The Effect of Nitrogen on the Reduction of Selenite to Elemental Selenium by *Pseudomonas stutzeri* NT-I

Job T. Tendenedzai, Hendrik G. Brink*

Water Utilisation and Environmental Engineering Division, Department of Chemical Engineering, University of Pretoria, Pretoria 0002.
deon.brink@up.ac.za

Selenium is a ubiquitously occurring natural mineral, however, an upsurge in anthropogenic sources has caused its release into the environment to aggravate thereby exceeding the proposed World Health Organisation (WHO) limit of 40 µg.L^{-1}. The soluble selenium oxyanions, selenate (SeO_4^{2-}), and selenite (SeO_3^{2-}), cause acute and chronic toxicity to living organisms in aquatic environments. In addition, they readily bio-accumulates, even at low concentrations. Therefore, the attenuation of effluent concentrations to acceptable levels prior to discharge into local water bodies is necessary. Selenium bioremediation is emerging as one of the most cost-effective treatment options.

In this article, the selenium reducing bacterium *Pseudomonas stutzeri* NT-I was studied for selenite reduction. Aerobic batch reduction experiments were carried out with the bacteria isolate suspended in glucose supplemented mineral salt media (glucose-MSM). The experiments were performed under previously determined optimum conditions of pH 7, temperature of 37 °C, salinity of 5 g.L^{-1} and glucose of 10 g.L^{-1}. To determine the effect of nitrogen addition on the reduction of selenite to elemental selenium (Se(0)), the reduction experiments were performed in the presence and absence of 1.604 g.L^{-1} ammonium chloride (nitrogen source). In the reduction of 0.5 mM SeO_3^{2-}, average biomass based selenite reduction rates of 0.0014 mmol.(g.h)$^{-1}$ in the presence of nitrogen as compared to 0.0012 mmol.(g.h)$^{-1}$ in the absence of nitrogen were measured. For a higher concentration such as 10 mM SeO_3^{2-}, average biomass based selenite reduction rates of 0.05 mmol.(g.h)$^{-1}$ in the presence of nitrogen as compared to 0.031 mmol.(g.h)$^{-1}$ in the absence of nitrogen were measured. This indicated that the increased initial reduction rate was a result of both increased biomass amount as well as increased biomass activity in response to increased selenite concentration.
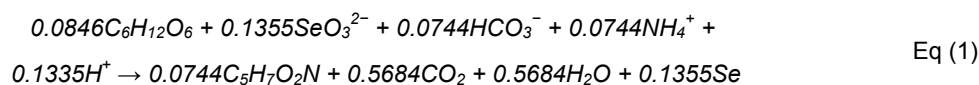
1. Introduction

Selenium (Se) is an essential element in living organisms and is present in the earth's crust at an estimated amount of 0.05-0.5 mg Se.kg−1 (Lemly, 2004). It is estimated that between 37.5 % and 40.6 % of total selenium emissions to the atmosphere are due to anthropogenic activities (Lenz and Lens, 2009). Slight changes in its concentration can easily be a tipping scale for it transforming from being safe to toxic (Brozmanova et al., 2010). The World Health Organization proposes a value of 40 µg.L^{-1} for countries without a specified legislative framework in place (Brink et al., 2018). Biological methods are emerging as the most cost-effective treatment option to remove selenium by either reducing or volatilising the oxyanions (Golder-Associates-Inc, 2009). The biological method considered in this research involves using *Pseudomonas stutzeri* NT-I, a selenium reducing bacteria, which converts soluble selenium ions found in water into their elemental state before being removed as a precipitate. *Pseudomonas stutzeri* NT-I is one of the many *Pseudomonas* species reported to be capable of aerobically metabolising selenium oxyanions (Lortie et al., 1992). Recent studies also confirmed the use of the same strain in an aerobic batch reduction of selenium oxyanions under optimum conditions of a temperature of 37 °C, pH 7-8 and salinity less than 5 g.L^{-1} NaCl (Brink et al., 2018).

Nitrogen makes up to 14 % of dry cell weight and is used to synthesise amino acids, DNA, and RNA (Kango, 2010). Hence, the absence of excess nitrogen is assumed to correlate to little or no increase in bacterial biomass. The effect of the NH₄Cl (nitrogen source) was investigated to establish whether or not selenite

reduction was depended primarily on microbial growth; usually the reduction of selenite (SeO_3^{2-}) to elemental Se involves the consumption of a carbon and nitrogen source coupled with the synthesis of biomass. The generic formula for the process according to (Ji and Wang, 2017) is:

$$0.0846C_6H_{12}O_6 + 0.1355SeO_3^{2-} + 0.0744HCO_3^- + 0.0744NH_4^+ +$$
$$0.1335H^+ \rightarrow 0.0744C_5H_7O_2N + 0.5684CO_2 + 0.5684H_2O + 0.1355Se$$

Eq (1)

If the reduction mechanism is found to proceed independent of nitrogen addition, this would imply a different, modified, or additional SeO_3^{2-}-reduction mechanism present in the system.

This study investigates the effect of the presence and absence of nitrogen in selenite reduction; SeO_3^{2-} is considered significantly more toxic than SeO_4^{2-} and is therefore of greater environmental concern (Ecimovic et al., 2018). The study was done at the previously determined optimal conditions (Brink et al., 2018), for SeO_3^{2-} concentrations of 0.5, 1, 2, 5, and 10 mM.

2. Materials and methods

2.1 Bacteria storage and cultivation

The bacterial strain was originally isolated from the drainage water of a selenium refinery plant in Hyogo, Japan by Masashi Kuroda and his team (Kuroda et al., 2011). The strain used in this study, *Pseudomonas stutzeri* NT-I, was furnished by the NITE Patent Microorganisms Depository (NMPD) in Chiba Ken, Japan. The culture was cultivated in Tryptone Soy Broth (TSB) for 24 h at 28 °C on a rotary shaker at 120 rpm (FSIM-SPO8, Labcon, Johannesburg), followed by glycerol addition before placing in the -70 °C storage chamber (Wessels and Chirwa, 2017). To revive the strain, the frozen vials were taken from the -70 °C storage chamber and allowed to thaw. The thawed strain was then inoculated it into the desired volume of TSB medium in a flask and capped with foil and cotton wool. The flask was placed on a rotary shaker at 120 rpm for 24 h at 37 °C.

2.2 SeO_3^{2-}-reduction rate batch experiments

For SeO_3^{2-}-reduction, two different batches containing the same SeO_3^{2-} concentrations in glucose-MSM were prepared. The first batch (glucose-MSM_A) had 1.604 g.L^{-1} NH_4Cl and the second batch (glucose-MSM_X) did not contain NH_4Cl or any other source of nitrogen. After 24 h of cultivation, the cells were concentrated and harvested by centrifugation (6,000 rpm, 15 mins, room temperature) and then washed with sterile physiological saline (0.85 % NaCl), before being re-suspended in 100 mL serum bottles, each containing either glucose-MSM_A or glucose-MSM_X (with different SeO_3^{2-} concentrations (0.5, 1, 2, 5, 10 mM)). Glucose-MSM at pH 7 consisted of (Brink et al., 2018): 2 g.L^{-1} glucose, 10 mM K_2HPO_4, 20 mM KH_2PO_4, 0.8 mM Na_2SO_4, and 0.2 mM $MgSO_4$, 50 nM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO), 25 nM $FeSO_4$, 0.1 nM $ZnCl_2$, 0.2 nM $CuCl_2$, 0.1 nM NaBr, 0.05 nM Na_2MoO_2, 0.1 nM $MnCl_2$, 0.1 nM KI, 0.2 nM H_3BO_3, 0.1 nM $CoCl_2$, and 0.1 nM $NiCl_2$). All chemicals were sourced from Merck (Merck, Darmstadt, Germany) unless otherwise specified.

To ensure that all other conditions were kept constant during the reduction of the different SeO_3^{2-} concentrations, the different batches, i.e. the batches with NH_4Cl and without, were run concurrently. In addition, the biomass of approximately 7 g.L^{-1} was added at the start of each reduction experiment and also kept constant for the varying concentrations. A control for each of the different concentrations was subjected to the same conditions in the absence of the bacterial strain to determine whether any chemical SeO_3^{2-}-reduction would occur.

The serum bottles were incubated on a rotary shaker (120 rpm, 37 °C). 1 mL samples were extracted at specific time intervals throughout the duration of the experiment and analysed for the total selenium before being centrifuged (6,000 rpm, 15 mins, room temperature). After centrifugation, the supernatant and the biomass-Se(0) pellet were separated and each stored in individual vials at -70 °C for subsequent analysis. The pellets were thawed and digested with concentrated hydrochloric- (HCl) and nitric acid (HNO_3) to re-dissolve the elemental selenium. The supernatant and dissolved pellets were analysed for Se concentrations.

2.3 Analytical methods

The total Selenium (Se) concentration was determined in the Varian AA–1275 Series Flame AAS (Varian, Palo Alto, CA (USA)) at 196.03 nm wavelength equipped with a 290 mA selenium lamp. For each specific time interval that a sample had been taken, the total Se was analysed in the sample before centrifugation. After centrifugation, the supernatant was separated from the formed pellet before analysis by the AAS once again

to ascertain the amount of SeO_3^{2-} that had not yet been reduced. The selenium pellet was then digested back to SeO_3^{2-} before being analysed as well. This was done in order to do a selenium balance and ensure that all the selenium absent in the supernatant had been converted to Se(0) in the pellet.

3. Results and interpretation

Figure 1 shows that within 0.5 h of commencing the reduction experiments, a red colour, which is an indication of the formation of Se(0) was more intense for experiments with NH_4Cl compared to those without. This remained the case throughout the duration of the experiment. This observation was attributed to a higher initial reduction rate in the presence of the nitrogen source. The rate was higher in the presence of a nitrogen source due to microbial growth because bacterial biomass is a nitrogen sink. As strain NT-I is the catalyst in selenium reduction, this meant that the amount of catalyst increased as well, resulting in a faster reaction rate. In the experiments without a nitrogen source, it was assumed that the rate of biomass change was negligible and therefore had little effect on the reaction rate.
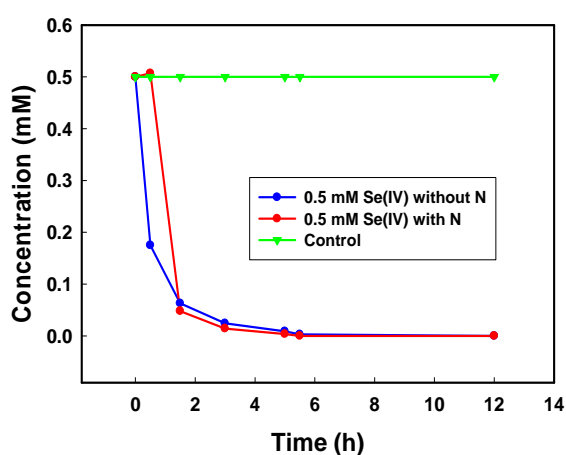
a

b

Figure 1: Shows the changes at different time intervals in the intensities of a red colour which is an indication of the formation of Se(0). (a) Experiments without NH_4Cl. (b) Experiments with NH_4Cl.
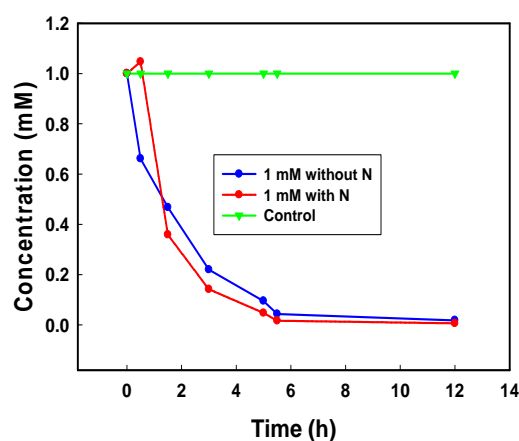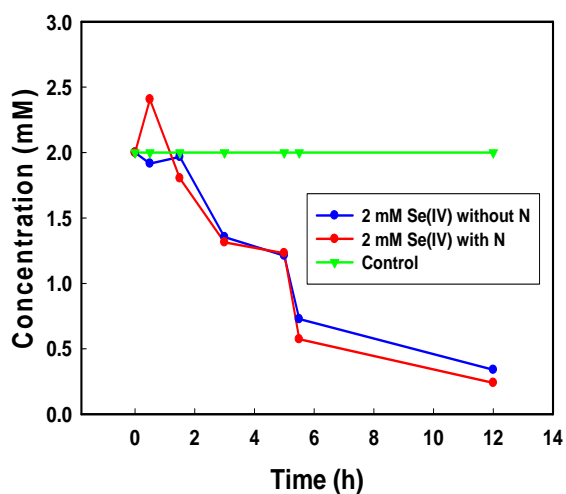
a

b

Figure 2: Results showing the effect of the absence and presence of nitrogen in reducing SeO_3^{2-} by strain NT-I. (a) Initial SeO_3^{2-} concentration of 0.5 mM. (b) Initial SeO_3^{2-} concentration of 1 mM.
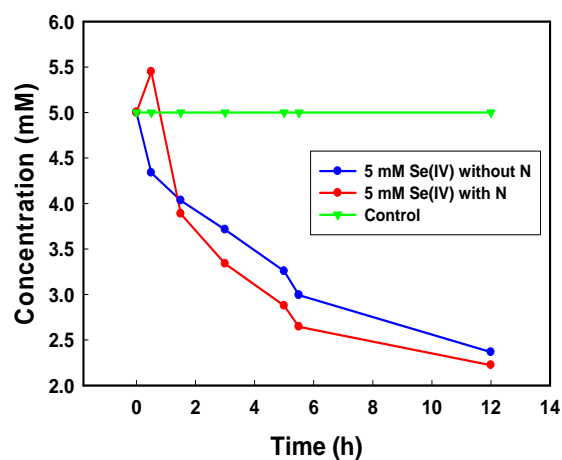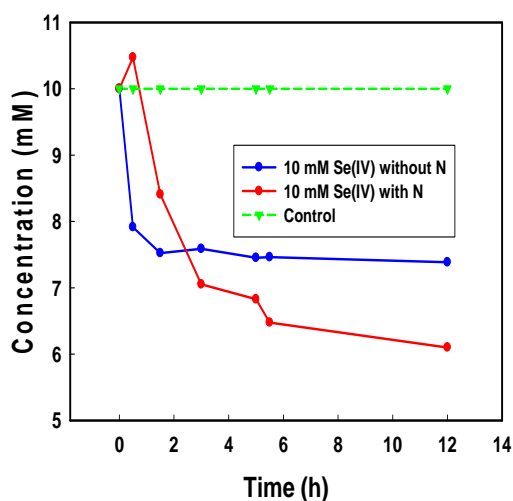
a

b

Figure 3: Results showing the effect of the absence and presence of nitrogen in reducing SeO_3^{2-} by strain NT-I. (a) Initial SeO_3^{2-} concentration of 2 mM. (b) Initial SeO_3^{2-} concentration of 5 mM.
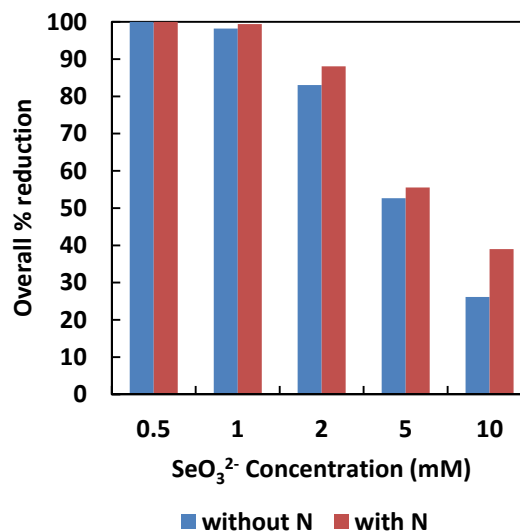
a

b

Figure 4: (a) Results showing the effect of the absence and presence of a nitrogen in reducing 10 mM SeO_3^{2-} by strain NT-I. (b) % reduction for different SeO_3^{2-} concentration at the end of the experiment.

Figures 2(a), (b); 3(a), (b) and 4(a) show the results of the comparison in reduction rate between the experiments with a nitrogen source and the ones without, for each of the different SeO_3^{2-} concentrations. The bacteria took a shorter period of time in reducing lower concentrations of SeO_3^{2-} as compared to the higher amounts. In the experiments in which both glucose and NH_4Cl (nitrogen source) were present, the results demonstrated an initial lag followed by a high rate of SeO_3^{2-}-reduction for each of the different concentrations. In comparison the runs which had no nitrogen source exhibited an immediate drop in Se followed by a rapid decrease in the reduction rate after 1 hour, most prominently seen at high concentrations (Figures 3(b) and 4(a)). In the reduction of 0.5 mM SeO_3^{2-} for example, the average biomass based SeO_3^{2-}-reduction rates of

0.0014 mmol.(g.h)$^{-1}$ in the presence of nitrogen as compared to 0.0012 mmol.(g.h)$^{-1}$ in the absence of nitrogen were measured. For a higher concentration such as 10 mM SeO_3^{2-}, average biomass based SeO_3^{2-}-reduction rates of 0.05 mmol.(g.h)$^{-1}$ in the presence of nitrogen as compared to 0.031 mmol.(g.h)$^{-1}$ in the absence of nitrogen were measured.

Figures 2(a), (b); 3(a), (b) and 4(a) also show that experiments with NH_4Cl exhibited an initial spike in SeO_3^{2-} concentrations which occurred within the first 0.5 h. The spike is a lag in selenium reduction which is likely a result of an increase in the bacterial biomass in the presence of a nitrogen source early on in the reaction. In addition, the assumption of an initial lag in the reduction of higher SeO_3^{2-} concentrations was also observed by Kuroda et al., (2011) when using the same *Pseudomonas* strain. However, the same phenomenon of an initial spike was not observed in the experiments which had no NH_4Cl (where an increase in biomass is negligible). A similar phenomenon of an initial spike was also observed in a recent study of bacterial Cr(VI) reduction using algae. Although the evidence was not conclusive, the spike was attributed to be a response of the algae towards Cr(III) in the solution, or a change in oxidation conditions (Roestorff and Chirwa, 2018).

The total average Se in the system did not remain constant but rather, it decreased as the reaction progressed. This decrease is an indication that not all the SeO_3^{2-} added at the start of reaction remained in solution or precipitated as Se(0). Kagami et al., (2013) found out that *Pseudomonas stutzeri* NT-I is capable of aerobically transforming SeO_4^{2-}, SeO_3^{2-}, and elemental selenium into dimethyl diselenide and dimethyl selenide. Their study showed that these organic species are temporarily accumulated in the aqueous phase and then transferred into the gaseous phase and lost into the atmosphere unless trapped. This, therefore, could explain why the total selenium decreased over time.

4. Conclusion

In this study, the effect of the presence and absence of a nitrogen source in the reduction of different SeO_3^{2-} concentrations ranging from 0.5 mM to 10 mM was investigated. The results obtained showed that the rate of reduction was higher in experiments which had a nitrogen source as compared to those which did not. However, the observed trend was that the reduction rate increased with increase in SeO_3^{2-} concentration as opposed to the overall percentage SeO_3^{2-}-reduction.

In the reduction of 0.5 mM SeO_3^{2-}, reduction rates of 0.0014 mmol.(g.h)$^{-1}$ and 0.0012 mmol.(g.h)$^{-1}$ were observed in the presence and absence of a nitrogen source respectively. In addition, the overall percentage reduction was 100% in both conditions after 12 h. For a higher concentration such as 10 mM SeO_3^{2-}, the difference in the reduction rates and overall percentage SeO_3^{2-} reduction became more significant. The reduction rate of 0.05 mmol.(g.h)$^{-1}$ and overall SeO_3^{2-}-reduction of 39 % was measured after 12 h in the presence of nitrogen. When nitrogen was not present, the reduction rate was 0.031 mmol.(g.h)$^{-1}$ and the overall SeO_3^{2-}-reduction was 26.17 %. It can therefore be concluded that *P. stutzeri* NT-I can successfully reduce SeO_3^{2-} to Se(0) in the presence or absence of nitrogen. Even though the amount of reduction observed in both conditions is comparable at lower SeO_3^{2-} concentrations, the reduction rate is initially faster in the presence of a nitrogen source.

The presence of nitrogen is attributed to correlate with an increase in biomass thus, an increase in the initial reaction rate. However, it is not clear whether this increase in biomass is directly proportional to cell metabolic activity. This is an area which still requires further investigation as it will assist in better understanding the mechanism employed *by Pseudomonas stutzeri* NT-I in selenium reduction. These results further question the application of the chemical mechanism presented by Ji and Wang (2017) to this organism, possibly indicating a non-growth SeO_3^{2-} mechanism present in the system.

In addition, this leaves further room to evaluate the effectiveness of a continuous system which can also be accounted for selenium losses due to volatilisation.

References

Brink H. G., Wessels C. E., Chirwa E. M. N., 2018, Pseudomonas stutzeri NT-I: Optimal Conditions for Growth and Selenate Reduction, Chemical Engineering Transactions, 70, 1651-1656.

Brozmanova J., Manikova D., Vickova V., Chovanec M., 2010, Selenium: a double-edged sword for defense and offence in cancer, Arch Toxicol, 84, 919-38.

Ecimovic S., Velki M., Vukovic R., Camagajevac S. I., Petek A., Bosnjakovic R., Grgic M., Engelmann P., Bodo K., Filipovic-Marijic V., Ivankovic D., Erk M., Mijosek T., Loncaric Z., 2018, Acute toxicity of selenate and selenite and their impacts on oxidative status, efflux pump activity, cellular and genetic parameters in earthworm Eisenia andrei, Chemosphere, 212, 307-318.

Golder Associates, Inc. 2009. Literature review of treatment technologies to remove selenium from mining influenced water. Report to Teck Coal Limited, Calgary, AB. <http://www.namc.org/docs/00057713.PDF> accessed 24.07.2018

Ji Y., Y.-T. Wang., 2017, Selenium Reduction by Batch Cultures of Escherichia coli Strain EWB32213. J. Environ. Eng, 143(6), 04017009.

Kagami T., Narita T., Kuroda, M., Notaguchi E., Yamashita M., Sei K., Soda S., Ike M., 2013, Effective selenium volatilization under aerobic conditions and recovery from the aqueous phase by Pseudomonas stutzeri NT-I, Water Res, 47, 1361-8.

Kango N., 2010, Textbook of Microbiology. New Delhi, Bangalore, I. K. International Pvt Ltd, 152.

Kuroda M., Notaguchi E., Sato A., Yoshioka M., Hasegawa A., Kagami T., Narita T., Yamashita M., Sei K., Soda S., Ike M., 2011, Characterization of Pseudomonas stutzeri NT-I capable of removing soluble selenium from the aqueous phase under aerobic conditions, Journal of Bioscience and Bioengineering, 112, 259-64.

Lemly A. D., 2004, Aquatic selenium pollution is a global environmental safety issue, Ecotoxicol Environ Saf, 59, 44-56.

Lenz M. & Lens P. N., 2009, The essential toxin: the changing perception of selenium in environmental sciences, Sci Total Environ, 407, 3620-33.

Lortie L., Gould W. D., Rajan S., McCready R. G., Cheng K. J., 1992, Reduction of Selenate and Selenite to Elemental Selenium by a Pseudomonas stutzeri Isolate. Appl Environ Microbiol, 58, 4042-4.

Roestorff M. & Chirwa E., 2018, Bacterial Cr(VI) Reduction with Internal Carbon Recirculation Using Freshwater Algae as Primary Producers, Chemical Engineering Transactions, 64, 457-462.

Wessels, C. E. & Chirwa, E. M. N. 2017. Reduction of selenium by Pseudomonas Stutzeri NT-I: Growth, Reduction and Kinetics, J bioremediat biodegrade, 8: 391.