

Measurement of selenite reduction to elemental selenium by *Stenotrophomonas maltophilia* OR02

By
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Abstract

Selenium is required by all organisms, but too much can be toxic. Environments that are contaminated with selenium contain large amounts of the oxyanions, selenite and selenate, which are toxic to living organisms. *Stenotrophomonas maltophilia* OR02 (*S. maltophilia* 02), which was isolated from a heavy metal contaminated site in Oak Ridge, TN, appears to reduce a clear solution of selenite to non-toxic red elemental selenium when grown in the presence of selenite. To test the ability of this strain to remove selenite from its environment, *S. maltophilia* 02 was grown to early log phase and then exposed to 1 mM selenite. Two control experiments containing 1) cells and growth medium and 2) growth medium and selenite were performed simultaneously. Growth over time was monitored by measuring turbidity in Klett units and viable cell counts. Selenium content in the growth medium and cells was monitored using Inductively Coupled Plasma (ICP) spectroscopy. Selenite affected the log phase of the cell growth, and the mechanism of resistance appeared to be reduction of selenite to elemental selenium. The concentration of selenium decreased in the growth medium and increased in pelleted cells. This reduction process can be a useful tool in bioremediation of selenite in the environment. From our results, when treated with 1 mM of sodium selenite the strain removed 17 % of the selenite.

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List of Symbols and Abbreviations

No Se	No selenium	Se	Selenium
°C	Degree Centigrade	K	Kelvin
min	Minutes	hrs	Hours
ml	Milli liter	μ	Micro
μl	Micro liter	α	Directly proportional
mol	Moles	sp.	Species
M	Molar	%	Percent
mM	milli Molar	fig	Figure
μM	micro Molar	LB	Luria-Bertani broth
l	Liter	mg	Milligram
kg	Killo gram	μg	Microgram
g	grams		
ICP – AES	Inductive Coupled Plasma - Atomic Emission Spectroscopy		
ICP – OES	Inductive Coupled Plasma - Optical Emission Spectroscopy		
<i>S. maltophilia</i> 02	<i>Stenotrophomonas maltophilia</i> OR02		

Introduction

Selenium:

In 1818 Swedish chemist Jons Jacob Berzelius discovered a compound and named it as Selene, which comes from the name of the Greek goddess of moon. Later it became selenium, which was identified as an essential element to animal health by Schwarz and Foltz. They found that trace amounts protected vitamin E deficient rats from liver necrosis[1]. On the other hand, selenium is very toxic to humans and animals at higher concentrations. The abundance of selenium varies throughout the world. In some regions it is artificially enriched and used as a diet supplement for health reasons and in some regions it is a pollutant and considered to be an environmental hazard.

Selenium is abundantly found in igneous rocks, sedimentary rocks and fossil fuels. Selenium bioavailability is affected by three means: 1) natural means which include weathering of rocks and earthquakes 2) human means such as coal mining and fuel refining and 3) industrial means such as manufacturing of chemicals, electronics and glass. The industrial and human means are both due to human activity.

The red amorphous form, black amorphous form and grey hexagonal form are the elemental forms of selenium which are interchangeable depending on temperature, on pH, and on red-ox conditions. Selenium also exists in the form of oxyanions, SeO_3^{2-} and SeO_4^{2-} [2]. The elemental form of selenium is not soluble in water and is non-toxic to the environment. The oxyanions are soluble in water and are toxic in the environment. Due to

this reason, scientists tried to find a biologically way to remove selenate and selenite by reduction to elemental selenium [2].

Uses of selenium:

Selenium acts an anti-cancerous agent and is present in glutathione peroxidase which prevents the cell from oxidative damage [3]. Selenium normally undergoes reduction to hydrogen selenite. Hydrogen selenide undergoes methylation to form its methylated derivative. The methylated derivative of selenium acts as a cancer preventing agent by inducing apoptosis in transformed cells [4]. S-adenosylmethionine which is present in liver, acts as a methyl donor for selenide [3].

Selenium Deficiency:

Selenium is an essential element in human nutrition with recommended daily allowance of 0.055-0.07 mg per day [5]. In humans, selenium deficiency causes, Keshan and Kashin-beck diseases. Keshan disease symptoms are heart failure and cardiac enlargement. Kashin-beck disease is characterized by atrophy and necrosis of cartilage tissue [6].

Health Hazards created by Selenium:

Inhalation and consumption are the two ways for selenium to enter humans or animals. Inhalation of selenium causes irritation of respiratory mucous membranes, bleeding from the nose, vomiting and bronchitis. Whereas oral intake of selenium

compounds causes pulmonary edema, lung lesions, tachycardia, diarrhea, abdominal pains, chills tremors and also effects the liver. [5]

Consumption of plants high in selenium by live-stock results in a disease called Blind Stagers which is characterized by aimless wandering and impaired vision. The consumption of selenium in huge amounts by livestock sometimes interfere with fetal development causing fetal malformations and even leads to fetal death. Chronic exposure effects fertility and reduces viability. [5]

Chronic effects of selenium poisoning in humans and animals include discoloration of skin, nails and teeth, loss of hair, loss of nails, tooth decay, bad odor in the breath and urine, restlessness and lack of alertness. Chronic exposure to selenium leads to alkali disease in livestock. It is characterized by anemia and erosion of joints [6].

Studies on selenite show that it is metabolized to dimethylselenide in the presence of glutathione and NADPH. Dimethylselenide is considered to be the fatal form of selenite as it is present in large amounts in the liver and kidneys of animals that died from selenium poisoning [7].

Areas with selenium pollution:

During World War II, uranium ore, which was used to manufacture nuclear weapons, was processed in the Y-12 plant near East Fork Poplar Creek in Oak Ridge, TN. Due to a lack of knowledge and time, heavy metal liquid wastes were stored in ponds which were not covered or lined. These heavy metals leaked into East Fork Poplar Creek

and the surrounding environment, creating many problems to the plant and animal life [8, 9, 10].

The Kesterson reservoir in San Joaquin valley, California is a good example of a natural water system that was polluted with oxyanions of selenium. Due to the bioaccumulation of selenium, the water body poses a threat to wild life [9]. Fish and birds in this reservoir accumulated selenium which caused severe problems and even lead to death. This reservoir was polluted with selenium due to the shale rocks which contain high amounts of selenium in the western states of America. Due to irrigation, large volumes of agricultural drainage water with high concentrations of selenium was pumped into ponds and allowed to evaporation leaving the patches of concentrated selenium. When floods occurred the selenium dissolved and overflowed into the Kesterson reservoir. In addition, coal mining caused higher concentrations of selenium in creeks in southern Idaho [10]. Scientists observed some double-headed trout and also observed fin, egg and facial transformations in some trout.

Due to the above factors, the US EPA (Environmental Protection Agency) is currently reviewing selenium rules. Previously, the US EPA set regulations for the selenium content in drinking water to not more than 0.05 ppm. But now they are looking to regulate the selenium waste released from various industries to save animals, especially aquatic organisms [11].

Ways to convert Selenite to Selenium:

Selenite can be converted to selenium by chemical processes, such as precipitation and coagulation. In precipitation reactions, it is subjected to changes in chemical and physical properties. Coagulation involves altering the charge of the particles so that they form agglomerates which can be removed by flocculation [12]. This is more costly because it uses chemicals in large amounts and also contaminates the environment [12].

Apart from *Stenotrophomonas maltophilia*, some other strains like *Enterobacter cloacae*, *Thauera selenatis*, [13] *Enterobacter hormaechi*, and *Klebsiella* are found to detoxify selenite and selenate. *Tetrathiobacter kashmirensis* also found to reduce toxic selenite to elemental non-toxic selenium which appears in red color [14].

The bacterial strain we used was *Stenotrophomonas maltophilia* OR02 (ATCC # 53510). It was isolated from the Y-12 plant in Oakridge, Tennessee and is an aerobic, non-fermentative and gram-negative bacterium that is resistant to several heavy metals, including selenium. The mechanism of resistance is most likely the conversion of selenite to elemental selenium because it produces a red color when grown in the presence of selenite [15]. We investigated the amount of selenite converted to selenium by this strain.

Measuring of Selenium concentrations:

We used Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) also known as inductively coupled plasma optical emission spectrometry (ICP-OES). It is an analytical technique used for the detection of trace metals [16]. It is a type of emission

spectroscopy that uses an inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element [17]. The intensity of this emission is indicative of the concentration of the element within the sample.

The ICP instrument that was used for this study is composed of 3 parts: ICP, optical spectrometer and torch. We used argon gas to create plasma The torch is ignited with the help of TESLA[18].

When the sample mixes with the argon gas, it reaches the torch through the nebulizer. When it reaches the torch it is converted to mist and enters an optical spectrometer, where it collides with electrons and charged ions and is itself broken down into charged ions. These charged ions are analyzed as the charge and size are based on the element properties.

Hypothesis

When grown, *Stenotrophomonas maltophilia* 02 demonstrates a typical growth curve with a lag phase, log phase, stationary phase and death phase. The bacterium was grown in two flasks containing R3A-TRIS medium to early log phase. At this point, 1 mM sodium selenite was added to one of the flasks and an equal amount of sterile water was added to the other flask. A third control flask contained R3A-tris medium with 1 mM selenite but no bacteria.

Turbidity was measured using a Klett colorimeter, viable cell counts are taken by plating the cultures on LB medium plates to observe the growth pattern and samples were collected every hour for 12 hours. The collected samples were centrifuged to separate the bacterial cells (pellet) and growth medium (supernatant). The centrifuged samples were subjected to digestion and concentration of selenium in the pellet and supernatant is measured by inductively coupled plasma (ICP).

Since we know that the bacterium, *Stenotrophomonas maltophilia* OR02, is resistant to selenite and the mechanism appears to be conversion to non-toxic elemental selenium, we expected the concentration of the selenium in the pellet to increase with time and the concentration of the selenium in the supernatant to decrease.

Methods

Bacterial Strain:

Stenotrophomonas maltophilia OR02 (ATCC # 53510) was isolated from East Fork Poplar Creek and plated on a LB medium with agar. For the regular growth, we used R3A-tris medium [19].

Growth Media:

LB medium was purchased from Fisher Scientific, (Fairlawn, NJ). It contains 10 g of tryptone, 5 g of yeast extract and 5 g of sodium chloride per 1000 ml of distilled water. R3A-tris medium consisted per liter 1 g of bacteriological grade yeast extract (Amresco, Solon, Ohio), 1 g of Bacto proteose peptone (Becton, Dickinson and Company, Sparks, MD), 1 g of bacteriological grade casamino acids (Amresco, Solon, Ohio), 1 g of biotechnology grade non-hydrate D-glucose (Amresco, Solon, Ohio), 1 g of Difco soluble starch (Becton, Dickinson and Company, Sparks, MD), 0.5 g of sodium pyruvate (Fisher Scientific, Fairlawn, NJ), 0.1 mL of ultrapure grade 1 M tris buffer pH 7.5 (Amresco, Solon, Ohio), and 0.1 g of magnesium sulfate hepta hydrate (Fisher Scientific, Fairlawn, NJ)

When required, media were supplemented with 1.6% agar (Amresco, Solon, Ohio) and sodium selenite (MP Biomedicals, LLC. Solon, Ohio).

Growth of *Stenotrophomonas maltophilia* OR02:

A single colony of *S. maltophilia* OR02 was added to 10 ml of R3A-tris medium and incubated for 30-36 hours in the incubator at 30 °C. Then 10 ml of the culture was mixed with 190 ml of R3A-tris medium and was incubated in the incubator at 30 °C. After 2 hours, sodium selenite was added to a concentration of 1 mM in the selenium flask and an equal volume of sterile water was added to the no selenium flask. The control flask with no cells also received 1 mM sodium selenite. Every hour the turbidity of the sample was recorded using a Klett Colorimeter (Cieceware, Belart, Pequannock, NJ) and the data was used to plot a growth curve. Also 10 ml of the sample was collected for measuring the amount of selenium using ICP, and cells were plated on the LB medium plate after necessary dilutions with 1 X M-9 salts to get cell counts which were plotted to monitor growth.

Centrifugation:

The samples were centrifuged using an Eppendorf centrifuge (5810 R, Brinkmann Instrumental INC., Westbury, NJ) for fifteen minutes and the supernatant was transferred to a digestion tube. The pellet was washed with distilled water and suspended in 10 ml water. The resuspended pellet and the supernatant were stored at -20°C after all the samples were collected, the pellet was mixed using a vortex and then transferred to a digestion tube.

Digestion:

For the digestion, we followed the procedure set by the US Environmental Protection Agency [20]. The samples were mixed with 2 ml of concentrated nitric acid (Fisher Scientific, Fairlawn, NJ) and 2 ml of concentrated hydrochloric acid (Fisher Scientific, Fairlawn, NJ) and heated in the Hot Block Pro digester (SC181) from Environmental Express (Mt. Pleasant, SC). The block was set at 108 °C to obtain a 95 °C sample temperature. The samples were digested for 2 hours after the sample reached 95 °C. The Instrument was connected to a controller (SC180, Environmental Express, Mt. Pleasant, SC).

Inductively Coupled plasma Atomic emission spectrometer (ICP-AES):

Selenium content was measured using an ICP-AES from Thermo Electro Corporation (Pittsburgh, PA). It was purged with 99.999% pure argon gas for 2 hours before running the samples. Yttrium was used as an internal standard which was run between every sample to make sure that the instrument was working properly. The instrument has a detection limit ranging from parts per billion (ppb) to parts per million (ppm).

We calibrated the instrument using standard solutions at regular intervals of time which can also be set in the auto sampler program. It was validated by loading a quality check standard and adjusting the limits of detection [21]. If the concentration of the standard fell outside the given limits of detection, the instrument automatically stopped analyzing the samples. In between samples, a spiked control containing equal amounts (in

ml) of known and unknown concentrations was analyzed so that the recovery of the instrument can be estimated.

Since the ICP data were given in parts per million (PPM), the data were converted to milli molar (mM) by dividing PPM with molecular weight of selenium (78.96). Error was calculated using the following equation for the Student T test [22]:

$$\text{Error} = t_{(95\% \text{ CI, (N-1) D.F})} * \text{Std Dev} / \sqrt{N-1} \dots \dots \text{Equation 1}$$

N is Number of observation

CI is Confidence interval

D.F is Degrees of Freedom

Std Dev is Standard Deviation

To calculate the error for amount of selenium reduced per cell, we followed the equation given for calculation of pooled errors [23].

$$\text{Error} = \sqrt{[(n-1) * r^2 + (N-1) * R^2 / (n+N)-2]} \dots \dots \text{Equation 2}$$

N number of observation of cell counts

R relative error of cell counts

n number of observation of selenium concentration

r relative error of selenium concentration

This gives the relative error which was then converted to absolute error by multiplying the actual value with relative error.

Results:

Growth Curve with Klett Readings:

Figure 1 shows the turbidity measurements of the cultures. The Y axis represents the logarithm of turbidity in Klett units, the X axis represents the time in hours, the blue line represents the turbidity of the culture with no selenite and the red line represents the turbidity of the culture with 1 mM sodium selenite. Until 1 hour, the untreated cells were in lag phase and then switched to exponential phase until 6 hours. After 6 hours these cells entered stationary phase.

After 2 hours, selenite was added to a concentration of 1 mM to the treated culture and an equal volume of sterile water was added to the no selenite culture flask. In the growth culture with selenite, the turbidity did not increase as rapidly as the culture that lacked selenite. After selenite was added at 2 hours, the exponential curve of the selenite treated culture was less steep than the exponential curve of the no selenite control. After 6 hours, the both cultures entered a stationary phase and growth for both was almost the same. The culture with selenium started developing a red color after 4 hours of growth. The generation time of the cultures were calculated using the following equations [24].

$$\text{Log } x = \log x_0 + (0.301/g) (t). \quad \text{Equation 3}$$

x = Klett Units

x_0 = Klett units at time 0

t = time

g = generation time (Time required for population to double)

The generation times were calculated using equation 3 and found to be 2.5 hours for the no selenium culture flask and 2.75 hours for the selenium culture flask. From the generation time it was evident that the selenite treated culture was growing slower because there was an increase in the generation time. The error bars for the graph were calculated using the Student's t test with a confidence interval of 95% and (N-1) degrees of freedom using equation 1. The error bars overlapped in the first 3 hours of the growth curve. Then, there was a statistically significant difference between the values of the selenite and no selenite cultures.

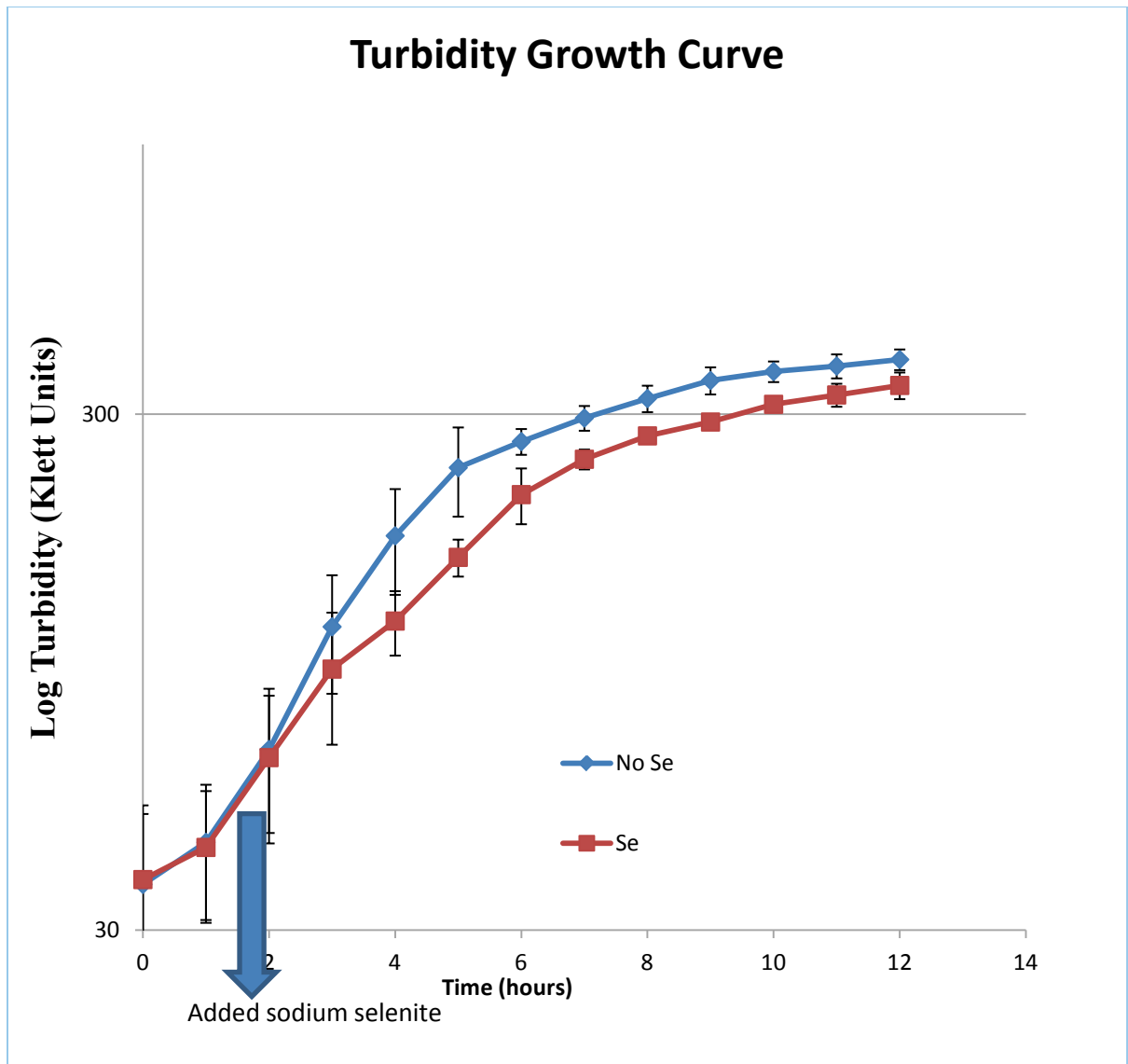


Figure 1: Graphical representation of Turbidity Changes over time. The Y-axis represents log of turbidity in Klett units and the x-axis represents time. The Blue line represents changes in turbidity of no selenium culture flask and the red line represents changes in turbidity of the selenite treated culture flask.

Growth Curve by Cell Counts :

Figure 2 represents the log of cell counts in the selenite and no selenite cultures versus time. The blue line represents the log of cells per milliliter in the no selenite flask and the red line represents the log of cells per milliliter in the selenite treated flask.

Until 2 hours both cultures had almost same characteristic lag phase of growth. At 2 hours, late lag phase, 1 mM sodium selenite was added to one culture (selenite flask) and an equal volume of sterile water was added to the second culture (no selenite flask). There was rapid increase in the cell counts in the no selenite flask till 5 hours, which contributes to an exponential phase. Between 5 and 9 hours the growth appeared to be exponential but at a slower rate. Stationary phase began after 9 hours of growth. Compared to the no selenite control flask, selenite had no effect on the growth rate of cells at 3 hours, 1 hour after it was added. Between 3 and 5 hours, growth in the selenite flask shifted into a lag phase. Then, after 5 hours it shifted back into an exponential phase but at a lower rate than in the no selenite flask. The culture in this flask was still in an exponential phase at 12 hours when the experiment was discontinued.

The error bars for the graph were calculated from Student's t test with a confidence interval of 95% and (N-1) degrees of freedom using equation 1. The error bars suggest that the values are statistically significant.

Generation time of the cultures were calculated based on the cell counts using equation 3 and were found to be 2.3 hours for the no selenite culture flask and 3 hours for the selenite treated culture. The generation time is calculated from the cell counts at 9

hours. The longer generation time for the selenite treated culture showed that selenite slowed the growth of *S. maltophilia* 02.

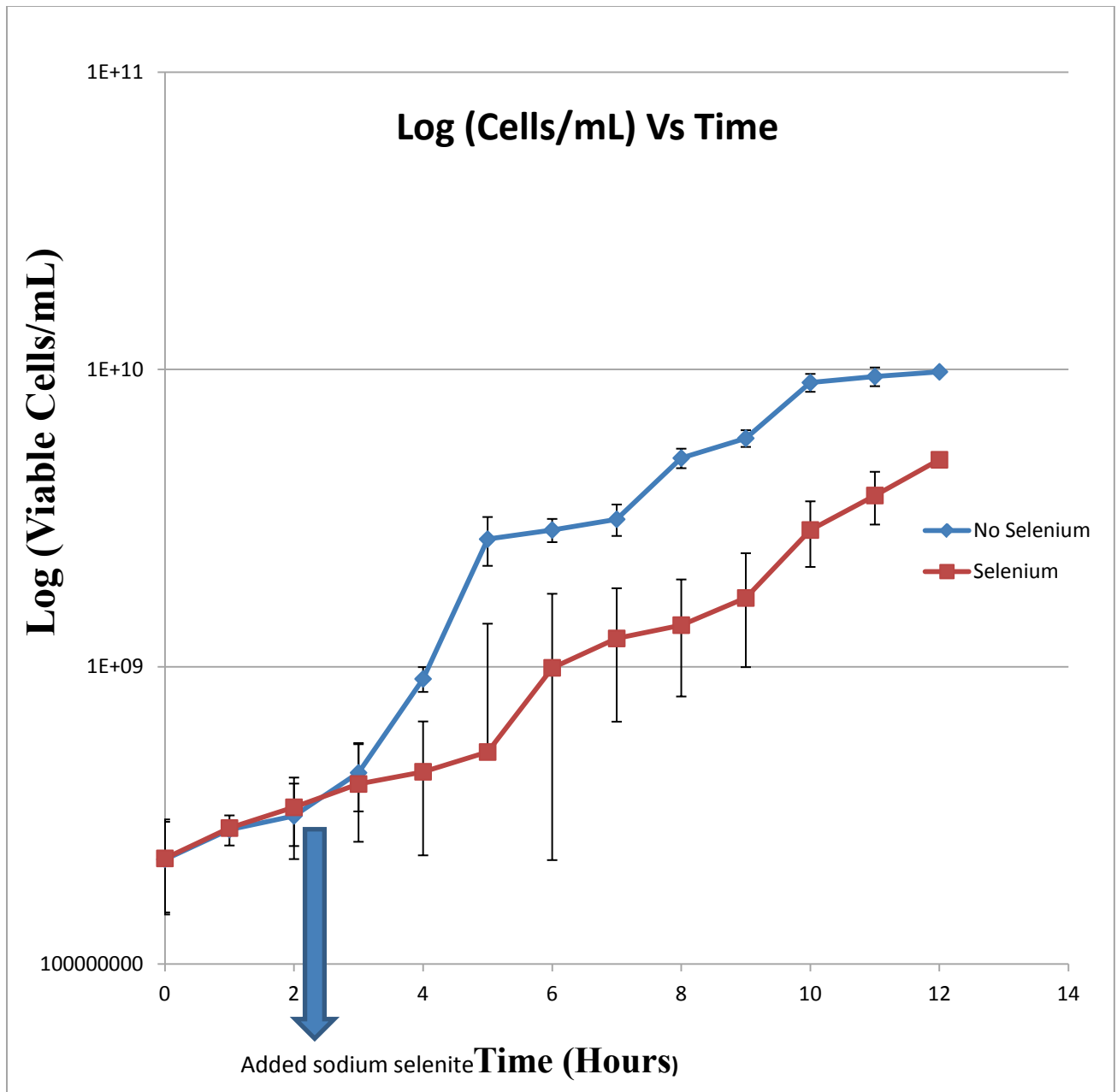


Figure 2: Graphical Representation of Log (Viable Cells/mL) vs Time. Y-axis represents turbidity in log of viable cells per milliliter, x-axis represents time. The blue line represents the number of viable cells in cultures lacking selenite and the red line represents the number of viable cells in cultures containing 1 mM selenite. .

Inductively Coupled Plasma (ICP) Spectroscopy:

The amount of selenium in the cells (pellet) and growth medium (supernatant) were analyzed by inductively coupled plasma. First 8 standard solutions of known concentrations along with a blank solution were analyzed which generated a calibration curve (Figure 3). The ICP instrument provided results in parts per million which were converted to millimolar for all the samples. The R^2 value of the graph represents the correlation of the data points on the straight line. A correlation of 1.000 represents that all the data points lie on the straight line. Any deviation of the values from the equation of straight line decreases the value significantly. The 0.9998 R^2 value suggested that there was a good correlation of the data points.

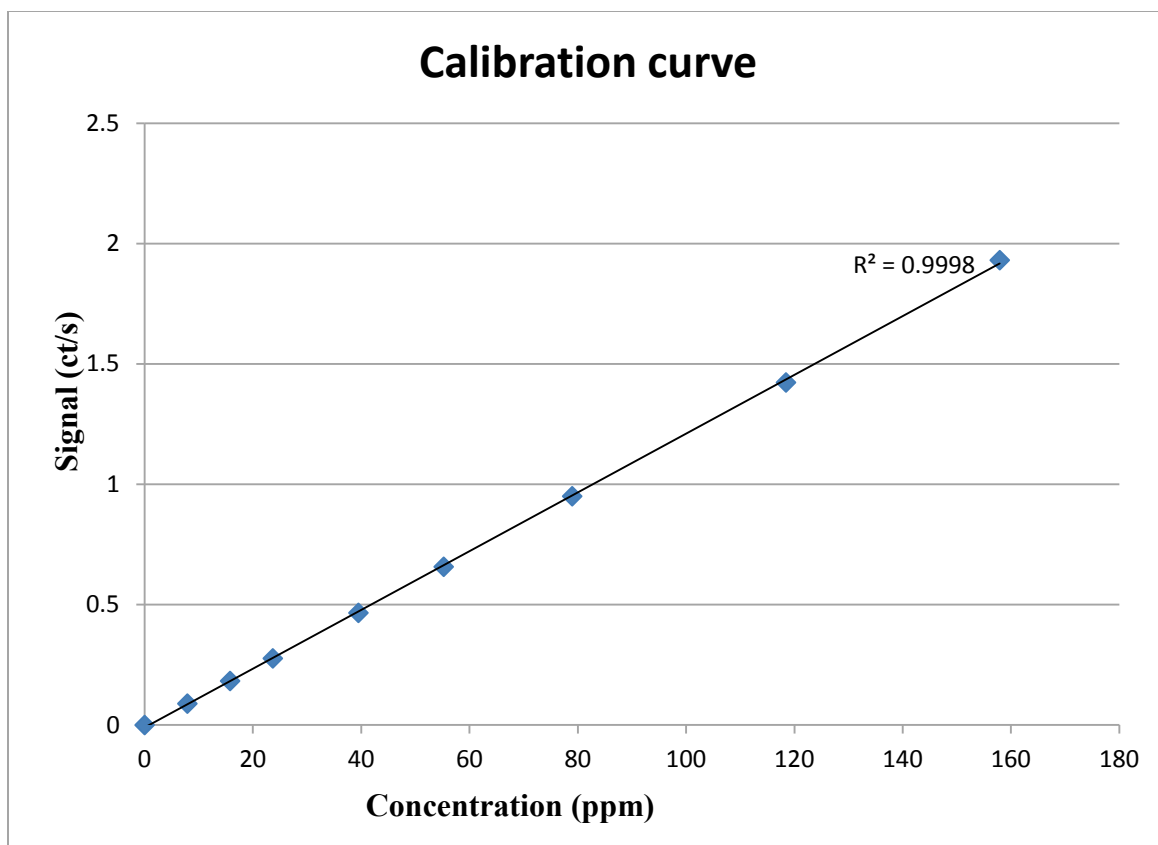


Figure 3: Calibration curve of standards. The Y- axis represents the ICP signal in counts per second, and the x-axis represents the concentration of selenium. The correlation is derived from the straight line which represents the accuracy of the data points compared to one another.

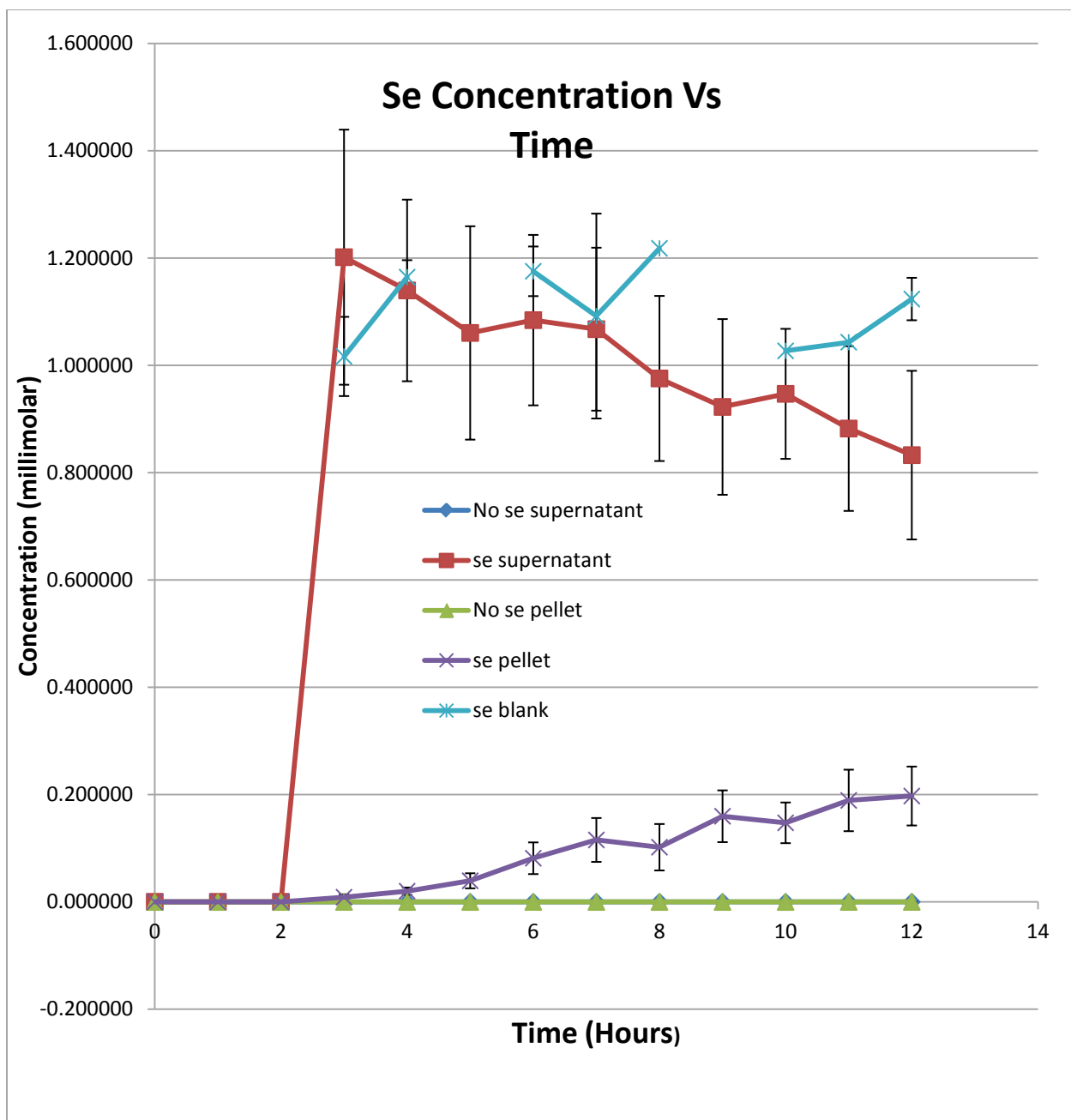


Figure 4 Graphical Representation of Selenium Concentration vs Time. The Y-axis represents the concentration of selenium in millimolar, the x-axis represents the time. The Red line represents the concentration of selenium in the supernatant, the violet line represents selenium concentration in the cells (pellet), the green line represents selenium concentration in cells (pellet) in cultures lacking selenite, the light blue line (covered by

the green line) represents the selenium concentration in the blank and the dark blue line represents the selenium concentration in growth medium that contains selenite but lacks cells.

Figure 4 follows the concentration of selenium in the bacterial cells (pellet) and the growth medium (supernatant). Selenium content in the two control flasks containing 1) growth medium, cells and no selenite (cells/ no selenite flask) and 2) growth medium, no cells and 1 mM selenite (selenite only flask) was also determined. In the graph, the y axis represents the concentration of selenium in millimolar units and x-axis represents the time.

The selenium concentration of the selenite only flask ranged between 1 and 1.22 mM selenite. It did not appear to decrease significantly over the 12 hour incubation period, although readings at all time-points would have provided better support for this observation. The selenium concentration in both the pellet and supernatant of the no selenite flask was 0 for all the times. In the flask containing cells and 1 mM selenite, there was a steep increase in the concentration of selenium in the growth medium from 0 mM to 1.2 mM at 2 hours when it was added. Then, it gradually decreased to 0.82 mM at 12 hours.

In the pellet of the culture containing cells and 1 mM selenite, the concentration of selenium increased from 0 mM at 2 hours when selenite was added to 0.2 mM selenite at 12 hours.

For all the data points the error range was calculated using the Student's t test with a confidence interval of 95% and (N-1) degrees of freedom using equation 1. After 3 hours there is no overlapping of error bars of pellet of the selenium culture flask, which suggests that the values obtained are statistically significant.

Amount of selenium per cell:

The selenium concentration per cell at each time point was calculated by dividing the average selenium concentration in the pellet by the average number of viable cells.

In figure 5, the y-axis represents the concentration (fM) of selenium per cell and the x-axis represents time. Sodium selenite was added after 2 hours. At 3 hours the amount of selenium increased from 0 to 0.02 fM/cell and continued to increase to 0.085 fM/cell at 7 at hours. At this point, the amount decreased rapidly to 0.040 to 0.045 fM/cell from 10-12 hours. The cells have switched to an exponential growth phase and may be switching from a reduction resistance mechanism to selenite efflux/exclusion resistance mechanism. The error bars, which were plotted using the absolute error, had a large error at every data point, suggesting that the data might not be statistically significant. Equation 2 on page 10 was used to calculate the error bars.

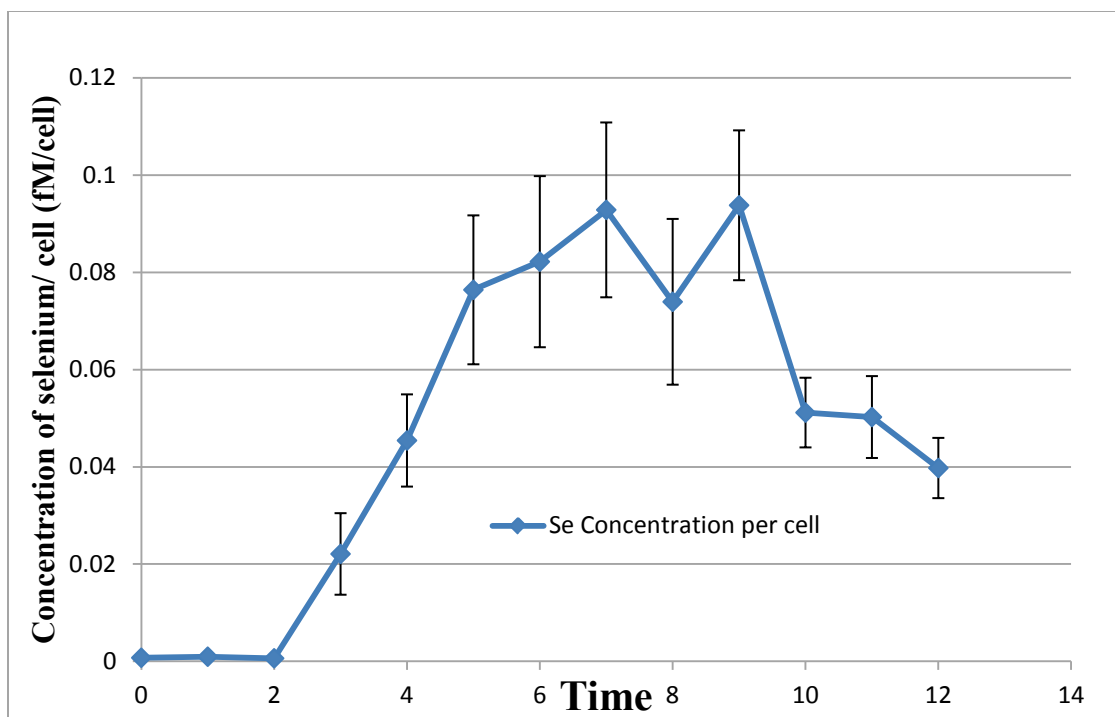


Figure 5: Graphical representation of fM of Se / cell vs Time The Y-axis represents fM of selenium per cell and the x-axis represents time. The graph shows the concentration of selenium in each viable cell of the selenite treated culture.

Discussion:

From the results, it can be concluded that the bacterial strain *S. maltophilia* 02 is resistant to selenite but the mechanism remains unknown. From my hypothesis, the expected mechanism was the conversion of selenite to elemental selenium by sequestration because of the appearance of a red color which had been observed previously [25]. The analytical data proved the presence of selenium inside the bacterial cells and the concentration of selenium in the cells was increasing over time.

In previous experiments done in our lab with 1 mM selenite, the turbidity in the selenite cultures surpassed the turbidity in the untreated culture. This occurred probably because the red precipitate contributed to turbidity rather than an increase in the number of cells. Viable cell counts showed that the cells in the selenite treated cultures never surpassed the number of viable cells in the untreated culture (figure 2). Although the turbidity of the selenite treated culture did not surpass the turbidity of the untreated culture after 12 hours in figure 1, expanding the growth period to 15 to 20 hours would have given the same result with higher Klett readings in the selenite treated culture than in the untreated culture. The viable cell counts in the untreated cultures followed a typical growth curve with a lag phase, exponential phase and stationary phase (Figure 2) [26]. A death phase would have been observed if growth had been followed for a longer time period. The growth curve of the selenite treated culture slowed upon the addition of selenite, forcing the cells to use some of their growth energy to combat its toxicity.

The concentrations in the pellet and supernatant were measured using inductively coupled plasma spectroscopy [27]. As expected, the concentration of selenium in the pellet increased over time, and the concentration of selenium in the supernatant decreased over time. The concentration in the growth medium and the selenite control without cells fluctuated between 1 and 1.2 mM but remained stable. This suggested that the selenite was not spontaneously precipitating and that the cells were responsible for most of the precipitation.

The initial mechanism appears to be selenite reduction between 2 and 5 hours of growth. From our observations, the expected mechanism of *S. maltophilia* 02 was to reduce [32,33] the selenite to elemental selenium, as the development of red color in the cultures was observed. Selenite reacts with glutathione and other reduced species, which may form different intermediates that may be nontoxic or toxic. [30] It was observed that *Rhodospirillum rubrum* pumps out elemental selenium across the plasma membrane. Selenium deposits were found in the cytoplasm [2,31], the periplasm and outside the cell.

Observations of *Bacillus subtilis* under a microscope after 6 hours of growth in the presence of selenite showed some atypical features which were spherical in shape and appeared on the surface of the cells as dark color granules. The observed dark color granules were present on 10% of the cells at 6 hours and it increased from then onwards [37]. The *Bacillus* species appeared to reduce the selenite and expel it as elemental selenium. Electron microscopy will be required to see if *S. maltophilia* 02 used a similar mechanism.

In *Clostridium .pasteurantium*, a cell free extract was reported to reduce selenite to elemental selenium with the help of a hydrogenase which acted as an electron carrier reduction system by oxidizing molecular hydrogen [38]. Although this is an anaerobic bacterium, the *S. maltophilia* 02 cells could be using a similar mechanism. A previous proteomic study [42] using *Enterobacter* sp. YSU showed that an outer membrane component hydrogenase is over-expressed in response to 40 mM selenite, and perhaps *S. maltophilia* 02 uses a similar hydrogenase for selenite reduction.

From the hypothesis, it was expected that the amount of selenium per cell would increase over time. This amount did increase until about 9 hours and then began to decrease from 10-12 hours. This decrease was due to an increase in viable cell number during this time period. This change may also have been caused by a shift in selenite metabolism from reduction to possibly methylation, exclusion or efflux.

According to Weiss, in the early stages of growth the growth rate of the cells treated with sodium selenite decreased as energy was used to convert selenite to selenium. The studies on *E. coli*, *P. vulgaris* and *S. thompson* showed that uptake of selenite decreased over time which might be due to changes in the selenium/ amino acid ratio [43]. In the case of *S. maltophilia* selenite uptake per cell also decreased after some time.

Proteomic studies revealed that selenite stress increased the synthesis of some chaperones, an elongation factor, and enzymes associated with oxidative stress [31]. Also Induction of oxidative stress enzymes in response to selenite stress was also observed in

E. coli [45]. The decrease in the selenite reduced per cell may be due to the proteomic factors which had effect of the selenite reduction.

Another selenite resistance mechanism could be methylation using a methyl transferase, which is the most common mode in eukaryotes [28] and prokaryotes [29]. The initial mechanism is most likely not methylation because the red color appeared, and no methyl derivative of selenite is known to produce a red color [34]. However, the bacterium may shift the mechanism to methylation if there are not enough resources available for the reduction. The presence of the garlicky smell at the end of 24 hours may be due to the methylation of selenium. Berzelius and Ghan noticed that the red color from elemental selenium gave garlicky smell when it was burned.

In previous experiments conducted in our lab [41], similar experiments were performed with 10 mM selenite. In those experiments, Klett turbidity measurements were followed to observe the change in the growth pattern, but due to the development of the red color, the actual growth pattern in the selenite culture flask was not determined. These results do not agree with the current results but the turbidity measurement agreed with the current turbidity measurements for 1 mM selenite. The cell pellet also sequestered selenium to about 0.2 mM which might be the limit for *S. maltophilia* 02 under normal conditions. The current results might be more consistent because viable cell counts were used along with turbidity. Also we improved the digestion technique. Previously, the samples were digested at high temperature which caused the complete evaporation of the liquid, leaving small amounts of debris. Only 5.0 mL of nitric acid was used for digestion in the previous experiments but we used a mixture of nitric and hydrochloric acid as suggested by the EPA. The new method resulted in a clear liquid

without any debris and improved the accuracy of the selenium concentration determinations.

Future Work

The transcriptome [44] from selenite treated and untreated cultures can be sequenced to detect changes in gene expression in response to toxic selenite levels. Similarly, microarray (RNA expression) studies may provide a better idea of the mechanism selenite resistance. Proteomic studies, especially, during the shift in growth rate at 3 hours after selenite was added and then, after the increase in growth rate at 5 hours may identify proteins involved in the reduction of selenite to selenium.

Selenite resistance genes can also be identified using transposon mutagenesis. The transposon is introduced by electroporation and randomly incorporates itself into the genome of *S. maltophilia* 02. Metal sensitive mutants can be identified by replica plating the transformants onto metal plates. Then, genomic DNA from the mutants can be digested, ligated and transformed into *E. coli*. Sequencing of the region flanking the transposon will identify the transposon interrupted genes.

Finally, from the data it can be concluded that *S. maltophilia* 02 can be a useful tool in bioremediation. If the resistance mechanism is known, efforts can be made to improve the amount of selenite reduced to selenium making the strain an important selenium bioremediation tool .

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