Quantitative determination of selenite transformation by *Enterobacter* sp. YSU and *Stenotrophomonas maltophilia* OR02

By

Nabeel A. Shaik

Submitted in Partial fulfillment of the Requirement

for the degree of

Master of Science

in the

Chemistry Program

Youngstown State University

December 2011

Quantitative determination of selenite transformation by *Enterobacter* sp. YSU and *Stenotrophomonas maltophilia* OR02

Nabeel A. Shaik

I hereby release this thesis to the public. I understand that this thesis will be made available from the OhioLINK ETD center and the Maag Library Circulation Desk for the public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research

Signature:

Nabeel A. Shaik, Student　　　　Date

Approvals:

Dr. Jonathan J. Caguiat, Thesis Advisor　　　　Date

Dr. Josef Simeonsson, Committee Member　　　　Date

Dr. Ganesaratnam K. Balendiran, Committee Member　　　　Date

Peter J. Kasvinsky, Dean of School of Graduate Studies & Research　　　　Date

Abstract

The Y-12 plant in Oak Ridge, TN processed uranium and lithium to produce nuclear weapons during World War II and the Cold War. Because the production of nuclear weapons during these time periods was urgent, waste from these processes was not properly contained and nearby East Fork Poplar Creek was contaminated with mercury and other heavy metals. *Enterobacter* sp. YSU and *Stenotrophomonas maltophilia* Oak Ridge strain 02 (*S. maltophilia* 02), which were isolated from East Fork Poplar Creek, are resistant to several heavy metals and selenite, an oxyanion of selenium.

The general resistance mechanism appears to be a reduction to elemental selenium. The sodium selenite that is added initially to logarithmically growing culture of these strains is clear in color but turns red when it reaches stationary phase. The change in color could be a result of the reduction of soluble selenite to insoluble elemental selenium. We examined the ability of *Enterobacter* sp. YSU and *S. maltophilia* 02 to remove 40 mM selenite and 10 mM selenite, respectively, from their growth.

Two cultures were prepared: one was exposed to selenite during early log phase and the control was treated with sterile water. Cells and media were collected at an hourly basis and digested using nitric acid. Then, the amount of selenium in each sample was determined using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Both strains appeared to sequester elemental selenium, but not at high enough levels to significantly decrease the amount of selenite in the growth medium.

Acknowledgments

From the bottom of my heart, I thank **Dr. Jonathan J. Caguiat** for all the support, help and guidance. He was always there to help me out no matter how big or small the problem was and made the lab a better and enjoyable place. He was always encouraging and comforting. I thank Dr. Caguiat's family (Mrs. Tani Spielberg, Emily and Max) for the affection they showed.

I also thank my committee members Dr. J. Simeonsson and Dr. G. K. Balendiran for their help and knowledge. I also would like to extend my gratitude to Dr. G. Yates and Dr. F. Armstrong for the instrumental and financial support.

I am much obliged to all my fellow students, who helped me in the lab work: the Undergraduate students (Mark, Emilie, Tara) and Graudate students (Samatha, Swarna and Ramesh).

I am grateful and thankful to GOD for being merciful in granting me all the success and also blessing me with great friends (Pradeepthi, Samatha, Sowmya, Swarna, Zubair, Yousif, Malik, Hamza, Vinod, Vivek, Saif, Naveed, Adeel, Awais, Munaf, Ramesh, Satya and Vamsi, who were ever there for me in all aspects of life). I am overwhelmed with gratitude for the relentless support by my friends.

I appreciate the Chemistry, Biology and Mathematics Departments, NSF (Grant DBI-0827205) and the diversity office for all the financial support.

I dedicate my thesis to my family, for this achievement would not have been possible without them.

Thanks Dad (**Mr. Shaik Abdul Jaleel**) for being there for me when I needed you the most and for being such a great person, Mom (**Mrs. Shaik Noorjahan**) for all the love and prayers, my lovely and adorable sister (**Shehnaz Shaik**) and the Best Brother (**Shakeel A. Shaik**) for their unconditional love and affection.

I also thank each and every one who directly or indirectly helped me and prayed for my Success.

Table of contents

TITLE PAGE i

SIGNATURE PAGE ii

ABSTRACT iii

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS v

LIST OF FIGURES vii

LIST OF TABLES ix

LIST OF SYMBOLS AND ABBREVIATIONS x

1. INTRODUCTION 1

1.1. Y-12 Plant 2

1.2. Properties of Selenium 2

1.3. Instruments used to Measure Selenium 4

1.3.1. Atomic Absorption Spectroscopy 4

1.3.2. Inductively Coupled Plasma – Atomic Emission Spectroscopy 5

1.4. Growth of S. maltophilia 02 and Enterobacter sp. YSU in the presence of Selenite 7

2. HYPOTHESIS 9

3. METHODS 11

3.1. Bacterial Growth 12

3.1.1. Bacterial strains 12

3.1.2. Growth Media 12

3.1.3. Growth of *Enterobacter* sp. YSU 13

3.1.4. Growth of *Strophomonas maltophilia* ORO2 13

3.2. Sample Preparation 14

3.2.1. Digestion 14

3.3. Analysis 14

3.3.1. Atomic Absorption Spectroscopy 14

3.3.2. Inductively coupled plasma – Atomic Emission Spectroscopy 16

4. RESULTS 19

4.1. Growth Curve 20

4.1.1. Growth of *Enterobacter* YSU 21

4.1.2. Growth of *S. maltophilia* 02 23

4.2. AAS 25

4.3. ICP-AES 27

4.3.1. ICP-AES Calibration Curve 27

4.3.2. ICP-AES Results for *Enterobacter* sp. YSU 31

4.3.3. ICP-AES Results for *S. maltophilia* 02 35

5. DISCUSSION 42

6. REFERENCES 47

Table of Figures

Figure Number	Title	Page number
1	**Schematic of AAS**	4
2	**Schematic of ICP – AES**	5
3	**Typical growth curve of bacteria, grown in a batch culture**	7
4	**Growth Curve of *Enterobacter* sp. YSU.**	22
5	**Growth Curve of *Stenotrophomonas maltophilia* 02**	24
6	**Calibration curve plotted for standards using AAS**	26
7	**Calibration curve plotted for standards using ICP-AES for *Enterobacter* sp. YSU**	28
8	**Calibration curve plotted for standards using ICP-AES for *Stenotrophomonas maltophilia* 02**	30
9	**Results of *Enterobacter* sp. YSU showing Turbidity in Klett Units and selenium concentration inside the cells that were exposed to selenium and cells not exposed to selenium obtained using ICP.**	32
10	**Results of *Enterobacter* sp. YSU showing Turbidity in Klett Units and selenium concentration in the media that were exposed to selenium and media not exposed to selenium obtained using ICP.**	34
11	**Results of *S. maltophilia* 02 showing Turbidity in Klett Units and selenium concentration inside the cells that were exposed to selenium and cells not exposed to selenium obtained using ICP.**	36

12	**Results of *S. maltophilia* 02 showing Turbidity in Klett Units and selenium concentration in the media that were exposed to selenium and media not exposed to selenium obtained using ICP.**	**38**
13	**Results of *S. maltophilia* 02 showing selenium concentration in the media and pellet that were exposed to selenium and not exposed to selenium obtained using ICP.**	**39**
14	**Graph plotted shows increase in se concentration per *S. maltophilia* 02 cells**	**41**

List of Tables

Table Number	Title	Page number
1	AAS temperature settings	15
2	Turbidity in Klett Units for *Enterobacter* sp. YSU grown with and without Selenium	21
3	Turbidity in Klett Units for *S. maltophilia* 02 grown with and without Selenium	23
4	AAS Results of calibration curve obtained using known standards	25
5	ICP Results of calibration curve for *Enterobacter* sp. YSU obtained using known standards	27
6	ICP Results of calibration curve for *S. maltophilia* 02 obtained using known standards	29
7	Results of Selenium Concentrations in *Enterobacter* sp. YSU obtained using ICP	31
8	Results of Selenium Concentrations in *S. maltophilia* 02 obtained using ICP	35
9	Amount of selenium in the bacterial cells and the Turbidity in Klett Units	40

List of Symbols and Abbreviations

mA	milli Ampere	$^{\circ}C$	Degree Centigrade
µ	Micro	K	Kelvin
L	Liter	hrs	Hours
mL	Milli liter	min	Minutes
µL	Micro liter	α	Directly proportional
mol	Moles	nm	nano Meter
M	Molar	%	Percent
mM	milli Molar	fig	Figure
µM	micro Molar	LB	Lauria broth
nM	nano Molar	No Se	No selenium
kg	Killo gram	Se	selenium
g	grams	sp.	Species
mg	Milligram	Abs	Absorbance
µg	Microgram	Pd	Palladium

ICP – AES	Inductive Coupled Plasma Atomic Emission Spectroscopy
AAS	Atomic Absorption Spectroscopy
S. maltophilia 02	*Stenotrophomonas maltophilia* OR02

Introduction

Y 12 plant

History stands evident for the devastation that nuclear weapons have caused, not only when they are used but also when they are manufactured. The Y-12 plant located at an origin of East Fork Poplar Creek in Oak Ridge TN processed the uranium that was used to make the nuclear weapons that destroyed Hiroshima in Japan during the Second World War. Four S-3 ponds near the Y-12 plant were used to discard heavy metal containing acidic liquid wastes[1]. As these ponds lacked coverings and linings, the waste leaked into East Fork Poplar Creek. In addition, this plant used tons of mercury to process hydrogen bombs during the Cold War. Of the 11,000,000 kg mercury used, around 920,000 kg of mercury was lost into East Fork Poplar Creek and the surrounding environment[2-4].

Two, East Fork Poplar Creek, bacterial strains that have been extensively studied in our lab are *Stenotrophomonas maltophilia* OR02 (*S. maltophilia* 02) and *Enterobacter* sp. YSU. These are both Gram negative bacteria that are resistant to salts of mercury, cadmium, zinc, silver, gold, arsenite and chromium[5]. Generally, *S. maltophilia* 02 exhibits higher minimal inhibitory concentrations (MIC) than *Enterobacter* sp. YSU for these metal salts[6]. In addition, these strains are resistant to selenite, an oxyanion of selenium. Selenium is an important cofactor in some enzymes of many organisms, but too much in the form of selenite can be toxic.[7]

PROPERTIES OF SELENIUM

Selenium, a group VI A element in the periodic table, was discovered by Jons Berzelius in 1817 and is a metalloid that can exist both in toxic and non-toxic forms[8, 9]. The toxic forms of selenium, selenate (SeO_4^{-2}) and selenite (SeO_3^{-2}) are water soluble and can bioaccumulate whereas the elemental selenium (Se^0) can be less or non-toxic as it is insoluble in water and

cannot bioaccumulate. Selenide (Se^{2-}) though highly toxic as a gas is unstable and gets readily oxidized to elemental selenium (Se^0) [10]. It is evident that oxidation of elemental selenium produces the toxic forms and bacteria act as electron donors thereby leading to reduction of the toxic forms to non-toxic form i.e., elemental selenium. The process of conversion of toxic forms of selenium to elemental Selenium could be dissimilatory reduction forming a red precipitate that could be an allotrope of Selenium. [8, 11]

There are 8 allotropes and several isotopes of selenium of which some are radioactive forms having half-life ranging from millisecond to millions of years. There are both stable and metastable allotropes of Selenium. The metallic gray allotrope is stable form that crystallizes in a hexagonal system. There are also two deep red crystalline monoclinic forms and two amorphous forms (one red and one black), which can interchange through temperature changes[12].

Selenium finds its ways into the environment by natural process like weathering of rocks and anthropogenic process[13]. Because of its chemical properties like photoconductivity, it is employed in manufacturing of electronic products like photocells, light meters, and solar cells. It is also used to remove color from glasses and enamels[12].

It is a trace element that is required by the body in minute quantities, approximately 55 µg per day for adults[14]. Selenium is used by the body to produce selenoproteins. Selenocysteine is a selenoprotein that exists as 21st amino acid and also an important component of mammalian glutathione peroxidase[15]. Selenium has been studied intensively because of its uses and toxic effects. Selenium in proper concentrations can be used to prevent gastrointestinal disorders, age related diseases, neurological disorder, cancer and heart disorders like congestive heart failure,

cardiovascular and muscle disorders[16]. Selenium can also act as an antagonist against arsenic and cadmium poisoning in rodents and cells cultures[14, 17].

Selenium deficiency can cause juvenile cardiomyopathy and osteoarthropathy[18]. High intake of selenium or selenium poisoning can cause blind staggers (subacute selenosis), alkali disease (chronic selenosis), nail deformation, hair loss, skin lesions, burning, irritation and tearing of eyes and also conjunctivitis[17]. Inhalation of selenium can cause various problems related to lungs like fluid accumulation causing pneumonitis, asthma, shortness of breath, fever, vomiting and diarrhea[8, 14, 19].

INSTRUMENTS USED TO MEASURE SELENIUM

Atomic Absorption Spectroscopy

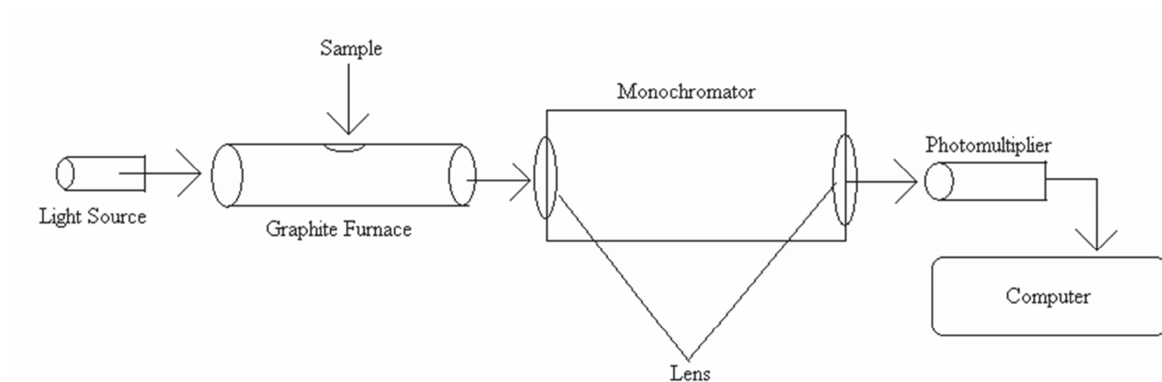

Figure 1: Schematic of AAS.

ICP and GFAAS are analytical instruments used for quantitative determination of elements. They involve atomization of element by using thermal energy. Every atom is capable of absorbing certain wavelength of radiation that is element specific. To provide that specific energy, a selenium hollow cathode lamp was introduced in to the path of AAS with a graphite

furnace and a detector (fig.1). To measure the intensity of the non-attenuated radiation and the radiation that is leaving the atomization device, two photomultiplier were used.

The various steps the sample undergoes in the furnace of an AAS are drying, pyrolysis, vaporization, and atomization. To prevent the sample from oxidation, Argon is used which is an inert gas. The samples are initially heated at low temperatures and gradually the temperature is increased to remove the unwanted residues. The selenite will still remain unaffected as the matrix modifier binds to it and prevents any loss or oxidation at high temperatures. When all the residues are removed by turning them in to ash, the sample is heated to very high temperatures that result in release of the selenium from the matrix and atomization of the sample takes place. At this point the attenuation of the lamp radiation was measured in the narrow volume of the graphite tube. The measured radiation reveals the absorbance and as the Beer's law states the amount of light absorbed is directly proportional to the number of atoms absorbing it and the quantity of the selenium can be determined.

Inductively coupled plasma – Atomic emission spectroscopy (ICP-AES)

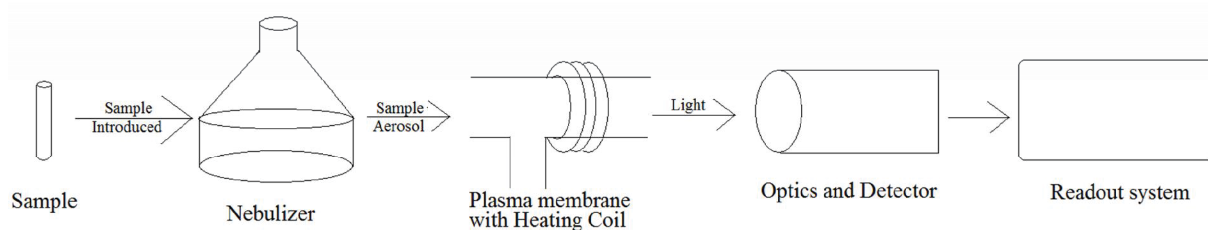

Figure 2: Schematic of ICP – AES.

Figure 2 represents different steps the sample undergoes. Initially it is introduced in to the nebulizer where the sample is converted to an aerosol and passed into the plasma atomizer chamber where the sample is subjected to high temperatures and the elements in the sample are

converted in to atoms and are excited into higher levels by absorbing element specific radiation. From higher levels, the atoms emit radiation and fall to lower levels where they are stable. The emitted radiation is collected, processed and converted to electrical signals that appear as absorbance on computer.

ICP-AES was obtained from Thermo Electron Corporation (Pittsburgh, PA.) and is also a very sensitive instrument with limits of detection ranging between parts per billion (ppb) to parts per million (ppm). It is equipped with a nebulizer that converts the liquid samples into aerosols. The aerosols undergo desolvation to form crystalline solids which on sublimation produces gaseous molecules. The rest of the unwanted sample will be released from the equipment as wastes. The gaseous molecules undergo atomization in the plasma atomizer that provides high thermal energy which is absorbed by the atoms. Upon the absorption of energy, the atoms are excited to higher levels. The excited atoms release energy and relax to lower energy states. The emitted radiation or energy hits the detector and a current is generated that is proportional to the amount of excited atoms.

To ensure the proper functioning of the equipment in generating the data for the samples an internal standard is used. The internal standard is injected simultaneously with the sample. Generally internal standards are selected that react the same way as the element of interest. The internal standard functions by performing a dynamic drift correction that corrects the physical difference between the sample and the standard by referring the sample to same element performance depending on the enhancement or suppression of the signal experienced by the internal standard. The internal standard used for this project was Yttrium. Argon gas of highest purity was used to purge the equipment and to carry the sample as it is an inert gas and does not react with the sample. An auto sampler was also used that could accommodate 240 samples

along with a blank and nine standards including a quality check standard. iTEVA was the software used to program the equipment regarding sample injection and treatment and also collect and process data.

Growth of *S. maltophilia* 02 and *Enterobacter* sp. YSU in the presence of selenite

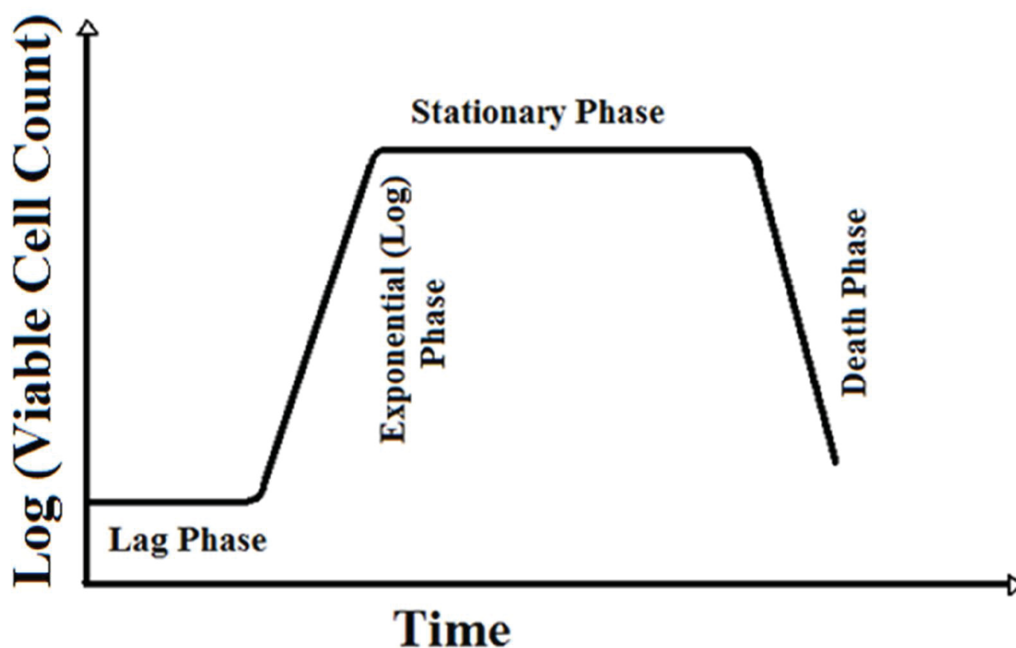

Figure 3: Typical growth curve of bacteria, grown in a batch.

Figure 3. Typical growth curve of bacteria, grown in a batch culture (cells grown in a flask without adding additional nutrients). The X-axis represents the time and the Y-axis represents log of the viable cell count. The lag phase is when the bacteria is just introduced in to the new culture and the bacteria acclimatizes with the new environment. In the exponential phase the growth rate is highest, in the stationary phase the growth is directly proportional to the death

phase and in the death phase the growth is lower than the death rate and the death rate is exponential[20].

In the Lag phase the bacteria is just introduced in to the culture and the bacteria acclimatizes with the new environment. In the log or logarithmic phase the bacteria grows exponentially and uses all the sources available in the media. In the stationary phase, as the nutrients deplete, the growth slows down and the growth is equal to the death of the cells so we see a plateau. In the death phase all the nutrients have been used and so the growth stops and cells tend to die, thereby a steady decrease in the curve[6, 21].

S. maltophilia 02 and *Enterobacter* sp. YSU, like other bacteria, followed the same growth pattern. They were grown to early log phase and exposed to selenite. It was assumed that the selenium would be converted to its allotrope forms during the exponential phase because as the bacteria grew in the liquid media a color change was observed with the change in time, after reaching a certain stage the color did not change further and remained red. Selenium content in the growth medium and cells was measured by ICP-AES after different time points after selenite addition.

Hypothesis

1) Selenium content will increase in the cells during stationary phase because the color is most intense during this phase
2) As the selenium concentration increases inside the cells during the stationary phase, the selenium content in the growth medium will decrease.

Methods

Bacterial Growth

Bacterial Strains

Stenotrophomonas maltophilia OR02 (*S. maltophilia* 02) was isolated from East Fork Poplar Creek and is resistant to salts of mercury, cadmium, zinc, copper, gold, chromium, arsenate and selenium. *Enterobacter* sp. YSU was also reported to resist to same metals but at lower concentrations than *S. maltophilia* 02.[7]

Growth Media

LB Broth (Fisher Scientific, Fair Lawn, NJ) media contained 10 g tryptophan, 5.0 g yeast extract, and 5.0 g sodium chloride per liter. 20 g of LB broth was mixed in a liter of Deionized water and 16.0g of Agar (Amresco, Solon, Ohio) and autoclaved at 121°C for 15.0 min. It was poured into culture plates, allowed to solidify and incubated dried at 37°C. LB – agar plates were used to streak out both the strains.

5X M-9 growth salts (DIFCO, Lawrence, KS) contained 26.6% (w/v) of 0.22 M KH_2PO_4, 60% (w/v) of Na2HPO4, 9% (w/v) of NH4Cl, and 4.4% (w/v) of NaCl. 56.4g of M-9 salts (DIFCO) was mixed in distilled water and autoclaved at 121°C for 15 min. M-9 salts medium was prepared by mixing 10 mL of 5x M-9 salts with 50 µL 1 mM MgSO4, 500 µL 4mg/ml of cysteine, 500 µL 0.2% glucose in 36.95 mL of sterile water.

R3A-Tris medium contained 1.0 g/L yeast extract, 1.0 g/L Difco proteose peptone no. 3, Casamino acids (1.0 g/L), Glucose (1.0 g/L), soluble starch (1.0 g/L), Sodium pyruvate (0.5 g/L), 10 mM Tris pH 7.5 (0.6g/L) and 25.0 mL $MgSO_4$. $7H_2O$.

When required growth medium was supplemented with sodium selenite (MP Biomedicals LLC, Solon, OH.).

Growth of *Enterobacter* sp. YSU

A single colony of *Enterobacter* sp. YSU was inoculated into 5 ml of M-9 minimal medium and grown overnight. The entire bacterial culture was then transferred to 45 ml of fresh M-9 medium (1:10 dilution) and shaken at 37 °C in a New Brunswick Scientific C24 incubator (Edison, New Jersey). Growth was followed by measuring turbidity every hour using a Klett Colorimeter (BEL-ART productions, Pequannock, New Jersey). After 1.5 hours of growth, 1.0 mL of sample was collected and sodium selenite (Company, city and state) or sterile water (control) was added to a concentration of 40 mM. Two sets of 1.0 mL samples were collected every hour for 4 hours and 3 more samples were collected at 24.0, 48.0, and 72.0 hours from the point when the culture was started. One sample was used for quantitative determination of selenium and the other was stored for future use.

Growth of *Stenotrophomonas maltophilia* OR02

A single colony of *Stenotrophomonas maltophilia* 02 was grown overnight at 30°C in 3.0 mL of R3A media. The overnight culture was mixed with 22.0 mL of the fresh R3A media and placed in the shaker (New Brunswick scientific, Edison, New Jersey) at 30°C. Density was measured every 1 hour using a Klett Colorimeter. After 1.5 hr, sample was collected and 2.0 mL of sodium selenite was added to the culture to a concentration of 10 mM. Two sets of samples 1.0 mL each were collected every hour for 12 hours and density was measured. One sample was used for quantitative determination of selenium and the other was stored for future use.

Sample preparation

Digestion:

The samples were centrifuged at 29,1000X g using an Eppendorf centrifuge 5417R (Hauppauge, New York) for two minutes and the supernatant was transferred to new tube. The pellet was washed with 1X M-9 salts and suspended in water keeping the volume same as the sample that was collected initially. The resuspended pellet and the supernatant were transferred to two different digestion tubes, mixed with 5.0 mL of concentrated nitric acid (Fischer Scientific, Fair Lawn, NJ) and incubated overnight at room temperature. The samples were digested using the digester (model # SC181) obtained from environmental express (Mt. pleasant, South Carolina) at 150.0^{o}C for an hour to evaporate all the nitric acid[22].

Depending on the equipment used for analysis, either water or 10% nitric acid was added to the digestion tubes. For atomic absorption spectroscopy, water was added, and for ICP-AES, 10 % nitric acid was added[22].

Analysis

Atomic Absorption Spectroscopy (AAS)

The digested samples were diluted so that the concentration ranged between 4 nM and 4 µM. 25.0 µL of samples was mixed with 4.0 µL of 200 µg/mL palladium nitrate. The samples were loaded in the AAS (Varian AA 220) and Argon gas (Praxair INC., Youngstown, OH) was used as carrier. The sample holder used could accommodate 50 samples and six additional wells for matrix modifier, bulk solutions, blank and make up solutions.

The sample volume to be collected was set to 20.0 µL and the following table was used to set different parameters that were required. The standards used were 4, 40 120, 400, 1200 and 4000 nM, that were prepared by diluting 1 M sodium selenite in sterile water.

Table 1: AAS temperature settings.

Step	Name	Temp (^{O}C)	Time (Sec)	Flow rate (L/min)	Gas Type	Read	Signal Storage
1	Dry	85	5.0	3.0	Normal	No	No
2	Dry	95	40.0	3.0	Normal	No	No
3	Dry	120	10.0	3.0	Normal	No	No
4	Ashing	1000	5.0	3.0	Normal	No	No
5	Ashing	1000	1.0	3.0	Normal	No	No
6	Ashing	1000	2.0	0.0	Off	No	Yes
7	Atomization	2300	0.8	0.0	Off	Yes	Yes
8	Atomization	2300	2.0	0.0	Off	Yes	Yes
9	Cleaning	2600	2.0	3.0	Normal	No	No

Table 1 shows the different temperatures and the duration sample treatment. The first three steps used lower temperatures that removed moisture from the sample. The next three steps i.e., steps 4, 5, and 6 burned the residue except selenium. Selenium was bound with the modifier (palladium nitrate) that prevents any loss at lower temperatures. Atomization of sample takes place during steps 7 and 8, wherein the selenium was transformed in to its atomic phase. 3.0 L of argon gas flowed through the equipment per minute for the first five steps that helped in

removing the vapors and ashes from the graphite tube. As the presence of selenium was required in the graphite tube in its atomic phase the flow of argon gas was cut off, so that the exact amount of selenium could be determined without any loss. At the end of each sample determination, the graphite tube was heated to 2600°C for 2 seconds with 3.0 L/min argon flow rate to remove any leftover sample to prevent cross contamination, so [23,24].

Inductively Coupled plasma Atomic emission spectrometer (ICP-AES)

After the digestion 10% Nitric acid (10.0 mL) was added to *S. maltophilia* 02 samples and a serial dilution (1 to 50) was done for *Enterobacter* sp. YSU with 10% Nitric acid to keep the concentration within the limits of detection. The samples were run on ICP using argon as carrier gas. Yttrium was used as internal standard. The samples were run in duplicates and the amount of sample required was approximately 3.0 mL.

The standard curve was obtained using 12, 10, 8, 5, 1, 0.5, 0.2 and 0.1 mM of selenite for *S. maltophilia* 02and 40, 20, 15, 10, 5, 1, 0.5 and 0.2 mM for *Enterobacter* sp. YSU. The standards were diluted the same way as the samples were. 8 mM selenite was used for quality check and was run after every 40 samples. A graph of calibration curve was plotted using the selenium concentration on the X- axis and absorbance on the Y-axis. Slope and correlation was obtained using the graph. All the absorbance of the samples was plugged in to the slope and the concentration of the samples was obtained.

$$Y = m x + c$$

Where Y = absorbance

X = concentration

m= slope

C= X- intercept

To determine the density of the culture, Klett colorimeter (BEL-ART productions, Pequannock, New Jersey) was used. The collected samples were centrifuged and the bacterial cells were separated from the media. Both the media and the bacterial cells were transferred to different digestion tubes and mixed with nitric acid. Nitric acid dissolved cells and media turning them in to clear solutions. The digestion tubes were placed in the digester at 150.0^{O}C and heated for one hour with reflux caps on the tubes to prevent loss or contamination of sample.

The Digester used was an Environmental express Hot Block that could accommodate 56 samples at a time and could heat up to 180°C. The Instrument was connected to a controller, which had the ability to run two Hot Blocks at a time maintaining different temperatures on both. A probe also was connected to the controller that showed. The Digested samples were diluted and used for quantitative determination of selenium. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) and Graphite Furnace Atomic absorption spectrophotometer (GF-AAS) were used.

The Varian AA 220 Atomic Absorption Spectrometer is a sensitive instrument that has a limit of detection ranging from 4 µM to 4 nM. It was equipped with a graphite tube that was pyro coated 10X partition tube (Reflex Analytical Corporation, Ridgewood, New Jersey, Product number 63-100012-00), GTA 110 graphite tube atomizer and a sample holder that could accommodate 50 samples and six additional wells for matrix modifier, bulk solutions, blank and make up solutions[25].

Measuring the quantity of a compound using AAS required an element specific lamp. As the element of our interest was selenium, we used selenium hollow cathode lamp that was operated at 15 mA and a wavelength of 196 nm with a spectral width of 1.0 nm. High purity(99.99%) liquid argon was used as carrier gas and flow rate was set to 3.0 L/min[25]. Field strength of 0.80 tesla was used and an injection rate of 15 injections per second was used to inject 15 µL of sample and 5 µL of matrix modifier. Palladium nitrate (Fluka Analyticals, Spruce St, St. Louis, MO. product code 32620P07) was used as a matrix modifier along with the samples that formed a complex with selenium and allowed the charring of selenite at very high temperature that allows us to remove all the impurities from the samples. 10.0 g/L of palladium nitrate (230.41 g/mol) was dissolved in 15% HNO_3.

SpectrAA worksheet software was used to control the instrument for results, data collection and signals of sample and standards. Peak area was chosen for the graphite furnace optimization and for quantification of selenite in the samples. To get the best results, the samples were run in duplicate and an average was taken.

To confirm whether the selenium is being reduced with the increase in growth of bacteria, the sample was diluted and grown on LB plate with overnight incubation at 30°C.

Results

Growth Curve

Growth curves were performed using two selenium resistant strains *Enterobacter* sp. YSU and *S. maltophilia* 02. Two overnight cultures of each strain were diluted in 1/10 ratio of fresh growth medium, For *Enterobacter* sp. YSU M-9 salt medium was used and for *S. maltophilia* 02 R3A-tris media was used. The cultures were incubated at $37^{O}C$ and after 1.5 hrs of growth, when the samples were in the early log phase selenite was added. For one culture of *Enterobacter* sp. YSU, selenium was added to reach final concentration of 40mM and for *S. maltophilia* 02 10mM was added. Control cultures contained equal volumes of sterile water.

Samples were collected hourly for 12 hours after the selenite was added to *S. maltophilia* 02 and for *Enterobacter* sp. YSU. Additional samples were collected at 24.0, 48.0 and 72.0 hours after the selenite was added. Along with sample collection, turbidity of the samples was measured using a Klett Colorimeter.

Table 2: Turbidity in Klett Units for *Enterobacter* sp. YSU grown with and without Selenium.

Time (hrs)	Turbidity	
	No selenium	Selenium
1.5	65	66
2.5	87	98
3.5	147	130
4.5	159	163
24	175	262
48	180	290
72	181	364

Table 2 shows the turbidity of the liquid culture, at each time point. As the turbidity increases, the number of cells in the culture increases. At 1.5 hour the turbidity is same in both cultures. This was the point where the selenium was added after collecting the sample. Later 2.5 hours it was evident that the turbidity in No selenium culture was lower than the cells in the culture having selenite. By the end of 72 hours the turbidity in selenite culture was twice the turbidity in the no selenium culture. From this it was clear that *Enterobacter* sp. YSU can survive in 40 mM selenite.

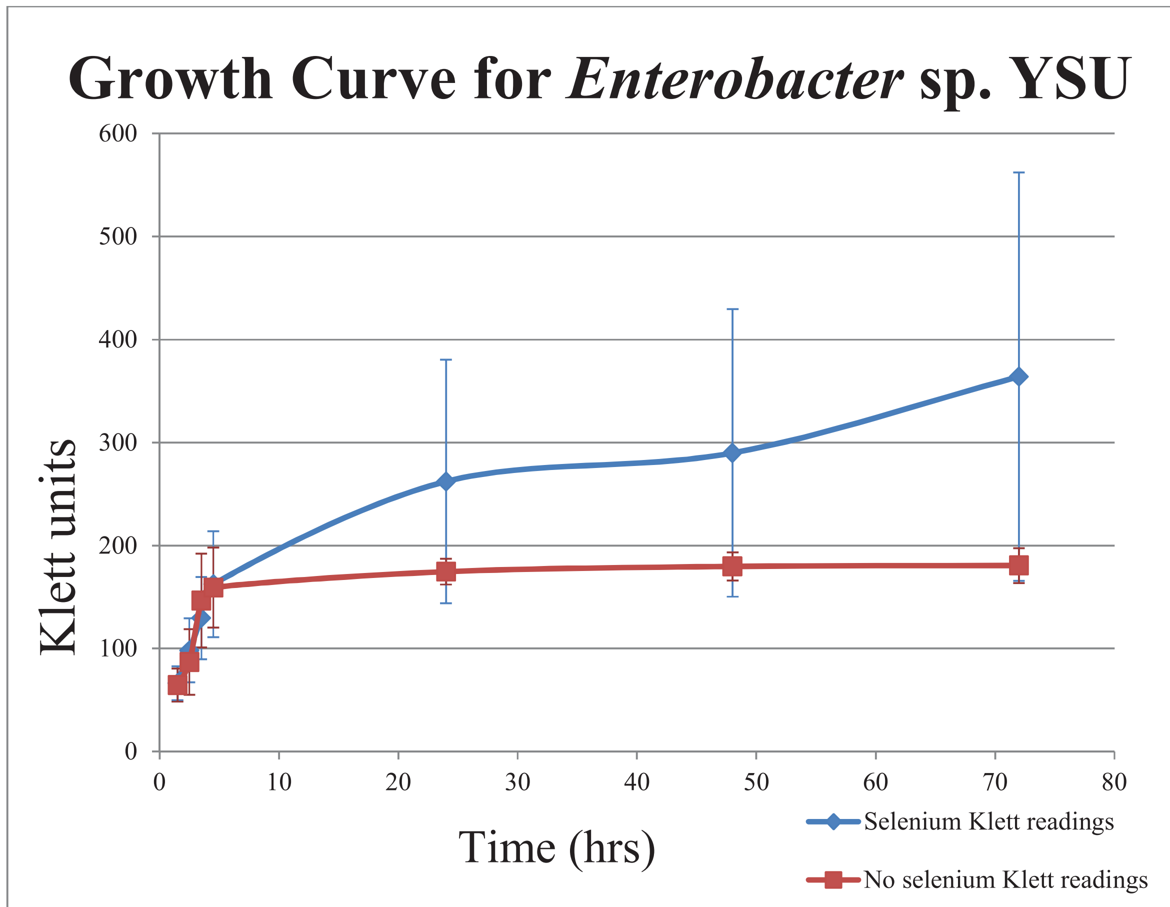

Figure 4: Growth Curve of *Enterobacter* sp. YSU.

Figure 4. Growth of *Enterobacter* sp. YSU in M-9 minimal medium supplemented with and without 40 mM selenite. Student T test was used for the error bars. The X-axis represents time in hours and Y-axis represents the turbidity in Klett Units.

From the Figure 4, it was clearly observed that after 24 hours the turbidity in Klett Units showed a plateau, indicating that the culture without selenium reached stationary phase, whereas the culture with selenium was still increasing. The increased turbidity for the selenium treated samples could be accounted by the elemental selenium that was precipitated by the bacteria.

Table 3: Turbidity in Klett Units for *S. maltophilia* 02 grown with and without Selenium.

Time (hrs)	Turbidity	
	No Selenium	Selenium
0	43	40
1	65	63
2	103	98
3	156	146
4	205	156
5	236	159
6	251	177
7	261	196
8	265	219
9	282	249
10	292	269
11	304	291
12	313	319
13	335	360
25	360	550

Table 3 and Figure 5, show the turbidity readings of *S. maltophilia* 02 in the presence and absence of selenite. Selenite was added at 2.0 hours after the culture was started. One hour after selenite was added (hour3), both cultures were at about the same turbidity. Because selenium is toxic, the culture without selenite initially increased in turbidity more rapidly. The selenite-treated culture re-entered into a lag phase for about 3 hours and then continued to grow. After 12 hours as the bacteria is acclimatized with the environment, the Klett readings of the culture with selenite increased more in turbidity than the culture without selenite. After 25 hours the turbidity of the No selenium culture was just 360 Klett units, whereas the culture with selenite had a turbidity of 550 Klett units. As the culture grew with time, a red precipitate started to accumulate in the culture. As selenite is water soluble and the elemental selenium is insoluble, the red precipitate could have been an allotrope of elemental selenium. This showed that once the *S. maltophilia* 02 acclimatized with the environment, it could not only survive but also reduced the selenite to al allotrope of elemental selenium

To confirm that the *S. maltophilia* 02 and *Enterobacter* sp. YSU can actually convert the selenite to non-soluble elemental selenium, the samples were analyzed by AAS and ICP-AES.

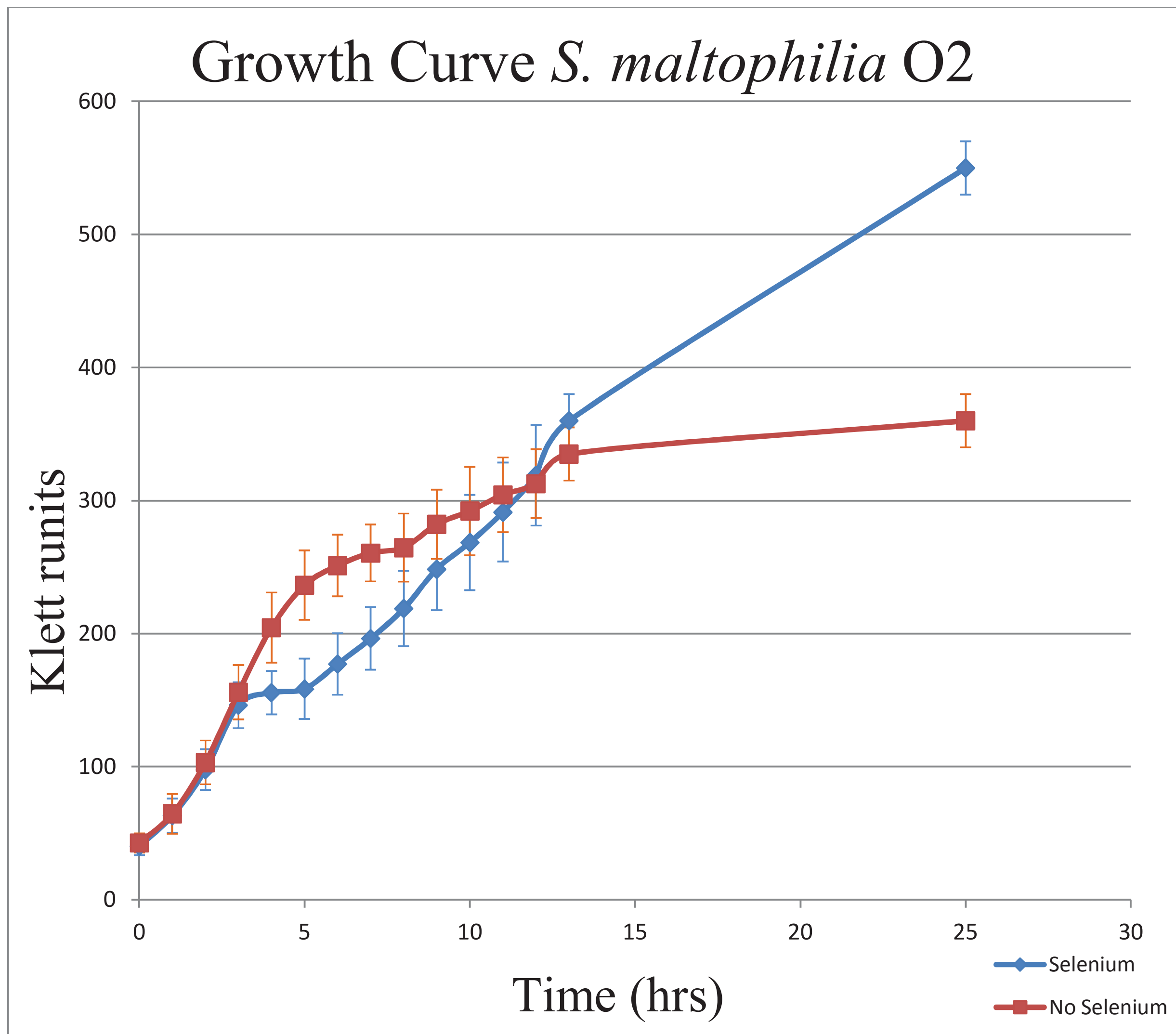

Figure 5: Growth Curve of *S. maltophilia* 02.

Figure 5. Growth curve of *S. maltophilia* 02 in the presence and absence of 10 mM selenite. Time is on the X-axis and turbidity in Klett units is on the Y-axis,. Though initially the culture without selenium grew better, the *S. maltophilia* 02 acclimatized with the new environment and after 12 hours the turbidity of the culture with selenium is higher than the one with no selenium.

AAS Results

Table 4: AAS Results of calibration curve obtained using known standards.

Standard Concentration (nM)	Average Absorbance at 196.0 nm
0	0.04223
4	0.21863
40	0.2945
120	0.31537
400	0.3121
1200	0.378
4000	0.46797

Table 4 shows different standards used for calibration curve and their emission measured at 196.0 nm wavelength. The absorbance does not increase with respect to the increase in standards. Figure 6 is a calibration curve that was plotted using the concentration of prepared standards on X- axis and the absorbance of the standards at 196.0 nm wavelength on Y- axis. Since the correlation was found to be 0.5 and as the correction was acceptable at 0.99, ICP was used for the analysis of samples instead of AAS.

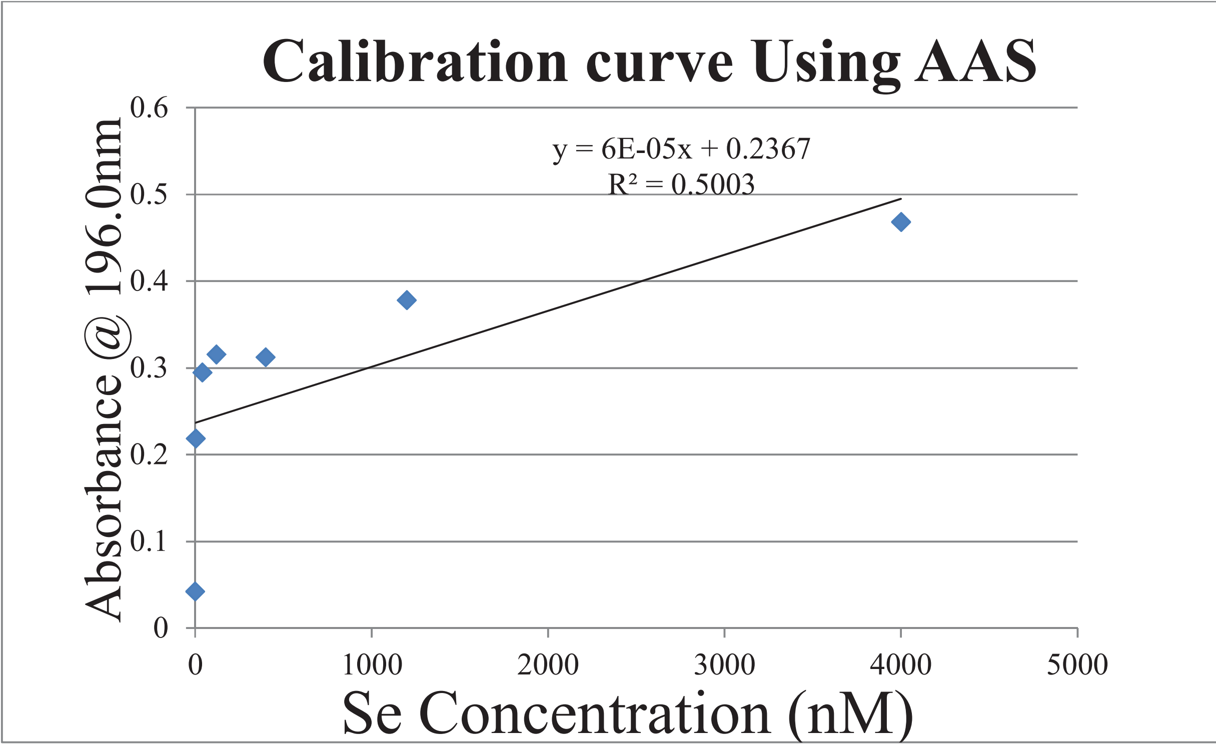

Figure 6: Calibration curve plotted for standards using AAS.

ICP-AES Results

ICP-AES Calibration Curves:

Table 5: ICP Results of calibration curve for *Enterobacter* sp. YSU obtained using known standards.

Standard		196.0 nm	203.9 nm	206.2 nm
ppm	mM			
0	0	1.099	-4.043	-0.972
0.32	0.2	38.82	21.99	7.773
0.8	0.5	97.36	61.19	19.25
1.6	1	210.5	135.6	41.44
8	5	1251	832.3	246.9
16	10	2628	1757	516.3
24	15	4114	2767	813.6
32	20	5707	3857	1133
64	40	10430	7104	2097

Table 5 shows different standards used for *Enterobacter* sp. YSU and their absorbance at different wavelengths. The emission increases with increasing concentrations of standards in a definite proportion. The standards column shows the mM concentration that is undiluted and the ppm concentration that involves the standards after dilution. Of all the wavelengths, 196.0 nm starts off with a positive number and the proportional increase of concentration to absorbance is better than other wavelengths. So 196.0 nm is used to determine the concentration of selenium in the samples.

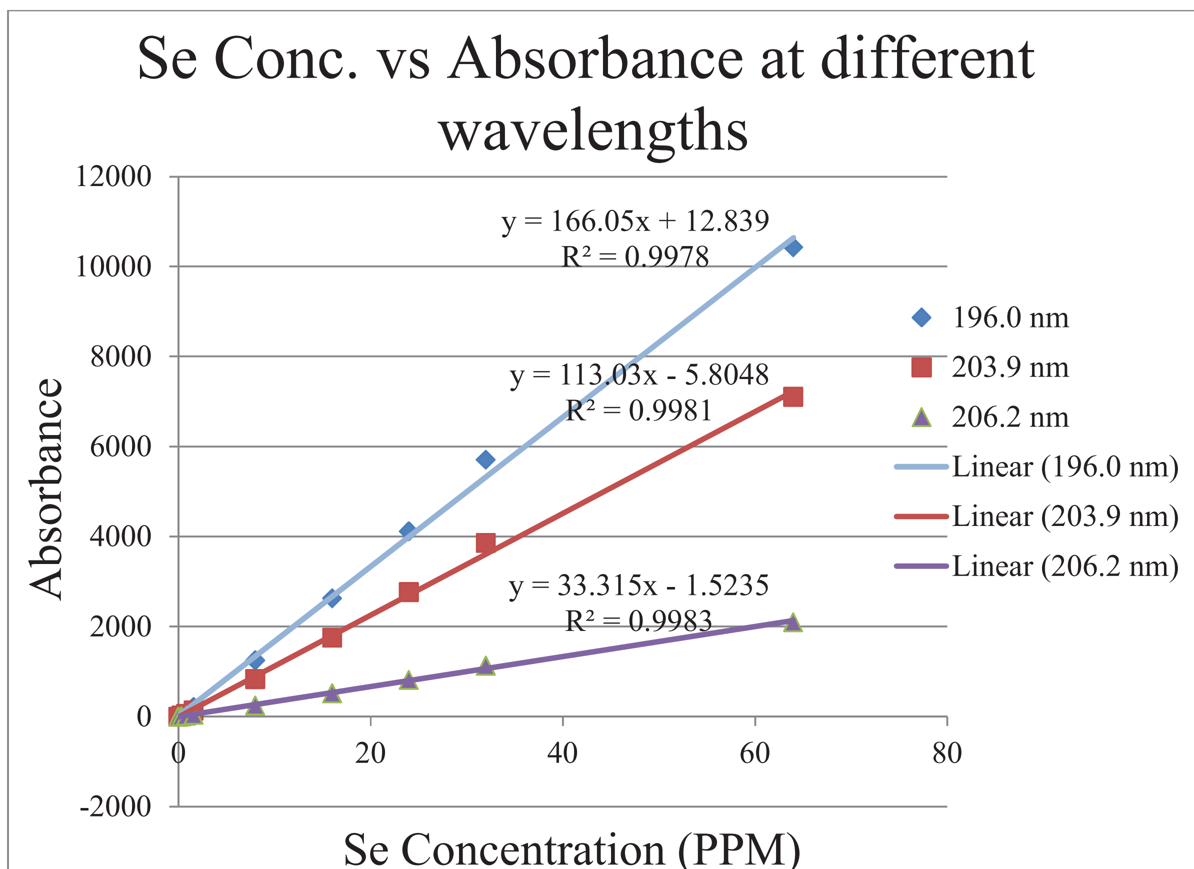

Figure 7: Calibration curve plotted for standards using ICP – AES.

Figure 7 was plotted using data from Table 5 and by taking selenium concentration on X-axis and Absorbance on Y-axis. The graph shows absorbance using three different wavelengths at which selenium shows good response. The slope and R-square was obtained using excel.

Table 6: ICP Results of calibration curve for *S. maltophilia* 02 obtained using known standards.

Standard		196.0 nm	203.9 nm	206.2 nm
ppm	mM			
Blank	0	6.883	1.407	1.407
0.8	0.1	6.674	1.54	0.8693
1.6	0.2	291.1	171	57.19
3.9	0.5	722.3	427.5	140.5
7.9	1	1437	850.2	279.9
39.5	5	7007	4212	1384
63.2	8	11280	6839	2255
79	10	14290	8687	2883
94.8	12	16750	10250	3403

Table 6 shows the concentration of the standards used and the emission at three different wavelengths. The standards column shows the undiluted mM concentration and the diluted ppm concentration. As the amount of selenium added to *S. maltophilia* 02 growth media was 10 mM different standards were prepared and used that would be more suitable.

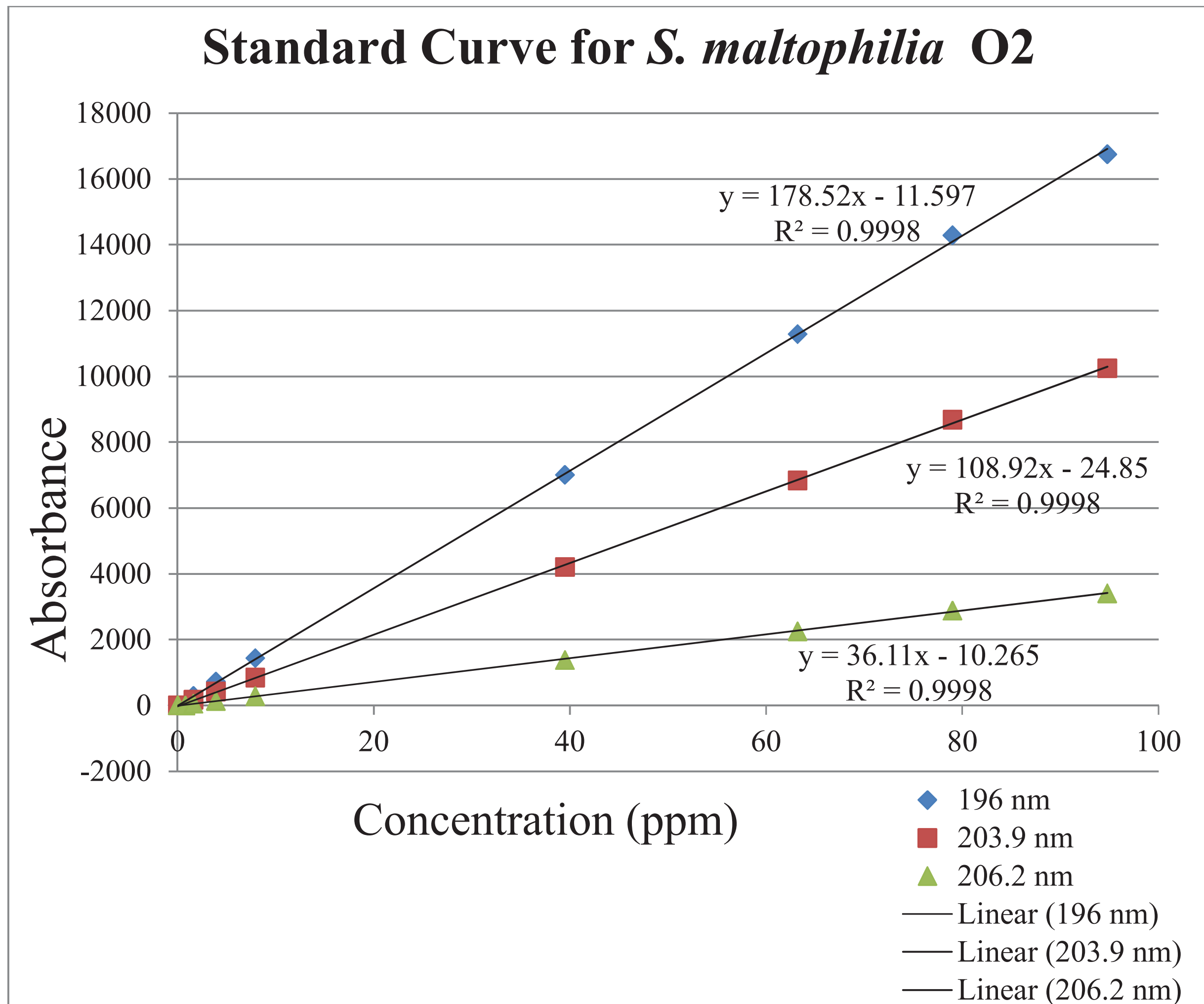

Figure 8: Calibration curve plotted for standards using ICP – AES.

Figure 8 shows the standards and their emission obtained with three different wavelengths for *S, maltophilia* 02. The R^2 and the slope is obtained using excel. The R^2 was found to be 0.998 which lay in the acceptable range. 196.0 nm wavelength was used to determine the concentration of selenium in the samples.

ICP-AES Results of *Enterobacter* sp. YSU

Table 7: Results of Selenium Concentrations in *Enterobacter* sp. YSU obtained using ICP.

Name/ Serial Number	Time (hrs)	Selenium Concentration (mM)	
		Selenium Pellet	No Selenium Pellet
1	1.5	0.013	0.017
2	2.5	0.499	0.023
3	3.5	0.551	0.028
4	4.5	0.547	0.026
5	24	0.541	0.023
6	48	0.699	0.027
7	72	0.676	0.023
		Selenium Supernatant	No Selenium Supernatant
1	1.5	0.014	0.011
2	2.5	33.952	0.017
3	3.5	34.009	0.019
4	4.5	33.342	0.017
5	24	33.500	0.017
6	48	34.148	0.014
7	72	33.781	0.012

Table 7 shows the different samples collected at different timings and average concentration of selenium in both the *Enterobacter* sp. YSU cells and M-9 media that were exposed to selenium and also the one that is not exposed. As the selenium was added after 1.5 hour, the selenium concentration in the culture is same as that of the culture not exposed to selenium. Comparing the pellets, it can be concluded that the amount of selenium increased in the cells that were exposed to selenium.

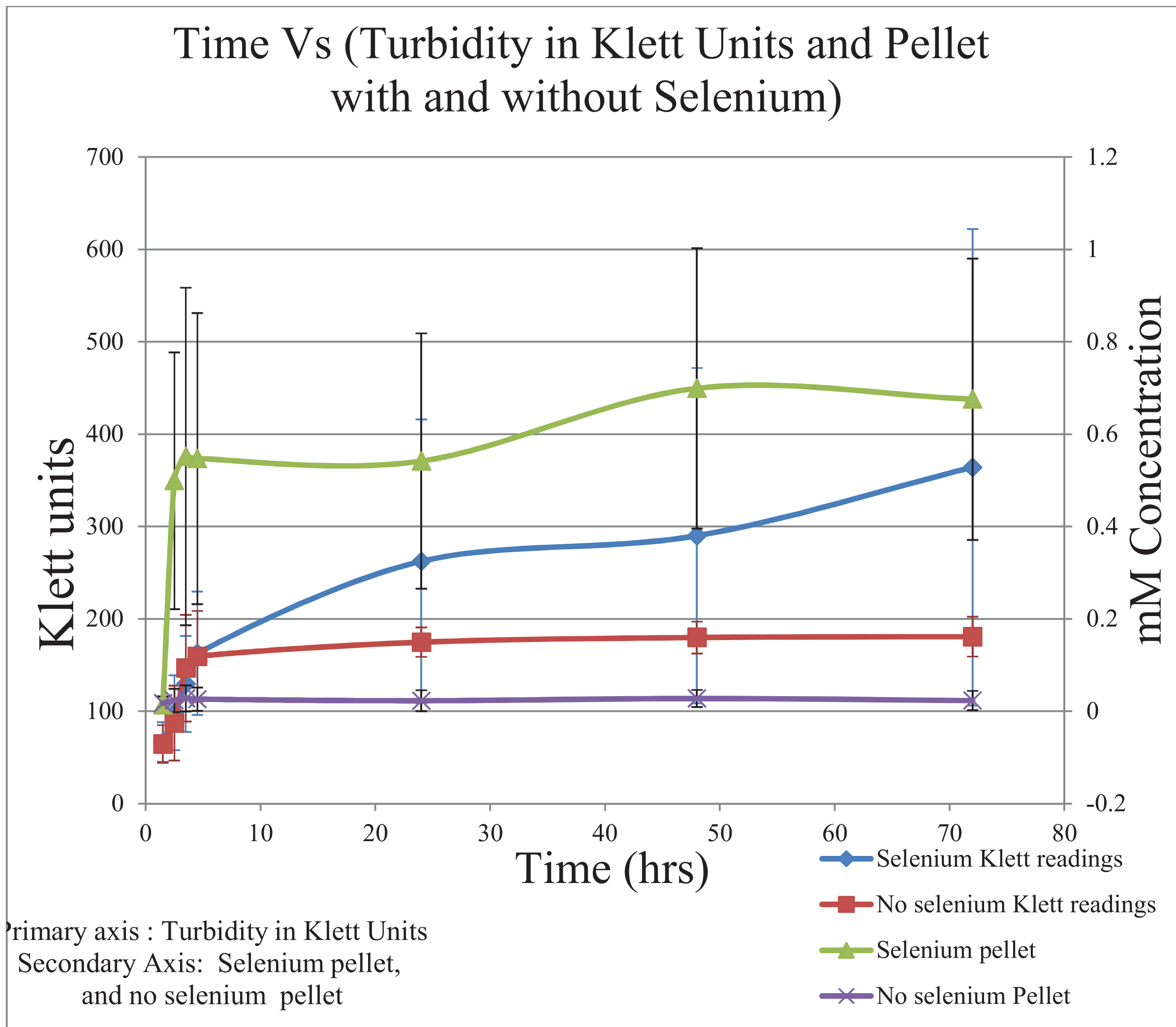

Figure 9: Results of *Enterobacter* sp. YSU showing Turbidity in Klett Units and selenium concentration inside the cells that were exposed to selenium and cells not exposed to selenium obtained using ICP.

Figure 9 shows the turbidity in Klett Units and selenium concentration in the cells of *Enterobacter* sp. YSU that were and were not exposed to selenium. The primary Y-axis corresponds to the turbidity in Klett Units and the secondary Y-axis to the selenium

concentration in the cells. The turbidity graph is same as the figure 4. The No selenium pellet is close to 0 and the selenium exposed pellet showed an increase in concentration.

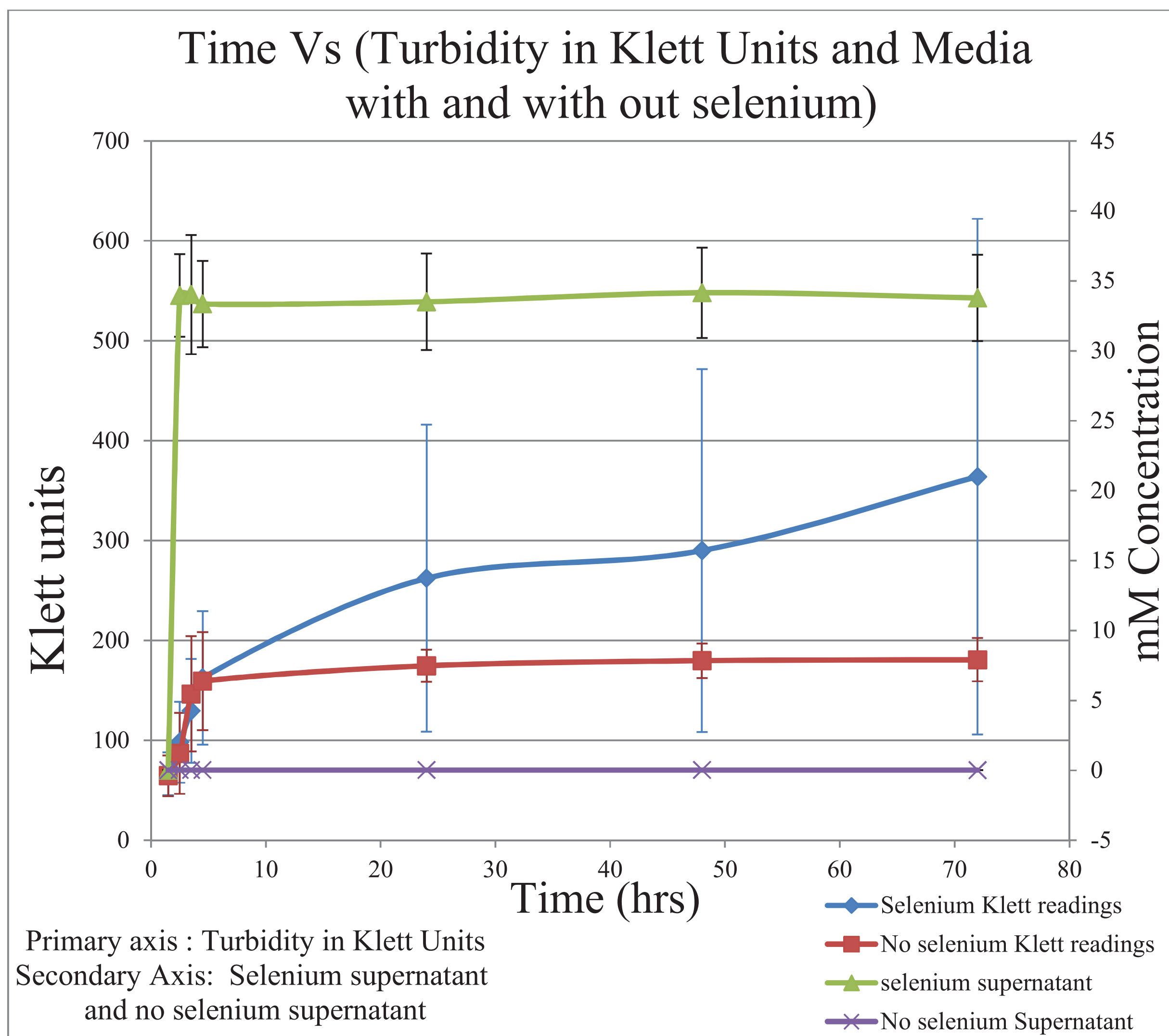

Figure 10: Results of *Enterobacter* sp. YSU showing Turbidity in Klett Units and selenium concentration in the media that were exposed to selenium and media not exposed to selenium obtained using ICP.

Figure 10 shows the different turbidity measurements in Klett units and selenium concentrations in the *Enterobacter* sp. YSU growth culture. From the graph it can be stated that the amount of selenium in the growth medium for the exposed one was ranged between 33-34 mM and the culture not exposed to selenite showed that the concentration of selenium was almost 0 mM.

Table 8: Results of Selenium Concentrations in *S. maltophilia* 02 obtained using ICP

Name/ Serial Number	Time (hrs)	Selenium Concentration (mM)	
		Selenium Pellet	No Selenium Pellet
1	0	0.0045	0.0042
2	1	0.0078	0.0031
3	2	0.0059	0.0026
4	3	0.0041	0.0040
5	4	0.0067	0.0095
6	5	0.0198	0.0120
7	6	0.0266	0.0120
8	7	0.0500	0.0088
9	8	0.0728	0.0096
10	9	0.0855	0.0023
11	10	0.1384	0.0015
12	11	0.1576	0.0053
13	12	0.1244	0.0116
		Selenium Supernatant	No Selenium Supernatant
1	0	0.0116	0.0018
2	1	0.0099	0.0018
3	2	0.0157	0.0019
4	3	7.8122	0.0030
5	4	8.1434	0.0051
6	5	7.6676	0.0056
7	6	7.2330	0.0065
8	7	7.5891	0.0063
9	8	7.8460	0.0056
10	9	7.8548	0.0025
11	10	7.3197	0.0054
12	11	7.8280	0.0065
13	12	7.2880	0.0165

Table 8 shows samples collected at different time intervals and their absorbance at 196.0 nm wavelength. As the Selenite was added after 2.0 hours the initial concentrations are about the same in the cells. With increasing time the selenium concentration inside the cells increased compared to the control. The Selenium concentration in the media exposed to selenium did not show a specific pattern.

ICP-AES Results of *Stenotrophomonas maltophilia* OR02

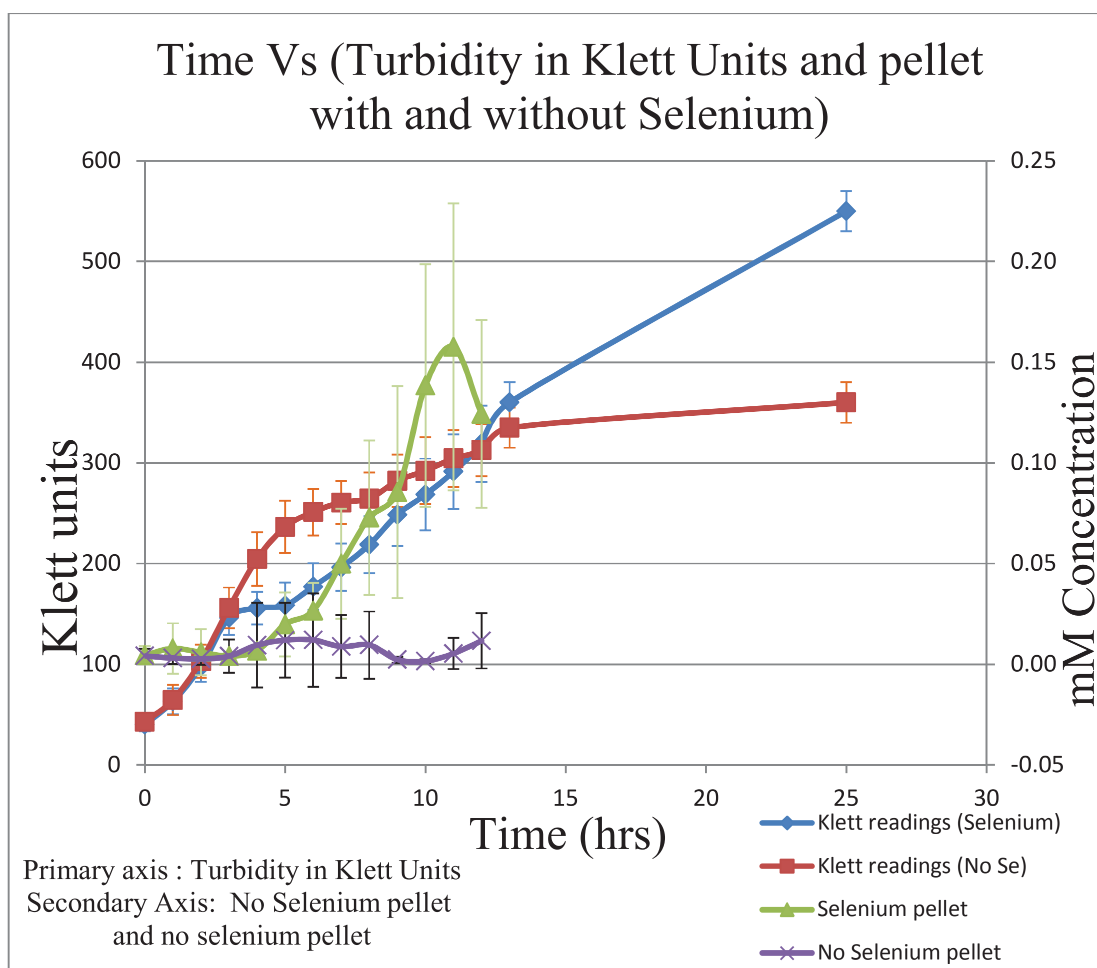

Figure 11: Results of *S. maltophilia* 02 showing Turbidity in Klett Units and selenium concentration inside the cells that were exposed to selenium and cells not exposed to selenium obtained using ICP.

Figure 11 was plotted using time on X-axis, the turbidity in Klett units on primary Y-axis and mM concentration of selenium on secondary Y-axis. It is evident that amount of selenium was increasing with increasing time in the cells that were exposed to selenite compared to the cells

that were not exposed to selenium. The decrease in the concentration of selenium after 12 hours of the cells that were exposed to selenium could be due to a decrease in the number of cells.

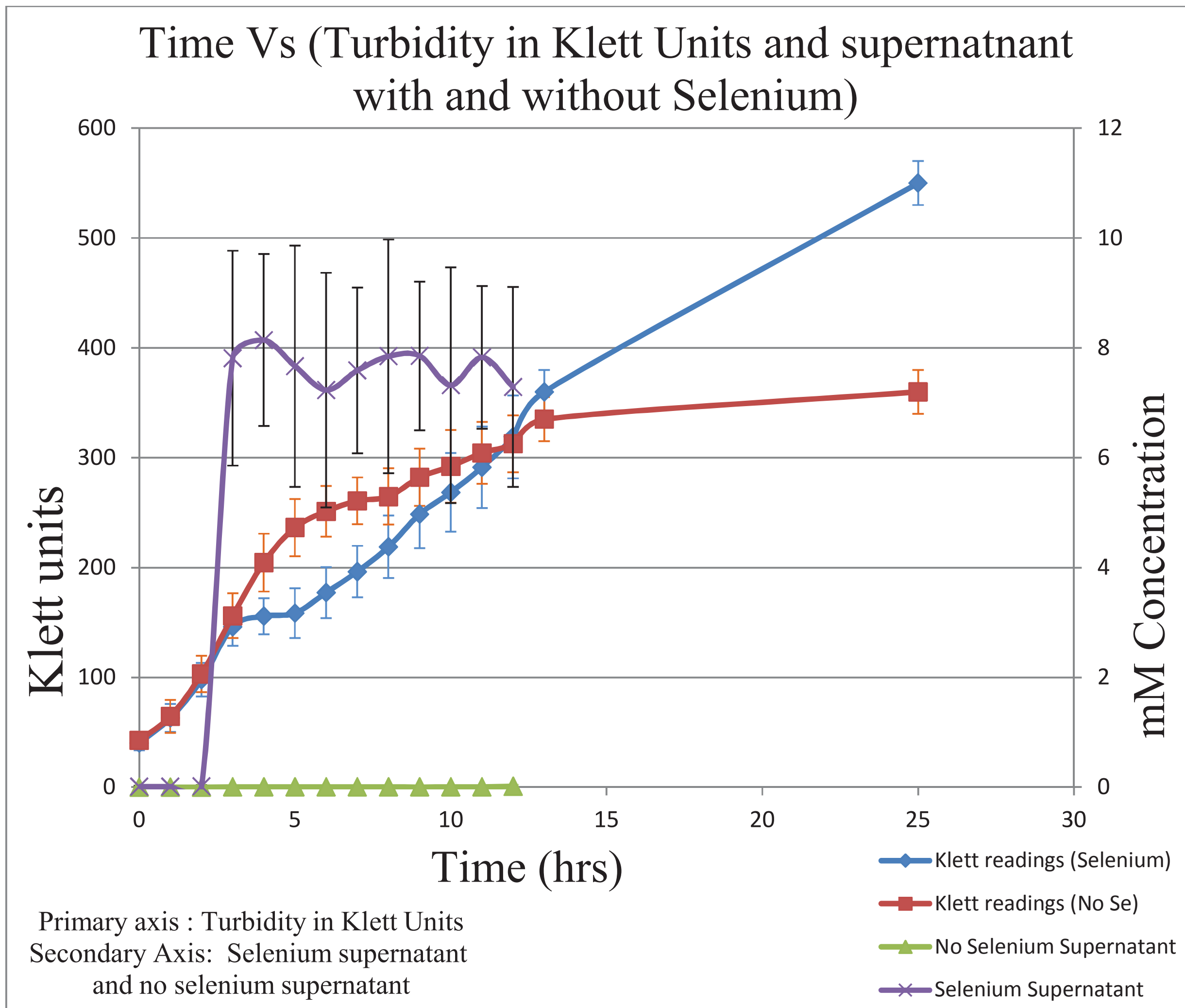

Figure 12: Results of *S. maltophilia* 02 showing Turbidity in Klett units and selenium concentration in the media that were exposed to selenium and media not exposed to selenium obtained using ICP.

Figure 12 was plotted using the time on X-axis, the turbidity in Klett units on the primary Y-axis and selenium concentration on secondary Y-axis. The graph shows the two cultures that were used. One was exposed to selenium and one was not exposed to selenium which is at 0.

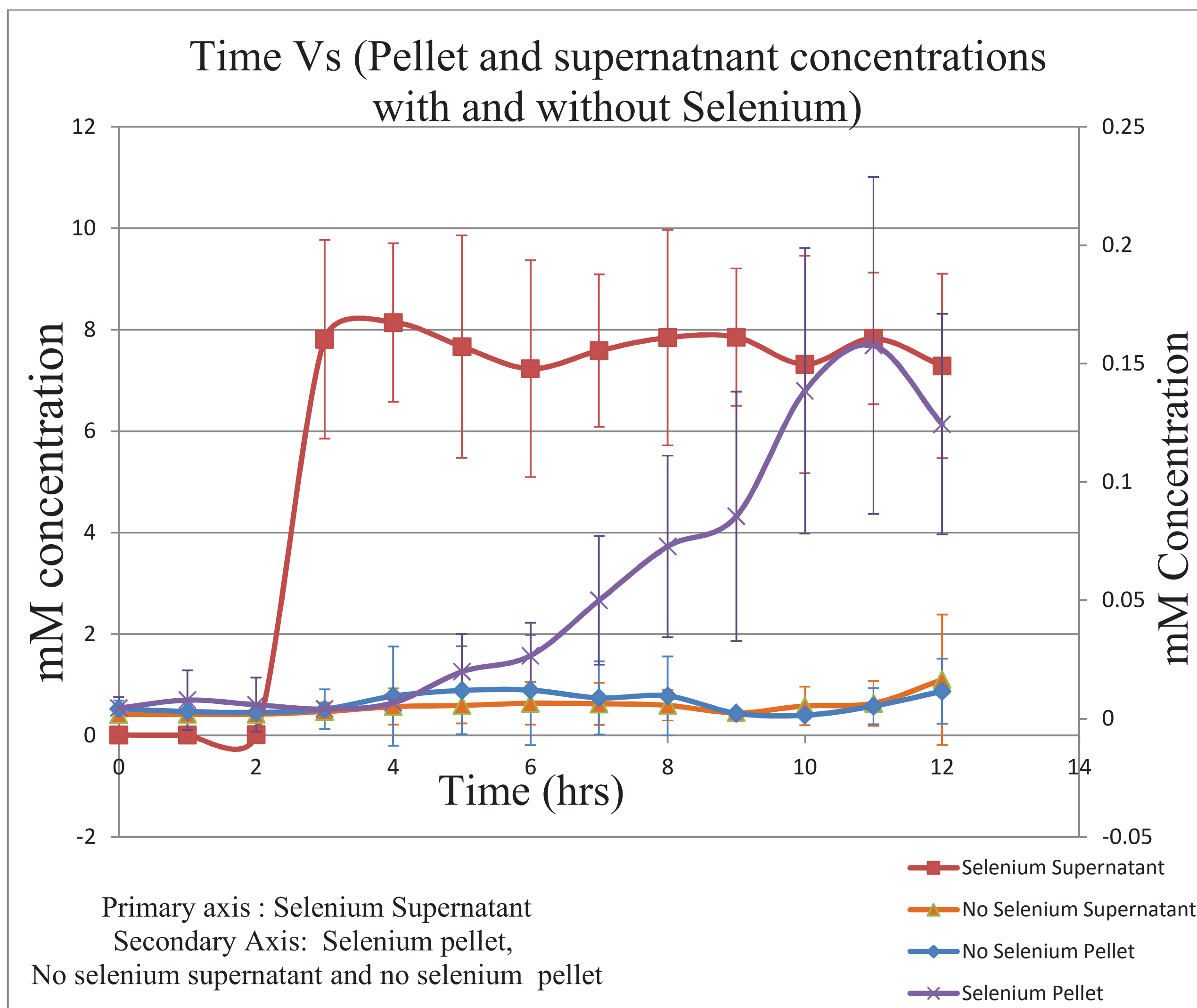

Figure 13: Results of *S. maltophilia* 02 showing selenium concentration in the media and pellet that were exposed to selenium and not exposed to selenium obtained using ICP.

Figure 13 is a cumulative graph in which the X-axis was used for time, the primary Y-axis was used for selenium concentration in the growth media that was exposed to selenium and the secondary Y-axis was used for the *S. maltophilia* 02 cells that were exposed to selenium and the control culture and cells. In this figure, it is evident that the cells in the culture exposed to selenium show a gradual increase in selenium concentration compared to the control.

Table 9: Amount of selenium in the bacterial cells and the Turbidity in Klett Units

Time (hrs)	Turbidity in Klett Units		Se Conc. in cells exposed to selenium	
	Exposed to selenium	Not Exposed to selenium	Exposed to selenium	Not Exposed to selenium
0	40	43	1.56E-04	5.85E-05
1	63	65	1.44E-04	8.45E-05
2	98	103	1.93E-05	3.21E-05
3	146	156	6.31E-05	2.86E-06
4	156	205	7.05E-05	9.44E-06
5	159	236	1.40E-04	7.42E-07
6	177	251	5.97E-04	1.87E-06
7	196	261	-	3.83E-05
8	219	265	1.05E-03	1.39E-04
9	249	282	1.42E-03	7.52E-05
10	269	292	-	-
11	291	304	2.50E-03	-
12	319	313	1.48E-03	-

Table 9 shows the turbidity in Klett units and the selenium concentration in the *S. maltophilia* 02 cells at particular interval of time. The diluted samples were grown on LB-agar plates and the colonies were counted. The total number of cells was used along with the ICP-AES results and the amount of selenium inside each cell was determined.

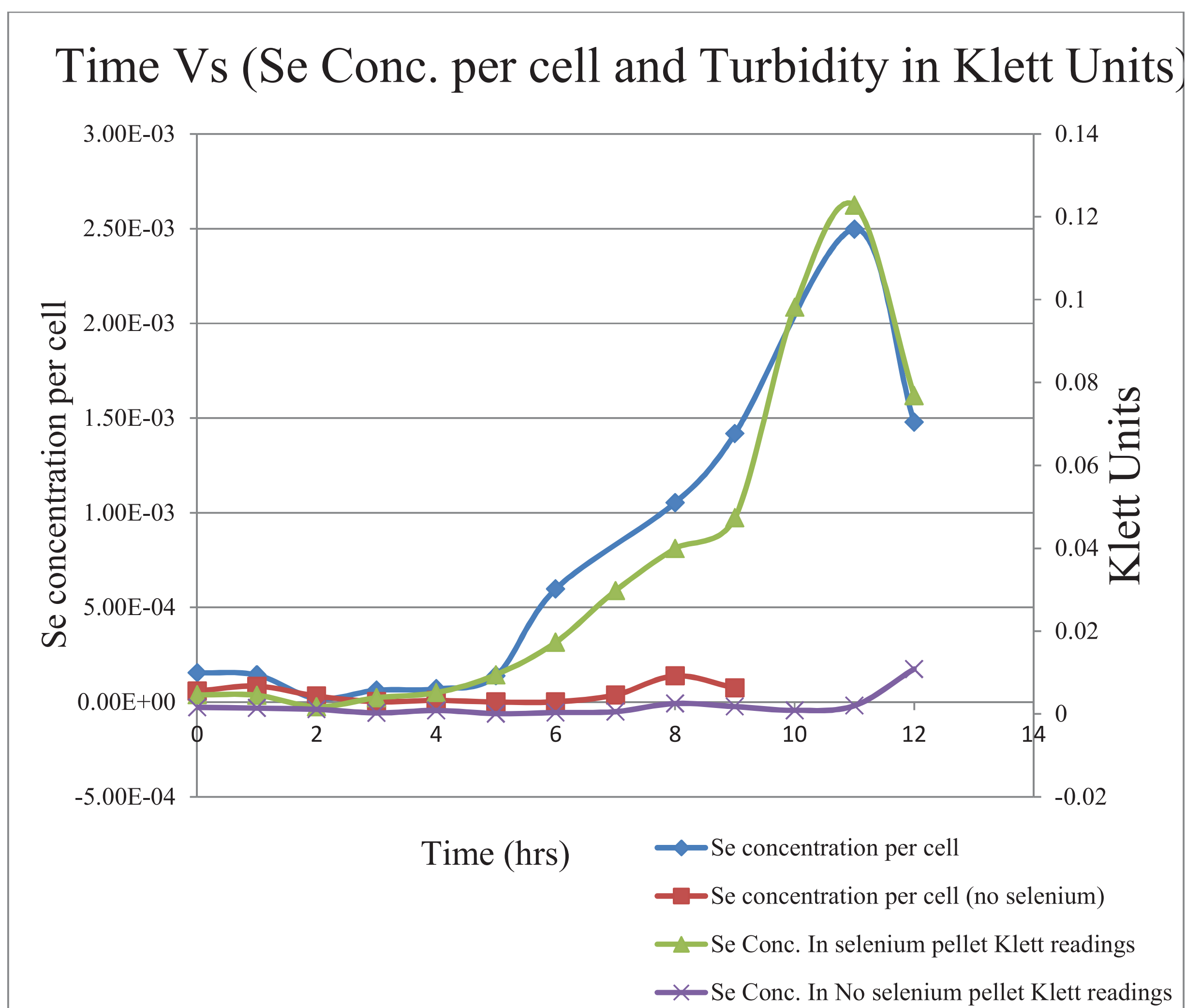

Figure 14: Graph plotted shows increase in se concentration per *S. maltophilia* 02 cell

Klett readings are based on turbidity. As the bacterial culture exposed to selenium grows with time the culture turns red. The turbidity in Klett units at this point could be a sum of the cells and red precipitate that appears in the culture. The samples were diluted and were grown on LB-agar plates. The amount of selenium was divided by the total number ofcells. The graph was plotted using the time on X-axis and selenium concentration per cell on Y-axis. The graph shows that the amount of selenium in each cell is increasing with time.

Discussion

Bacteria have the ability to not only survive in highly contaminated soil but also can transform the contaminants in them. Two such bacteria were isolated from East Fork Poplar Creek, *Stenotrophomonas maltophilia* OR02 (*S. maltophilia* 02) and *Enterobacter* sp. YSU showed resistance to high concentrations of selenite and they also transformed it to elemental selenium. It was postulated that the selenium content in the cells would increase during the stationary phase of their growth as the media turns to deep red. In addition, as the selenium concentration increased inside the cells, it was proposed that selenium content in the growth media would decrease. Selenium content did increase inside the cell but not enough to decrease the amount of selenite in the growth medium

Analytical instruments like Atomic Absorption spectroscopy (AAS) and Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) can be used to quantify the amount of selenium in both the culture and the bacterial cells. To monitor bacterial growth, Klett Colorimeter can be used. Klett Colorimeter determines the turbidity of the culture which is directly proportional to the growth of bacteria. It does not depend on any other factor. The cells probably converted the selenite to a red allotropic form, which may have contributed to the turbidity. Thus, the Klett readings could be a sum of the bacterial cells and the red allotrope. To overcome this, the samples were plated on LB-agar medium to get the specific number of cell count and calculate the amount of selenium reduced per cell.

To perform quantitative analysis of cells, the cells have to be lysed or digested to extract selenium. The samples can be directly introduced into AAS but the culture can clog the injection tube. However for ICP, direct injection may not only clog the tube but the presence of the whole cells could also decrease the uniformity during nebulization and compromise the reading. The digestion procedure requires high temperatures and acids, as the boiling point of elemental

selenium is 685°C heating it up to 150°C should not affect the sample however the chances of selenium changing into allotropic form are higher.

Both the analytical instruments used have a higher limit of detection in ppm, so any higher concentration of selenium can saturate the equipment thereby that particular element can never be used to analyze that particular element. From table 4 (calibration curve using AAS), it was clear that there was a problem with AAS. The standards did not show a constant and proportionate increase in absorbance with increasing concentrations of selenium. Sometimes it was also noted that the absorbance for the blank was too high. After a couple of trials, it was concluded that AAS was saturated with selenium and was not useful for quantitative analysis of selenium in these samples.

ICP-AES was then used for the analysis of selenium. The advantages of ICP over AAS were that the sample does not need to be diluted to nano molar concentrations, ICP can work on different wavelengths at a time and use of modifier is not necessary. The only disadvantage was a larger volume of sample was required, which in fact benefited us, as the sample collected was 1.0 mL. Diluting the samples by a factor of 10 or 50 was not tedious work and the complete sample could be diluted using 10 % nitric acid. As nitric acid can be injected into ICP without any problem, the samples were diluted with 10 % nitric acid. The sample holder for ICP can accommodate 240 samples at a time compared to 50 samples in AAS. ICP can run more samples at a stretch than AAS. ICP also does a quality check at specified intervals, so any problem during the sample run can be easily identified and fixed which is not possible with AAS.

After digestion, red precipitate or the red allotrope of selenium was insoluble in water and even 10% nitric acid. From the results of selenium concentration in the media, it can be said that the

red precipitate formed can account for the initial decreased selenium concentration in the growth medium. Otherwise, the digestion procedure seemed apt for the separation of selenium from the samples. The only problem was that the red precipitate settled by the time the sample was injected by the autosampler. Manually injecting the samples would be more suitable as uniform injection of the sample can be achieved. The average amount of selenium in the culture as shown in Figure 10 and 12 was 33 mM and 8 mM for *Enterobacter* sp. YSU and *S. maltophilia* 02, respectively and could be due to settling of red precipitate in the samples as the amount of selenium added was 40 mM to *Enterobacter* sp. YSU and 10 mM to *S. maltophilia* 02.

Any procedure that would dissolve instead of resuspend the red precipitate will yield good and more accurate results. As the boiling point of selenium is 685°C it is clear that heating the samples will not evaporate selenium however might transform it in to its allotropic form and using a combination of hydrogen peroxide and hydrochloric acid instead of nitric acid would be more favorable as hydrogen peroxide might oxidize the elemental selenium or the red allotrope of selenium into selenite or selenate that are more water soluble. After evaporating all the hydrogen peroxide and HCl from the sample and then using 10 % HNO_3 as a solvent, the samples could be analyzed using ICP. If not, then it is also possible that the red precipitate can be separated out by centrifuging the samples and then carry out a detailed analysis of the red precipitate or else establish a suitable method to dissolve the red precipitate by trial and error technique or else determine the concentration of selenium that is present in the form of red allotrope and then sum of the selenium concentration in the cells, media and the red precipitate might give the total selenium added to the culture initially.

It was expected that the amount of selenium inside in the culture and cells would add up to the amount of selenium added initially to the culture, but this was not achieved as the red precipitate

was formed. From Figures 9 and 11 pertaining to *Enterobacter* sp. YSU and *S. maltophilia* 02 it can be concluded that the amount of selenium in the cells is more when compared to the control. It can also be said from the increased levels of selenium inside the cells that the bacteria might be taking in selenium, reducing selenite to elemental selenium and then releasing it back in to the culture. However it is an assumption and more work would be needed to prove the assumption and identify the definite pathway responsible for this process. The main reason for this assumption is the solubility, selenate, selenite and salts of selenium are water soluble and elemental selenium and its allotropic forms are not.

It was also noted that heating the samples with red precipitate at 180°C for three hours resulted in the formation of gray precipitate that could be another allotrope of selenium. For this reason, the heat applied and the duration of heating was reduced to prevent such transformation.

Future work could be a detailed analysis of the red precipitate or development of a new procedure that would prevent the formation of red precipitate. Use of lower concentration of selenium that would eliminate dilution process and might help in determining the total amount of selenium, as the total selenium added to the culture was 40 mM and the amount of selenium found in the culture was 33 mM and in the cells it was 0.15 mM, so using 0.5 mM of selenium might improve the results. Instead of using Klett Colorimeter for determining the cell growth, growing the sample on LB-agar culture plate and determining the cell count would be more accurate.

REFERENCES

1. Brooks, S. C., Waste characteristics of the former S-3 ponds and outline of uranium chemistry relevant to NABIR field research center studies. **March 2001**.

2. www.y12.doe.gov.

3. Carroll, K. J.; Robinson, R. C.; Hogle, W. M., *Oak Ridge Y-12 Plant Review of Lessons Learned of the Tokaimura Criticality Accident*. 2000; p Medium: ED; Size: 6 pages.

4. Widner, T. E. R., Stephen R.; Buddenbaum, John E, Identification and Screening Evaluation of Key Historical Materials and Emission Sources at the Oak Ridge Reservation. . *Health Physics* **October 1996,** 71, (4), 457-469.

5. Dungan, R. S.; Yates, S. R.; Frankenberger, W. T., Transformations of selenate and selenite by Stenotrophomonas maltophilia isolated from a seleniferous agricultural drainage pond sediment. *Environmental Microbiology* **2003,** 5, (4), 287-295.

6. Jasenec, A.; Barasa, N.; Kulkarni, S.; Shaik, N.; Moparthi, S.; Konda, V.; Caguiat, J., Proteomic profiling of L-cysteine induced selenite resistance in Enterobacter sp. YSU. *Proteome Science* **2009,** 7, (1), 30.

7. Holmes, A.; Vinayak, A.; Benton, C.; Esbenshade, A.; Heinselman, C.; Frankland, D.; Kulkarni, S.; Kurtanich, A.; Caguiat, J., Comparison of Two Multimetal Resistant Bacterial Strains: <i>Enterobacter</i> sp. YSU and <i>Stenotrophomonas maltophilia</i> ORO2. *Current Microbiology* **2009,** 59, (5), 526-531.

8. Ikram, M.; Faisal, M., Comparative assessment of selenite (SeIV) detoxification to elemental selenium (Se0) by <i>Bacillus</i> sp. *Biotechnology Letters* 32, (9), 1255-1259.

9. http://en.wikipedia.org/wiki/Selenium. http://en.wikipedia.org/wiki/Selenium

10. Turner, R. J.; Weiner, J. H.; Taylor, D. E., Selenium metabolism in Escherichia coli. *BioMetals* **1998,** 11, (3), 223-227.

11. Carlos, G.; Donald, C.; Mike, A.; Boihon, C. Y.; Joshua, H. W.; Edgar, R.; Terrance, L.; Bob, B. B., Morphological and biochemical responses of Bacillus subtilis to selenite stress. *BioFactors* **1999,** 10, (4), 311-319.

12. Butterman, W. C.; Brown, R. D. J., Mineral Commodity Profiles : Selenium. **2004**.

13. Savard, D.; Bédard, L. P.; Barnes, S.-J., Selenium Concentrations in Twenty-Six Geological Reference Materials: New Determinations and Proposed Values. *Geostandards and Geoanalytical Research* **2009,** 33, (2), 249-259.

14. Zwolak, I.; Zaporowska, H., Selenium interactions and toxicity: a review. *Cell Biology and Toxicology*, 1-16.

15. Wu, J.; Lyons, G. H.; Graham, R. D.; Fenech, M. F., The effect of selenium, as selenomethionine, on genome stability and cytotoxicity in human lymphocytes measured using the cytokinesis-block micronucleus cytome assay. *Mutagenesis* **2009,** 24, (3), 225-232.

16. Heras, I.; Palomo, M.; Madrid, Y., Selenoproteins: the key factor in selenium essentiality. State of the art analytical techniques for selenoprotein studies. *Analytical and Bioanalytical Chemistry* 400, (6), 1717-1727.

17. http://www.lenntech.com/periodic/elements/se.htm.

18. Foster, L. H.; Sumar, S., Selenium in health and disease: A review. *Critical Reviews in Food Science and Nutrition* **1997,** 37, (3), 211-228.

19. Fan, A. M.; Kizer, K. W., Selenium. Nutritional, toxicologic, and clinical aspects. *West J Med.* **1990,** 153, (2), 160-167.

20. Willey, J. M. S., L. M.; Woolverton, C. J., *Microbiology*. 7 ed.; Prescott, Harley, Klein,; McGraw-Hill.

21. Willey, J. M.; Sherwood, L. M.; Woolverton, C. J., *Microbiology*. 7 ed.; Prescott, Harley, Klein,; McGraw-Hill; .

22. Eskilsson, H.; Haraldsson, C., Reductive stripping chronopotentiometry for selenium in biological materials with a flow system. *Analytica Chimica Acta* **1987,** 198, (0), 231-237.

23. Zhang, B.; Zhou, K.; Zhang, J.; Chen, Q.; Liu, G.; Shang, N.; Qin, W.; Li, P.; Lin, F., Accumulation and species distribution of selenium in Se-enriched bacterial cells of the Bifidobacterium animalis 01. *Food Chemistry* **2009,** 115, (2), 727-734.

24. Robles, L. C.; Feo, J. C.; de Celis, B.; Lumbreras, J. M.; GarcÄ±Ì a-Olalla, C.; Aller, A. J., Speciation of selenite and selenate using living bacteria. *Talanta* **1999,** 50, (2), 307-325.

25. Stephan, C.; Fournier, M.; Brousseau, P.; Sauve, S., Graphite furnace atomic absorption spectrometry as a routine method for the quantification of beryllium in blood and serum. *Chemistry Central Journal* **2008,** 2, (1), 14.