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Reduction of Selenite by use of *Pseudomonas stutzeri* NT-I Cell-free Extract

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The soluble selenium oxyanion, selenite (Se(IV)) is toxic and it bioaccumulates at low concentrations which is why its presence in aquatic systems has to be regulated. *Pseudomonas stutzeri* NT-I is one of the numerous microorganisms capable of reducing Se(IV) into the relatively less toxic Se(0). Previous studies using strain NT-I have mainly focused on Se(IV) reduction occurring in the presence of bacterial biomass for the entirety of the reaction. However, it has been reported that some pseudomonas strains are capable of secreting biomolecules or metabolites into their surrounding environments which aid Se(IV) reduction. In this study, aerobic batch reduction of 2 mM Se(IV) was carried out in two stages, that is, firstly in the presence of bacterial biomass for 1 h (in order to initiate the reaction) and secondly in the absence of biomass by use of the bacterial supernatant (cell-free extract), post biomass removal. This was done to investigate the selenium reducing capabilities of the cell-free extracts. Obtained results showed that the total Se(IV) reduction within the first hour prior to biomass removal was rapid, translating to an average reduction rate of 0.45 mmol.h^{-1} and a 22 % reduction in the Se(IV) concentration. However, Se(IV) reduction still occurred post biomass removal by use of the cell-free extract, albeit at a slower rate. The average reduction rate was 0.01 mmol.h^{-1} and the Se(IV) removal was 58 %. From these results, it was concluded that the bacterial biomass likely secreted metabolites which remained in the medium after the removal of biomass. It is these secreted metabolites which are thought to possess selenite reducing capabilities.

1. Introduction

Selenium (Se) is a naturally occurring element and it is known for its dual toxic and beneficial effects on plants and animals (Fernández-Martínez and Charlet, 2009). It is an essential micronutrient at low concentrations but can be toxic at higher ones. A Se concentration of 55 µg.d^{-1} amounts to selenium deficiency whereas once it exceeds 400 µg.d^{-1} ,it can result in toxic effects (Nancharaiah and Lens, 2015b). Although Se is ubiquitous, anthropogenic activities such as phosphate mining, coal combustion, and oil refining (Lemly, 2004) have led to high selenium concentrations being detected in both surface and groundwaters. In the aquatic environment, Se is largely present as either selenate (Se(VI)), or selenite (Se(IV)). These two inorganic forms of Se are readily bioavailable, toxic, mobile, and soluble in water . Although Se(IV) is generally more toxic (Ecimovic et al., 2018), both oxyanions can easily bioaccumulate in the food chain, thereby posing a potential threat to humans and animals. However, Se(IV) and Se(VI) can be reduced to elemental selenium (Se(0)) which is considered to be biologically inert and relatively less toxic (Garousi, 2015).
Several methods have been developed over the years for the remediation of selenium laden environments. However, biological methods are preferred due to their eco-friendly nature, ability to use self-generating catalysts, and low capital, operational and maintenance costs (Lenz and Lens, 2009).
Therefore, microorganisms are essential, and they play a vital role in selenium remediation. Numerous selenium resistant microorganisms have been reported to reduce the toxic Se oxyanions to Se(0). These include; *Clostridium*, *Citrobacter*, *Flavobacterium* (Lovley, 1993), *Pseudomonas putida*, *Pseudomonas fluorescens, Pseudomonas stutzeri* NT-I (Kuroda et al., 2011)*, Pseudomonas aeruginosa* (Lortie et al., 1992b, Kagami et al., 2013), *Bacillus selenitireducens,* and *Enterobacter cloacae* (Avendaño et al., 2016), among others.

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The reduction can be conducted under aerobic, microaerophilic, and anaerobic conditions (Nancharaiah and Lens, 2015a) depending on the Se oxyanion to be reduced and the bacterial species employed. For many Se(IV) reducing microorganisms, it has remained largely unclear which reductive mechanisms are at play.
However, others have reported that the conversion of Se(IV) to Se(0) can occur via a number of different mechanisms. These include thiol-mediated reactions and reductase catalysed reactions (Eswayah et al., 2016) or biotransformation through direct enzymatic reaction or indirectly with metabolites (Camargo et al., 2003). Moreover, other studies have reported that Se(IV) can be reduced aerobically through a detoxification mechanism independent of dissimilatory reduction (Lortie et al., 1992a). To add on, Tendenedzai and Brink , (2019) recently demonstrated that *P. stutzeri* NT-I can reduce Se(IV) to Se(0) in the absence of an added carbon source (electron donor) and/or nitrogen source (synthesis of biomass). Furthermore, different pseudomonas species secrete biomolecules which can reduce selenium oxyanions into elemental selenium nano-particles (SeNPs) extracellularly. An example is that of a nitrate reductase with selenium-reductase-activity (Hunter, 2014). In addition, Zawadzka et al., (2006) found that the siderophore; pdtc, secreted by several pseudomonas species had the ability to reduce selenium and tellurium oxyanions in self-defence. The abovementioned studies point to the possibility of other mechanisms which could be employed by different pseudomonas strains for selenium reduction.
In order to investigate other likely selenium reducing mechanisms in strain NT-I (used in this study), aerobic batch reduction of 2 mM Se(IV) was carried out in two stages. The reduction was initially in the presence of bacterial biomass before its removal after 1 h. Thereafter, Se(IV) reduction was monitored with the use of the remaining bacterial supernatant. The other parameters which were monitored for the duration of the experiments were; the glucose concentration, the cell metabolic activity (MA) and variations in the total organic carbon (TOC).

2. Materials and methods

2.1 Bacteria storage and cultivation

The bacterium 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 from the NITE Patent Microorganisms Depository (NMPD) in Chiba Ken, Japan. Thereafter, it was cultivated in Tryptone Soy Broth (TSB) for 24 h at 28 °C on a rotary shaker at 120 rpm (FSIM-SPO8, Labcon, Johannesburg) then glycerol was added 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 Tryptone Soy Broth (TSB) medium in a flask and capped with foil and/or cotton wool. The flask was placed on a rotary shaker at 120 rpm for 24 h at 37 °C.

2.2 Aerobic batch reduction experiments

Aerobic batch reduction experiments were carried out in conical flasks. Firstly though, the bacteria cultures were grown in TSB at 35±2 °C and on a rotary shaker (120 rpm). After 24 h (log phase), the cells were harvested and concentrated by centrifugation (6 ,000 rpm, 15 mins, room temperature). Approximately 5 $g.L^{-1}$ (dry weight) of bacterial biomass was obtained after harvesting and was used as the inoculum. The flasks contained bacterial biomass and glucose supplemented mineral salt medium (MSM) described by Brink et, al (2018) spiked with 2 mM Se(IV) (added as Na_2SeO_3). The reduction was divided into two stages, one with bacterial biomass (5 $g.L^{-1}$) present in the MSM and the other after its removal. Stage 1 (in the presence of bacterial biomass) was approximately an 1 h long and during this period, 3 samples were taken at specific time intervals namely; 0, 0.5 and 1 h respectively. Stage 2 required the removal of biomass from the medium by filtration and centrifugation. Thereafter, the bacterial supernatant (cell-free extract) was monitored for Se(IV) reduction. For the post biomass removal stage, 9 more samples were taken from 1.5 h until the termination of the experiment at time 48 h. The starting pH was 7 and for the duration of the experiment, the temperature was maintained at 35±2 °C, and all flasks were subjected to shaking on a rotary shaker at 120 rpm. The samples which had been taken at various time intervals were centrifuged first and the supernatant was analysed for the various parameters.

2.3 Analytical methods

Several parameters were measured at the different extraction time intervals mentioned earlier, namely the total selenium (Se) concentration, the total organic carbon (TOC), the cell metabolic activity (MA) and lastly the glucose concentration. The total Se was quantified using a Varian AA–1275 Series Flame AAS (Varian, Palo Alto, CA (USA)) at 196.03 nm wavelength equipped with a 290 mA selenium lamp. Se(IV) which had not yet been reduced to Se(0) was measured in the liquid phase (supernatant). The TOC was also measured in

the supernatant using TOC analyser (TOC-VWP, Shimadzu). This was necessary in order to track the variations in TOC prior to and post biomass removal. The amount of glucose that had not yet been used up was measured in the supernatant using a CONTOUR®PLUS blood glucose monitoring system (CP BGMS, Bayer) which proved to be accurate. A study by (Dunne et al., 2015) also concluded the CP BGMS had a lower mean difference from the reference value than all other systems tested across all glucose ranges. The MA of the bacteria was measured using the MTT colourimetric assay which employs reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) to measure cellular metabolic activity as a proxy for cell viability. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple colour (Wang et al. 2010). The resulting intracellular purple formazan was solubilized, and the absorbance measured at 550 nm using a spectrophotometer (WPA, Light Wave II, Labotech, South Africa).

3. Results and interpretation

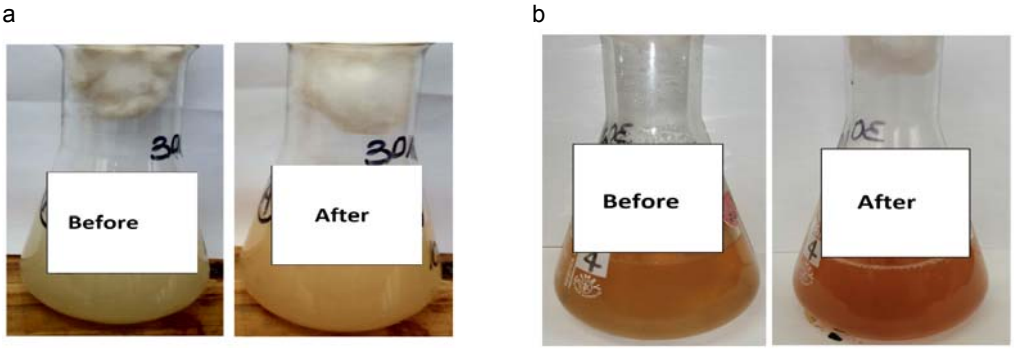

Figure 1: Colour changes at the start and end of (a) Stage 1: prior to biomass removal (b) Stage 2: post biomass removal

Figure 1 shows the colour changes for the two stages. The red colour, which became more pronounced as the reaction proceeded is an indication of the formation of Se(0) confirming the occurrence of Se(IV) reduction. Figure 2 (a) shows the observed trend in the reduction of 2 mM Se(IV) prior to and post biomass removal. The generally observed trend was that selenite reduction was rapid for the first 1 h (prior to biomass removal). During this period, approximately 0.445 mM Se(IV) was reduced, translating to a 22 % removal and a reduction rate of 0.45 mmol.h^{-1}. A slight change in colour as depicted in figure 1 (a) corresponded with the observed reduction. For the duration of the post biomass removal stage, obtained results indicated that approximately 0.543 mM (35 %) Se(IV) was reduced and the reduction rate was 0.01 mmol.h^{-1}. Figure 1 (b) shows a more intense red colour confirming that more reduction was taking place even when biomass had been removed. The overall selenite reduction after 48 h was 0.989 mM which was almost 50 % of the initial 2 mM Se(IV) concentration. The disparity in the two reduction rates is evidence that the presence of biomass influences rate at which Se(IV) is reduced. The other main observation from these results was that Se(IV) reduction continued with the bacterial supernatant alone, after the biomass had been removed. This was taken as an indication of the presence of selenite reducing biomolecules secreted into the supernatant by the bacterial cells prior to their removal. Furthermore, it was used as a confirmatory test showing that *P. stutzeri* NT-I strains do not reduced selenite inside the cell but rather in the external environment by secreting metabolites. In one study, selenite reducing abilities of cell-free extracts of certain bacterial species were also reported. Of the four species which were investigated in the study, *Pseudomonas pseudoalcaligenes* was found to have the highest percentage reduction. Moreover, they used these results as confirmation that the strains released a reductase protein which reduced selenite outside the cell (Saima Javed et al., 2015). Another reason why the reduction was able to occur after biomass removal may be due to the presence of outer membrane vesicles (OMVs) which can be released by gram-negative bacteria such as *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*. OMVs have been found to mediate diverse functions such as enabling bacterial survival during stress conditions (Schwechheimer and Kuehn, 2015) such as in the presence of toxic metals.

In order to prove that there was indeed no more bacterial cells in the supernatant after the post biomass removal stage, the metabolic activity (MA) was also monitored throughout the duration of the reaction. The variation of MA over the 48 h period is shown in figure 2 (b). The MA was measured throughout the two stages

using the MTT colourimetric assay described earlier and it served as a proxy for cell viability and growth. The trend observed was that the MA gradually declined within the first hour prior to biomass removal and it reduced by 22 % of its initial value. The decrease was attributed to low cell viability as the bacterial cells required time to acclimatise to the high selenite concentrations. For this study, the cells were removed after an hour before any growth was noticeable. Post biomass removal , the cell MA plummeted below zero as expected because there were no longer any cells to produce the NAD(P)H-dependent oxidoreductase enzymes required to react with the MTT reagent. In the absence of bacterial cells (zero MA), selenite reduction was no longer influenced by the cell activity. This further cemented the idea of the presence of a selenite reduction mechanism which was not reliant on the presence of bacterial biomass but rather on what was in the supernatant.

a b

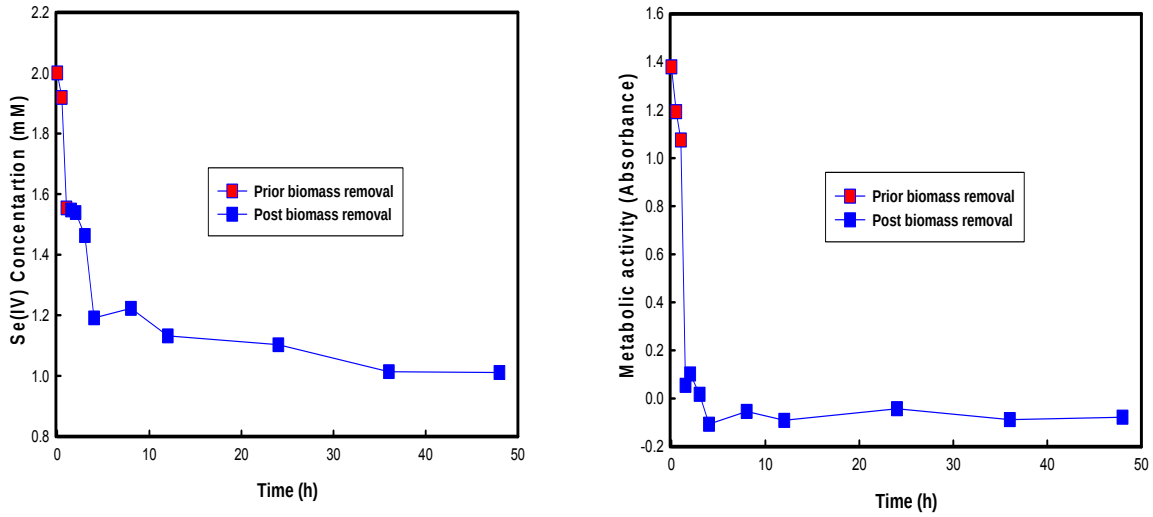

Figure 2: Results showing variations in (a) Selenite reduction and (b)Metabolic activity prior to and post biomass removal.

The TOC was also measured in the supernatant and its variation is depicted in figure 3 (a). Results from measuring the TOC showed a 32 % (2.757 g) increase within the first hour when the biomass was still present, but it remained relatively constant post biomass removal. The increase was assumed to be due to carbon-rich extracellular metabolites secreted by the bacterial cells. Moreover, carbon containing compounds secreted by microbes may sometimes be utilised by the cells as detoxifying agents (Anderson and Appanna, 1993). Therefore, they could have been secreted by the bacterial cells as a way of protecting themselves from the prevailing selenite concentrations. The TOC increase could also have been due to OMVs mentioned earlier as they are made up of mainly proteins and lipids (Schwechheimer and Kuehn, 2015). Consequently, their release into the medium before biomass removal could have been a contributing factor in TOC increase. After biomass removal, there was no longer any source for these carbon-rich metabolites which is why the TOC remained relatively constant. It can be argued that if the metabolites in the supernatant were taking part in Se(IV) reduction, the TOC should have decreased post biomass removal, but it did not. It was assumed that the participation of the metabolites in Se(IV) reduction led to their deactivation rather than their degradation, hence the constant TOC.

Glucose is one of the many organic substrates (carbon sources) utilised as an energy source by microorganisms (Golder-Associates-Inc, 2009). It also promotes growth and activity of selenite-reducing microorganisms. That is why monitoring its concentration in this study was essential as it would give an indication of the microorganisms' activity. In addition, glucose is known to serve as an electron donor for the oxidation-reduction reactions in the microbial reduction of selenium. The Se oxyanions serve as electron acceptors (Huber et al., 2000) and are therefore reduced to Se(0). Figure 3 (b) shows the changes in the glucose concentration and there was a 13 % (1.313 g) decrease in the glucose concentration within the first hour which shows that it was being used by the microbes. However, once the biomass was removed, glucose concentration remained relatively constant even though selenite reduction continued. This lack of decrease in the glucose concentration was an indication that there were no longer any microbes present to consume it.

Hence the selenite reduction taking place was entirely dependent on other mechanisms likely the presence of an alternative electron donor possibly of biological origin.

a b

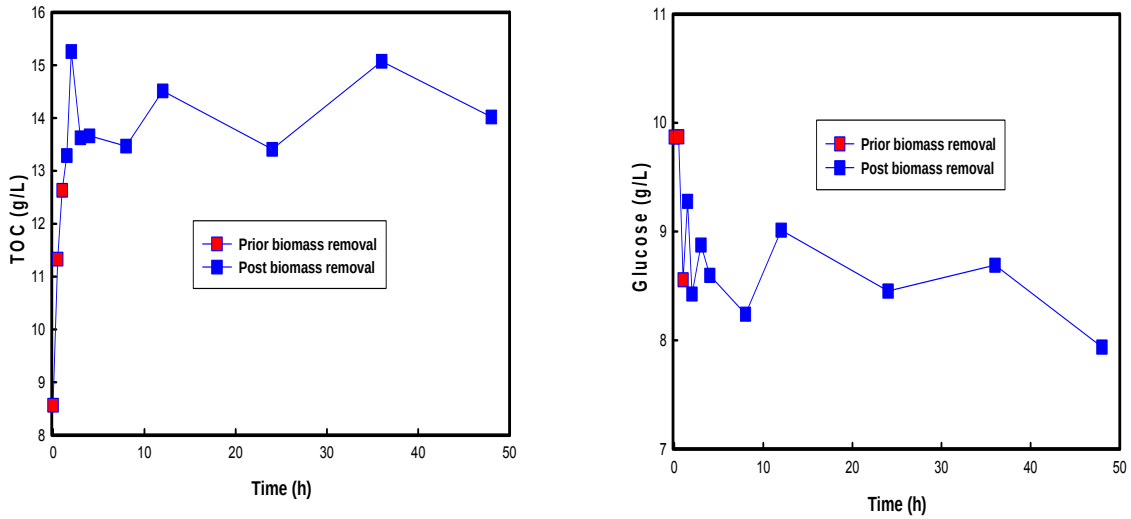

Figure 3: Results showing variations in (a) Total organic carbon and (b) Glucose consumption prior to and post biomass removal

4. Conclusion

Findings from this study demonstrated the ability of *P. stutzeri* NT-I cell-free extract (bacterial supernatant) to reduce selenite, albeit at a rate slower than when biomass is present. Of the total Se(IV) reduction observed, 22 % was by the bacterial biomass (within the first hour) and 35 % by the cell-free extract. Moreover, the reduction rates were 0.45 mmol.h^{-1} and 0.01 mmol.h^{-1} prior to biomass removal and post biomass removal respectively. Data obtained from measuring the total organic carbon and glucose indicated that the bacterial biomass likely secreted carbon-containing metabolites into the medium and also utilised glucose before its removal. These conclusions were backed by a 32 % increase in the total organic carbon and a 13 % glucose reduction prior to biomass removal. Both concentrations became relatively constant afterwards indicating that the changes in the concentrations had been influenced by the microbial activity. To add on, cell metabolic activity was not detected post biomass removal thus proving that reduction at the later stage did not depend on the microbes. Further analysis will be undertaken using liquid chromatography-mass spectrometry (LC-MS) and Fourier-transform infrared (FTIR) spectroscopy. LCMS on the cell-free extract will enable the identification and characterisation of the metabolites. FTIR spectroscopy conducted on selenite-stressed cells can show the changes in functional groups on the cell surface. This will help to further understand and validate the suggested mechanism for selenite reduction in strain NT-I.

References

Anderson, S. & Appanna, V. D. 1993. Indium detoxification in Pseudomonas fluorescens. *Environmental Pollution,* 82**,** 33-37.

Avendaño, R., Chaves, N., Fuentes, P., Sánchez, E., Jiménez, J. I. & Chavarría, M. 2016. Production of selenium nanoparticles in Pseudomonas putida KT2440. *Scientific Reports,* 6**,** 37155.

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, 2018.

Camargo, F. a. O., Bento, F. M., Okeke, B. C. & Frankenberger, W. T. 2003. Chromate reduction by chromium resistant bacteria isolated from soils contaminated with dichromate. *J. Environ. Qual. ,* 32**,** 1228–1233.

Ecimovic, S., Velki, M., Vukovic, R., Stolfa Camagajevac, 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.

Eswayah, A. S., Smith, T. J. & Gardiner, P. H. E. 2016. Microbial Transformations of Selenium Species of Relevance to Bioremediation. *Applied and environmental microbiology,* 82**,** 4848-4859.

Fernández-Martínez, A. & Charlet, L. 2009. Selenium environmental cycling and bioavailability: a structural chemist point of view. *Reviews in Environmental Science and Biotechnology,* 8**,** 81-110.

Garousi, F. 2015. The toxicity of different selenium forms and compounds – Review.

Golder-Associates-Inc 2009. LITERATURE REVIEW OF TREATMENT TECHNOLOGIES TO REMOVE SELENIUM FROM MINING INFLUENCED WATER.

Huber, R., Sacher, M., Vollmann, A., Huber, H. & Rose, D. 2000. Respiration of arsenate and selenate by hyperthermophilic archaea. *Syst Appl Microbiol,* 23**,** 305-14.

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.

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. *J Biosci Bioeng,* 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. 1992a. Reduction of Selenate and Selenite to Elemental Selenium by a Pseudomonas stutzeri Isolate. *Appl Environ Microbiol,* 58**,** 4042-4.

Lortie, L., Gould, W. D., Rajan, S., Mccready, R. G. L. & Cheng, K. J. 1992b. Reduction of Selenate and Selenite to Elemental Selenium by a Pseudomonas stutzeri Isolate. *Applied and Environmental Microbiology,* 58**,** 4042-4044.

Lovley, D. R. 1993. DISSIMILATORY METAL REDUCTION. *Annu. Rev. Microbiol.,* 47**,** 263-9.

Nancharaiah, Y. V. & Lens, P. N. L. 2015a. Ecology and Biotechnology of Selenium-Respiring Bacteria. *Microbiology and Molecular Biology Reviews,* 79**,** 61-80.

Nancharaiah, Y. V. & Lens, P. N. L. 2015b. Selenium biomineralization for biotechnological applications. *Trends in Biotechnology,* 33**,** 323-330.

Saima Javed, Arslan Sarwar, Mohsin Tassawar & Faisal, M. 2015. Conversion of selenite to elemental selenium by indigenous bacteria isolated from polluted areas. *Chemical Speciation & Bioavailability,* 27:**,** 162-168.

Schwechheimer, C. & Kuehn, M. J. 2015. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol,* 13(10)**,** 605–619.

Tendenedzai, J. T. & Brink, H. G. 2019. The Effect of Glucose and Nitrogen Supplementation on Cell Metabolic Activity and the Reduction of Selenite to Elemental Selenium by *Pseudomonas Stutzeri* NT-I. *Chemical Engineering Transanctions,* 76**,** 1315-1320.

Wessels, C. E. & Chirwa, E. M. N. 2017. Reduction of Selenium by Pseudomonas stutzeri Nt-I: Growth, Reduction and Kinetics. *J Bioremediat Biodegrad 8: 391.*

Zawadzka, A. M., Crawford, L., Andrzej, J. & Paszczynski, A. J. 2006. Pyridine-2,6-Bis(Thiocarboxylic Acid) Produced by Pseudomonas stutzeri KC Reduces and Precipitates Selenium and Tellurium Oxyanions. *Applied Environmental Microbiology,* 72**,** 3119–3129.