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# The Impact of Nano-Silver on Bacterial Aerobic Nitrate Reductase

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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 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, it seems that entering certain concentration of Ag-NPs in environments has no significant impact on microbial aerobic denitrification as a certain part of nitrogen cycle.

**Keywords:** Silver nanoparticles; Denitrification; Nitrate reductase; Bacteria

## Introduction

Although Water is a source of life and considered as the most essential natural resources, a growing number of contaminants are entering water supplies from industrialization and human activity [1]. Since the 1970, contamination of groundwater by nitrate ($NO_3^-$) 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 waters [2-4]. According to The European Union and World Health Organization (WHO), drinking contaminated water with more than 11.3 mg/l nitrogen are considering as unsafe water source especially for infants [5]. Hence, Nitrogen removal is one of the crucial step in wastewater treatment and remediation of groundwater system contaminated with nitrate has been extensively studied over last decades. Chemical methods for nitrate reduction can be divided into two groups: nonspecific methods in which many metals can reduce nitrate and methods designed for nitrate decomposition [6]. Biological processes including microbial denitrification is considered as other promising alternative for remediation of nitrate methods [1,7]. Aerobic nitrate respiration as a part of denetrification process has been detected in numbers of facultative anaerobic heterotrophic bacteria such as *Pseudomonas sp* [7]., *Paracoccus pantotrophus* [8]., *Pseudomonas aeruginosa*, *Rhodobacter sphaeroides f. sp.* denitrificans *[7,8].* Typically, Nitrate reductase as one of major enzyme in aerobic nitrate respiration located in periplasmic or cytoplasmic membran parts of denitrifiers [9].

Recent studies show that metal ion concentration is a critical parameter that affects the ability of denitrifying bacteria to remove nitrate and nitrite [10,11]. Over the past few decades, because of antibacterial and remarkably unusual physical, chemical and biological properties [12-15], 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 [16,17]. However, these wide ranges of applications of Ag-based products caused an increasing in the risk of Ag- ions entering to the aquatic environment including marine, water supplies, and waste-water treatment systems. Therefore, Silver (Ag) can be a potential pollutant in water environment due to its toxicity, chemistry and bioavailability [12,18,19]. Few reports are available concerning the interaction of Ag-based material and ions with environmental bacterial communities [13]. 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, in *Azotobacter* and *Rhizobium* isolates. To our knowledge, the effect of Ag-based material on denitrification has not been investigated yet. This study demonstrates the aerobic reducrion of nitrate by *Rhizobium sp* and *Azotobacter sp* isolates. Afterwards, the effect of Ag-NPs on the nitrate reduction process catalyzed by native cells, cell free and periplasmic extracts of selected strains was studied. In addition to, the bactericidal activity of Ag-NPs on the studied bacteria was also evaluated.

## Materials and Methods

### Bacteria, preparation of media and nano-silver

*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 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 [20].

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 (MIC) 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 nanosilver 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 (28ºC and 37ºC) for 24 h according to each strain. Minimal bactericidal concentrations (MBC) were determined by agar plate method [21-23]. 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 ($1.5\times10^8$ 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 28ºC for 24 h with mild shaking (130 rpm). The process of nitrite assay was carried out 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 28ºC with mild shaking (130 rpm). Cells were harvested by centrifugation (4,830g for 10 min) in sterile 50mL 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 [24].

### 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 (10000 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 $NO_3$--N. Afterward, the following conditions were conducted for each isolates: (1) 0.5 g/l sodium thioglycolate and 0.2 ppm Ag-NPs were added to suspension, (2) sodium thioglycolate free samples, (3) nanosilver free samples, and also sodium thioglycolate and nanosilver 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 [5,11].

### 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 (2683g 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 (11180g, 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 tube kept at room temperature for 60 min. Nitrite was then assayed according to [25].

### Preparation of periplasmic extract and nitrate reduction activity

Bacterial cells were harvested using centrifugation at 4920g for 10 min and 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 -70ºC for further analysis as mentioned above [26]. The activity of nitrate reductase was assayed as described above.

## Results and Discussion

### Screening of nitrate reductase positive-isolates

Denitrification typically occurs under anaerobic or anoxic conditions [11,27-29]. In addition, microbially aerobic nitrate reduction has also been reported by soil bacteria such as *Pseudomonas*, *Arthrobacter*, *Moraxella* and *Aeromonas* [30-32]. 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 reducing nitrate aerobically (Table 1). Further studies were carried out using these strains.

### MIC and MBC determination of Ag-NP

Cho et al. discovered the MIC of Ag-NPs for *Staphylococcus aureus* and *E.coli* were 5 and 10 ppm, respectively [21]. In other research by Kim [12], 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 silver ion-containing Ag NPss having greater bactericidal activity against E. coli compared with *S.aureus*. Petica et al. [33] showed that stable solutions containing up to 35 ppm of Ag have significant antimicrobial and antifungal properties [33]. Choi et al. showed that 0.5 mg/L Ag has the inhibitory effect on the growth of *E.coli* PHL628-gfp in the forms of Ag NPs, $Ag^+$ ions, and AgCl colloids by 5578%, 100%, and 6676%, respectively [11]. In this study, the bactericidal activity of Ag-NP on 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 at 8 ppm. 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).

| Isolates | Nitrite content (mg N/L) | Isolates | Nitrite content (mg N/L) | Isolates | Nitrite content (mg N/L) |
|---|---|---|---|---|---|
| *Rhizobium 4R* | 0.05 | *Rhizobium PS2* | 5 | *Azotobacter P11* | 12.73 |
| *Rhizobium A3* | 11.52 | *Rhizobium A1* | 13.15 | *Azotobacter P81* | 0.05 |
| *Rhizobium 6R* | 6.36 | *Rhizobium Sin1* | 0.26 | *Azotobacter A1* | 0.1 |
| *Rhizobium A4* | 5.68 | *Rhizobium D1* | 0.1 | *Azotobacter D1* | 13.42 |
| *Rhizobium A2A* | 3.78 | *Rhizobium 1R* | 1.89 | *Azotobacter D2* | 0 |
| *Rhizobium A2B* | 10.1 | *Rhizobium 2R* | 11.36 | *Azotobacter* PHB$^+$ | 14.82 |

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

Briefly, it was found that 0.2 ppm of Ag-NPs had no effect on growth of soil bacteria. Therefore, this concentration was applied as an effective concentration of Ag-NPs for evaluation the effect of these NPs on the responsible enzyme in reduction of nitrate which is one of the key enzyme of nitrogen cycle.

| 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*.

## Effect of Ag-NPs on bacterial growth and Nitrate reduction

The effect of Ag-NPs on bacterial growth and nitrate reduction activity in culture medium was investigated. We discovered that using Ag-NPs in the concentration of 0.2 ppm had no significant effect on nitrate reduction 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 role of some metals in nitrate reduction and also by the catalytic properties Ag-based particles [6,34].

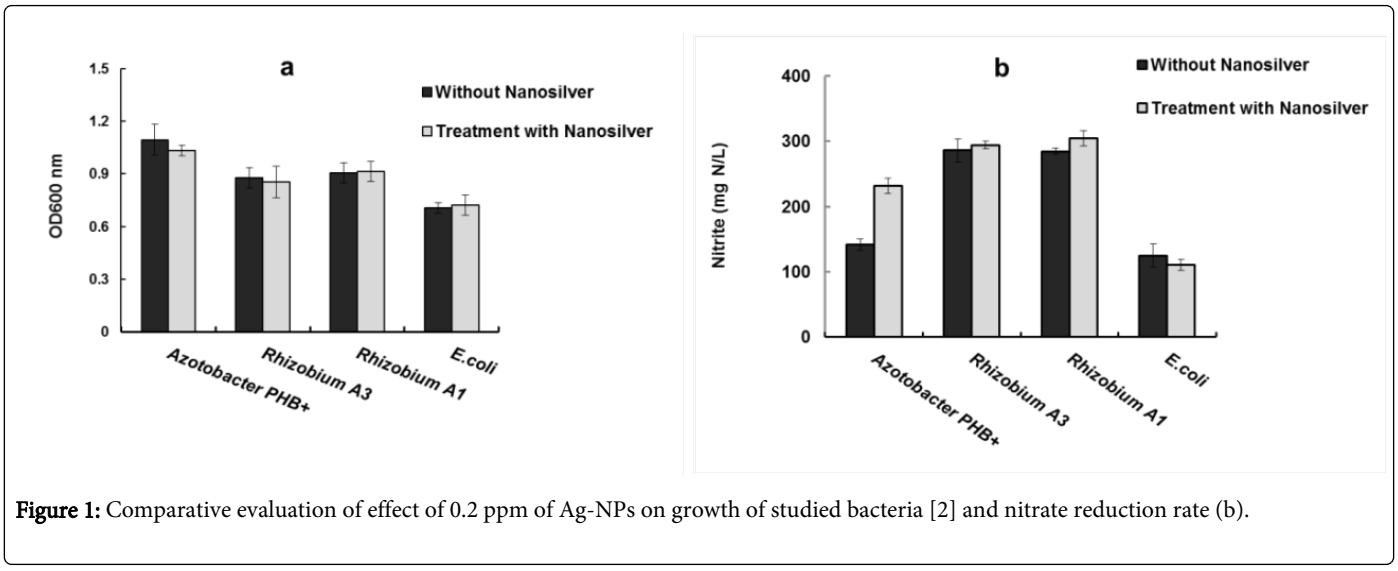


**Figure 1:** Comparative evaluation of effect of 0.2 ppm of Ag-NPs on growth of studied bacteria [2] and nitrate reduction rate (b).

## Impact of Ag-NPs with Nitrate reduction in native cells

Kariminiaae-Hamedaani et al. and Pintathong et al. were studied the nitrate reduction activity of native cells of *Pseudomonas sp.* ASM-2-3 and *Paracoccus pantotrophus* P16 [5,11]. In our study, aerobic reduction of nitrate in native cells of the strains was investigated. To evaluate the effects of a reducing agent on reduction potential of the studied strains, sodium thioglycolate was selected and its effect with or without the presence of Ag-NPs was evaluated. Figure 2 represents that the nitrate reduction activity of the selected native bacterial cell were still active after 60 minutes. Unlike *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 AgNPs 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 since it has been

confirmed that this strain is not able simply to reduce nitrate in aerobic condition.

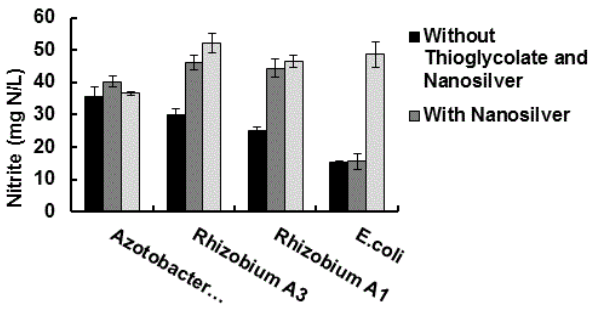


**Figure 2:** Comparative evaluation of nitrate reduction in native cells, cells treated with nanosilver and sodium thioglycolate, cells treated by AgNPs, and cells without any treatments.

## 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 cell 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. In this method by using non-physiological electron donors such as benzyl viologen ($BV^+$) and methyl viologen ($MV^+$) dyes, the location of enzymes responsible for nitrate reduction can be determined. In whole-cell assays, $BV^+$ acts as an electron donor to both membrane-bound and periplasmic nitrate reductases, whereas $MV^+$ donates electrons mainly to the periplasmic enzyme [30,35].

Periplasmic nitrate reductase has been characterized as a responsible enzyme for aerobic nitrate reduction [5,34]. 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 (but not *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 (Figures 3 and 4).

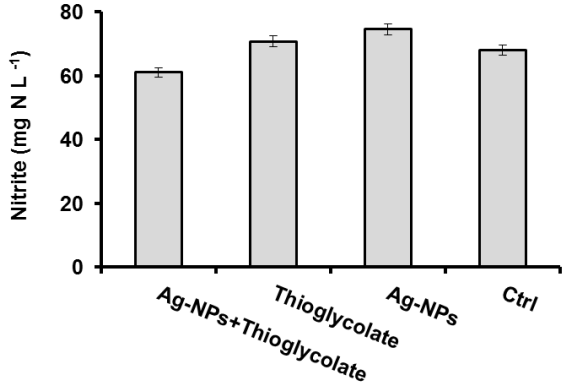


**Figure 3:** Investigation of nitrate reduction activity in the periplasmic extract of *Rhizobium* A1 in different conditions including: (1) addition of Nanosilver and Sodium thioglycolate, (2) sodium thioglycolate free samples, (3) nanosilver free samples and (4) controls, without any treatment.

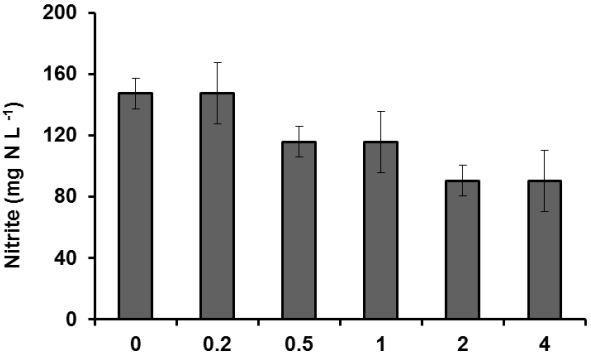


**Figure 4:** The periplasmic nitrate reductase activity of *Rhizobium* A1 in presence of different concentration of Ag-NPs.

## Conclusions

In conclusion, the 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 of nanosilver have no significant effect on this activity. Therefore, the results of this study suggest that Ag-NPs at low concentration (1 or 2 ppm) can be a promising biocide for applying in recirculating wastewater treatment systems. In another point of view, the results of this paper can be concluded that although entering low concentration of Ag-NPs may not affect biological processes in wastewater treatment systems, increasing in the concentration of Ag-NPs calls for more extensive considerations. 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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