Study of Selenite Resistance in *Stenotrophomonas maltophilia* Oak Ridge Strain 02 and *Pseudomonas sp.* PC37

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ABSTRACT

Stenotrophomonas maltophilia Oak Ridge strain 02 (*S. maltophilia* 02) and *Pseudomonas* sp. PC37 (PC37) were isolated from a heavy metal contaminated site in Oak Ridge, TN, and demonstrated the ability to grow in the presence of toxic levels of different metals. This study focused on the tolerance or resistance of bacteria to selenite, an oxyanion of selenium. When grown in the presence of 0.5 mM selenite, *S. maltophilia* 02 produced a red precipitate, which is most likely, elemental selenium. However, PC37 did not produce the red precipitate when grown in 0.5 mM selenite. From these observations, we hypothesized that *S. maltophilia* 02 used a selenite reduction mechanism for resistance to 0.5 mM selenite, whereas PC37 used a selenite exclusion mechanism. The growth of both strains was measured in the presence of 0.5 mM selenite by turbidity measurement and by counting viable cells. Selenium in the cells and in the growth medium was measured by inductively coupled plasma (ICP) atomic emission spectroscopy. During the 10 hour growth period of *S. maltophilia* 02, the concentration of selenite in the growth medium decreased from 0.5 mM to 0.3 mM, while the selenium content in the cells increased from 0 to 0.1 mM. As expected, ICP quantitative analysis of the selenium concentration in PC37 did not show any significant change in the concentration of selenite in the growth medium or in the cell mass.

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LIST OF SYMBOLS

mM	milliMolar
mg	milligram
kg	kilogram
µg	microgram
l	liter
µl	microliter
g/l	gram/liter
cfu/ml	colony forming unit/milliliter
g/mol	gram/mole

INTRODUCTION

Various human activities such as industrialization, mining, refining of ores, manufacturing and agricultural practices have been contaminating the environment with toxic heavy metals and metalloids over the past 200 years (Kozdrój & Van Elsas, 2001). Heavy metals are widely scattered in the environment in many ways through run-off from mining activities, irrigation in agricultural practices, water wastes from metal processing, industrial processes (e.g. tanning) that use metallic components and deliberately-applied chemical agents (e.g. wood preservatives). In addition, traditional extraction of coal releases iron-rich wastes into the environment (Macaskie et al., 2010). Metals are naturally present in soil, water, sediments and living organisms and constantly cycle between biotic and abiotic compartments of the Earth. Microorganisms especially play a very important role in the cycling of metals and non-metals (Bruins et al 2000).

Trace Metals as Enzyme Cofactors

Some metals such as copper, zinc, nickel, and chromium are required in trace amounts to sustain vital functions of life. For example, the enzymes, nitrate reductase, xanthine dehydrogenase, aldehyde oxidase and sulfite oxidase, catalyze redox reactions that use molybdenum as a cofactor (Kaiser et al, 2005). Glutathione peroxidase, a selenium containing enzyme which is found in many body tissues, catalyzes the breakdown of the highly reactive metabolite, hydrogen peroxide (Parkman & Hultberg, 2002).

High Metal Concentrations Are Toxic

However, these metals become toxic above certain threshold concentrations. Pollution due to arsenic, barium, cadmium, chromium, lead, mercury, nickel, zinc, selenium and other metal salts not only cause various diseases but also affect the environment adversely. In general, metals are non-biodegradable, unless they are transformed by microorganisms (Lovley, 1995). Microbes transform metals and metalloids by reduction, oxidation, methylation, dealkylation, and other mechanisms which make them either more soluble and bioavailable or less soluble and immobile. These processes contribute to the natural biogeochemical cycling of metals (Lloyd & Lovley, 2001). Over the last two decades there has been considerable interest in the use of biological agents for remediation of soils.

Resistance Mechanisms

Microbes interact with metals in many ways. The three main mechanisms are i) reduction to a less toxic oxidation state, ii) intracellular precipitation or iii) efflux. i) *Bacillus licheniformis, Bacillus subtilis, Bacillus cereus, Bacillus pumilus* and *Exiquobacterium* sp. reduce selenite, a toxic oxyanion, to nontoxic elemental selenium aerobically and precipitate it from the medium (Ikram & Faisal, 2010). Plasmid encoded mercury resistance catalyzes a biochemical reaction that converts toxic Hg(II) into Hg(0) which is nontoxic and volatilizes it from the medium (Misra, 1992). (ii) In response to Cu or Cd, *Saccharomyces cerevisiae* synthesizes γ-glutamyl peptides which bind to and form precipitates with these metals (Mehra et al, 1988). (iii) Efflux, where the bacteria expel metals before they can interfere with metabolic processes, is one of the commonest metal resistance mechanisms. (Bruins et al., 2000). *Escherichia coli* and *Staphylococcus aureus*

exhibit resistance towards arsenate and arsenite ions by removing them outside the cells through an energy-dependent efflux mechanism (Belliveau et al, 1987) .

Previous studies have shown that a large number of metals and metalloids are either less soluble or more volatile in the reduced state than they are in oxidized state (Lovley, 1995). The process in which a microorganism converts or transforms a soluble oxidized form of metal contaminant to an insoluble reduced forms is known as dissimilatory metal reduction through anaerobic respiration (Lovley, 1995).

Occurrence of Selenium

Selenium (Se) was first reported in 1817 by a Swedish chemist Jons Jakob Berzelius (Fordyce, 2007). Selenium naturally occurs in the native state with sulfur in the form of seleniate in minerals such as eucairite (CuAgSe), claustalite (PbSe), naumanite (Ag_2 Se) crookesite ($(CuTlAg)_2$ Se) and zorgite (PbCuSe) (Ikram & Faisal, 2010) The main selenium sources are copper ores, from which it is recovered as a by-product in the processes of electrolytic refining. The largest world producers are in the USA, Canada, Sweden, Belgium, Japan and Peru. This element is widely distributed on earth. According to the USGS, US Department of Interior report conducted in 2004 there are no economically significant selenium ores or ore deposits (Butterman W. C, 2004). Selenium is present in high-sulfur coal found in the USA, Russia and China with selenium content as high as 43 mg/kg (Yudovich & Ketris, 2006). Selenium is also found in black slate and volcanic tuff in the Daba region of China at concentration of 22 and 32 mg/kg, respectively (Kunli et al 2004). Some parts of Northwestern India display considerably high selenium

concentrations, where native plants are found to accumulate more than 100 mg/ kg, dry weight (Dhillon & Dhillon, 2009).

Selenium is a Required Trace Metalloid

A trace amount of selenium is required for the health of both plants and animals (Fordyce, 2007). Selenium is found to be essential for proper functioning of the thyroid gland (Köhrle, 1999). Selenium compounds such as sodium selenate and sodium selenite are used in the prevention of many nutritional deficiency diseases (Abdo, 1994). The daily requirement for selenium depends on age and life stage. According to the National Institutes of Health's Office of Dietary Supplements, children ages 1 to 14 need between 20 and 40 µg per day. The average daily intake in the United States is 153 µg for men and 109 µg for women. While this daily amount of selenium is higher than the minimum requirement of 55 µg a day, it is well below toxic levels. In general, the concentration of selenium in natural water is less than 10 µg/l and the concentration of selenium in soil ranges from 0.010 to 2 mg/kg (Fordyce, 2007).

Selenocysteine and selenomethionine are two special amino acids that are formed in some bacterial species such as *E. coli* (Turner et al 1998). Once selenium (selenite or selenate) is inside the cells, it is then utilized to form selenocysteine or selenomethionine. Selenium oxyanions are first reduced to selenide by either the sulfate reduction pathway or by an undefined carrier which is yet to be identified. Selenide is then incorporated into selenocysteine. In *E. coli*, selenocysteine is incorporated into the enzyme, formate dehydrogenase, at a stop codon (Turner et al., 1998).

Selenium Contamination

Health problems in animals and humans caused by exposure to an excess amount of selenium from natural sources g is rare (Fordyce, 2007). However, selenium toxicity due to anthropogenic reasons have been associated with birth defects in aquatic birds and freshwater fishes (Hamilton, 2004). In the United States there are two main factors that cause selenium mobilization and introduction of selenium at toxic levels into the aquatic systems. The first factor is through industries such as the combustion of fossil fuels. Coal fly ash released from power stations are one of the main sources of selenium contamination into aquatic systems (Parkman & Hultberg, 2002). The second factor is agricultural irrigation of seleniferous soils for crop production (Parkman & Hultberg, 2002). Agriculture drainage, industrial effluents and sewage sludge are other major sources of selenium contamination (R L Seiler et al, 1992). It has also been associated with mining, the storage and disposal of radioactive wastes and most recently the manufacturing and application of selenium-based nanomaterials (He & Yao, 2011).

The Kesterson Reservoir in California is well known for selenium contamination, which was linked to embryonic deformities of aquatic birds and ultimately resulted in closure of the Reservoir in 1985 (Ohlendorf et al, 1987). Selenium bioaccumulation in waterfowl eggs was reported (Skorupa & Ohlendorf, 1991). In the western side of California's San Joaquin Valley, agriculture irrigation waters leached naturally elevated levels of selenium from the soil. The drainage waters were then disposed of in evaporation ponds, where selenium accumulated to toxic levels (Ohlendorf et al., 1987). Belews Lake in North Carolina is one of the most extensive and prolonged cases of selenium poisoning in freshwater fish (Lemly, 2002). This lake was contaminated by selenium in wastewater from a coal-fired power

plant in the mid-1970. Selenium bioaccumulated in the aquatic food chain and caused severe tissue pathology and reproductive damage to the resident fish community. Selenium poisoning in fishes remain invisible until the time of hatching. Selenium in the female's diet accumulated in eggs and was stored until hatching, whereupon developing larval fish metabolized it. If the concentration in eggs are great enough (about 10 µg/g or greater), biochemical functions may be disrupted, and teratogenic deformity and death may occur. Adult fish can survive and appear healthy despite the fact that extensive reproductive failure occurs. As a result of selenium contamination, 19 of 20 fish species in Belews Lake were eliminated.

In 2007, Wen and Carignan estimated that between 37.5% and 40.6% of the total selenium emissions to the atmosphere are due to anthropogenic activities. One solution to this environmental problem is to remove selenium from the contaminated area before it is transported further. Selenium exists in different oxidation states: highly water soluble, selenate (Se [VI]) and selenite (Se[IV]); insoluble elemental selenium (Se[0]); and methylated and unmethylated selenide (Se[-II]) (Tamari, 1998). The oxidized states, selenate and selenite, are frequently found in surface waters (Lenz & Lens, 2009) which also makes it bioavailable to many different microorganisms.

Selenium Bioremediation

Since selenium is less soluble and less biologically available in the reduced elemental state, reduction of selenate and selenite to elemental selenium can be used to detoxify and remove selenium from a contaminated site. The chemical strategy requires a strong

reducing agent which reduces selenate to selenite. Selenate is converted to selenite in the presence of 10 M HCl at 60°C according to the following reaction (Bye & Lund, 1988):

$$SeO_4^{2-} + 4H+ + 2Cl^- \rightarrow H_2SeO_3 + 2H_2O + Cl_2\ (g)$$

Then, the selenite can be reduced directly to elemental selenium by ferrous hydroxide (Andenbohede et al, 1988). Bioremediation, which exploits the metabolism of microorganisms, can also be used to remove toxic selenium. In this case, elemental selenium, selenate and selenite can serve as electron acceptors during anaerobic respiration (Lovley, 1995). The advantage of using a biological method is that it uses a renewable organic electron donor such as lactic acid as a reducing agent. Furthermore, the biological reaction takes place under natural environmental condition and does not require an external energy input (Hageman et al, 2013).

Biological transformation of selenium oxyanions can be categorized as assimilatory and dissimilatory reduction, alkylation, dealkylation and redox reactions (Lenz & Lens, 2009). Several studies have shown that selenium undergoes oxidation and reduction reactions mediated by microorganisms. There are a wide variety of microorganisms which have the ability to reduce selenium oxyanions (Frankenberger and Karlson, 1994). Generally, the dissimilatory pathway involves the anaerobic reduction of water soluble selenite and selenate to insoluble elemental selenium (Lenz, Janzen, & Lens, 2008). It sometimes also involves non-specified reduction via dissimilatory sulfate reduction (Tucker, Barton, & Thomson, 1998) or via dissimilatory nitrate reduction (Lenz et al., 2001). Also, selenium oxyanions reduction can take place either in strictly anaerobic or microaerophillic conditions by different organisms (Lenz & Lens, 2009). *Thauera selenatis* a selenite-

respiring bacterium has been used for the bioremediation of selenium in drainage water from the Westland water district, San Joaquin Valley (Macy, Lawson, & Demoll-decker, 1993). This bacterium respires selenate anaerobically using acetate as the preferred electron donor. Spore forming *Bacillus subtilis* and *Microbacterium arborescens*, other isolates from Kesterson Reservoir soil in California, reduced selenite to form red elemental selenium (Se^0) (Combs et al., 1996).

A strain of *Stenotrophomonas maltophilia* isolated from a selenium-contaminated evaporation pond sediment in the Tulare Lake Drainage District, California, is a non-fermenting, aerobic gram-negative rod which was studied for its potential for bioremediation. This organism reduced selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) to red amorphous elemental selenium (Se^o) only upon reaching stationary phase, when O_2 levels are less than 0.1 mg/l (Dungan, Yates, & Frankenberger, 2003). It also produced volatile alkylselenides when both oxyanions were present in the medium.

Similarly, *Sulfurospirillum barnesii, Bacillus arsenicoselenatis* and *Selenihalanaerobacter shriftii* are all examples of selenate reducing bacteria that form elemental selenium as end products. This insoluble form can also be converted into selenide by the selenite respiring bacterium *Bacillus selenitireducens* (Herbel et al., 2003). Anaerobic respiratory pathways where microorganism use electron acceptors other than oxygen is termed as "dissimilatory metal (loid) reduction".

Stenotrophomonas maltophilia OR02 and *Pseudomonas* sp. PC37

During World War II and the cold war, the Y-12 plant in Oak Ridge, TN, processed uranium and lithium to produce nuclear weapons and waste which contaminated nearby

East Fork Poplar Creek with mercury and other heavy metals. *Stenotrophomonas maltophilia* OR02 (*S. maltophilia* 02) and *Pseudomonas* sp. PC37 (PC37), were isolated from East Fork Poplar Creek, which exhibit resistance to salts of selenium (Holmes et al., 2009). When *S. maltophilia* 02 cells growing at exponential phase are introduced to 0.5 mM selenite, they appear to reduce it to elemental selenium and methylate it because they turn red and produce a strong stale garlic odor. Exponentially growing PC37 cells, on the other hand, do not turn red or produce the stale garlic odor. They appear to be excluding the selenite from entry into the cell. My research will examine the differences between these two phenotypes using inductively coupled plasma (ICP) resonance to measure the levels of selenite reduction.

EXPERIMENTAL DESIGN:

Initially, a single colony was inoculated into R3A-tris medium. After growing it overnight, it was diluted 1:50 into fresh medium. The growth was detected by viable cell counts using a spread plate technique and by measuring the turbidity of the sample using a Klett Colorimeter. During early exponential phase, selenite was added to a concentration of 0.5 mM. Samples were removed every hour, centrifuged to separate pellet and supernatant, digested and analyzed for selenium content by ICP.

HYPOTHESIS:

S. maltophilia 02 appears to enzymatically reduce the selenite to elemental selenium and methylate it whereas PC37 appears to exclude the selenite from the cells. Thus, in *S. maltophilia* 02, we expect to see a decrease in selenite concentration in the growth medium and an increase in concentration in the cell mass. Since PC37 appears to exclude the selenite from the cells, we expect to observe little or no change in the selenite concentration of either the cell mass or growth medium. Because of *S. maltophilia* 02 appears to rapidly transform selenite into elemental selenium, it may be useful for removing selenite from seleniferous agricultural wastewater.

METHODS

Bacterial strains

Stenotrophomonas maltophilia strain OR02 (ATCC # 53510) was obtained from the American Type Culture Collection (Manassas, VA) and is resistant to many different metals, including selenite (Holmes et al 2009). PC37 was isolated from Poplar Creek and tentatively identified as a species of *Pseudomonas* by 16s rRNA sequencing (unpublished data).

Growth media**:**

Each strain was grown in R3A-tris medium containing 1.0 g/l yeast extract (Amresco, Solon, OH), 1.0 g/l Difco proteose peptone no. 3 (Becton, Dickinson & Company, Sparks, MD), 1.0 g/l casamino acids (Amresco, Solon, OH), 1.0 g/l D-glucose (Aresco, Solon, OH), 1.0 g/l soluble starch (Becton, Dickinson & Company, Sparks, MD), 0.5 g/l sodium pyruvate (Fisher BioReagents, Fisher Scientific, Fair Lawn, NJ), 10.0 ml/l 10.0 mM Tris pH 7.5 (Amresco, Solon, OH) and 0.1g/l $MgSO_4.7\ H_2O$ (Enzyme Grade, Fisher Chemicals, Fisher Scientific, Fair Lawn, NJ). 1X M-9 minimal salts were purchased from Fisher Scientific (Fair Lawn, NJ) and contained 42.3 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 18.6 mM NH_4Cl and 8.6 mM NaCl. When required, sodium selenite (MP Biomedical, LLC, Solon, OH) was added to a concentration of 0.5 mM.

Lennox Luria-Bertani (LB) medium was purchased from Fisher Scientific (Fair Lawn, NJ) and contained 10 g tryptone, 5 g yeast extract and 5 g sodium chloride per liter of distilled

water. When required, each liter was supplemented with 16 g of agar (Amresco, Solon, OH) and then autoclaved before use.

Growth Curves:

For starting inoculum, single colonies from pure culture plates were inoculated into two sterile test tubes containing sterile R3A tris medium. The tubes were then incubated at 30^{o}C overnight. The following day, the overnight cultures were diluted 1:50 into 2 side arm flasks containing fresh R3A tris medium. A third flask which contained growth medium but lacked bacteria served as a negative control. Then, all three flask were incubated at 30^{o}C with shaking at 120 rpm for 10-12 hours. After 2.5 hours of incubation two flasks, one with culture and one without culture, were supplemented with 0.5 mM sodium selenite (Na_2SeO_3).

Turbidity

The growth was monitored in each flask using a Klett Colorimeter (BEL-ART productions, Pequannock, New Jersey) at 1 hour intervals. The same procedure was followed for both bacterial strains.

Viable Cell Count

For viable cell count, 0.1 ml of sample from each flask was diluted in 1X M-9 salts to the desired dilution and plated in duplicate onto Luria-Bertani (LB) medium petri plates. For each incubation hour, there were 4 plates with two different dilutions in 1X M-9 minimal salts. For *S. maltophilia* 02, 10^{-5} and 10^{-6} dilutions were plated at times 0, 1 and 2 hours of

incubation. Then, 10^{-6} dilutions were plated at times 3-5 hours. Finally, 10^{-6} and 10^{-7} dilutions were plated at times 6-10 hours. The same procedure was performed for PC37.

Plating of these diluted samples was done using a spread plate technique with an L-shaped spreader on sterile LB agar petri plates. All the inoculated plates were incubated at 30^{o}C for 24-30 hours. After incubation, the colonies were counted manually. Colony counts between 30 cfu/ml to 400 cfu/ml per plate were recorded.

Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES)

ICP-AES (Thermo Electro Corporation, Pittsburgh, PA) spectroscopy was done to determine the concentration of selenium content present in our samples. Selenium was analyzed using three different wavelengths to confirm response of Se concentrations. In addition Yttrium was used as an internal standard to adjust response to instrumental changes over time.

Preparation of Calibration Curve

In further analysis, ICP-AES was used to measure the amount of selenium present in the cell mass (cell pellet) after digestion and in the growth medium (supernatant). Sterile growth media was also tested for selenite concentration as negative controls. The samples were read against a standardized concentration curve. Calibration standards were prepared for the generation of a multipoint standard curve covering the range of analyte concentrations anticipated in our samples from both the bacteria under study. , Different standards (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM and 0.5 mM) were prepared from a 1 M

stock solution of sodium selenite using a mixture of 5% hydrochloric acid, 5% nitric acid and water as the solvent. For quality control, a concentration near the midpoint (0.3 mM selenite) of the calibration curve was used. Since the ICP data is produced in parts per million (PPM), the data was converted to millimolar (mM) by dividing PPM by 78.96 g/mol, the molecular weight of selenium. The output produced by the ICP-AES analysis was provided as a detailed Excel spreadsheet. Later the data of interest was exported and converted into mM. The experiments were performed more than once and an average value was recorded for further calculations and result interpretations.

Standard error was calculated using the following equation for the Student T test (Levie, 1986):

$$\textbf{Error} = \frac{t_{(95\%CI,(N-1)d.f.)} * \textbf{Std Dev}}{\sqrt{N-1}} \qquad \textbf{.... Equation 1}$$

Where, N is Number of observation, CI is Confidence interval, d.f. is Degrees of Freedom and Std Dev is standard deviation.

Sample Preparations for ICP

Centrifugation:

For ICP, 10 ml samples were removed at each hour of incubation and centrifuged at a relative centrifugal force (rcf) of 7,000 for 15 minutes using an Eppendorf centrifuge (5810 R, Brinkmann Instrumental INC., Westbury, NJ). The supernatant was poured into a new tube and both the pellet (cells) and supernatant were stored at -20^{o}C till all the samples were collected. The same procedure was followed for both the cultures.

Digestion:

Frozen samples were thawed, vortexed, transferred to a digestion tube and digested using a laboratory protocol provided by the United States Environmental Protection Agency. 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 at 110°C to obtain a 90°C sample temperature for two hours in a Hot Block Pro digester (SC181), which was connected to a SC180 Controller (Environmental Express, Mt. Pleasant, SC). For normalization purposes, distilled water was added up to 10 ml for all digested samples. ICP-AES was then used to measure the amount of selenium present in the cell mass (cell pellet) and in the growth medium (supernatant).

RESULTS

Turbidity Growth Curve of *S. maltophilia* 02

Growth of both bacterial strains, *S. maltophilia* 02 and *Pseudomonas sp.* PC37, in the selenite supplemented medium (medium is singular and media is plural. Since we only used 1 type of medium, R3A tris, please use the term medium and not media) showed that both are tolerant to 0.5 mM selenite. *Figure*-1(a) shows the growth of *S. maltophilia* 02 with and without selenite as measured by turbidity in Klett units.

In Figure 1(a) the Y-axis represents the logarithm of cell turbidity in the media, and the X-axis represents incubation time in hours. Sodium selenite was added 2.5 hours after inoculation. The blue curve with open circle data points shows the growth of the *S. maltophilia* 02 positive control without selenium. It demonstrated typical growth curve with lag, log and a more prominent stationary phase. The figure does not show a death phase, since the experiment was monitored hourly only for the first 10-11 hours. Turbidity started at about 20 Klett units and increased to 350 Klett units after 10 hours. There was no change in color of the media except that it turned from a clear medium to off-white colored turbid media, indicating cell growth. For the negative control, the same experiment was conducted with no inoculation but with 0.5 mM selenite in the growth medium. The colorimetric data showed a negligible change in the turbidity of the media without bacteria, and there was also no change in the color of the medium. The red curve with the closed square data points in Figure 1(a) showed the turbidity of *S. maltophilia* 02 cells supplemented with 0.5 mM selenium.

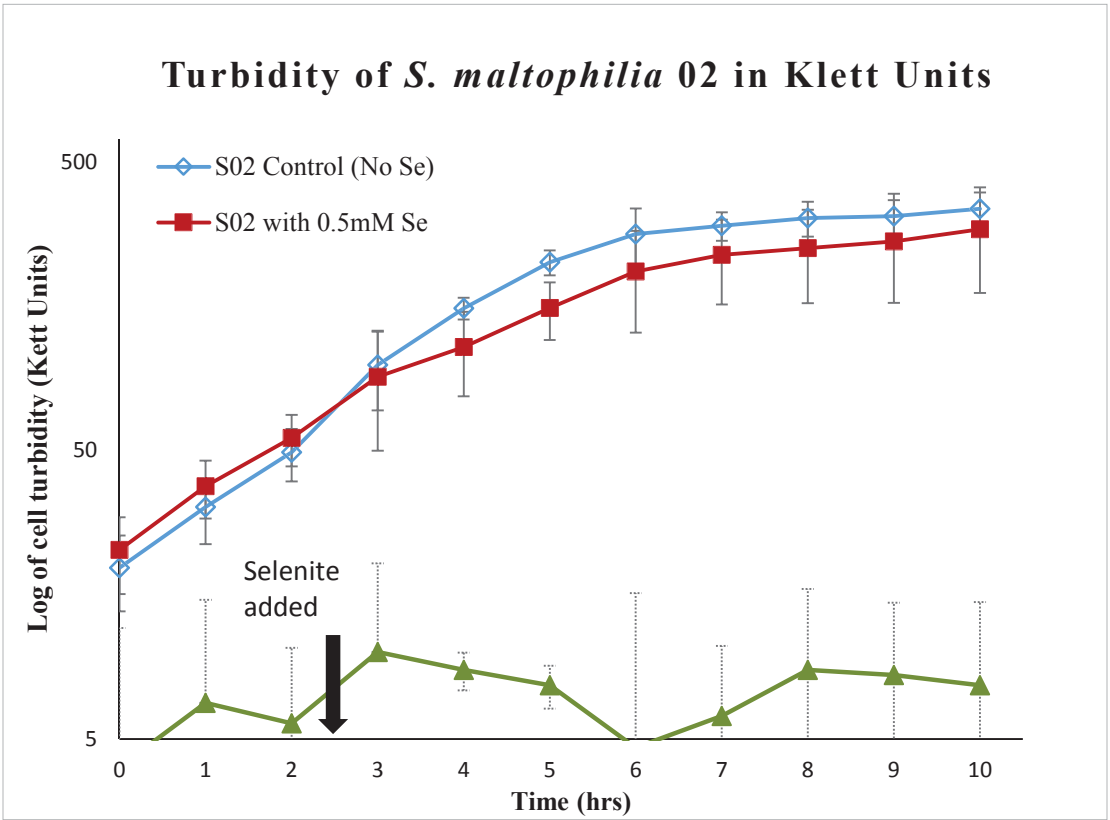

Figure 1(a): The measurement of growth of *S. maltophilia* 02 expressed as turbidity in Klett units. The error bars represents the standard error of 5 different experiments, Red square = turbidity of *S. maltophilia* 02 with Se, Blue circle = turbidity of *S. maltophilia* 02 (No Se) and Green triangle = turbidity of sterile media with 0.5 mM. The error bars were calculated using the Student-t test with a 95% confidence interval and N-1 degrees of freedom (equation 1, page 22) where N=5.

Turbidity was in the range of about 20 Klett units to about 350 Klett units and increased to ~400 Klett units after 24 hours (data not showed in the figure 1a). After time point 4 hours, the selenite treated *S. maltophilia* 02 culture started to form a red precipitate.

The turbidity of the selenite treated *S. maltophilia* 02 culture concomitantly increased with the appearance of a reddish color and a pungent stale garlic-like odor, which was probably due to selenium methylation. In addition, the coloration of the medium was probably due

to bacterial reduction of the soluble selenite into insoluble elemental selenium which was indicated by the formation of red precipitated. After 6 hour of incubation, the precipitate started to deposit on the side of the flask and the growth medium appeared to be more turbid and reddish in comparison to the control flask without any selenite. The growth of the selenite treated culture appeared to be slightly retarded compared to the growth of the untreated culture, but the difference was not statistically significant.

Turbidity Growth Curve of *Pseudomonas sp*. PC37

Figure 1(b) represents the growth of *Pseudomonas sp.* PC37 with and without selenite. The positive control culture contained fresh sterile medium inoculated with an overnight culture of PC37 but no selenite. It was plotted as blue colored open circle data points showing the growth of PC37 without selenite. It demonstrated a typical growth pattern exhibiting a brief lag, exponential and stationary phase (Figure 1b). The first hour between time zero and time one hour was the lag phase.

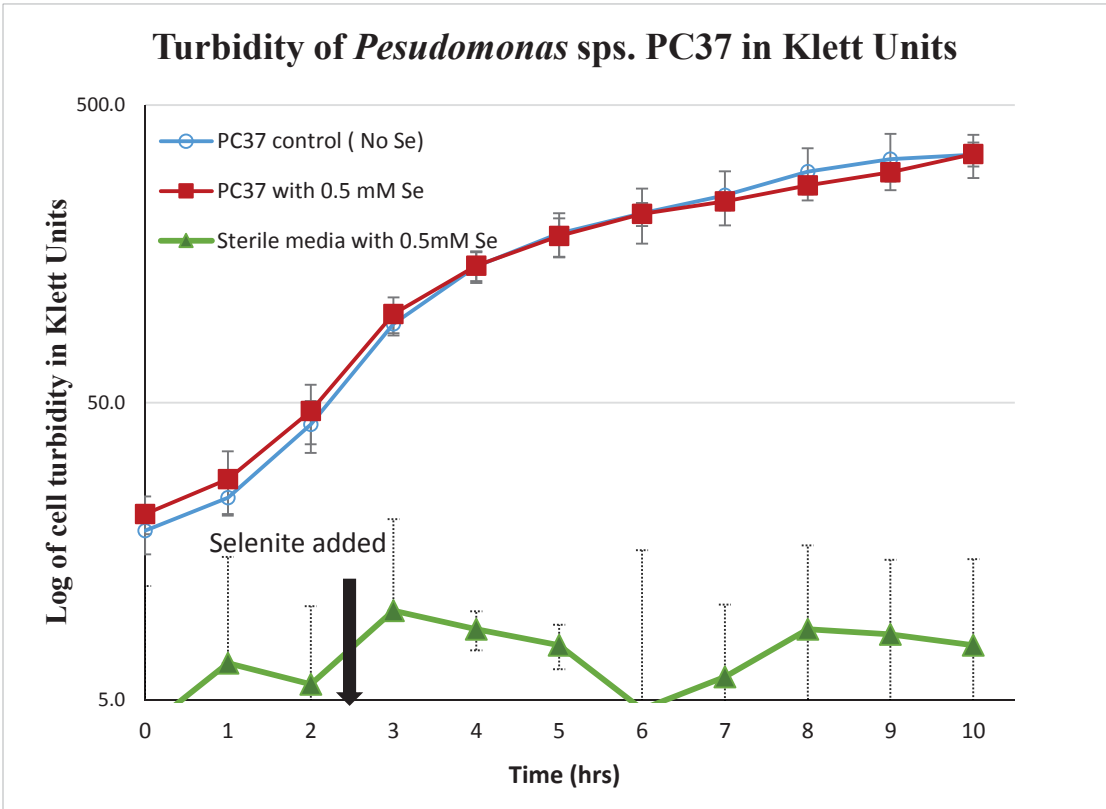

Figure 1(b): The measurement of growth of *Pseudomonas sp.* PC37 expressed as turbidity in Klett units. The error bars represents the standard error of 7 different experiments. Selenite was added to 0.5 mM 2 ½ hours after the culture was started. Red squares = turbidity of PC37 with Se, Blue open circles = turbidity of PC37 without Se. Green triangles = turbidity of sterile media with 0.5 mM.

Then, the cells entered the exponential phase between time one hour and time four hours with increasing turbidity followed by the stationary phase between time 4 hours and time 10 hours. Since the experiments were only monitored for 10 hours, the death phase was not observed. The initial turbidity of PC37 without selenite at zero hours was approximately 19 Klett units which after 10 hours of incubation increased up to approximately 350 Klett units.

For the negative control, the same experiment was conducted with no cells but with 0.5 mM selenite in the growth medium. In Fig 1(b), green colored curve with closed triangle symbols shows the turbidity of sterile media with 0.5 mM for 0-10 hour incubation period.

The colorimetric data showed a negligible change in the turbidity of the medium without bacteria, and there was also no change in the color of the media. The growth of PC37 exposed to 0.5 mM selenite is shown by closed red square symbols, where selenite was added 2 ½ hours after the culture was started.

Graphically, it is evident that the turbidity of both treated and untreated cells did not show any significant difference. Unlike *S. maltophilia* 02, in the presence of selenite, PC37 did not produce any red precipitate or pungent, rancid garlic odor. Thus, in contrast to *S. maltophilia* 02, PC37 appeared to not reduce selenite, and statistically, its growth was not impeded by 0.5 mM selenite.

Viable Cell Count Growth Curve of *S. maltophilia* 02

Since, the production of red elemental selenium contributed to the turbidity, it was necessary to determine the viability of the cells in the cultures to measure actual growth. Thus, at each hour of incubation dilutions of each culture were plated on LB agar medium to measure viable cells.

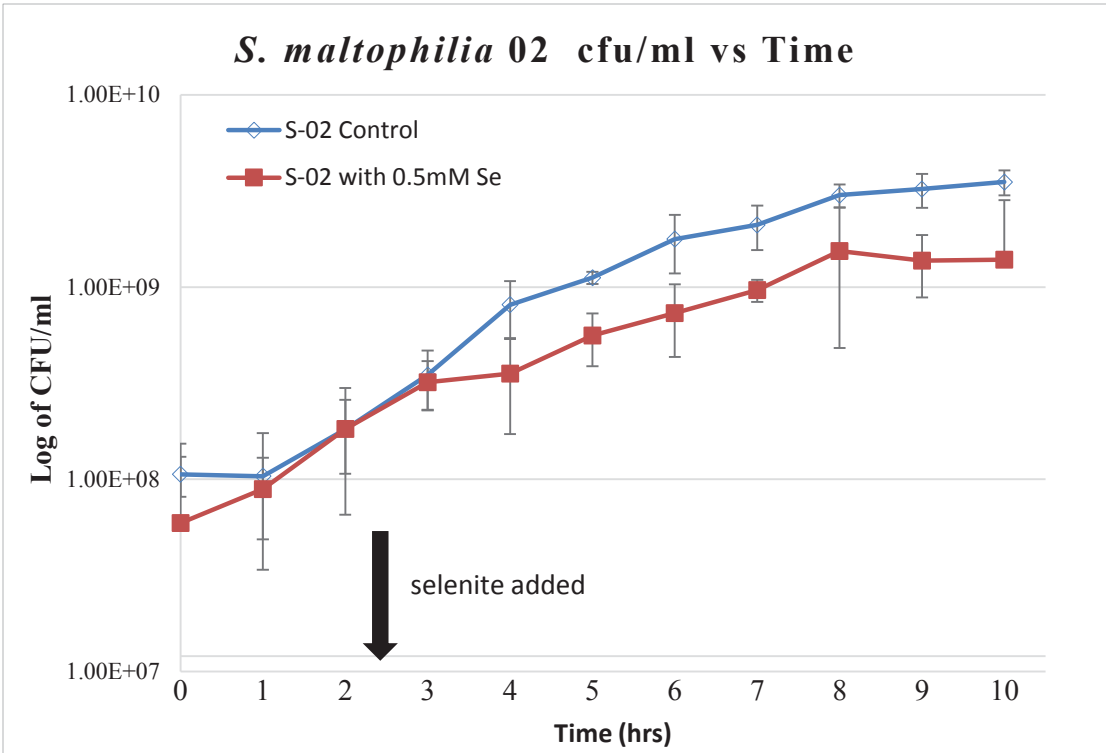

Figure 2(a): The measurement of growth of *S. maltophilia* 02 expressed as CFU/ml. The error bars represents the standard error of 5 different experiments. Selenite was added after 2 ½ hours of incubation to 0.5 mM concentration. Open circles = No selenite and filled squares = selenite. The negative control was not plotted because it is not possible to plot zero values on a logarithmic scale.

Figure 2 (a) follows the growth of *S. maltophilia* 02 cultures in the presence and absence of 0.5 mM selenite as measured by viable cells count. The curve with the open circles represents the positive control, which contained growth medium and cells but no selenite. There was a very brief lag phase between time zero and one hour. Then, the next four hours of incubation from time one hour to time four hours demonstrated exponential growth. The culture entered stationary phase after time five hours. Overall, *S. maltophilia* 02 demonstrated a typical growth curve in the absence of selenite.

The negative control, containing medium and selenite but no cells yielded no growth on any of the plates as expected and could not be plotted on a logarithmic scale. The closed squares show the viable cell count of *S. maltophilia* 02 in the presence of 0.5 mM selenite, which was added to the medium after 2 ½ hours of incubation. During the first 2 hours of incubation period the growth of *S. maltophilia* 02 with and without selenite looks similar. Then, immediately after selenite was added, the growth rate of *S. maltophilia* 02 in the presence of selenite decreased compared to the positive control. The cell count from time three hours to time ten hours was significantly lower than that of the control cell count at time points 5, 6, 9 and 10 hours, indicating inhibition by selenite. However, this was not observed in turbidity measurements, possibly because the formation of red precipitate was contributing to the turbidity.

Viable Cell Count Growth Curve of *Pseudomonas sp.* PC37

Figure 2(b) shows the viable cell count growth curve of for PC37 with and without 0.5 mM selenite. The blue curve with open circle data points shows the cell count of PC37 without selenite. The incubation period between time zero and time two hours was the lag phase. Then, the rapid increase in viable cells count between time two hours and time six hours demonstrated an exponential phase. After time six hours, the PC37 culture entered stationary phase. Overall, the growth of PC37 without selenite demonstrated a typical growth curve.

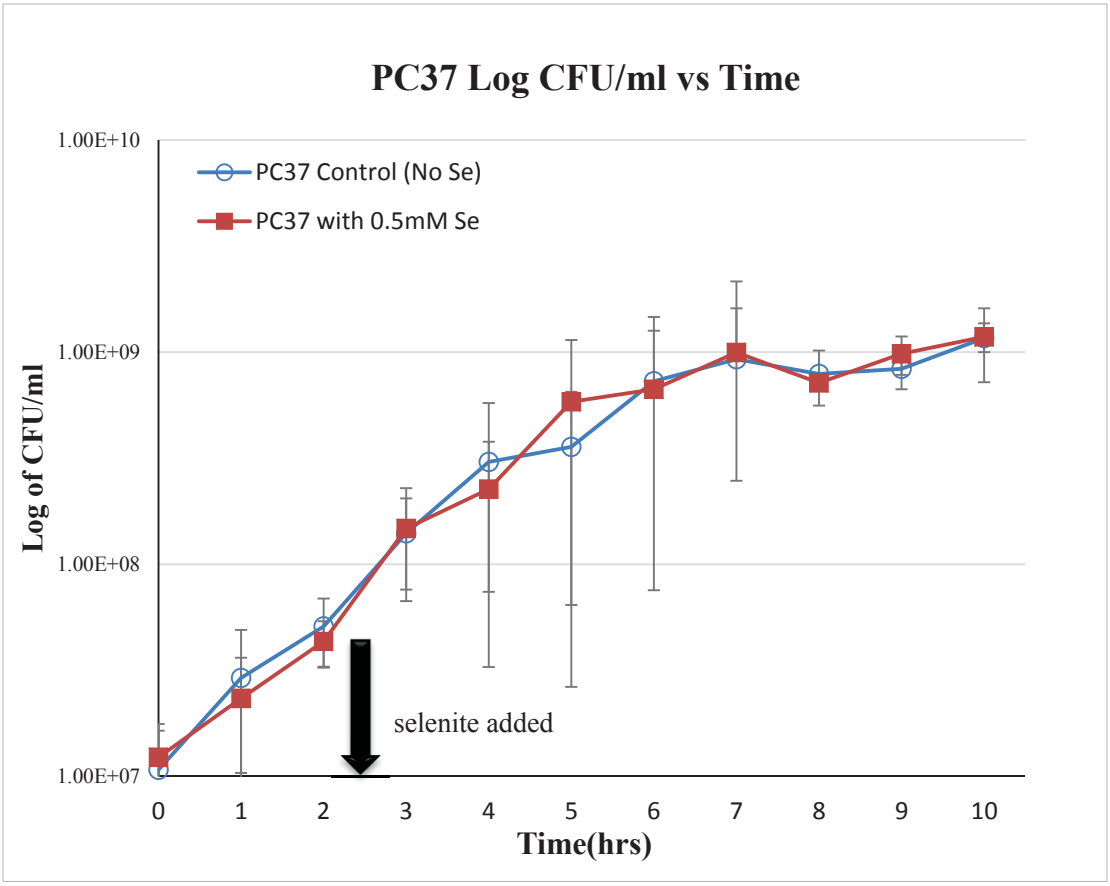

Figure 2(b): The measurement of growth of *Pseudomonas sp.* PC37 expressed as CFU/ml. The error bars represents the standard error of 6 different experiments. Selenite was added after 2 ½ hours of incubation at 0.5 mM concentration. Open circles = No selenite and filled square = selenite. The negative control was not plotted because it is not possible to plot zero values on a logarithmic scale.

The curve red closed squares follows the cell count growth curve of the selenite treated PC37 bacterial strain. The graphical representation of the cell count of PC37 with and without selenite did not reflect any significant difference. The error in the cell count of both with and without selenite was not only overlapping but large. After selenite was added, there was no significant difference between the growth curves of the selenite treated and untreated cultures. PC37 does not appear to reduce selenite or produce the stale garlic odor.

Measurement of concentration of Selenium by ICP

The ICP instrument was calibrated using standard solutions of sodium selenite at concentrations of 0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM and 0.5 mM. As a quality check, a calibration was performed in between the sample run at regular intervals of times. First, a calibration blank followed by 5 different calibration standards were introduced into the ICP. Then using linear regression analysis, the ICP software automatically plotted relative response vs concentration in mM and determined the slope (m), intercept (b) and correlation coefficient (r) of the calibration curve. In Figure 3, the correlation curve (R^2) of our standards was 0.9939, which is acceptable and can be further used to analyze the unknown samples. The output produced by the ICP was in PPM, which was later converted into mM by dividing each observation by 78.89 g/mol, the molecular weight of selenium. Also, the efficiency of the ICP was checked by running spiked samples in between the unknown samples which was found to be equivalent to 80% recovery.

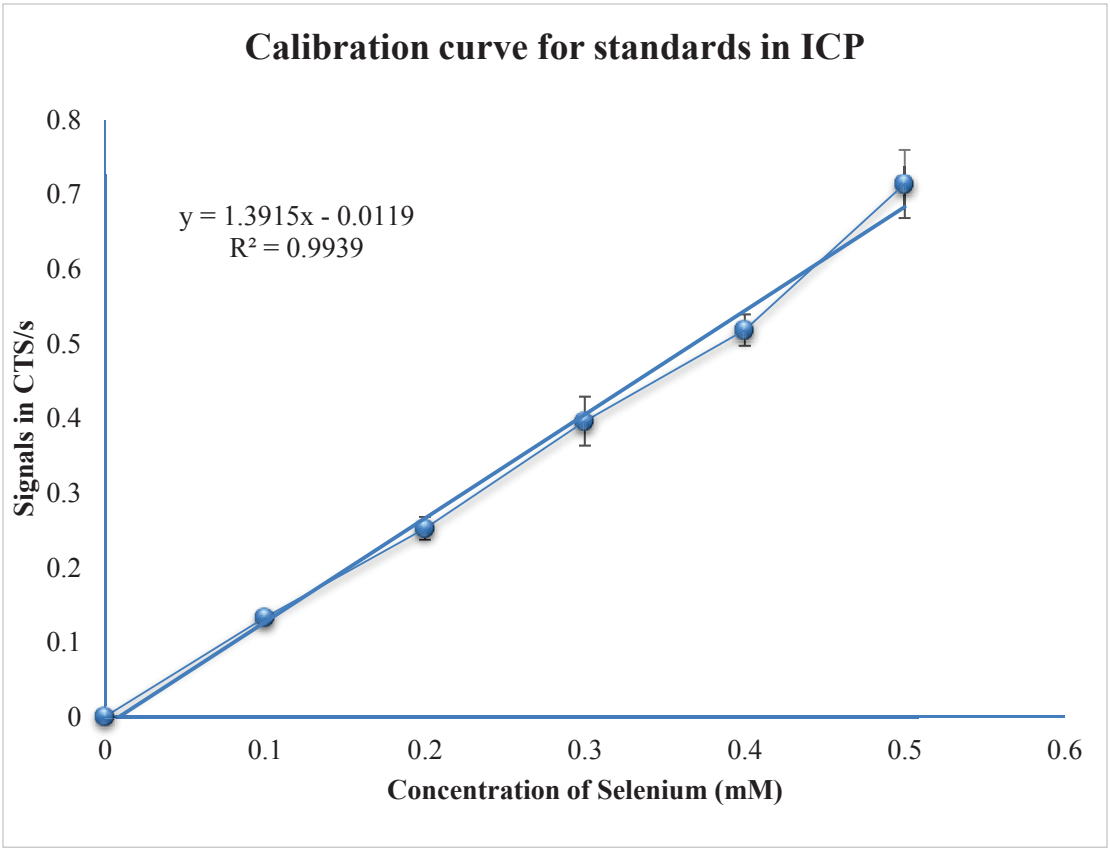

Figure 3: Standard Curve of the standard Se solutions generated for ICP calibration. The error bars represents the standard error of 6 different experiments.

Concentration of Selenium in ***S. maltophilia* 02**

In Figure 4 (a), the X- axis is time in hours and primary Y-axis is the concentration of selenium in mM. There are five different data series shown in the figure. First, the blue colored curve with asterisk symbols shows the concentration of selenium in the sterile media. Selenite was added to the sterile media after 2 ½ hours of incubation. Hence, the hourly sample ICP analysis of sterile media showed an absence of selenium in the first 2 hours of incubation. Then, at three hours, it was measured at 0.53 mM selenium and gradually increased to 0.6 mM at time ten hours. As expected the selenium concentration in the sterile media did not change significantly.

Second, the red curve with open circle symbols shows the concentration of selenium present in the supernatant of *S. maltophilia* 02 cells grown in absence of selenite. The concentration of selenium is shown on the vertical Y-axis. Selenium present in the selenite untreated *S. maltophilia* 02 supernatant was zero or negligible throughout the 10 hours incubation period.

Similarly, the third green colored curve with open circle symbols shows the concentration of selenium plotted against the vertical Y-axis present in the *S. maltophilia* 02 culture with no selenite. As expected, ICP analysis of the *S. maltophilia* 02 cells with no selenite showed that there was no selenium in the supernatant or in the cell pellet.

Fourth, the red colored curve with closed diamond symbols shows the selenium concentration present in the supernatant of *S. maltophilia* 02 cells with selenite. The concentration of selenium was first detected at time three hours and gradually decreased through time ten hours from 0.54 mM to 0.31 mM selenium.

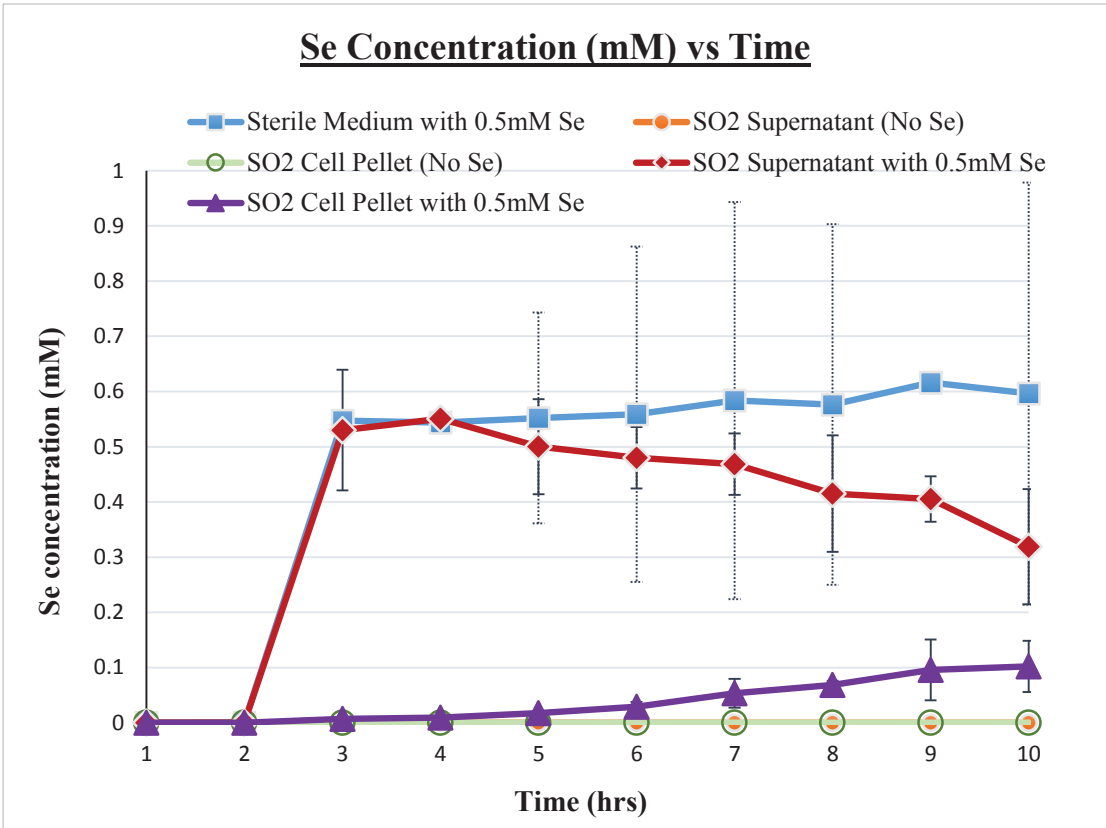

Figure 4 (a): The concentration of Selenite in *S. maltophilia* 02 cell pellet and supernatant. Data points represents average of 3 different experiments. Selenite (0.5 mM) was added after 2 ½ hours of incubation. Error bar were calculated at 95% CI at (N-1) d.f., where N=3. The error was calculated using the Student-t test at 95% confidence interval and (N-1) degrees of freedom, where N is 3. Blue curve filled square symbol = Concentration of Se (mM) in sterile medium, Red curve with filled diamond symbol = Concentration of Se (mM) in *S. maltophilia* 02 supernatant with Se, Purple curve with filled triangle symbol = concentration of Se (mM) in *S. maltophilia* 02 cell pellet with Se, Green empty circle shaped symbols = concentration of Se (mM) of *S. maltophilia* 02 cell pellet without Se , Red colored curve with empty circles symbol = concentration of Se (mM) in *S. maltophilia* 02 cell supernatant without Se.

Lastly, the purple colored curve with closed triangle symbols shows the concentration of selenium present in the cell pellet of *S. maltophilia* 02 cells incubated with 0.5 mM selenite. The concentration of selenium increased from 0 mM to 0.1 mM in the cell pellet of the selenite treated cells of *S. maltophilia* 02. The increasing concentration in the cell pellet and decreasing concentration in the growth media suggested that the reduction of soluble selenite was biologically mediated. At time ten hours, the total increase in selenium concentration in the cell pellet increased from 0.0 mM to 0.1 mM. The concentrations of selenium in the growth medium and pellet did not add up to the total selenium concentration that was added at time 2 hours. Some of the selenium may have escaped as a volatile alkylselenide gases (Dungan et al., 2003).

Concentration of Selenium in *Pseudomonas sp.* PC37

Figure 4(b) represents the concentration of selenite present in the PC37 with and without selenite. There are four different curves in this figure. Selenium concentration of the PC37 cell pellet and supernatant without selenite treated were shown on secondary Y-axis. ICP analysis of PC37 cell pellet samples collected from hourly incubation without selenite is plotted as open green triangle symbols and shows that the concentration of selenite in the cell pellet of PC37 without selenite was zero or negligible.

Similarly, no selenite was detected in the supernatant (growth medium) hourly samples of PC37 with no selenite as observed by the purple open circle symbol curve in the figure.

The red colored curve with closed square symbols shows the concentration of selenite present in the supernatant of selenite treated PC37. After it was added at time 2 ½ hours, the selenite concentration decreased from 0.4 to 0.33 mM selenite.

Unlike S. *maltophilia* 02, the ICP analysis showed that the PC37 lacked cell associated selenium. It did not appear to be inside the cells or associated with the cell mass.

Although the error was large for the growth medium containing selenite, they do not overlap with the any of the error bars for the other curves. Thus, this data support the hypothesis that the soluble selenite was not undergoing any transformation inside or outside the cells.

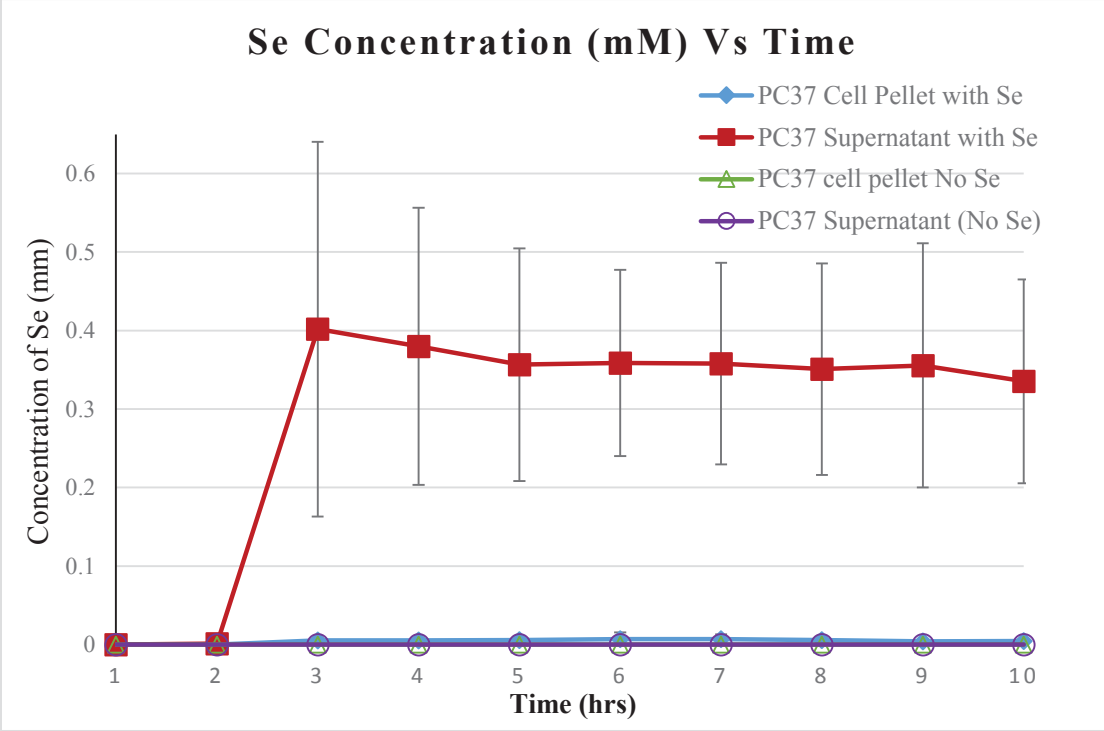

Figure 4 (b): The concentration of Selenite in PC37 cell pellet and Supernatant. Data points represents average of 3 different experiments. Selenite (0.5 mM) was added after 2 ½ hours of incubation. Error bar were calculated at 95% CI at (N-1) d.f., where N=3. The error was calculated using Student-t test at 95% confidence interval and (N-1) degrees of freedom, where N is 3. Red curve with filled square symbol = Concentration of Se (mM) in PC37 supernatant with Se, Dark blue curve with filled diamond symbol = concentration of selenium (mM) in the PC37 cell pellet with selenium, Green empty triangle symbols = concentration of Se (mM) of PC37 cell pellet without selenite plotted on the secondary vertical axis, Purple colored curve with the empty circle symbol = concentration of Se (mM) in *S. maltophilia* 02 cell supernatant without selenium plotted on secondary vertical axis.

DISCUSSIONS

The present research focused on the selenite resistance of *S. maltophilia* 02 and *Pseudomonas* sp. PC37. Because cultures of *S. maltophilia* 02 turn red when exposed to 0.5 mM selenite and PC37 did not, I originally hypothesized that S. *maltophilia* 02 reduced selenite to elemental selenium, whereas PC37 excluded it from its cells. The results from this study support this hypothesis. The turbidity growth curves and ICP measurements of the selenite treated S. *maltophilia* 02 culture showed that this bacterium removed the selenite from the growth medium and sequestered it in the cells. It appeared that the S. *maltophilia* 02 cells detoxified it via reduction. The resulting slower growth rate was probably caused by the energy consumption required for the reduction reaction. However, in the case of PC37, selenite addition to the media did not seem to affect the growth. PC37 did not transform selenite by reducing it, but appeared to exclude it from the cell. If the selenite were being expelled, the cells would have to put energy into pumping it out of the cell, and there probably would have been a decrease in the number of viable cells in the selenite treated culture compared to the untreated culture.

Inductive coupled plasma spectroscopy results showed an increase in the selenium content with the growth of *S. maltophilia* 02 cell mass and a decrease in the total selenium concentration from the growth medium. Only ~20% of the total selenium content was found to be present in association but there was a ~40% decrease of total selenium in the growth medium during the 10 hour incubation period. The remaining selenium which could not be traced might have escaped as volatile alkylselenide gas after the reduction, hence could not be detected in ICP. Other species of *Stenotrophomonas* were previously studied

at 0.5 mM and found to be capable of removing 81.2% selenate and 99.8% selenite from the growth media in 50 hours of incubation (Dungan et al., 2003).

In a similar study, *Stenotrophomonas maltophilia* 02 and *Enterobacter sp.* showed resistance at 10 mM and 40 mM selenium concentration, respectively (Shaik, 2011). Then again in another 2013 work, *S. maltophilia* 02 was studied for selenium resistance using an improved digestion procedure (Gudavalli, 2013). Another strain of *S. maltophilia* has been reported to biotransform toxic oxyanions of selenium into nontoxic elemental selenium (Dungan et al., 2003). Similarly, another bacterial strain of *Stenotrophomonas sp.* isolated from the rhizospheric region of *Astragalus bisulcatus* was found to be resistant to 50 mM of selenite in nutrient and mineral salts medium and also precipitated it to form elemental selenium (Gregorio et al, 2005).

Pseudomonas sp. have also been studied extensively for heavy metal resistance and found to have various established mechanisms to adapt against different heavy metal toxicity. One of the ways to cope with copper toxicity in *Pseudomonas syringae* is by efflux, where a set of *cop* structural genes encoded a P-type efflux ATPase to pump Cu(II) from the cell. (Ji & Silver, 1995). *Pseudomonas fluorescens* LB300 was reported to be the first chromate resistant bacterial strain (Nies, 1999). This bacteria was isolated from a chromate rich environment from the Hudson River, and the mechanism involved in chromate-resistance was plasmid mediated (Bopp et al, 1983). Similar efflux mechanisms are observed for copper resistance in *Pseudomonas syringae* (Ji & Silver, 1995). This strategy would probably result in a decrease in cell growth due to extra energy input in efflux process. However, this decrease in growth was not observed in *Pseudomonas* sp. PC37. The concentration of selenium measured by ICP in the growth medium and cells indicated that

there was no significant change in the concentration of selenium either in the cell pellet and or in the growth medium. Thus, this bacterium appears to simply exclude the selenite from the cells.

Several earlier research have reflected the view that the selenium resistance is biologically mediated reduction of the soluble selenium oxyanions into insoluble elemental selenium, and it is the only understood defense mechanism that has been reported by various microorganisms. There are a wide variety bacteria which transform selenium oxyanions (selenite or selenite) into elemental selenium either aerobically or anaerobically. *Stenotrophomonas maltophilia* (Gao et al, 1997), *Escherichia coli* (Harrison and Turner, 2004), *Enterobacter cloacae* SLD1a-1 (Yee et al, 2007), *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruoginosa* ATCC 27853 (Harrison et al., 2004) and *Thauera selenatis* (Oremland et al., 1999) are examples. Formation of red colored precipitation demonstrated the ability of the selenite treated *S. maltophilia* 02 cell to reduce soluble selenite into elemental insoluble selenium. This reduction is most probably enzymatically driven. There are a few enzymes which are either membrane bound or are present in the periplasmic space that catalyzes the reduction of selenium oxyanions into elemental selenium. For example, the selenite respiring bacterium, *Thauera selenatis,* contains a periplasmic selenate reductase which catalyzes the reactions involved in the removal of the selenium oxyanions from the environment (Oremland et al., 1999) and *Sulfurospirillum barnesii* SES3 contains a membrane bound selenate reductase (Oremland et al., 1999).

The stale-garlic odor produced by *S. maltophilia* 02 in the presence of selenite suggested that it released a volatile organic complex of selenide during the detoxification of soluble selenite. This hypothesis is supported by the observation that at time ten hours, the

concentration of selenium in the *S. maltophilia* 02 cell pellet and growth medium did not add up to the total concentration of selenite that was added at time 2.5 hours. In addition to reduction of selenium oxyanions, its transformation into volatile compounds is known to be the more effective way to bioremediate selenium contamination (Dungan et al., 2003). Some selenium tolerant plants use a similar mechanism (Kamaludeen & Ramasamy, 2008). These volatile methy-selenide compounds can be measured using Gas Chromatography (Chasteen & Bentley, 2003).

To understand the efficiency of a given bacteria for its reduction of toxic selenite into nontoxic elemental selenium, the elemental selenium was measured by ICP. An alternative method can be applied. Elemental selenium can be converted into a red-brown solution by reacting it with sodium sulfide which can then be measured at 500 nm in a spectrophotometer, where the absorbance value of the red-brown solution is directly correlated to the amount of elemental selenium present in the media. (Biswas et al., 2011).

Future work:

Since the results in this study showed that *S. maltophilia* 02 removes selenite from its growth medium, it may be a useful tool for bioremediation. This may be examined by repeating this study with different selenite concentrations and by extending the length of the growth curve to see if it can completely remove the selenite from the medium. Further, protein analysis could be performed on both bacteria to investigate the selenite-resistance mechanisms. Bacteria express different proteins to survive the metal stress. 2-D gel electrophoresis could be done to identify the different proteins involved in the reduction process. Also by transposon mutagenesis we can generate mutations in the wild type strain and identify the mutated genes through gene rescue (Caguiat, 2014). The sequencing of the genome of the selenite treated *S. maltophilia* 02 cell could be done and further investigated for specific sequences which codes for enzymes that are involved in selenium reduction. Finally, RNA sequencing may be used to identify genes involved in selenite resistance. Each strain is grown in the presence and absence of selenite. Extracted RNA is then converted to cDNA and sequenced using Next Generation Sequencing. Differences in the number of transcripts produced in the presence and absence of selenite will suggest which genes are expressed in response to selenite. These results can then be verified by quantitative PCR.

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