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The Effect of Glucose and Nitrogen Supplementation on Cell Metabolic Activity and the Reduction of Selenite to Elemental Selenium by Pseudomonas Stutzeri NT-I

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Selenite (Se(IV)) readily bio-accumulates as compared to elemental selenium (Se(0)) which is considered to be biologically inert and relatively less toxic, therefore its microbial reduction from selenium laden waters is imperative. This study investigated the effect of glucose (carbon source), NH_4Cl (nitrogen source) and the metabolic activity on the reduction of 2 mM Se(IV) by Pseudomonas stutzeri NT-I in order to establish the mechanism employed the bacterium. The experiments were performed aerobically under previously determined optimum conditions. Four batches were compared. These were A) glucose (10 $g.L^{-1}$) and ammonium chloride (1.604 $g.L^{-1}$), B) glucose only (10 $g.L^{-1}$), C) no added glucose or nitrogen, and D) 1 M of the metabolic inhibitor, sodium azide (NaN_3). The highest average biomass-based selenite reduction rate of 0.1061 $mmol.(g.h)^{-1}$ was in the presence of glucose and nitrogen as compared to 0.0638 $mmol.(g.h)^{-1}$ in the presence of glucose alone. The reduction rate decreased in the batches which did not have either of the nutrients. The lowest average biomass-based reduction rate of 0.026 $mmol.(g.h)^{-1}$ was measured for the batch which had the NaN_3. The overall Se(IV) removal followed a similar trend to the one observed with reduction rate, with highest reduction of 90.83 % recorded in the first batch and the lowest of 35.68 % being in the presence of NaN_3.

1. Introduction

Selenium (Se) is an unusual metalloid with a very narrow safe range of intake which necessitates the attenuation of effluent concentrations to acceptable levels prior to discharge into local water bodies. The World Health Organization proposes a value of 40 $\mu g.L^{-1}$ for countries without a specified legislative framework in place (Brink et al., 2018). Of all the treatment methods currently in use, biological methods are emerging as the most cost-effective treatment option to remove selenium by either reducing or volatilising the oxyanions. A recent example of the possibility of sustained biodegradation was demonstrated by bacterial growth simultaneous with biodegradation of a metal mixture by aerobic metal-resistant bacteria Brevundimonas diminuta (Islas-Espinozaa et al., 2018).

Se(0) is considered to be biologically inert and relatively less toxic because of the low bioavailability (Garousi, 2015). The soluble selenium oxyanions; selenate (SeO_4^{2-}), and selenite (SeO_3^{2-}); readily bio-accumulate and even though SeO_4^{2-} is most prevalent of the two, SeO_3^{2-} is considered the more toxic species (Ecimovic et al., 2018). The biological method considered in this study involves using Pseudomonas stutzeri NT-I, a selenium reducing bacteria, which converts soluble SeO_3^{2-} ions found in water into their elemental state before being removed as a precipitate.

Selenite can be reduced in three ways, mainly; detoxification, dissimilatory, and assimilatory reduction (Wessels and Chirwa, 2017). The most widely accepted mechanisms are that of microbial detoxification and dissimilatory reduction in which Se oxyanions act as the terminal electron acceptor. The reduction of Se oxyanions depends on oxidation-reduction reactions in the presence of a substrate, usually a carbon source. The carbon source acts an electron donor while selenite serves as the electron acceptor. This is considered to take place in conjunction with the synthesis of biomass requiring the presence of a nitrogen source, usually ammonium.

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The generic formula for the process according to (Ji and Wang, 2017) is:

$$0.0846C6H12O6 + 0.1355SeO32- + 0.0744HCO3- + 0.0744NH4+ + 0.1335H+ \rightarrow 0.0744C5H7O2N + 0.5684CO2 + 0.5684H2O + 0.1355Se \quad \text{Eq (1)}$$

In this study, the aerobic batch reduction of Se(IV) was done under previously established optimum conditions of a temperature of 37 °C, pH 7 – 8 and salinity less than 5 $g.L^{-1}$ NaCl (Brink et al., 2018).
This study investigated how the absence or presence of an organic substrate (glucose) and/or a nitrogen source (NH_4Cl) affected Se(IV) reduction. This would establish whether Pseudomonas stutzeri NT-I employs other mechanisms in Se(IV) reduction which are not necessarily depended on the availability of a known carbon/nitrogen source. Furthermore, sodium azide (NaN_3), which has been reported by various studies as a metabolic inhibitor and does not compromise the cellular structure of Gram-negative bacteria (Akel et al., 2006), was added in one of the batches as an active metabolic inhibitor to assess the impact of metabolic activity on Se(VI) reduction.

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 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 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 Selenite reduction rate batch experiments

Four batch reduction experiments were carried out, in triplicate, for the Se(IV) concentrations of 2 mM in a mineral salt medium (MSM) described elsewhere (Brink et al., 2018), the only variation being the constituents of the medium in which reduction took place. The first batch ($batch_A$) contained 10 $g.L^{-1}$ glucose, 1.604 $g.L^{-1}$ NH_4Cl, in addition to the 7 $g.L^{-1}$ biomass suspended in MSM and 2 mM Se(IV). The second batch ($batch_B$) contained 10 $g.L^{-1}$ glucose and 7 $g.L^{-1}$ biomass suspended in MSM and 2 mM Se(IV). $Batch_C$ only contained 7 $g.L^{-1}$ biomass suspended in MSM and 2 mM Se(IV). The final batch ($batch_D$) contained 1 M NaN_3 and 7 $g.L^{-1}$ biomass suspended in MSM and 2 mM Se(IV).
Before suspending the biomass in each of the different batches, 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 re-suspended in 100 mL serum bottles containing the constituents for $batch_A$ - $batch_D$. During the reduction experiment, the different batches were incubated on a rotary shaker (120 rpm, 37 °C). 1 mL aliquots were extracted at specific time intervals throughout the duration of the experiment and used in colourimetric assay to determine the metabolic activity. In addition, 2 mL aliquots were extracted at the same time intervals, centrifuged and analysed for dissolved selenium.

2.3 Analytical methods

The metabolic activity of the bacteria was measured using the MTT colourimetric assay. The assay 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 reduces 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).
The total Se was determined 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. Each sample was centrifuged, the supernatant was separated from the pellet and analysed by the AAS, to ascertain the amount of Se(IV) that had not yet been reduced.

3. Results and interpretation

To ensure that all other conditions were kept constant during the reduction of the 2 mM Se(IV), a triplicate set for each of the different batches was run concurrently. In addition, the biomass of approximately 7 $g.L^{-1}$ was added at the start each reduction experiment for all batches. Control experiments for each of the different batches were subjected to the same conditions in the absence of the bacterial strain to determine whether any abiotic reduction would occur.

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) for the batch containing both glucose and nitrogen.

Figure 2: Shows the changes at different time intervals in the intensities of a red colour which is an indication of the formation of Se(0) for the batch containing glucose only.

Figure 3: Shows the changes at different time intervals in the intensities of a red colour which is an indication of the formation of Se(0) for the batch containing biomass only.

Figures 1-4 show the different batches at various time intervals throughout the reduction of 2 mM Se(IV). Figures 1 and 2, the batches with the carbon and nitrogen sources, exhibited a colour change in the shortest time. In contrast, Figures 3 and 4 showed a similar colour change (red) at a slower rate. As the red colour is an indication of the formation of Se(0), this translates to the rate of Se(IV) reduction being faster in the first two batches compared to the second pair. This visual evidence is backed by data from Figure 5 and Table 1, which illustrates

the amount of Se(IV) in the system as well as the Se(IV) removal percentages at specific times, respectively. The highest rate of reduction of 0.1061 mmol.(g.h)$^{-1}$ was observed in batch$_A$ (glucose and nitrogen), followed by batch$_B$ (glucose only) with a rate of 0.0638 mmol.(g.h)$^{-1}$. Batch$_C$ (no added nutrients) exhibited the third highest reduction rate of 0.0284 mmol.(g.h)$^{-1}$ and lowest reduction rate of 0.026 mmol.(g.h)$^{-1}$ was observed in batch$_D$ which contained the inhibitor.

Figure 4: Shows the changes at different time intervals in the intensities of a red colour which is an indication of the formation of Se(0) for the batch containing NaN_3.

The overall percentage reduction also followed a similar trend. The summary of the results is shown in Table 1 below.

Table 1: Overall percentage reduction of Se(IV)

Time (h)	Batch$_A$	Batch$_B$	Batch$_C$	Batch$_D$
0	0 %	0 %	0 %	0 %
0.5	10.95 %	11.64 %	1.98 %	0.29 %
3.5	53.24 %	44.72 %	25.26 %	8.69 %
5.5	69.46 %	51.27 %	40.43 %	13.97 %
12	90.83 %	85.60 %	63.68 %	35.68 %

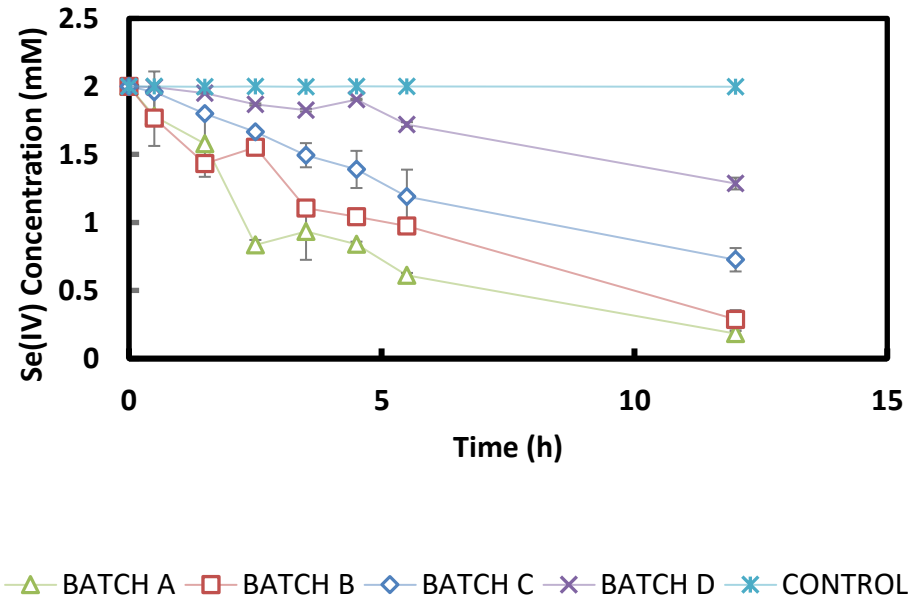

Figure 5: Results showing the effect of different media on Se(IV) reduction by strain NT-I.

Figure 6 shows a comparison between the metabolic activities, an indication of cell viability, between the different batches. The generally observed trend showed that the cell metabolism increased to a peak level in $batch_A$ and $batch_B$ within the first 4.5 h of the experiment followed by a decrease until termination of the experiment. The changes in metabolism for $batch_C$ and $batch_D$ remained nearly constant for the first six hours before decreasing. It was concluded from this data that a correlation exists between the metabolic activity and reduction rate; the absence of a carbon and nitrogen source translated to a lower initial metabolic activity corresponding to a lower rate of reduction in $batch_C$ and $batch_D$.

It is interesting to note that the reduction of Se(IV) still proceeds, albeit much slower, in the absence of an added nitrogen and/or carbon source (electron donor), indicating that the reduction of Se(IV) does not require ammonium as a nitrogen source and glucose as an electron donor as described by equation (1).

This could indicate an alternative mechanism responsible for Se(IV) reduction, possibly an alternative electron donor originating from the microbe.

Alternatively, a surface adsorption mechanism could be responsible for the removal of selenium from solution, followed by a chemical reduction of Se(IV) on the surface of the microbe. To assess the possibility of the latter, a modification of $batch_D$ was made in which the centrifuged biomass was spiked with 1M NaN_3 and left for an hour before addition of Se(IV). In this case, no reduction or removal of Se(IV) was observed. Therefore, it can be resolved that the Pseudomonas stutzeri NT-I bacterial cells need to be sufficiently viable for Se(IV) reduction to occur when no known carbon source is present as an electron donor.

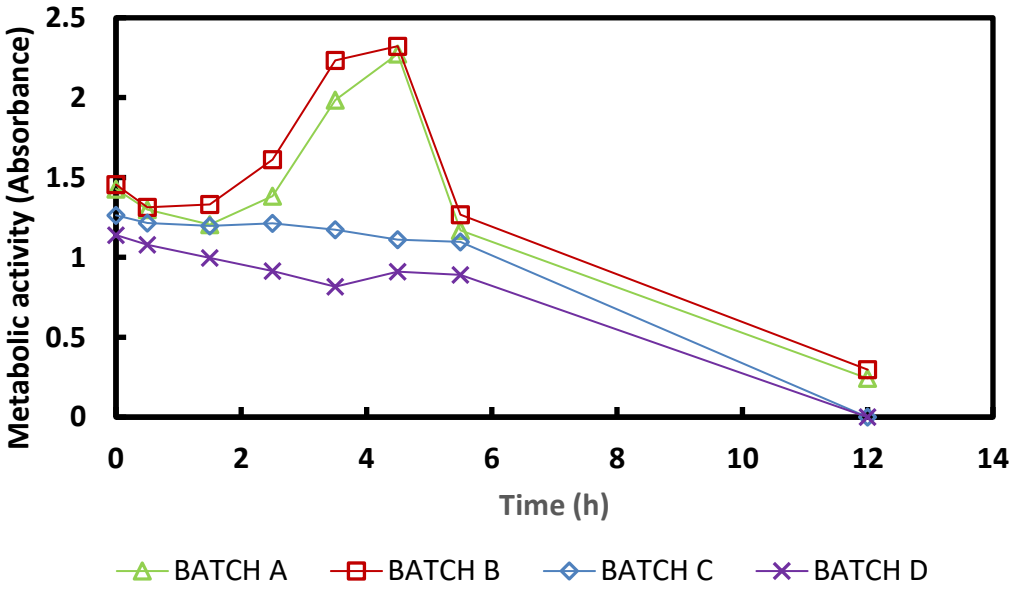

Figure 6: Results showing variations of metabolic activity in the different batches.

4. Conclusions

The results showed that the presence of nitrogen and carbon sources translates to high reduction rates, high overall Se(IV) reduction and high cell metabolic activity. This was evident for $batch_A$ (glucose and nitrogen) and $batch_B$ (glucose only) which had the highest average biomass-based selenite reduction rates of 0.1061 $mmol.(g.h)^{-1}$ and 0.0638 $mmol.(g.h)^{-1}$, respectively. Moreover, the overall Se(IV) removal in both exceeded 85 % after 12 h. $Batch_A$ and $batch_B$ also exhibited the highest peaks in metabolic activity during the reduction reaction. Lower average biomass-based reduction rates of 0.0284 $mmol.(g.h)^{-1}$ and 0.026 $mmol.(g.h)^{-1}$ were measured in $batch_C$ (no added glucose or nitrogen) and $batch_D$ (actively inhibited), respectively. The cell metabolic activity in these remained nearly constant for the first 6 h before reducing to almost zero by the time the experiment was terminated. The overall Se(IV) removal was also lower in the absence of a carbon source and it did not surpass 65 % after 12 h. It can be concluded that Pseudomonas stutzeri NT-I can successfully reduce Se(IV) to Se(0) even in the absence of carbon and nitrogen sources as shown in $batch_C$ and $batch_D$ in which Se(IV) reduction still occurred, though at a significantly slower rate. These results indicate the presence of an alternative electron donor in the system, likely of biological origin, which facilitated the Se(VI) reduction. Zawadska et al., (2006) found that a different strain of P. stutzeri, strain KC

excretes a siderophore (pdtc) in apparent self-defence, which has the ability to reduce selenium and tellurium oxyanions extracellularly. This was further validated as an alternative adsorption/chemical reduction mechanism of Se(IV) removal is unlikely; Se(IV) reduction requires viability of bacteria as opposed to merely presence of bacterial cells.

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