Ag NPs at this concentration has no significant effect on the nitrate reduction activity of periplasmic extracts of the selected isolates in aerobic conditions. Thus, entering certain ation of Ag NPs in environments has no significant impact on microbial aerobic denitrification as an important part of nitrogen cycle.

Keywords: Silver nanoparticles; Denitrification; Nitrate reduction; Bacteria

Introduction

Since the 1970, contamination of groundwater by nitrate (NO3-) has become a great concern for human health and harmful effect on environmental ecosystems due to carcinogenic influence and high rate of accumulation in surface water [1]. According to The European Union and World Health Organization (WHO), drinking contaminated water with more than 11.3 mg L^{-1} nitrogen are considering as unsafe water source especially for infants [2]. Hence, remediation of groundwater system contaminated with nitrate has been extensively studied over last decades. Biological processes including microbial denitrification has been considered as promising alternative for remediation of nitrate methods [1]. Aerobic nitrate respiration as a part of denetrification process has been detected in numbers of facultative anaerobic heterotrophic bacteria such as *Pseudomonas* sp., [3] *Paracoccus pantotrophusm*, [4] *Pseudomonas aeruginosa, Rhodobacter sphaeroides* f. sp. denitrificans [3]. Typically, Nitrate reductase as one of key enzyme in aerobic nitrate respiration is located in periplasmic [4].

Recent studies show that metal ions concentration is a critical parameter that affects the ability of denitrifying bacteria to remove nitrate and nitrite [5,6]. Over the past few decades, silver nanoparticles (Ag NPs) have been applied in wide range of commercial product such as catalysts, electronics, printing industry, photographic manufacturing, clothing, food industry, paints, cosmetics products and medical consumptions [7]. However, these wide ranges of applications of Ag-based products caused an increasing in the risk of Ag- ions entering to the wastewater treatment systems [8]. Few reports are available concerning the interaction of Ag-based material and ions with environmental bacterial communities [9]. Therefore, it is essential to consider whether the entering silver compound and ions in the wastewater system can impact the activity of biological processes or not. This research aims to study the impact of Ag NPs on the aerobic reduction of nitrate, first step of the denitrification processes, in *Azotobacter* and *Rhizobium* isolates. To our knowledge, the effect of Ag-based material on denitrification has not been investigated yet. In this study the aerobic reduction of nitrate by *Rhizobium sp.* and *Azotobacter sp*. isolates was studied. Afterwards, the effect of Ag NPs on the nitrate reduction process catalyzed by native cells, cell free and periplasmic extracts of selected strains was evaluated. In addition to, the bactericidal activity of Ag NPs on studied aerobic denitrifies was also examined.

Materials and Methods

Bacteria, preparation of media and silver nanoparticles

Rhizobium A1, Rhizobium A3, Azotobacter PHB$^+$ (previously isolated from meliloti nodules and soil of Isfahan respectively) and *Escherichia coli* ATCC 1339 (Gram negative model bacterium with ability of anaerobic nitrate reduction) were obtained from the microbial culture collection of microbiological laboratory of the University of Isfahan. All isolates were grown in the medium containing the following ingredients g per liter: 0.5 KH_2PO_4, 0.5 K_2HPO_4, 0.2 $MgSO_4.7H_2O$, 3.0 $CaCO_3$. $2H_2O$, 0.001 $MgSO_4.5H_2O$, 1 yeast extract, 0.1 NaCl and 10 glucose and consequently the pH of the medium was adjusted to 7.5 [10].

Ag NPs (average size: 40 ± 10 nm) was obtained from Nanocid Company, Iran.

MIC and MBC assay of Ag NPs

To evaluation the minimum inhibitory concentration of Ag NPs on our strains, a serial dilution of Ag NPs (0.25, 0.5, 1,2, 4,8 ppm) was prepared. 80 µl of sterile nutrient broth was added to 96-well microtitre plates followed by adding 40 µl of different concentration of Ag NPs to each row of the microtitre plates. 80 µl of the bacterial suspension (adjusted to 0.5Mac Farland level) was then added to each row (resulting the final volume of 200 µl cell suspension for each well). The microplate was sealed with plastic film and incubated at suitable temperatures

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(28°C and 37°C) for 24 h according to each strain. Minimal bactericidal concentrations (MBC) were determined by agar plate method [11,12]. All experiments were performed in triplicate.

Growth in presence of Ag NPs

Bacterial cell were exposed to Ag NPs by inoculating desired concentration of each strains (1.5×10^8 CFU/ml) of all strains in to medium contained 25 ml nutrient broth supplemented by 0.01% KNO_3 and 0.2 ppm Ag NPs. All of flasks were incubated at 28°C for 24 h with mild shaking (130 rpm). Nitrite reduction was assayed as described below. Growth of bacteria were monitored by measuring optical density of cells at 600nm (OD_{600}) over times.

Nitrite assay

The bacteria were grown in nutrient broth supplemented with 0.01% KNO_3 at 28°C with mild shaking (130 rpm). Cells were harvested by centrifugation (4,830g for 10 min) in sterile 50 mL centrifuge tubes. One ml of supernatant was transferred to 3 ml cuvettes. Consequently, 0.02 ml of nitrite determination reagent was added, mixtures were kept at room temperature for 15 min. The pink color was measured spectrophotometrically at 540 nm against the blank [13].

Nitrate reduction activity of native cells

The nitrate reduction activity of aerobically grown bacterial cells was measured in the presence of nitrate (100 mg/L NO_3--N). The cells were harvested by centrifugation (10,000 g, 10 min) at the middle of their exponential growth (OD_{660} about 0.8), washed 3 times and re-suspended in 0.1 M phosphate buffer, and pH 7.2. The concentration of cell suspensions was adjusted to 1.0 (OD_{600}) with 0.1 M potassium phosphate buffer.

Five ml of each cell suspension was transferred into reaction tubes and nitrate solution was added in final concentration of 30 mg L^{-1} NO_3--N. Afterward, the following conditions were conducted for each isolates: (1) 0.5 g L^{-1}sodium thioglycolate and 0.2 ppm Ag NPs were added to suspension, (2) sodium thioglycolate free samples, (3) Ag NPs free samples, and also sodium thioglycolate and Ag NPs free controls.

The tubes were kept at room temperature for 60 min. Nitrite was assayed in supernatant (harvested from biomass by centrifugation at 4,830 g for 10 min) as described before [2,6].

Nitrate reduction activity in cell free extract

Cytoplasmic enzyme were obtained by following procedure, fresh biomass of cells were harvested from nutrient broth media containing 0.01% KNO_3 by centrifugation (2,683g and 30 min). Pellets were re-suspended in 0.1M phosphate buffer, pH 7.2. Consequently, cells were disrupted using an ultrasonic processor (GmbH VP200H) in (50 Hz, 7 times, 30s) in an ice-water bath). After bacterial cell disruption, supernatant were separated by centrifugation (11,180g, 15 min). One ml of supernatant was exposed to nitrate solution with final concentration of 30 mg/l NO_3--N. Suspension was treated for nitrite assay as mentioned in pervious part. The tubes kept at room temperature for 60 min. Nitrite was then assayed according to Emtiazi et al. [14].

Preparation of periplasmic extract and nitrate reduction activity

Bacterial cells were harvested using centrifugation at 4,920g for 10 min. The supernatant were carefully removed from cells pellet. Pellets were re-suspended in ice-cold extraction buffer (20 ml of 1X TES buffer). Extraction buffer were contained: 0.2 M Tris-HCl, 0.5 mM EDTA, 0. 5 M sucrose pH = 8.00. Thirty three ml of 1/5 X TES was mixed with obtained biomass from each liter of cell culture. The re-suspended cells were kept on ice for 30 min. The obtained supernatant was carefully transferred (containing periplasmic extract) to tube and stored at -20 to -70 °C for further analysis as mentioned above [15]. The activity of nitrate reductase was assayed as described above.

Results and Discussion

Screening of nitrate reductase positive-isolates

Denitrification is typically occurring under anaerobic or anoxic conditions [6,16-18]. In addition, microbially aerobic nitrate reduction has also been reported by soil bacteria such *as Pseudomonas, Arthrobacter, Moraxella* and *Aeromonas* [19-21]. In this study, a comparative evolution in reduction of nitrate by different strains of Rhizobium and *Azotobacter* showed that *Rhizobium* A1, A3 and *Azotobacter PHB*$^+$ were the best strains in aerobically reducing nitrate (Table 1). Further studies were carried out using these strains.

MIC and MBC determination of Ag NPs

Cho et al. [11] discovered the MIC of Ag NPs for *Staphylococcus aureus* and *E. coli* were 5 and 10 ppm, respectively [11]. In other research by Kim [7], the antimicrobial activities of Ag ions and Ag NPs against a Gram-negative *E. coli* and a Gram-positive strain such as *S. aureus* were investigated and they found Ag NPs having greater bactericidal activity against *E. coli* compared with *S. aureus*. Petica et al. [22] showed that stable solutions containing up to 35 ppm of Ag have significant antimicrobial and antifungal properties [22]. Choi et al. [8] showed that 0.5 mgL^{-1} Ag has the inhibitory effect on the growth of *E. coli* PHL628-gfp in the forms of Ag NPs, Ag^+ ions, and Ag Cl colloids by 5578%, 100%, and 6676%, respectively [8]. In this study, the bactericidal activity of Ag NPs on *E. coli* and different soil isolates with ability of nitrate reduction, *Rhizobium* and *Azotobacter,* was determined. Exposing of these bacteria to different concentration of Ag NPs solution revealed that the growth of all strains was completely inhibited by 8 ppm of Ag NPs. In general, results showed that the MIC of Ag NP for Rhizobium and Azotobacter was 1 ppm and in case of *E. coli* was 2 ppm (Table 2). Briefly, it was discovered that 0.2 ppm of Ag NPs had no effect on growth of soil bacteria. Therefore, this concentration can be applied as an effective concentration of Ag NPs for further experiments regarding the the effect of these NPs on the responsible enzyme in reduction of nitrate as one of the key enzyme of nitrogen cycle.

Effect of Ag NPs on bacterial growth and nitrate reduction

Isolates	OD_{543nm}	Isolates	OD_{543nm}	Isolates	OD_{543nm}
Rhizobium 4R	0.001	*Rhizobium* PS2	0.095	*Azotobacter* P11	0.242
Rhizobium A3	0.219	*Rhizobium* A1	0.250	*Azotobacter* P81	0.001
Rhizobium 6R	0.121	*Rhizobium* Sin1	0.005	*Azotobacter* A1	0.002
Rhizobium A4	0.108	*Rhizobium* D1	0.002	*Azotobacter* D1	0.255
Rhizobium A_2A	0.072	*Rhizobium* 1R	0.036	*Azotobacter* D2	0.000
Rhizobium A_2B	0.192	*Rhizobium* 2R	0.216	*Azotobacter* PHB$^+$	0.282

Table 1: Comparative evaluation of nitrate reduction by different strains of Rhizobium, Azotobacter.

Bacteria	MIC	MBC
Rhizobium A1	1 ppm	8 ppm
Rhizobium A3	1 ppm	8 ppm
Azotobacter PHB$^+$	1 ppm	8 ppm
E.coli	2 ppm	8 ppm

Table 2: MIC and MBC assay of Ag NPs on different strains of *Rhizobium, Azotobacter and E.coli.*

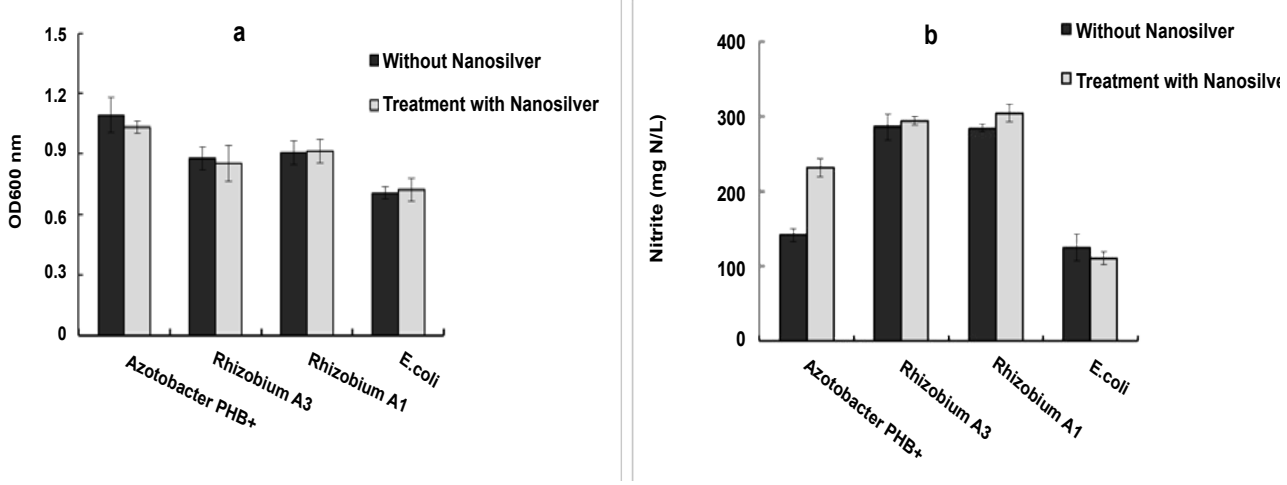

Figure 1: Comparative evaluation of effect of 0.2 ppm of Ag NPs on growth of studied bacteria [2] and nitrate reduction rate (b).

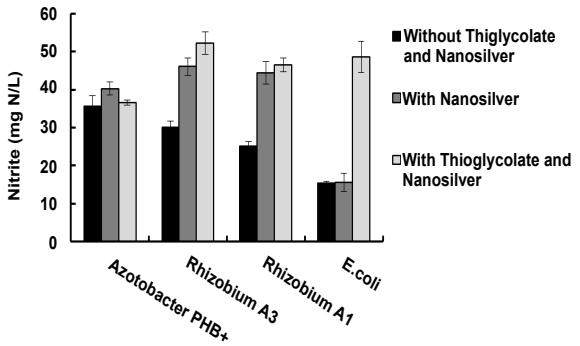

Figure 2: Comparative evaluation of nitrate reduction in native cells, cells treated by Ag NPs and sodium thioglycolate, cells treated by Ag NPs and cells without any treatments.

The effects of sodium thioglycolate as reducing agent and Ag NPs on the enzymatic activity of the strains were investigated. We discovered that using 0 Ag NPs in the concentration of 0.2 ppm had no significant effect on nitrate reductase activity of *Rhizobium* A1 and A3. Interestingly, an increasing up to 20% in the nitrate reduction activity of *Azotobacter PHB*$^{+}$ was observed in compare with untreated cell (Figure 1). An increase in the amount of nitrite in these samples may be explained by the catalytic properties of Ag-based particles in reduction of nitrate [23].

Impact of Ag NPs on the nitrate reduction process in native cells

Kariminiaae-Hamedaani et al. [2] and Pintathong et al. [6] were studied the nitrate reduction activity of native cells of *Pseudomonas sp.* ASM-2-3 and *Paracoccus pantotrophus* P16 [2,6]. In our study, aerobic reduction of nitrate in native cells of the strains was investigated. Figure 2 represents that the nitrate reductase of selected bacterial cell were still active after 60 minutes. In contrast with the *E.coli*, nitrate reduction activity in soil isolates was not significantly affected by sodium thioglycolate and even this activity was higher in the presence of Ag NPs in compared to the controls. A significant increase in the activity of nitrate reductase of *E.coli* in the presence of sodium thioglycolate suggested that it may provide a suitable reduced condition for more efficient activity of this enzyme. It can be confirmed that this strain was not able simply to reduce nitrate in aerobic condition.

Impact of Ag NPs with nitrate reduction activity in the cell free and periplasmic extract compartments

Nitrate reductase activity in the cell free and periplasmic extract was tested under aerobic conditions. Extracted enzyme from sonicated cells showed a significant decreasing in activity in compare with blanks. This can be related to the sensitivity of these enzymes to oxygen or disturbing the appropriate order of the enzyme subunits in the cytoplasmic membrane.

It has been found that periplasmic nitrate reductase play a main role in aerobic nitrate reduction. To define the membrane-bound and periplasmic nitrate reductase of bacteria such as *Rhodobacter capsulatus, Rhodobacter sphaeroides f.* sp. *denitrificans, Alcaligenes eutrophus, Paracoccus denitrificans* PD1222 and GB17, *Pseudomonas putida* and *Escherichia coli* the non-physiological electron donor (BV^{+} and MV^{+}) method have been applied [19, 24].

Periplasmic nitrate reductase has been characterized as a responsible enzyme for aerobic nitrate reduction [2,23]. To our knowledge, the effect of Ag NPs on the activity of aerobic nitrate reductase has not been investigated yet. Unlike the previous studies, this study for the first time demonstrates the activity of periplasmic nitrate reductase in the periplasmic extract of the cells. Interestingly, we observed that all soil isolates, excluding *E.coli*, have had significant high periplasmic nitrate reductase activity in the presence of oxygen while adding sodium thioglycolate had no significant effect on its activity (Figure 3). Therefore our result showed that the presence of thioglycolate and anaerobic condition is not necessary for activity of this enzyme.

Increasing in the concentration of Ag NPs up to 4 ppm caused a 40% decrease in enzyme activity of *Rhizobium* A1 (Figure 3 and 4).

Conclusions

In conclusion, Ag NPs can enhance the activity of nitrate reductase in native bacteria cells while have not such effect on cell free extracted enzyme. According to our finding, periplasmic nitrate reductase is responsible for aerobic nitrate reduction and specific concentrations

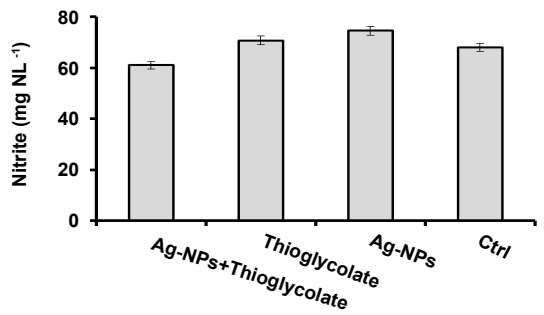

Figure 3: Investigation of nitrate reduction activity in the periplasmic extract of *Rhizobium* A1 in different conditions including: (1) addition of Ag NPs and sodium thioglycolate, (2) sodium thioglycolate free samples, (3) Ag NPs free samples and (4) controls without any treatment.

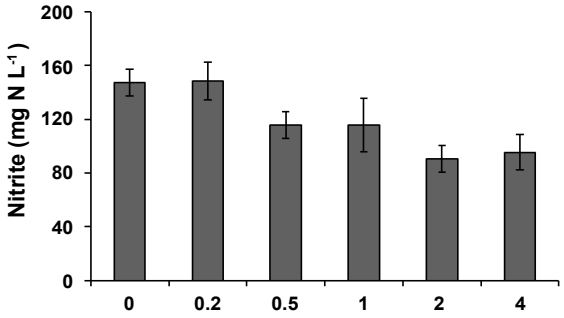

Figure 4: The periplasmic nitrate reductase activity of *Rhizobium* A1 in presence of different concentration of Ag NPs.

of Ag NPs have no significant effect on this activity. Therefore, the results of this study suggest that Ag NPs at certain concentration can be a promising biocide for applying in recirculating wastewater treatment systems. Moreover, this study introduced an efficient method for extracting periplasmic nitrate reductase enzyme. This enzyme can apply for further applications in wastewater treatment, applying in reduction of metals and formation of nanoparticles and etc.

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