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Formation of Elemental Selenium Nanoparticles (SeNPs) from the Reduction of Selenite (SeO_3^{2-}) by a Pure culture of *Pseudomonas stutzeri* NT-I

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Selenite (SeO_3^{2-}), the most toxic and most reactive selenium (Se) oxyanion can be reduced to elemental selenium (Se^0) by a variety of bacteria, including *Pseudomonas stutzeri* NT-I. Se^0 is in the form of selenium nanoparticles (SeNPs), which are relatively less toxic. The development of SeNPs has gained commercial interest as they have various applications. Examples include uses in the photoelectrical and medicinal fields. In this study, *Pseudomonas stutzeri* NT-I, a bacterium exhibiting high selenite tolerance and reduction capacity, was used in aerobic batch reduction experiments (36 h, 120 rpm, 35±2 °C, pH ≥7). Thereafter, the SeNPs were harvested, recovered and analysed. Transmission Electron Microscopy (TEM) analysis indicated the biogenesis of extracellular spherical electron-dense SeNPs, as confirmed by abiotic control. Other key results indicated that more Se^0 was recovered when reducing higher SeO_3^{2-} concentrations. This implies that more SeO_3^{2-} had been reduced for the 10 mM SeO_3^{2-} concentration compared to the 0.5 and 2 mM SeO_3^{2-} concentrations, even though there was constant biomass (5 $g.L^{-1}$) in all experiments. Findings from this study confirm that strain NT-I can reduce SeO_3^{2-} to Se^0 which can be recovered for other applications. Moreover, it sheds more light on the mechanism of reduction employed by the bacteria as it appears to occur extracellularly as per the TEM images.

1. Introduction

Selenium (Se) is an essential micronutrient for both plants and animals at low concentrations. However, concentrations below 40 $\mu g.d^{-1}$ are considered a dietary deficiency and once its concentration exceeds 400 $\mu g.d^{-1}$, it becomes toxic to humans (Nancharaiah and Lens, 2015). Industrial practices such as petrol refining and mining as well as other agricultural discharges (Dungan and Frankenberger, 1998) lead to selenium laden water, which is a major environmental concern. In the aquatic environment; the selenium oxyanion, selenite ((SeO_3^{2-}) is known to be toxic, soluble in water and it bioaccumulates in the food chain, even at low concentrations (Stewart et al., 2010, Kuroda et al., 2011). Due to an increase in anthropogenic sources, various treatment technologies have been developed overtime. Selenium treatment technologies can be divided broadly into three categories mainly; physical treatment, chemical treatment; and biological treatment (Gusek et al., 2009).

Some bacteria are known to reduce the SeO_3^{2-} to elemental selenium (Se^0) which is biologically inert and relatively less toxic because of its low bioavailability (Garousi, 2015, W. J. Hunter and Manter, 2009). The microbially produced Se^0 always forms elemental selenium nanoparticles (SeNPs). These are typically nanospheres with a diameter of up to 400 nm (Oremland et al., 2004). SeNPs are of particular interest because once recovered, they can be used in a wide variety of applications. These include in enhancing absorption, cytotoxicity and antioxidant activity of other materials (Mehdi et al., 2013, STDA, 2010). In addition, they can also be used as medical devices for drug release, additives in skincare products and agricultural supplements (Schrauzer, 2001). In this study, Se^0 was recovered from selenite reduction by *Pseudomonas stutzeri* NT-I.

Upon recovery, the Se^0 were quantified and analysed in order to assess the suitability of bioremediations as a potential source for SENPs as wee as to establish the potential reduction mechanisms employed by the bacteria.

2. Materials, methodology and analytical methods

The experiments were done in triplicate. The control had a desired selenite concentration but without cells and the conical flasks had selenite, cells and glucose supplemented mineral salt medium with ammonia. Before the experimental runs, the bacteria were cultivated for 24 h. Thereafter, 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 resuspended in the different conical flasks. Selenite was added as Na_2SeO_3 (Sigma-Aldrich, St. Louis, MO) and different selenite concentrations (0.5, 2, and 10 mM) were added at the start of each experimental run. Aerobic batch reduction experiments were conducted (36 h, 120 rpm, 35±2 °C, pH ≥7). Samples were taken at different time intervals throughout the 36 hours the experiment was run. Prior to going through various analytical methods, each of the extracted samples was centrifuged and the supernatant was separated from the pellet.

The total Se was quantified using a Varian AA–1275 Series Flame AAS (Perkin Elmar, Varian, Palo Alto, CA (USA)) at 196.03 nm wavelength equipped with a 290 mA selenium lamp. SeO_3^{2-} which had not yet been reduced to Se^0 was measured in the supernatant. In order to quantify the amount of elemental selenium in the solid phase formed from the reduction of the various selenite concentrations, the pellets extracted after centrifugation of each sample were used. After centrifugation, the pellets containing both biomass and red elemental selenium were resuspended in ultrapure water. Thereafter, the mixtures were poured into digestion vials before adding 2 ml of both 70 % HNO_3 and 32 % HCl. The vials were then thoroughly mixed by vortexing before putting them in a thermoreactor for digestion (60 min, 100 °C). After digestion, this re-oxidised form was analysed using AA analysis for total Se. This was necessary in order to do a Se balance and quantify the Se^0 recovery.

For TEM (transmission electron microscopy) analysis, the bacterial cultures were grown in both the control (without selenite) and in the presence of 10 mM selenite. The cells were harvested using centrifugation (6000 rpm, 5 min) and fixed with 2.5 % glutaraldehyde in 1.5 M phosphate-buffer for 30 mins. The cells then underwent post-fixation treatment in 1% osmium tetroxide for 30 min. The samples were subsequently processed using standard procedures (Tizro et al., 2019) and mounted on copper grids. The analysis was conducted at 200 kV FE (Field Emission) with a FEGTEM: Jeol 2100 (Peabody, MA, USA) transmission electron microscope.

Cellular metabolism comprises of controlled biochemical processes that allow organisms to grow and reproduce, maintain their structures as well as respond to environmental variations. The metabolic activity (MA) of the bacteria was measured using the MTT colorimetric assay as described by (Tendenedzai and Brink, 2019). The glucose amount was measured using a CONTOUR®PLUS blood glucose monitoring system (CP BGMS, Bayer, Ascensia Diabetes Care, Basel) as described by (Tendenedzai et al., 2020). The Oxidation Reduction Potential (ORP) was measured by an ORP probe (PL700AL, Pacific Sensor Technologies, Neutron Place Rowville, Victoria).

3. Results and interpretation

3.1 SeO_3^{2-} reduction and Se^0 formation assay

During selenite reduction by *Pseudomonas stutzeri* NT-I, it is converted to elemental selenium. Figure 1 shows the red colour at 36 h which is an indication of the formation of Se^0. The trend observed during SeO_3^{2-} reduction between the various initial concentrations was not uniform. This nonuniformity is an indication that the trends and the amount of reduction seem to be influenced by the amount of selenite to be reduced, like an increased biomass activity in response to increased selenite concentration (Tendenedzai and Brink, 2019b). Moreover, strain NT-I appeared to respond the higher selenite concentrations, and this is likely a defense mechanism in which the bacteria try to detoxify its surroundings as rapidly as possible. This may also be reason why the SENPs are located outside the cells (Figure 3).

The nonuniformity was also observed visually by the colour changes observed during the reduction of the different selenite concentrations. The red colour, which is an indication of the formation of Se^0, was more defined for the 10 mM SeO_3^{2-} concentration than it was for the 0.5 mM concentration. This suggested that more selenite had been reduced for a higher concentration compared to the lower one, for the same time period. The results were backed by measurements obtained from the AA analysis and they are shown in Figures 2 (a), (b) and (c). The ORP (Oxidation Reduction Potential) was also monitored in tandem with SeO_3^{2-} reduction because like other metals, selenium's redox status often has bearing on its toxicity. The variations in the ORP values observed across the three selenite concentrations are indicated in Figure 2 (d). An increase in ORP correlated with a decrease in the SeO_3^{2-} concentration and vice versa. This was explained by the fact that the lower the

ORP, the greater the concentration of the reducing agent (glucose). As more reduction occurred, this would result in an increase of the oxidising agent (Se^0), hence the higher ORP. Recent studies have shown the use of redox potential measurements as a monitoring technique for metal reduction (Ioka et al., 2016). Therefore, more research has to be conducted to find ways of using the SeO_3^{2-}/Se^0 redox couple in the same manner.
As stated earlier, the pellets obtained after centrifugation were digested before analysis with the AA. The red colour disappeared after digestion indicating that all the red elemental selenium had been reoxidised back the selenium oxyanions. Measurements for Se^0 are also displayed in Figures 2 (a), (b) and (c).

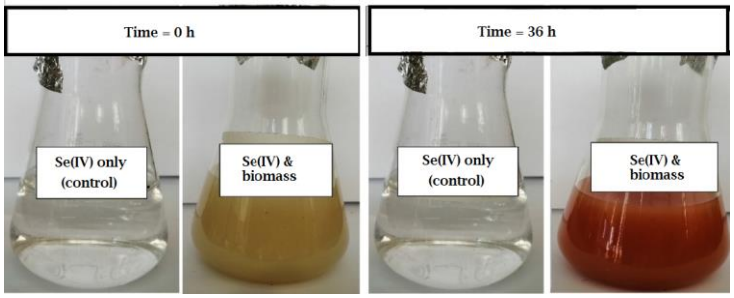

Figure 1: Colour variations in SeO_3^{2-} reduction at the start (0 h) and the end (36 h)

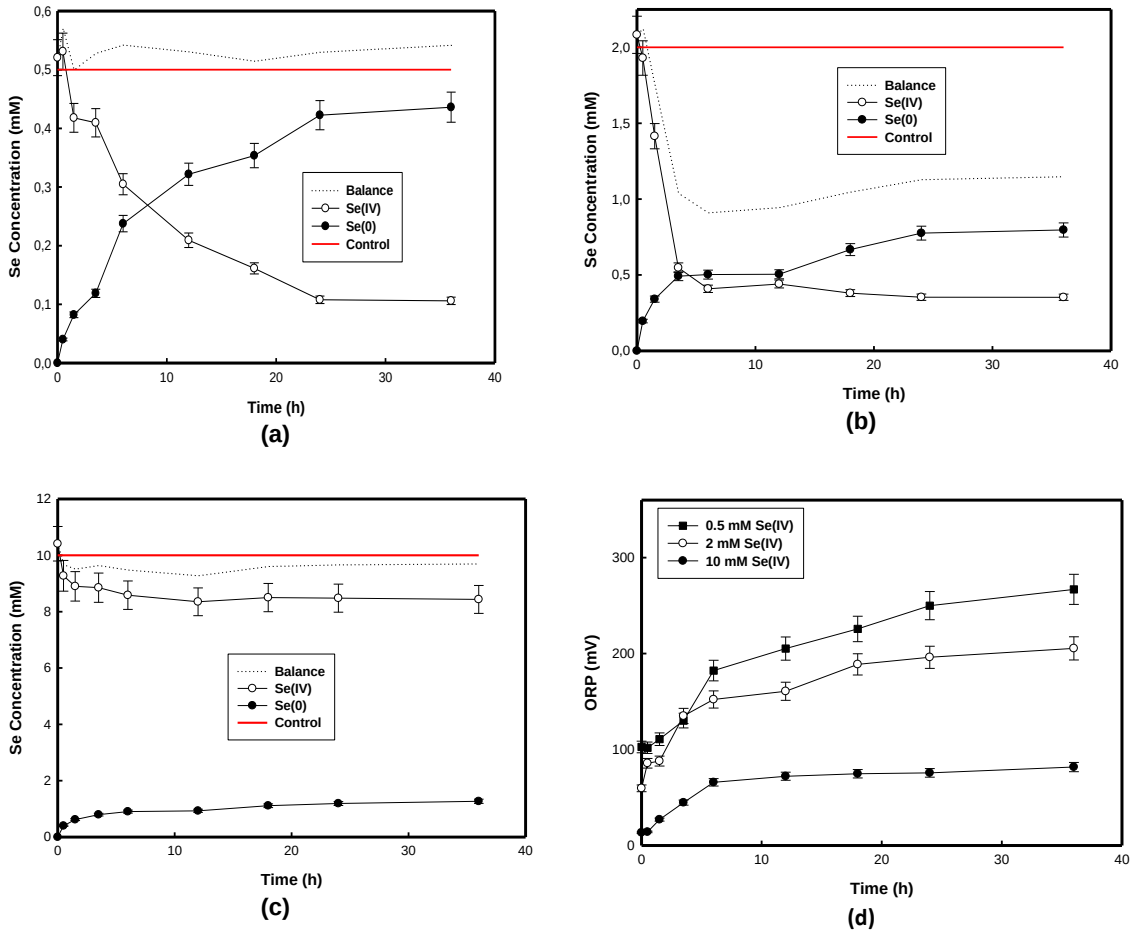

Figure 2: (a), (b) and (c); SeO_3^{2-} reduction and Se^0 formation profiles; (d) Variations in ORP across; the different SeO_3^{2-} concentrations.

3.2 Selenium balance

Table 1: Se balance for the different SeO_3^{2-} concentrations

SeO_3^{2-} concentration (mM)	SeO_3^{2-} not reduced (mM)	Se^0 recovered (mM)	Total Se (mM)
0.5	0.101	0.418	0.519
2	0.339	0.765	1.104
10	8.102	1.217	9.319

Table 1 shows the total selenium balance done across the different initial concentrations. For the 0.5 mM SeO_3^{2-}, the total Se is above the initial 0.5 mM concentration. The difference is marginal and the anomaly was attributed to experimental errors during measurement and digestion. Therefore, it was concluded that for the 0.5 mM concentration, approximately all of the reduced SeO_3^{2-} was able to be recovered as Se^0.

For the 2 mM concentration, the total Se did not add up to the initial concentration. Approximately 0.896 mM SeO_3^{2-} could not be accounted for. Similarly, 0.681 mM SeO_3^{2-} could also not be accounted for the 10 mM concentration. It was concluded that the values were too high to be attributed to experimental error alone and the large differences may have been due to volatisation.

The assumption of volatisation has also been demonstrated by Kagami et al., 2013 who concluded that *P stutzeri* NT-I was capable of volatising selenium oxyanions after prolonged incubation. They suggested that the bacteria transforms the oxyanions into DMDSe and DMSe (Kagami et al., 2013). These compounds would then accumulate in the aqueous phase prior to the gaseous phase. This therefore can explain the high margin of differences in the selenium balance as the system employed in this study did not trap any gaseous substances during the reduction process.

TEM analysis was also conducted in order to verify the location and presence of SeNPs. Figure 3 (b) confirms the presence of electron-dense SeNPs after 36 h. Similar findings from another study using a different strain also revealed that the majority of the SeNPs were distributed outside the cell (Wang et al., 2018).

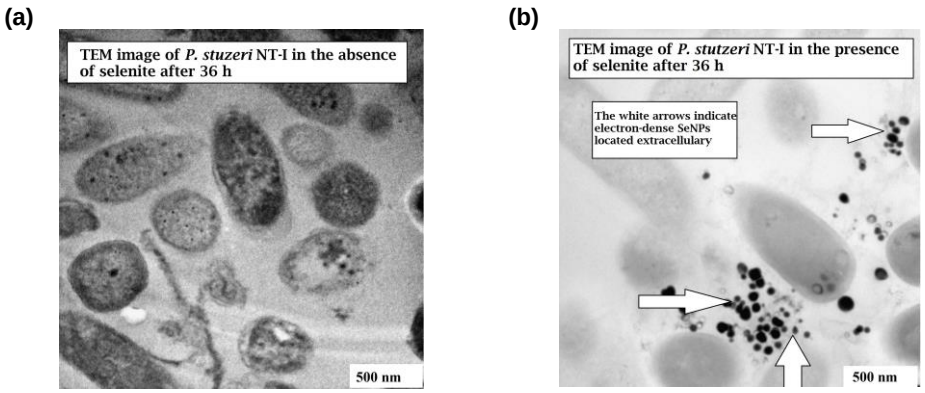

Figure 3: TEM images for P. stutzeri NT-I grown in the;(a) absence of selenite, (b) presence of 10 mM selenite after 36 h.

3.3 Cell viability assay

The variations in metabolic activity (MA) are depicted in Figure 4 (a). The MA was used as a proxy for viable cell count for the different selenite concentrations that were reduced. The metabolic activity was also used as a depiction of the growth profiles for the bacteria. The cells used for the reduction of the selenite were not selenium-adapted prior to their use. Therefore, an inhibition in growth was noticeable for all the different selenite concentrations. In the reduction of 0.5 mM SeO_3^{2-}, the lag due to inhibition was noticeable for only the first 0.5 h. Thereafter, the metabolic activity rose by 47 %.

For a slightly higher selenite concentration of 2 mM, the bacteria took approximately 3.5 h to acclimatise to the selenium concentration. During this time, the metabolic activity reduced by 21.2 %. Thereafter, the MA increased by more than 100 % and remained high for a large portion of the 36 h long experiments. There was a rapid decrease in the MA for the 10 mM SeO_3^{2-} concentration within the first 3.5 h. Approximately 80 % of metabolic activity was lost within this period. Thereafter, the activity remained somewhat stationary with the only notable decrease being at the end of the run. Therefore, it can be concluded from the results that the amount of

selenite to be reduced impacted the metabolic activity of the cells. Cell activity was higher with low SeO_3^{2-} concentration and it was lower for higher SeO_3^{2-}. This might be because the growth of bacteria cells was inhibited in the presence of elevated selenite concentrations.

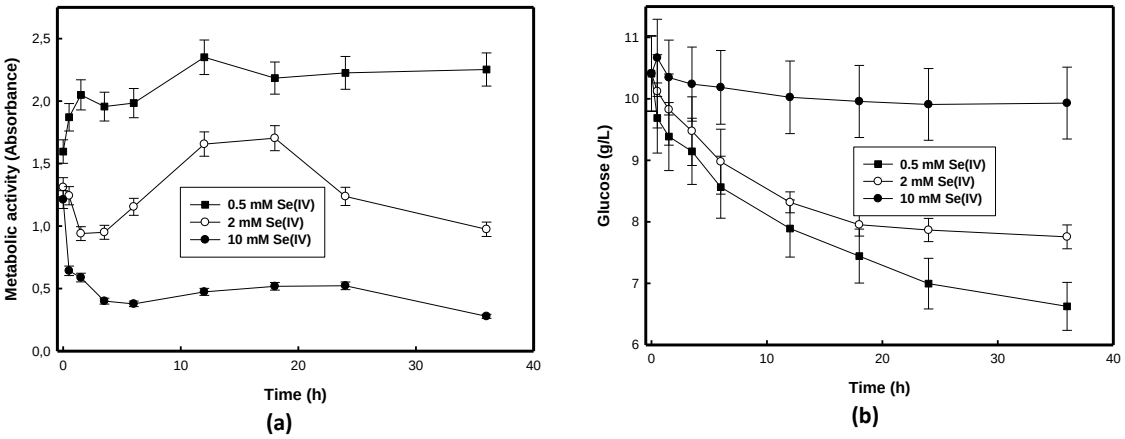

Figure 4: (a) Metabolic activity and (b) Glucose consumption variations; across the different SeO_3^{2-} concentrations

3.4 Glucose consumption assay

Glucose was not a limiting substrate as it was never depleted regardless of the initial SeO_3^{2-} concentration to be reduced. To add on, the amount of glucose consumed by the microbes was inversely proportional to the SeO_3^{2-} concentration to be reduced. Of the 10 $g.L^{-1}$ of glucose added at the start of each reduction experiment, an average amount of approximately 36 %, 29 % and 5 % was utilised in the reduction of 0.5, 2 and 10 mM SeO_3^{2-} concentrations respectively. The observed trend was likely due to a variety of factors.

Firstly, less glucose might have been utilised in the reduction of higher selenite concentrations because of the reduced number of viable bacterial cells. This is because according to the metabolic activity trends described earlier, the death rate of the cells was evidently higher in the presence of elevated selenite concentrations. This is why more glucose was consumed for the 0.5 mM and 2 mM selenite concentrations as compared to the 10 mM concentration. The trends in glucose consumption for the 0.5, 2 and 10 mM concentrations are shown in Figure 4 (b). Furthermore, the specific glucose consumption rates across the different concentrations were calculated using Equation 1 below. The glucose consumption rate was 0.103, 0.074 and 0.013 $g.(L.h)^{-1}$ for the 0.5, 2 and 10 mM SeO_3^{2-} concentration respectively. The calculations show that the glucose consumption rate was higher in the presence of more viable cells.

$$\mathbf{q_g} = \text{concentration of glucose consumed}/(\mathbf{t}) \quad (1)$$

where; $\mathbf{q_g}$= specific glucose consumption rate $(g.(L.h)^{-1})$ and **t** = time (h)

4. Conclusions

This study showed that elemental selenium can be recovered from reducing selenite using *P. stutzeri* NT-I. It also established that a defense mechanism might be the one being employed by this bacterial strain mainly because more selenite was reduced for a higher initial concentration than a lower one, in the same time period. Moreover, TEM analysis indicated the presence of electron-dense SeNPs located extracellularly which further validates the mechanism suggested. Other discoveries include that the ORP measurements correlated with the trends observed in selenite reduction indicating that the measurements can be used as a monitoring technique for metal reduction. The metabolic activity indicated how the presence of a high selenite concentration affects bacterial growth and viability which in turn affect the substrate utilisation. Findings from this study leave room to expand this research further. Analysis such as Energy Dispersive X-Ray Analysis (EDX) in order to identify the elemental composition of the SeNPs are necessary. To add on, Dynamic light scattering (DLS) might also be necessary to determine the mean size distribution of the particles. Finally, (Fourier-transform infrared spectroscopy) FTIR analysis can also shed more light on the functional groups on the surface of the SeNPs which could be useful in establishing more details on the mechanisms of reduction. In short, this study has established that selenium bioremediation is a potential resource for SeNPs.

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