Studies on the role of microorganisms in mobilization of selenium in seleniferous soils

A thesis submitted in fulfillment of the
Requirement for the award of the degree of
DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY
by
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October 2012

Certificate

Certified that the thesis entitled "**Studies on the role of microorganisms in mobilization of selenium in seleniferous soils**" which is submitted by Mr. Saurabh Gupta in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy in the Department of Biotechnology & Environmental Sciences, Thapar University, Patiala, is a record of candidate's own independent and original research work carried out by him under our supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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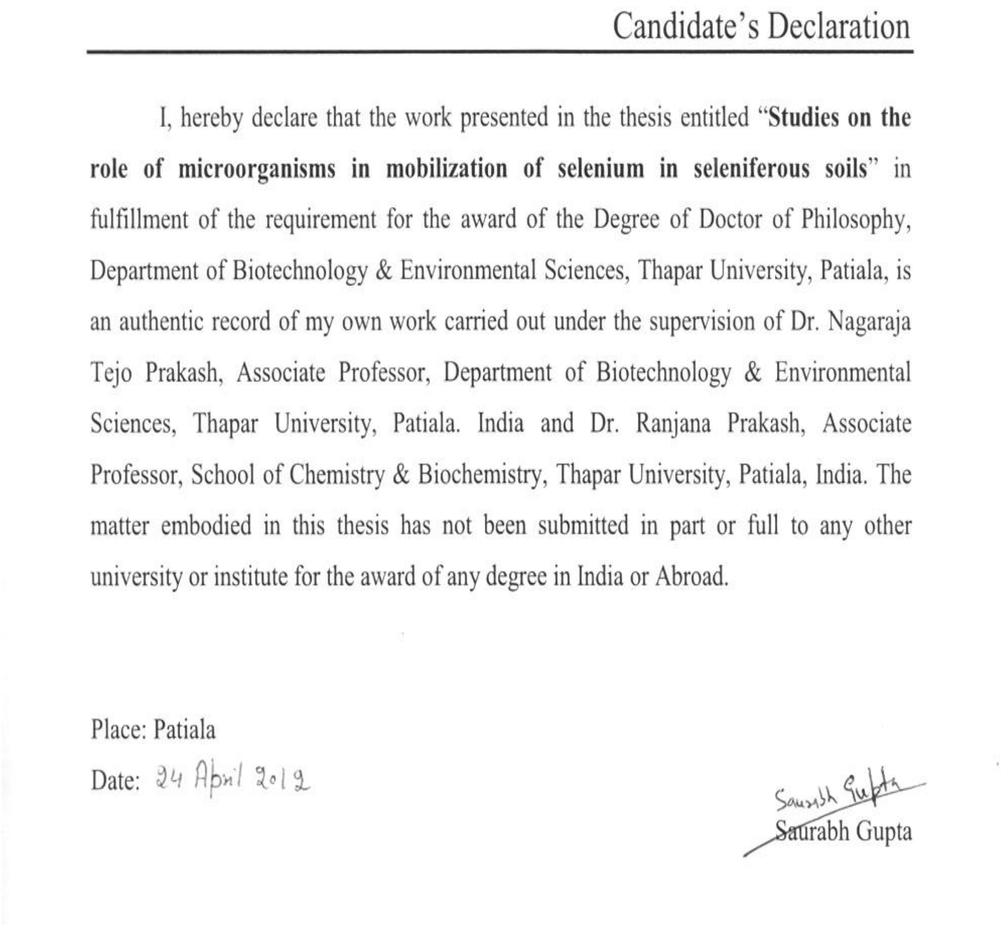

Candidate's Declaration

I, hereby declare that the work presented in the thesis entitled "**Studies on the role of microorganisms in mobilization of selenium in seleniferous soils**" in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy, Department of Biotechnology & Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work carried out under the supervision of Dr. Nagaraja Tejo Prakash, Associate Professor, Department of Biotechnology & Environmental Sciences, Thapar University, Patiala. India and Dr. Ranjana Prakash, Associate Professor, School of Chemistry & Biochemistry, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

Place: Patiala

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Acknowledgements

A statement of thanks is not evident to inculcate my deep sense of gratitude and obligation to all those who helped me in one way or another in completion of thesis. I would like to take this opportunity to thank many people who have helped and encouraged me throughout this study. It is a pleasant aspect that I have now the opportunity to express my gratitude to all of them.

I take this opportunity to thank my esteemed supervisor Dr. N. Tejo Prakash, Associate Professor, Department of Biotechnology & Environmental Sciences, Thapar University, Patiala and Dr. Ranjana Prakash, Associate Professor, School of Chemistry & Biochemistry, Thapar University, Patiala for providing me an opportunity to work under their guidance and supervision, assisting with all kinds of support and inspiration, wide counsel, constant encouragement, prudent advices, valuable suggestions and comments, expertise and fruitful advice throughout this investigation and preparation of the thesis. I extend my heartfelt thanks to them forever. It's a great privilege to experience a sustained enthusiastic and involved interest, constructive criticism, tremendous enthusiasm and meticulous supervision from his side throughout the period of my work.

I express my gratitude to Dr. Abhijit Mukherjee, Director, Thapar University, Patiala, for all the facilities which have been immensely helpful for the completion of my work.

I express my regards and gratitude to Dr. N. Das, (PhD committee member- Subject expert) for providing suggestions and taking interest in the progress of work along with lab facility.

I am highly thankful to Dr. Susheel Mittal, (PhD committee member- Subject expert) for his encouragement and motivation during my PhD work.

I feel a special gratitude towards Dr. M. S. Reddy, Head, Department of Biotechnology & Environmental Science for support for providing facilities.

No amount of words can adequately express the debt I owe to Mr. Anoop Verma, for his kind support that cultivated endurance in me to do the work throughout the course of project.

I feel highly obliged to Dr. Swaranjeet Singh Cameotra, Deputy Director, IMTECH, Chandigarh for providing support and lab facilities required during course of research. I extend my sincere thanks to Ms. Abha (Research Scholar) IMTECH, Chandigarh for her conscientious efforts done in analysis.

I am highly obliged to Dr. Sarabjeet Singh Alhuwalia, Assistant Professor, Department of Biotechnology, Khalsa College, Patiala for his valuable suggestions and constant encouragement during my doctorate work.

I extend my thanks to all the faculty members of Department of Biotechnology & Environmental Sciences, Thapar University, Patiala for providing necessary guidance during my research work. I am thankful to the Mr. Ashwani Aggarwal, Mr. Harish, Department of Biotechnology & Environmental Sciences for the constant official help and cooperation. I also convey my thanks to Mr. Mukesh Aggarwal, R & D, Thapar University, Patiala for his un-conditional and timely help.

I deem it a pleasure to express my heartfelt thanks to Mr. Anshu Bansal, Mr. Amit Polyesta, Mr. Siddharath Sharma and Ms. Honey Aggarwal for their kind concern and critical observations in designing and planning experiments.

I am highly thankful to Mr. Babban, Mr. Phool Chand, Mr. Iqbal, Mr. Mohinder, Mr.Bharat Verma, Mr. Satpal for timely assistance.

I take this opportunity to thank Mr. Satnam Singh and Mr. Sumit Jaiswal for their discussions and timely suggestions. I owe a word of thanks to all research scholars, at the Department of Biotechnology & Environmental Sciences, for providing constant support, encouragement and a good working atmosphere that has given me necessary time to relax from my work.

I am especially indebted to Mohit garg, Vidya for their help, constant encouragement, love and support. Life at Thapar University, Patiala has been enjoyable with my friends I thank them all for their great company.

I want to mention the names of M.Sc students Mr. Neelabh Anand, Mr. Jessey Sameuel, Ms Sonika, and Mr Parveen in our Biotechnology lab for their cheerful company.

The acknowledgement would be incomplete without the mention of constant encouragement as well as support of my college colleagues. Their affection, faith and noble guidance played a key role in the completion of this project work.

I feel a deep sense of gratitude for my parents and siblings who have always showered unconditional love on me, encouraged and supported me in every aspect. They formed a part of my vision and taught me the good things that really matter in life. Along with them I would like to mention the name of niece Sanchi Aggarwal who encouraged me to complete the work.

I express my gratitude to Dr. R.S. Kahlon for development of a scientific aptitude and courage to go ahead in this field along with his amicable endeavours, sustained encouragement, throughout and always.

Above all, I'm grateful to Almighty God for blessing me to complete this work successfully.

Saurabh Gupta

List of Symbols/Abbreviations

%	Percentage
bp	Base pair
cfu	Colony forming units
d	Days
DNA	Deoxyribonucleic acid
dNTP	2’-deoxynucleoside-5’-triphosphate
e.g.	For example
EDTA	Ethylene diamine tetraacetic acid
etc.	Extra
FAD	Flavin adenine dinucleotide
g	Gram
GF-AAS	Graphite-furnace atomic absorption spectroscopy
h	Hours
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
i.e.	that is
Kb	Kilobase
L	Litre
LB	Luria broth
LiCl	Lithium chloride
mg/kg	milligram per kilogram
mg/L	Milligrams per liter
min	Minute
mL	Millilitre
mM	milli molar
N	Normality (normal)
NAD	Nicotinamide adenine dinucleotide (oxidized)

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
nm	Nanometer
OD	Optical density
PCR	Polymerase chain reaction
rDNA	Ribososmal deoxyribonucleic acid
RDP	Ribosomal database project
RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
Se (IV)	Sodium selenite
Se (VI)	Sodium selenate
TBE	Tris borate EDTA
TE	Tris-EDTA
Tris	Tris (hydroxymethyl) amino methane
XRD	X-Ray diffraction
μg/g	Microgram per gram
μg/m	Microgram per millilitre
μL	Microlitre
μS/cm	Microsiemens per cm

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1.0 Introduction

The pressure on soil and ground water caused by harmful substances, especially heavy metals, is a widespread problem in dumping sites and agricultural soils. Soils act as a primary sink of all contaminants including heavy metals either present naturally or that are released due to anthropogenic activities. Out of 106 elements identified so far in nature, 80 elements are categorized as metals. These metals are divided into two broad classes on the basis of their requisite and non-essential nature for the living beings. In addition to alkali and alkaline earth metals, some d-block metals find their major role in vital physiological functions in the biological system. Trace metals such as Cu, Mn, or Zn are necessary for plant growth. However, when taken up in excessive quantities from the soil along with other nutrients during natural uptake processes, these elements get accumulated in the plants. Thus these elements enter in the food chain where these may have adverse effects on the health of animals and humans. Unlike organic contaminants, which can be degraded to harmless chemical species, heavy metals cannot be destroyed or metabolically degraded. Remediation of the pollution caused by these inorganic contaminants can therefore only be envisioned as their immobilization in a non-bioavailable form, or their re-speciation into less toxic forms (Valls and Lorenzo 2002).

Selenium is a naturally occurring d-block element placed in VI-group and possesses both metallic and non metallic properties with an atomic weight of 78.96. Selenium is abundant in the earth's crust with normal concentrations of 50 to 90 µg/kg, Often selenium is found in association with sulphur and sulphur containing compounds. Although selenium is found widely distributed in the earth's crust, but its concentration varies in different regions. Selenium is commonly present in rocks and soils with higher concentrations generally found in volcanic, sedimentary and carbonate rocks. Chemistry of selenium is quite complex due to presence this element in several oxidation states such as selenate (6^+), selenite (4^+), selenides (2^-), and elemental selenium (0). Selenium when exist in its pure form of amorphous red, metallic gray to black hexagonal crystals, is often referred as elemental selenium or selenium dust. However, in the environment, selenium is seldom found in its pure form. It is usually present in the form of compounds in rocks with other substances, such as sulfide minerals, silver, copper, lead, and nickel minerals. Selenium also forms chemical compounds analogous to those of sulphur (Amdur et al 1991). The salts of selenous acid (H_2SeO_3) and selenic acid (H_2SeO_4) represent selenite (Se^{4+}) and selenate (Se^{6+}) forms of selenium respectively. The chemical reactions of selenium resemble those of sulfur and are typically non-metallic in nature. Owing to its unique properties, selenium and its compounds were exploited largely in different industrial applications as well as pharmaceutical preparations. Selenium compounds are used as a decolorizing agent in the glass industry, as a vulcanizing agent in the rubber industry and in insecticides, and photoelectric cells as well. Selenium compounds also find their wide application in the toning baths used in photography and xerography. Selenium sulfide (SeS) is an antidandruff agent and used in shampoo preparations. The most widely used selenium compound in industry is selenium dioxide (SeO_2) (HSDB 1994). It is produced by the oxidation of Se with nitric acid followed by evaporation or by burning Se in oxygen.

Selenium is virtually present in all the materials on earth (McNeal and Balistrieri 1989). Both anthropogenic and natural processes play an important role in distribution of Se in different ecosystems. A variety of physical, chemical, and biological activities such as volcanic activity, combustion of fossil fuels, weathering of rocks, soil leaching, groundwater transport, bioaccumulation in biological systems followed by its release after death results in recycling of selenium. However both abiotic and biotic transformation of selenium in these processes is determined by the particular chemical species of Se (Nriagu 1989). Nearly 90% of the selenium content in ambient air is emitted during the burning of fossil fuels (Kut and Sarikaya 1981). Selenium is particularly concentrated in the soils of the arid and semi-arid regions where the soil tend to be more alkaline (Sharma and Singh 1983). A wide range of selenium is reported in various soils throughout world (Ure and Berrow 1982). Selenium contaminated soils have been reported in China, South Africa, Columbia, Argentina, Venezuela, Spain, Bulgaria, Ireland, Algeria, Morocco, Australia, and New Zealand and in some drier regions of former Soviet Union. Weathering of rocks is the major source of environmental Se. Generally, limestone and sandstone contain lower concentrations of Se (0.1 μg/g), whereas shale tends to contain higher Se concentrations (0.6 μg/g). The distribution of Se in rocks and soils of the United States was studied extensively during 1930–1950 in connection with Se toxicity in livestock in the Western states (Larkin and Byers 1948). A generalized summary of Se distribution in soils of the United States, associated with white muscle disease (Muth and Allaway 1963) indicates that soils in areas of the rocky mountains and extending into the Great Plain states are high in Se, and soils of both the Eastern and Western coastal states are low in Se. In California, the US Geological Survey (Presser and Barnes 1984) and Tanji et al (1986) reported that the rocks of the Coastal Ranges contain FeS_2 (pyrite). Pyrite containing rocks of marine origin occur along the entire western margin of the San Joaquin valley and some of these contain significant amounts of Se. Selenium concentrated drainage water overflowed into the grasslands and Kesterson national wild refuge and caused deleterious ecological problems (Ohlendorf et al 1986). Generally, natural waters have Se concentration less than 0.01 mg/l. Alkaline water have a natural tendency of selenium leaching from seleniferous rock which lead to elevated Se concentrations in soils. Most bio-available Se in the soil is the water-soluble forms and their concentration may also get enriched by supplementation of phosphate fertilizers obtained from Se-containing phosphate rocks leading to increase in its concentration in agriculture drainage.

In acidic, clay soils and those containing high organic matter, selenium is present as selenides and selenium sulfides (Kabata and Pendias 1984). These species are slightly soluble in water and therefore have reduced potential for selenium bioavailability to plants. In alkaline and well-oxidized soils selenium occurs as selenate. As selenium oxyanions are highly soluble and therefore available to plants and potentially toxic (White et al 1991). The most important soil factors affecting the availability of Se are fixation capacity, pH, and microbial activity. Soluble Se oxyanions represented the major fraction of Se in the presence of oxygen in the root zone (Tokunaga et al 1991). Clay minerals play an important role in the retention of soluble form of selenium such as selenite in the soil. Selenite has a stronger adsorptive affinity for particles, particularly amorphous iron oxyhydroxide than manganese dioxide and this affinity is far less for selenate and other forms of selenium. Adsorption of selenium oxyanions is also strongly influenced by pH and presence of other competitive oxyanions (Merrill et al 1987). The other process is the formation of metal selenides or Se-sulfides. Sulfide minerals such as galena and pyrite tend to act as carriers for Se. Selenium is found as a constituent of more than 40 different minerals and some of these sulfide minerals contained selenium in an abundance of up to 20% (Nazarenko and Ermakov 1972). Therefore, adsorption and mineral formation limit the mobility and bioavailability of Se in the environment. Organic matter naturally binds the metals/metalloids and has more affinity of selenite binding than clay soil. Selenium in the organic fractions is found in the form of complexes with organic compounds or it is converted into amino acids and proteins by microorganisms and plants.

Bio-availability and toxicity of different selenium compounds is related to its oxidation state and largely to their solubility. Like sulfates, selenates (Se^{+6}) are highly soluble and are readily taken up by living beings. In contrast, selenites (Se^{+4}), selenides (Se^{-2}) and elemental selenium are relatively insoluble. The most acutely toxic selenium compound reported is hydrogen selenide (H_2Se). Hydrogen selenide is formed by the reaction of acids or water with metal selenides or by the contact of nascent hydrogen with soluble selenium compounds (Clayton and Clayton 1982). Hydrogen selenide has no reported commercial use. Although not true in all cases, the relative toxicity of various chemical forms of selenium generally follows this order (from most to least toxic): hydrogen selenide > selenite > selenate > elemental selenium ~ metal selenides ~ methylated selenium compounds. Elemental selenium is believed to be of low toxicity because of its insolubility in biological media followed by methylated selenium compounds due to their high volatile characteristics.

Since its discovery by Berzelius in 1817, selenium was broadly known as a toxic element due to ailments associated with uptake of this element. As the research progressed on this element during the recent past, selenium was observed to be an essential nutrient and harbour properties associated with treatment of many serious deficiency disorders. From a biochemical point of view, selenium is an essential component of the various enzymes such as glutathione peroxidase, superoxide dismutase, catalase and biochemical reactions involving vitamin E. These enzymes protect cellular components against damage by utilization of peroxides, hence prevent the accumulation of peroxides in the tissue. Selenium also helps in the prevention of cancer and ageing along with treatment of atopic dermatitis and asthma. Several microbiological systems with Se containing enzymes have been also identified, e.g. glycine reductase, formate dehydrogenase, hydrogenase, nicotinic acid hydroxylase, xanthine dehydrogenase and thiolase (Pinsent 1954; Stadtman 1987; Tappel 1987). Selenium is an essential nutrient for animals including humans. It is associated with normal growth in humans and rats along with an important role in reproduction in rats (Casey and Hambidge 1980). Severe deficiency of selenium has been found responsible for damage to heart muscles especially in growing children and women of reproductive age (Hira et al 2004). A chronic and endemic osteochondropathic disease (Kashin-Beck disease) associated with selenium deficiency is widely reported in Northwestern to southwestern China along southeast Siberia and North Korea (Yao et al 2011). Recent study reported more than one million people suffering from this disease (Wang et al 2011). Uptake of food supplemented with selenium has been reported to overcome this disease (Ovaskainen et al 1993). Most of the biological systems have the tendency to take up and utilize both inorganic as well as organic selenium compounds except some plants which can take up only inorganic selenium compounds. Although selenium is an essential trace element in many species, but the dose differential between acute toxicity and chronic deficiency is very narrow (Amdur et al 1991). The oral intake level of selenium associated with chronic deficiency is 20 µg Se/day with "normal" oral intake of 70 µg Se/day and the lower limit for acute oral selenium toxicity is 200 µg Se/day in humans. Dietary intake of 55 µg/day selenium in adult humans has been observed to fulfill the selenium requirements for optimum enzyme activity (Moghadaszadeh and Beggs 2006). Selenium is an essential trace element and is necessary for the functioning of the enzyme glutathione peroxidase (GPx), which protects against oxidative damage to intracellular structures. Selenium gets incorporated in other enzymes or proteins, such as phospholipid hydroperoxide, glutathione peroxidise (PHG-px), Selenoprotein P (Se-p), and type- I iodothyronine 5-deiodinase (5’-ID-I) which play important human health functions (Tan et al 2002). Eleven seleno-proteins such as cellular glutathione peroxidases; seleno-protein P; types 1, 2, and 3; iodothyronine deiodinase; seleno-protein W; thioredoxin reductase; and seleno-phosphate synthetase have been identified which play an important role in normal metabolism of various organisms including humans (Holben and Smith 1999). Other selenoproteins include peripheral terathiodothyronone 5'-I-deiodinase I which converts thyroxine to T3 in the thyroid and other peripheral organs. Seleno-protein P is a plasma protein containing selenium and may possess transport as well as an antioxidant function but its precise role is unclear. The reduced activity of GPx in selenium deficient individuals is also accompanied by a reduction in the levels of GPx protein (Sunde 1990). Conversely during selenium repletion, the increase in GPx activity paralleled the increase in GPx protein. Selenothioredoxin reductase, a seleno-protein plays an important role in regulation of metabolic activity. This protein catalyzes NADPH-dependent reduction of 5, 5’-dithiobis (2-nitrobenzoic acid) (DTNB) along with reduction of insulin in the presence of thioredoxin (Trx) (Tamura and Stadtman 1996). A second major class of seleno-proteins are the iodothyronine deiodinase enzymes which catalyse 5′, 5-mono-deiodination of the prohormone thyroxine (T4) to the active thyroid hormone 3, 3′, 5-triiodothyronine (T3) and the conversion of inactive reverse T3 to 3, 3′-di-iodothyronine (Brown and Arthur 2001).

Clinton (1947) reported eye, nose and throat irritation and headache in workers briefly exposed to high, un-quantified concentrations of selenium fumes. Buchan (1947) reported first time the signs of acute intoxication of selenium such as irritation of the respiratory tract, severe bronchitis, bronchial pneumonia, and pulmonary edema due to occupational exposure to 0.21 ppm (0.7 mg/m³) H_2Se. In an another case study, workers accidentally exposed to selenium oxide reported initial symptoms of bronchospasms, irritation of the upper respiratory passages, violent coughing, and gagging with nausea and vomiting (Wilson 1962). Chronic selenosis results in severe effects on keratinized tissue such as loss of hair and lesions on the nails, claws, horn and skin (O`Toole and Reinbeck 1995).

In India, chronic selenium poisoning of plants, animals and humans has been reported in seleniferous soils of Northwest India (Dhillon and Dhillon 1997). Plant and animal uptake and accumulation of selenium from the environment is influenced by the concentrations and chemical forms of selenium; the medium in which it occurs (e.g. water and/or diet); the period of exposure; and, in water, the chemical and other characteristics of the water (e.g., dissolved oxygen content, hardness, pH, redox state, salinity, and temperature), including the presence of other chemicals (e.g. sulfate or cadmium). Deposition of alluvium carried by the flowing river water of the Indus resulted in formation of highly fertile agricultural soil of this region. A well defined path followed by the water currents flowing from the Shiwalik range along the elevation of the Himalayan mountain range deposited all the minerals in low-lying areas of the affected region (Dhillon and Dhillon 1991). Agricultural soils in this seleniferous region are alkaline, calcareous, silty loam to silty clay loam in texture and are well drained (Dhillon et al 1992). Maximum selenium contents in these soils were reported in low lying areas of this region where selenium oxyanions are transported by rain water through seasonal rivulets from nearby hills of the Siwalik range and deposited in the low lying areas (Dhillon and Dhillon 1991). The toxic sites are located at the dead ends of the seasonal rivulets. Due to this reason, a scattered seleniferous region has been developed in agricultural soils of 4-5 villages of the Northeastern Punjab. A wide variation in selenium concentration in these soils has been observed due to deposition of minerals that are mainly composed of polymictic conglomerates of variable composition containing many unstable materials such as granite, basalt, limestone that play an important role in partitioning of particulate selenium (Dhillon and Dhillon 2003b). The underground water drawn from tube-wells located in the seleniferous region of Northwestern India was found fit for irrigation on the basis of electrical conductivity and residual sodium carbonate (RSC). Se content of groundwater varied drastically at or near the toxic sites ranging between 0.25 µg/l and 69.5 µg/l. Nearly 5% and 10% of these samples contained more selenium than permissible limits for either irrigation or drinking purposes (Dhillon and Dhillon 2003a). Uptake of soluble selenium from tube-well water played an important role in bioaccumulation and bio-magnification of selenium in the plants and food chain. Substantial increases in stem and leaf selenium (5-160 mg/kg and 4-66 mg/kg respectively) contents was observed in Sorghum, a major fodder crop grown in the region along with wheat and rice grown in these sites (Dhillon and Dhillon 1991; 1997).

Although selenium (Se) is an essential trace element for both animals and bacteria, but whether it is essential for plants still remains to be determined. At concentrations beyond trace amounts, Se is generally toxic to plants and other organisms. To overcome this toxicity, mechanism such as reduction and volatilization predominately operate in the biological systems and reported in both microorganisms and plants (Kahakachchi et al 2004). Certain plants like Indian mustard (*Brassica juncea*) are known for hyper-accumulation and volatilization of this metalloid (Di Gregorio et al 2006). Bacteria have been shown to play a key role in the biological cycle of selenium using various mechanisms such as reduction and volatilization of inorganic selenium (Stolz and Oremland 1999; Gadd 1993a; 1993b). The extent of the environmental impact on the transformation and mobilization of selenium by microbial dissimilatory processes has also been reviewed critically for efficient bio-removal of excess selenium (White et al 1997). Bioremediation is defined as "the use of living organisms to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals and other hazardous wastes" (Gibson and Sayler 1992). These days bioremediation is exploited in combination with different technologies that accelerate natural processes for degrading and/or detoxifying harmful chemicals in soil, groundwater and wastewater. Microbial bioremediation is the application/use of microorganisms to clean up hazardous contaminants in soil, surface or subsurface waters, or wastewater. Modern biotechnology has selectively adapted naturally occurring microbes for their ability to detoxify specific toxic chemicals. When combined with nutrients, pH stabilizers, oxygen, and surfactants, these microbes attack the offending materials at a rapid rate to minimize contamination and reduce or eliminate the environmental hazard. Most microbial bioremediation processes take advantage of indigenous microorganisms. The objective of a microbial bioremediation program is to immobilize or to transform toxic compounds in to chemical products no longer hazardous to human health or the environment.

Bioremediation of selenium contaminated sites generally involves reduction of the bio-available selenium oxyanions into elemental selenium along with bio-methylation. The former product is insoluble, non-toxic where as the latter belong to volatile forms of selenium. Potential microbial transformations of selenium oxyanions that can be useful in bioremediation strategies, includes (i) bio-reduction of selenium oxyanions to insoluble forms such as elemental Se (Se0) or (ii) to assimilated organic forms such as seleno-amino acids and (iii) simultaneous reduction and methylation, which yield volatile forms, primarily dimethyl selenide (Oremland 1994; Thompson-Eagle and Frankenberger 1992; Doran 1982). Primarily microorganisms reduce selenium oxyanions under anoxic conditions by using selenate as a terminal electron acceptor during metabolism. Some microorganisms convert these biologically available forms of selenium into elemental selenium naturally as a mechanism to overcome toxicity. Microbial activity is thought to be the primary means by which soluble active form of selenium can be reduced to elemental selenium and thus got precipitated (Stolz et al 2002). Microorganisms reduce toxic soluble selenium oxyanions species (selenate and selenite) to a much-less toxic insoluble forms, namely elemental selenium (Garbisu et al 1995). A number of reports have been published on microbial selenite reduction in both aerobic and anaerobic conditions by various researchers (Klonowska et al 2005; Watts et al 2003; Lovley 1993). Several genera of bacteria are adapted to thrive in soil polluted with heavy metals, such as *Bacillus, Pseudomonas, Thaurea, Ralstonia,* and *Enterobacter*. A wide variation in the redox reactions carried out by these microorganisms have resulted in selenium species dependent oxidation (Dowdle and Oremland 1998) and reduction mechanisms (Stolz et al 2002) to overcome selenium toxicity. Turner et al (1998) reported reduction of both selenate and selenite into elemental selenium using *Escherichia coli* while *Rhodobacter sphaeroides* and *Ralstonia metallidurans* were reported to reduce only selenite (Bebien et al 2001; Roux et al 2001). A number of reports have been published with reference to distribution of biologically reduced selenium in and out of the cell. Particles of elemental selenium accumulate in the periplasm (Gerrard et al 1974), in the cytoplasm (Silverberg et al 1976) or outside the cell (Yamada et al 1997), depending upon the species and the associated mechanisms.

A significant number of reports have been published on the reduction and volatilization of selenium oxyanions especially selenite by bacteria under anaerobic conditions, sparse data is available on the reduction of selenate or selenite under aerobic conditions. The biological mechanisms and enzymes involved in the reduction of selenate and selenite are still to be characterized. Till date only one reductase enzyme has been reported from *Thaurea selenatis,* an anaerobic bacterium for reduction of selenate. Keeping these lacunae in mind, present work was carried out with objective (a) to isolate and characterize aerobic bacterial strains from the tropical regions; and (b) to understand the efficacy of bacterial mobilization/removal of selenium from rhizospheric soils. As on date, the literature dominantly indicated the role of anaerobic bacteria on *in situ* removal of selenium from contaminated environments with limited studies on aerobic organisms, which was the important component of this study.

Anthropogenic activities have been contaminating the environment by adding both organic and inorganic materials for a long time. Among these, toxic heavy metals and metalloids played a very important role in deterioration of the ecosystems as these are never degraded, only transformed from one state/form to another resulting in severe disturbance of ecological balance in most ecosystems (Kozdroj and Elas 2001). Because of their relative abundance at contaminated sites and potential toxicity to plants or animals, elements such as aluminum (Al), antimony (Sn), arsenic (As), barium (Ba), beryllium (Be), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), silver (Ag), strontium (Sr), thallium (Tl), vanadium (V), and zinc (Zn) are of major concern for the environmental scientists. Unlike the other elements listed, antimony, arsenic, molybdenum, selenium, and vanadium generally occur as oxyanions in waters and soils, and not as cations. These inorganic pollutants are present as anions/complexes in soil and groundwater systems and pose a problem due to limited sorption as most soil minerals also have negative surface charge near neutral pH (Langmuir and Klusman1997). Majority of soils wide spread over the world have a neutral or slightly alkaline pH. Similar charges on both materials results in electrostatic repulsion which results in high mobility of these anionic contaminants in the natural soil environment. These properties exacerbate the problems associated with their presence in both saturated and unsaturated subsurface environments. All these elements share a common feature i.e. ability to form stable oxyanions under oxidizing conditions along with the property to form insoluble precipitates under reducing conditions (Simonton et al 2000). Soil contamination is particularly a serious environment concern as majority of superfund sites are highly contaminated with these heavy metals (Peters 1991).

2.1 Selenium: occurrence and use in industry

Selenium (Se) is an element with an atomic weight and number of 78.96 and 34, respectively (Table 2.1). It occupies group VIA in the periodic table of elements along with other elements such as sulphur (S). Selenium and sulphur being present in same group, shares similar electronic configuration and chemical properties which accounts for many of the interactions between these two elements (Reilly 2006). Selenium in nature exists in six stable isotopes with varying degrees of their abundance. ^{74}Se (0.87%) exist in least amount while ^{80}Se (49.82%) and ^{78}Se (23.52) are present in abundance with other short-lived isotopes (e.g.^{75}Se) commonly used in radiology (Reilly 2006; USEPA 2004). Selenium is a naturally occurring mineral in the earth`s crust that is re-distributed in the environment through multiple natural and anthropogenic processes and scattered widely in all the natural environments including most of the rocks and soils (Chapman et al 2010).

Table 2.1: Chemical and physical properties of selenium (Cooper and Glover 1974; Rosenfeld and Beath 1964)

Properties	**Values**					
Relative atomic mass	78.96					
Atomic number	34					
Atomic radius	0.14 nm					
Covalent radius	0.116 nm					
Electro-negativity (Pauling's)	2.55					
Electron structure	$[Ar]3d^{10}4s^{2}4p^{4}$					
Oxidation states	-2, 0, +2, +4, and +6					
Stable isotopes						
Mass	74	76	77	78	80	82
Natural abundance (%)	0.87	9.02	7.85	23.52	49.82	9.19

Selenium is considered as metalloid as it possesses characters of both metals and non-metals (Wilber 1983). Elemental selenium exists as metallic gray to black hexagonal or monoclinic crystals and amorphous powder in its pure form, but in nature it usually exist in complex forms, combined with both non-metal such as sulfide or with metals like silver, copper, lead and nickel. It has the tendency to interact with both organic and inorganic compounds to form complexes. Selenium and its compounds may act as either an oxidant or reductant depending on the redox potential of the site. Chemical adaptability of selenium accounts for its widespread occurrence in abiotic and biotic environment such as soils, plants, animals and human beings (Bauer 1997). Selenium is widely distributed in nature with relatively small concentrations in rocks, plants and fossil fuels. Selenium is chiefly found in igneous rocks, volcanic sulfur deposits, hydrothermal deposits and sedimentary rocks. The major geologic sources of selenium include crustal rocks such as black shale, phosphate rocks and coal. Black shales and phosphate rocks are the materials known to have highest selenium concentrations. Run-off water from these sites carries significant amount of dissolved selenium species which get adsorbed on the clay particles and are deposited into soils (Bauer 1997). These sources have the potential to convert normal sites to seleniferous sites.

Selenium find its wide application in the electronic industry, glass industry; as a component of pigments in plastics, paints, enamels, inks, and rubber; in the preparation of pharmaceuticals; as a nutritional feed additive for poultry and livestock; in pesticide formulations; in rubber production; as an ingredient in antidandruff shampoos; and as a constituent of fungicides. Radioactive selenium is used in diagnostic medicine.

2.2 Chemistry of selenium and its compounds

Selenium chemistry is complex, as selenium exists in several oxidation states. Chemical reactions of selenium resemble those of sulphur and are typically metalloid in nature. Selenium is listed as one of 129 priority pollutants by the Environmental Protection Agency. Selenium occurs in several forms, including multiple oxidation states, which vary depending on ambient environmental conditions (such as pH, Eh [oxidation/reduction potential], and microbial activity) and medium (such as water, sediments and biological tissue). Biologically significant oxidation states include selenide (Se^{-2}), elemental selenium (Se^0), selenite (Se^{4+}) and selenate (Se^{6+}). The toxic and essential concentration of selenium depends on its chemical form as toxicity of different oxidation states are strictly related to their degrees of water solubility and hence bioavailability (Banuelos et al 1999). Different selenium species exist either in dissolved or suspended particulate forms (Chapman et al 2010). Selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) are the two water soluble species of selenium found mostly in aerobic habitats. Both oxyanions are toxic and tend to bio-accumulate in the biological systems (Dungan and Frankenberger 1999). Hydrogen selenide, a highly toxic gas is readily oxidized to elemental selenium in presence of air. These compounds are toxic for biological systems at the level of parts per million (ppm). In normal aquatic conditions with pH values between 6 and 8, dominating species of selenium are elemental Se (0), HSe^-, $HSeO_3$, and SeO_4^{2-}. Selenates (SeO_4^{2-}) are found in the upper part of the pe-pH diagram and are stable under alkaline oxidizing conditions. The redox behavior of selenium is similar to that of sulfur in that (1) Se (VI) oxyanions predominate under oxidizing conditions, (2) the element has a stability field under reducing conditions, and (3) metal cations react with Se(-II) to form insoluble selenides. Selenate is highly stable and not readily reduced by H_2S or Fe^{2+}. These Se (-II) forms are very insoluble metal selenides and act as an important sink for dissolved Se in reducing environments (Takeno 2005).

2.3 Distribution of selenium

Selenium in environmental and biological systems exists as inorganic, organic, methylated and low molecular mass species (Table-2.2). Behavior of selenium in the environment and biological system is largely influenced by its oxidation state as well as physical factors such as geology, climate and hydrology. Selenium bioavailability, however, is determined by many factors including soil pH, the redox potential, soil texture, organic-matter contents, as well as the presence of competitive ions, use of fertilizers, rainfall and other human activities such as industrial emissions, sewage sludge and fly ash etc (Seby et al 1997). Selenium is often more abundant in different environments (soil, water and air) in areas with upper cretaceous marine sedimentary rocks and other formations naturally high in selenium (USDI 1998). Climate also affects selenium distribution because it behaves differently in arid climates than in humid or wet climates. Areas that have a local geologic source of selenium, concentrations and the potential for toxic effects generally increase as aridity increases (NSMP 2009).

Table 2.2: Selenium species present in environmental and biological systems (D'Ulivo 1997)

Type	Selenium species
Inorganic species	Selenite, selenate, Se^0, Se^{-2}
Simple organic and methylated species	MeSeH, Me_2Se, Me2Se2, Me_3Se^+, Me_2Te, Me_2Te_2, Me_2SeO_2, MeSeO(OH), Me_2SeO, MeSSeMe, $Se=C(NH_2)_2$
Amino acids and low molecular mass species	Selenomethionine, selenocysteine, selenocystine, Se-methylselenocysteine, selenocysteic acid, Se-methylselenomethionine, selenomethionine selenoxide, selenoniocholine, selenobetaine
Other compounds	Selenoproteins, selenoenzymes, Se–metal metallothioneins (e.g., Se–Hg, Se–Zn)

Hydrology can increase selenium contamination by acting as a transporting agent, and certain receiving water bodies may become sinks for the mobilized selenium. Selenium is transported via rivers, streams, creeks, groundwater, and irrigation drainage water. Terminal water bodies may become contaminated due to evaporative enrichment and sequestering over several seasons of runoff. These physical factors influence the fate and transport of selenium in various ecosystems (NSMP 2009). Selenium partitioning to amorphous iron oxy hydroxides and maganese dioxide particles also play an important role in sequestration of selenium (Balistrieri and Chao 1990). In freshwater environments, selenium is adsorbed onto iron-maganese oxyhydroxides at sediment surfaces released by the reduction of those oxy-hydroxides mineralized with organic matter and removed from pore water as Se [0] and as selenopyrites (Sandy and Disante

2010). In acidic, clay soils and soils containing high organic matter selenium is present as selenides and selenium sulfides (Kabata and Pendias 1984). In acidic soils, selenium occurs mainly as selenite (SeO_3^{2-}) which strongly adsorb to iron oxides under these acidic conditions. Se (IV) may also form highly insoluble iron compounds such as ferric selenite or iron selenide. This form has an extremely low solubility and remains unavailable for plants (Goldberg and Glaubig 1988). Alkaline conditions favor the conversion of elemental Se to selenate (SeO_4^{2-}), which is not fixed in the soil and hence makes selenium more available to plants (Lakin and Davidson 1973). In alkaline and well-oxidized soils, selenium occurs as selenate. These selenium oxyanions are highly soluble in water and therefore are potentially toxic to biological systems due to easy uptake by the plants (White et al 1991). As elemental Se is formed under reducing conditions from SeO_3^{2-} and SeO_4^{2-}, Se can become unavailable for plants. Elemental selenium [Se (0)] is slightly soluble in water and therefore has reduced bioavailability potential for plants.

The availability of Se to plants is also affected by soil moisture: the element is most available to plants when there is less precipitation and low soil leaching (Gissel-Nielsen 1998). Plants were observed to accumulate high amounts of Se in the seleniferous soils which are typically alkaline with little rainfall and drought situation. These conditions reduce leaching of selenium salts away from the root zone (Banuelos et al 2011). Selenate is the most prevalent bioavailable form of soluble Se for the plants. Selenium speciation of water-saturated extracts from seleniferous San Joaquin Valley soils revealed that 98% of total Se was present as selenate followed by selenite. The hydrophobic organic Se was less than 1% and was associated with the humic acid (Fio and Fujji 1990). Organic forms of Se such as seleno-amino acids (selenomethionine and selenocystine) represent an important source of available Se for plants.

2.4 Selenium: An essential nutrient

The question, whether an element is essential for human health is rather complex. An element was recognized as being essential when: (1) a dietary deficiency of the element led to consistent and adverse changes of a biological function from optimal and (2) these changes are prevented and reversed by dietary intake (Nielsen 2007). Since its discovery, role of selenium remained controversial in context with its requirement and health implications associated with both, its deficiency and excess in the food chains respectively. Concerns about the health hazards from overexposure tend to become overwhelmed by a bewildering discussion of the potential benefits (Flohe and Gunzler 2000). Since its discovery more than a century ago, selenium was first coined as an essential element in 1957 (Mayland 1994). Selenium has been reported as an essential nutrient for almost all animals as well as some microorganisms. Many reviews described various biological functions of selenium including its nutritional importance (Birringer et al 2002; Mugesh et al 2001). It has been identified as a key component in anti-oxidant defences at cellular and tissue level (Reilly 2006). In vertebrates, after absorption, Se can be incorporated into functional seleno-proteins, Se-containing proteins and amino acids (Daniels 1996). Selenium plays an important role in maintaining human health as an integral unit of several seleno-enzymes that catalyze the reactions of intermediate metabolism and attenuates the toxic effects of heavy metals such as arsenic, cadmium, mercury, and tin (Magos and Webb 1980). The recommended daily dietary allowance for men (70 µg) and women (55 µg) has been established (Lavender 1991) along with maximum uptake of 10 µg/l in drinking water (Pyrzynska 1998). Selenium has been recognized as an essential nutrient for humans based on its presence in enzyme glutathione peroxidase that leads to cell protection against oxidative damage (Zingaro and Cooper 1974). The natural selenium-containing antioxidant enzymes like glutathione peroxidase and thioredoxin reductase have the potential to reduce the effect of free radicals on the ageing process. Several other selenoproteins have also been identified with specific role in vital life processes. Deiodinases harbour selenium and play an important role in the activation/inactivation of the thyroid hormone. Similarly selenoprotein P, a metal binding protein in blood, helps in transportation of Se from the liver to other organs and tissues. Seleno-phosphate synthetase produces seleno-phosphate, an intermediate molecule in the synthesis of Se-Cys (Reilly 2006). In addition, there are many other seleno-proteins whose functions are still not clear (e.g. selenoprotein T; Reilly 2006). Selenium forms the active center for seleno-enzymes that carry out redox reactions such as glutathione peroxidase (GPx), thioredoxin reductase and thyroid hormone deiodinase families. Selenium-containing proteins can incorporate exogenous Se-Met non-specifically in the place of methionine. SeCys residues incorporated into seleno-proteins are not of an exogenous intact one but of endogenously prepared SeCys residue, which is synthesized from the activated selenium, seleno-phosphate and activated serine (ser) residue on ^{SeCys}tRNA to SeCys^{SeCys}tRNA (Suzuki 2005). Although there is no evidence of specific Se storage in vertebrates, these Se-containing proteins are largely found in skeletal muscle, liver, kidney, stomach and erythrocytes and contribute greatly to the Se pool (Reilly 2006; ATSDR 2003; Schrauzer 2000). Nutritional requirements for fish, amphibians and birds are less than 0.5 mg selenium per kilogram in the diet (Sandy and Disante 2010). Furthermore, the abundance of seleno-proteins (e.g. seleno-protein P) has been reported for higher selenium accumulation in zebra fish (Kryukov and Gladyshev 2000). Fish also appear to utilize seleno-proteins to a larger extent than other vertebrates (Kryukov and Gladyshev 2000). Selenoneine, an organic selenium compound possesses a strong free radical scavenging activity and plays an important role in improving the antioxidant potential of tissues and cells. Yamashita et al (2011) have reported the accumulation of selenium in the form of selenoneine in muscles of different species of tuna.

Selenium appears to have completed its metamorphosis in biology and medicine. It is necessary for the proper functioning of structural proteins and cellular defenses against oxidative damage and selenium deficiency is a more widespread problem as compared to toxicity caused by excess selenium (Ralston et al 2008; Raisbeck et al 2000; Chapman 1999). Edible portion of most of plants contain selenium; however, its concentrations vary greatly, depending upon amount of selenium in the soil. In several parts of world nutritionally inadequate amount of selenium in forage and feed resulted in selenium deficiency in livestock, which require supplementation of selenium to alleviate the problem. The first report of a disease caused by deficient daily intake of selenium was in 1935 from Keshan country, Heilongjiang Province in northeast China (Reilly 1996).

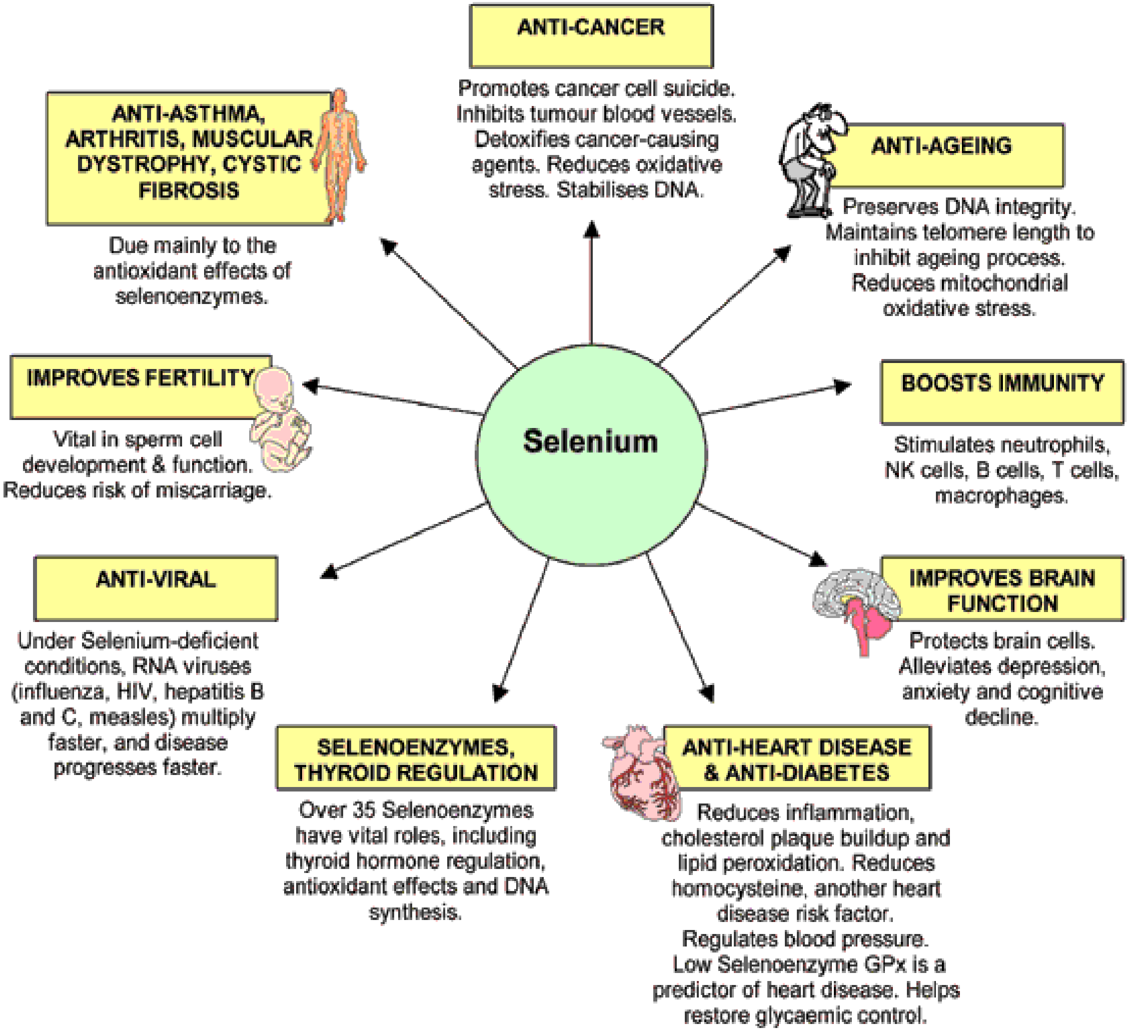


Fig 2.1: Health benefits of selenium

(Source: http://1phil4everyill.files.wordpress.com/2010/12/selenium_benefits.gif)

In 1957, Schwarz and Foltz discovered that selenium was contained in “factor 3”, a still ill-defined compound isolated from hog kidney, which efficiently prevented the experimental fatal liver necrosis of rats that were fed a diet consisting of *Torula* yeast and sucrose. In the 1960s, this view was corroborated by veterinary scientists, since various syndromes such as white muscle disease in cattle, mulberry heart disease in pigs and exudative diathesis in poultry can be attributed to insufficient selenium supply (Edens 1996). In the 1970s, Chinese scientists observed that severe selenium deficiency causes, or at least predisposes to, diseases in humans such as Keshan disease, which is a fatal cardiomyopathy, and Kashin–Beck disease, a disabling chondronecrosis (Levander 1987). Selenium deficiency causes pulmonary odema, abdominal pain, jaundice, chronic gastrointestinal, hair loss and fatigue in humans (Yang et al 1983). Adequate alimentary selenium supply is claimed to delay the onset of ageing, cardiovascular diseases and cancer, to enable an optimum immune response, to guarantee an appropriate function of the endocrine system (Duntas 2006; Oster and Prellwitz 1990). Selenite (SeL) is an important dietary antioxidant is now recognized as an essential component of selenoenzymes (Shen et al 2001; Behn et al 1995). It possesses the pharmacological properties to lower blood glucose level alone and in combination under both *in vitro* and *in vivo* conditions (Xu et al 2011). Variety of other biological roles of selenium (Fig. 2.1) resulted in enhanced research interests on this metalloid across the globe.

2.5 Selenium: An un-avoidable toxin

Although selenium (Se) is an essential element for many organisms, but it is also toxic at higher concentrations. This element is essential because seleno-Cys (Se-Cys) is present in the active site of certain seleno-proteins which are involved in oxidative stress resistance (Stadtman 1996). Several researchers have established the essentiality of selenium for humans (Thomson 2004; Goldhaber 2003), however NRC has established safe and adequate daily intake of selenium @ 50-200 µg/day for an adult. Small concentration above this level can cause the health implications. Wu et al (2003) suggested that high concentration of selenium accumulation in the food chain was the cause of death and deformities of embryos of the waterfowl nesting at the wetland habitat at Kesterson reservoir national wildlife refuge in the central valley of California. Accumulation of selenium in hyper accumulator plants or Beath indicators has been historically associated with toxicity symptoms to animals that feed on these plants

(Beath et al 1937; Franke 1934). Preliminary reports on chronic selenium toxicity as a cause of hoof and horn disease in livestock of seleniferous regions of Punjab (Hoshiarpur and Nawanshar districts) were given by Gupta et al (1982). Dhillon et al (1997) reported the malformations appearing in livestock and attributed these to the feeding of fodder grown in seleniferous soils. High doses of selenium (more than 900 µg/day) produce a toxic syndrome of dermatitis, loose hairs, diseased nails and peripheral neuropathy. Selenosis (selenium toxicity) has been observed in both humans as well as animals. Signs and symptoms of selenosis include loss of hairs and nails, skin rashes and gastrointestinal disturbance and effects on central nervous system. Endemic selenium intoxication with 49% morbidity was reported in villagers where the daily intake of selenium was about 5 mg (Yang et al 1983). The main symptoms were brittle hair with intact follicles, lack of pigment in new hair, thickened and brittle nails and skin lesions. O`Toole et al (1996) has also observed alkali diseases and blind staggers disease in horses and cattle after prolonged consumption of non-accumulator and hyper-accumulator plants respectively. Ironically, China has been the site of both endemic chronic selenium toxicity and severe selenium deficiency in humans. Yang and co-workers (1983) reported an outbreak of selenosis caused by drought and crop failure which forced villagers to consume increased amounts of vegetables and corns containing high levels of selenium.

2.6 Absorption and selenium uptake in biological systems

The essential nature of selenium as well as its toxicity depends on the concentration and the chemical forms of this element present in a given sample. Animal and human studies have established that the chemical form of selenium in foods and supplements influences not only the bioavailability of this element but also its distribution in the body (Pyrzynska 2002). Bioavailability can be defined as a quantitative measure of the utilization of a nutrient in a food or meal, to support normal structural and physiological processes occurring in the body. Generally selenium compounds are very efficiently absorbed in different parts of human body and their absorption is not found under homeostatic control (WHO 1987). For example, absorption of the selenite form of selenium is more than 80 percent, whereas more than 90 percent of selenium absorption was observed when supplemented with selenate or selenomethionine (Patterson et al 1993; WHO 1987). Therefore, the rate limiting step determining the overall bio-availability of selenium is not likely to be its absorption, but rather its conversion within tissues to the metabolically active forms (e.g. its incorporation into GPx or 5'-deiodinase) (Contempre et al 1996). Different bioavailabilities and differences in non-specific incorporation of selenium compounds from different sources such as cereals, meat, fish along with organic and inorganic supplements results in differential total intake of selenium determined in whole blood (Alexander and Meltzer 1995). A number of depletion-repletion experiments have been carried out on animals to estimate the bio-availability of selenium in human foods (Mutanen 1986). Bio-availability of selenium in wheat was determined on the basis of restoration of GSHPx activity in selenium depleted rats after feeding on Se-enriched wheat grains wherein more than 80% of original GSHPx activity was found to be restored. Brazilian nuts and beef kidney also contain more than 90 % of total selenium as biologically available selenium. Only 20-60% of total selenium accumulated in tuna fish is in the bioavailable form of selenoneine whereas the availability of selenium from other sea foods such as shrimp, crab, and baltic herring was found quite high. The selenium contents present in different mushrooms had low availability to rats. Although data on the nutritional bio-availability of selenium to humans is sparse, a supplementation study carried out on Finnish men of relatively low selenium status showed that selenate was as effective as the selenium contained in seleniferous wheat in increasing platelet GSHPx activity (Levander 1983). The wheat selenium, however, increased plasma selenium levels more than did selenate selenium and once the supplements were with-drawn, platelet GSHPx activity declined less in the group given wheat. This study showed the importance of estimating not only short-term availability but also long-term retention and the convertibility of tissue selenium stores into biologically active forms.

2.7 Mechanism of selenium toxicity

Selenium toxicity can occur by three major mechanisms: oxidation of thiol (S-H) groups followed by redox-cycling (Fenton-type reaction) and generation of free radicals (e.g. $O^{2\bullet -}$), substitution of Se for S in proteins, and inhibition of Se methylation (Spallholz and Hoffman 2002). Cells cannot discriminate between Se and S and therefore, when selenium is present in excessive amounts, Se is substituted for S during protein synthesis (Lemly 1997). Selenium and S have similar chemical properties; however at biological pH, Se is reduced while S is oxidized. This alternation of redox state produce breaks in the disulfide bonds (S-S) necessary for proper protein function (Lemly 1997). Number of symptoms such as damage to hairs, nails, feathers and hoofs has been attributed to the substitution of Se for S in structural protein (Spallholz and Hoffman 2002). In addition, inhibition of the Se methylation process (a detoxification pathway) also results in an increase in available selenium (Se) to substitute for sulphur (S) during protein synthesis and/or formation of superoxide radicals (Spallholz and Hoffman 2002). Hence selenium becomes toxic at higher levels due to incorporation of Se into sulfur (S)-containing molecules, especially the non-specific replacement of Cys by Se-Cys in proteins. Due to structural and chemical similarity of selenium with sulfur and replacement of sulfur with selenium; most enzymes involved in S metabolism can catalyze the analogous reaction with the corresponding Se substrates with similar affinity for both substrates (Anderson 1993). On the other hand, Se-specific enzymes tend to have a much higher affinity for the Se substrate than for the S analog (Mihara et al 2000).

2.8 Seleniferous soils

Selenium is widely distributed throughout the world and occupies 69th position in order of abundance among naturally occurring elements in earth’s crust (Dhillon and Dhillon 2003b). Selenium exist in many natural soils and aquatic environment around the world, but anthropological activities such as irrigated agriculture on selenium laden soils has created many ecological problems with respect to this element. It combines with both metals and non-metals directly and hydro-chemically forming both organic and inorganic compounds. Heterogeneity in its distribution results in movement of selenium in various environments such as air, water and soil (Nriagu 1989). Selenium is particularly concentrated in the soils of the drier regions where the soil tend to be more alkaline (Sharma and Singh 1983). Concentration of selenium in soils is variable ranging from >0.1 to 8000 mg/kg with an average selenium concentration of 0.40 mg/kg for 1623 soils throughout the world (Ure and Berrow 1982). Total soil selenium concentration in Kesterson reservoir, California was found to range from 2.0-4.0 mg/kg to 40-70 mg/kg (Wu et al 1993). In China the mean total selenium concentration was found in a range of 0.022 to 3.806 mg/kg (Tan et al 2002).

In India, high concentrations of selenium have been observed in various regions. These sites include parts of Northwestern region of Punjab (Dhillon and Dhillon, 1991); Haryana (Arora et al 1975); Assam; Meghalaya (Dey et al 1999) and west Bengal (Ghosh et al 1993). In Haryana state, a few acres of land was identified as seleniferous in the village Chamarkhera near Karnal based on development of symptoms of selenosis in livestock after consuming fodder. In the sub- Himalayan west Bengal soils, an increase in Se toxic pasture has been located in the Jalpaiguri district due to deposition of soluble selenium compounds carried by heavy rainfall. Visible and specific toxic effects of selenium were observed in wild animal species from various locations of Assam and Meghalaya states (Dey et al 1999). Amongst all the locations identified, the agriculturally rich belt along border of Nawanshahr-Hoshiarpur districts of Punjab has gained prominence due to extensive research and agri-extension activity (Dhillon and Dhillon 2003a). The affected villages, covering over 1000 acres are Barwa, Bhano Majra, Sikandarpur, Rakker, Simbli, Jainpur, Mahenpur and Nazarpur (Dhillon and Dhillon 1997). These high selenium areas were initially identified on the basis of visible symptoms associated with selenium toxicity among domestic animals and human beings like hair fall, softening of nails and horns, etc. Further analysis of water and soil samples along with the plants grown in this region confirmed higher selenium content in these areas. Chronic selenium poisoning of plants, animals and humans has been reported in seleniferous soils of Northwest India (Dhillon and Dhillon 1997).With reference to Se uptake by the human population, the average selenium intake of both men and women in the region was more than nine times than that of the non-endemic area. More than 60% of male population exceeded the maximum tolerable limit. Mean selenium content of hairs, nails and urine of both men and women was ten times higher than in the non-endemic area (Hira et al 2004). The soils of this region originated by the deposition of alluvium carried by the flowing river water of the Indus system that has periodically completely covered the old land surface. This alluvial tract is developed on troughs formed concomitantly with the elevation of the Himalayan mountain range. Further an additional deposition of alluvium has been transported by rainwater runoff through seasonal rivulets from nearby hills of the Shiwalik range and deposited in low-lying areas of the affected region (Dhillon and Dhillon 1991). These terminal ends has been developed as the patches of highly selenium contaminated sites which has resulted in spread of seleniferous soils in more than 1000 hectare region of the Northeastern Punjab. Soils found in this seleniferous region are alkaline, calcareous, silty loam to silty clay loam in texture and are well drained (Dhillon et al 1992). Selenium concentration in these soils is relatively variable ranging from 0.32 to 4.55 mg/kg. Parent material of soils in the seleniferous region is derived from upper Shiwalik rocks that are mainly composed of polymictic conglomerates of variable composition containing many unstable materials such as granite, basalt, limestone (Dhillon and Dhillon 2003b). The quality of underground water drawn from tube-wells located in the seleniferous region of northwestern India was evaluated on the basis of Se content, electrical conductivity and residual sodium carbonate (RSC). These water samples have been reported fit for irrigation on the basis of total dissolved salts. With respect to RSC contents, 86% of the samples were marginally fit and 12% of the samples were unfit for irrigation purposes. A wide variation in the selenium content of tube-well waters has been observed at or near the toxic sites ranging between 0.25 µg/l and 69.5 µg/l with an average value of 4.7 µg/l. Besides the variation in selenium content associated with different sites, a significant difference in selenium concentration has been observed with depth of ground water collected. The maximum contamination level (MCL) of 10 µg Se /l for drinking purposes was exceeded by 11.1% of the tube-well waters and the maximum permissible level (MPL) of 20 µg Se/l for irrigation purposes was exceeded by 4.4% of the waters (Dhillon and Dhillon 2003b). As tube-wells are the main source of irrigation in the Se-toxic regions of Punjab, selenium has started accumulating in the plants and food chain. Se content of fodder and grains varies from 5-160 mg/kg and 4-66 mg/kg, respectively (Dhillon and Dhillon 1991; 1997). Typical Se toxicity symptoms of snow-white chlorosis with pink colouration on the lower side of the leaves were observed in the wheat grown in these seleniferous regions (Rosenfeld and Beath 1964).

2.9 Remediation of seleniferous sites

Applications of physical and chemical methods for selenium removal are limited due to high cost and low efficiency. A number of physical and chemical methods such as precipitation, filtration, ion-exchange, oxidation reduction, electrochemical recovery, membrane separation and other techniques were exploited for removal of selenium and other inorganic pollutants from contaminated sites (Sandy and Disante 2010). These processes are applicable only at comparatively high concentrations of metals. Biological

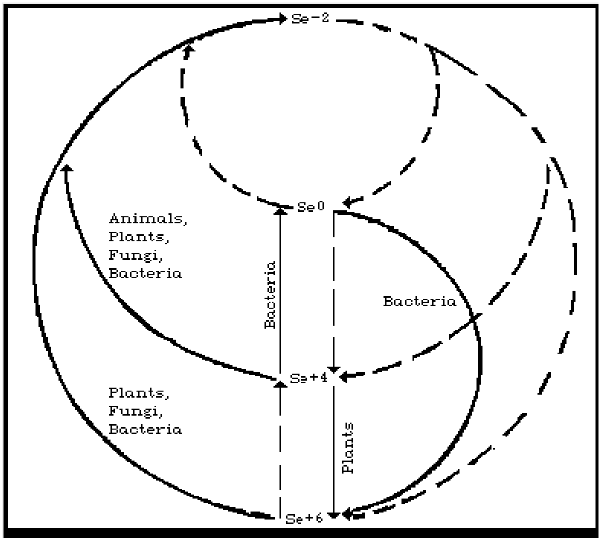


Fig 2.2: Biological selenium cycle. Well-established pathways are indicated by solid lines, those needing further substantial by dotted lines.

(http://www.inchem.org/documents/ ehc/ehc/e58_1.gif) methods of selenium removal are found promising and have been studied most extensively. Use of biological system for the transformation of toxic and bioavailable form of inorganic pollutants is an upcoming area with its wide applications. Hence a number of bacterial species have been exploited for conversion of bioavailable forms of selenium oxyanions into either the non-available elemental form or less toxic volatile organic form. Although some microorganisms have been reported with well established role of selenium in their cellular mechanisms, most microorganisms metabolize selenium to circumvent its toxicity. Selenium cycle in different biological entities has been represented in Fig. 2.2.

2.10 Mechanisms of biotransformation

Transformation of heavy metals and metalloids through oxidation-reduction reactions play a significant role in geochemical cycling of these elements. These processes modify the mobility and toxicity of these elements and are also of great bioremediation potential with use of technology (Lovley 1993; Karlson and Frankenberger 1993; Gadd 1993a, b). Significant work has been documented on different mechanisms of transformation of inorganic selenium compounds, i.e., reduction (Doran and Alexander 1977), oxidation (Sarathchandra and Watkinson 1981; Dowdle and Oremland 1998) and methylation (Barkes and Fleming 1974) by various workers. In natural environments, microbial activity is thought to be the primary mean by which selenium oxyanions (selenate and selenite) are reduced to less toxic insoluble elemental form of selenium (Stolz et al 2002; Garbisu et al 1995). Some microorganisms are naturally reported to convert these biologically available forms of selenium into elemental selenium as a part of their detoxification mechanisms. Various potentially useful microbial transformations towards remediation of selenium contaminated sites includes (i) bio-reduction of soluble selenium forms to insoluble forms such as elemental selenium (Se^0) or assimilation of selenium into organic components of bio-molecules such as seleno-amino acids and (ii) reduction and methylation, which yield volatile forms, primarily dimethyl selenide (Oremland 1994; Thompson- Eagle and

Frankenberger 1992). A hypothetical reaction for reduction of selenium oxyanions illustrating the mechanism may be represented as follows.

Se (VI) + bacteria +organic carbon → Se (IV) → Se^0
Selenate Selenite Elemental selenium
(Soluble) (Soluble) particles

Anaerobic bacteria may utilize selenium oxyanions as terminal electron acceptor which results in dissimilatory reduction of these oxyanions. Principally, microorganisms reduce selenium oxyanions under anoxic conditions using selenate or selenite as a terminal electron acceptor during metabolism. Reduction of both SeO_4^{2-} and SeO_3^{2-} (both toxic) to elemental selenium (Se^0) results in immobilization and detoxification (Oremland et al 1990). Aerobic selenium respiring bacteria when exposed to higher doses of selenium oxyanions, convert these oxyanions into insoluble form of elemental selenium as a part of their detoxification mechanism (Oremland et al 2003). A number of reports have been published for microbial selenite reduction in both aerobic and anaerobic conditions by various research groups (Dungan 2003; Rajnard 2003). Despite of the ubiquity of selenium reducing bacteria in diverse terrestrial and aquatic environments, mechanism of elemental selenium bio-mineralization is still poorly understood (Yee et al 2007). Various enzymatic systems, such as nitrite reductases, sulphite reductases, and glutathione (GSH) reductases (GR), have been proposed for or suspected to be involved in the reduction of selenite in bacteria, especially during dissimilatory reduction. The reduction of the bio-available selenium oxyanions into elemental selenium, which is insoluble and non-toxic, is of great interest for bioremediation.

Assimilatory reduction of selenium oxyanions result in their incorporation in the proteins. Methylation of selenium compounds results in volatilization (Karlson and Frankenberger 1993; Tamaki and Frankenberger 1992). Several bacteria, including *Escherichia coli* (Turner et al 1998) are able to reduce both selenate and selenite into elemental selenium (Se^0), while certain species like *Rhodobacter sphaeroides* (Bebien et al 2001; Fleet-Stalder et al 2000) or *Ralstonia metallidurans* (Roux et al 2001) only reduce selenite into elemental selenium.

2.11 Bio-reduction of selenite

Selenite (SeO_3^{2-}) is the most toxic of selenium oxyanions often encountered in soils that are oxidizing and acidic (Plotnikov 1958). It is highly mobile and irrigation of seleniferous soils with selenite rich water can result in its movement into ground water table. Biological remediation approaches involves microbial biotransformation and offer an effective and inexpensive method to remove selenite from water. Some aerobic soil bacteria like *Bacillus subtilis* (Garbisu et al 1999) or the purple non-sulfur bacterium *Rhodospirullum ruburum* has been reported for bio-reduction of selenite (Kessi et al 1999). Fleet-Stalder et al (2000) reported the accumulation of elemental and organic selenium along with small fractions of volatile selenium by *Rhodobacteium sphaeroides,* a purple non-sulfur bacterium under phototrophic conditions. Selenite has been reported to be either used as an electron acceptor in anaerobic respiration (Macy et al 1989) or reduced independently of dissimilatory electron transport (Lortie et al 1992). Garbisu et al (1995) reported *Pseudomonas fluorescens* and *Bacillus subtilis* reduced selenite to elemental selenium, independent of dissimilatory Se reduction. Detoxification of selenium oxyanions, especially selenite, is carried out independently of nitrite and sulphite as terminal electron acceptor but depends upon substrate provided for growth (Garbisu et al 1996). Many of these microorganisms are not resistant to high level of selenite. Growth of *Bacillus subtilis* (Garbisu et al 1999), *Ralstonia metallidurans* (Roux et al 2001), *Rhodobacter sphaeroides* (Van fleet et al 2000) and *Rhodobacter rubrum* (Kessi et al 1999) was inhibited by 2-6 mM selenite. Hunter et al (2007) reported the reduction followed by complete inhibition of growth in *Rhizobium* species on its exposure to selenite between 8-18 mM concentrations. Comparatively high resistance for selenite i.e. up to the extent of 16-50 mM was observed in *Aeromonas salmonicida; Azospira oryzae* and *Stenotrophomonas maltophila* (Hunter 2007; Hunter and Kuykendal 2006; Di Gregorio et al 2005). Andreoni et al (2000) reported some species of *Lactobacillus* with high tolerance for selenite along with reduction during exponential phase of growth. Recently Hunter and Mantor (2008) reported one bacterial strain which was able to tolerate high levels of selenite (up to 64 mM). This culture was capable of reducing selenite only under aerobic conditions and was characterized as *Tetrathiobacter kashmirensi.* Under anaerobic conditions, this strain was not able to reduce either selenite or selenate and was also unable to use selenite, selenate or nitrate as respiratory electron acceptors for growth. Native gel electrophoresis of the strains cell free extracts revealed a protein band with a molecular weight of ≅120 kDa which reduces selenite to elemental selenium. The type strain of *T. kashmirensis* was originally isolated on reduced sulfur compounds and was described as a facultative sulfur chemolithotroph (Ghosh et al 2008).

2.12 Bio-reduction of selenate

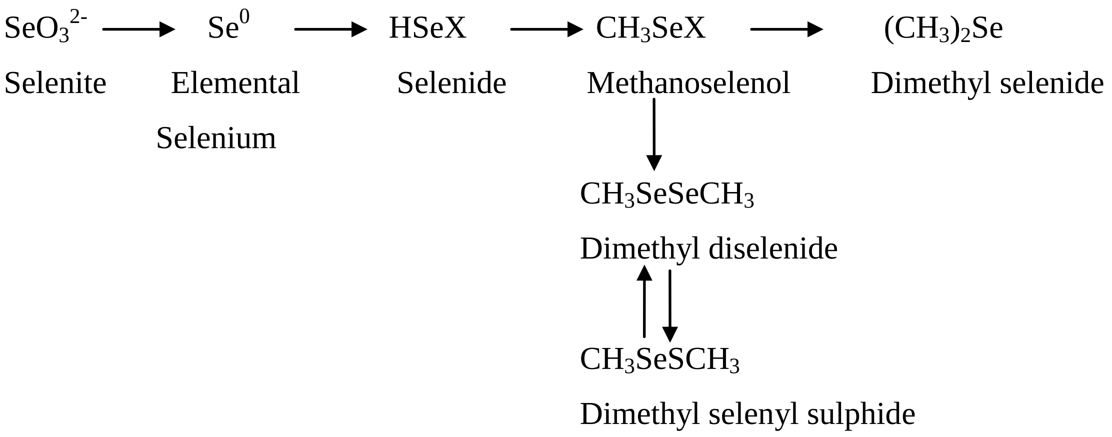
A number of reports describing microbial transformation of selenium oxyanions were focused mainly on reduction and/or biomethylation of SeO_3^{2-} while reports describing SeO_4^{2-} biotransformation were less common. Reports on microorganisms that reduce SeO_4^{2-} are comparatively fewer in number than those that can reduce SeO_3^{2-} (Doran 1982). Selenate can be used as a terminal electron acceptor for respiration, a process termed dissimilatory selenate reduction (Oremland et al 1989). A Gram negative rod shape bacteria of β- subclass protobacteria, *Thaurea selenatis*, isolated from selenate contaminated waste water in San Joaquin valley reduces selenate to selenite under anaerobic conditions (Macy et al 1989). *Thaurea selenatis* was the first characterized organism used for reduction of selenate to selenite in a biological reactor for removal of selenium oxyanions (Lawson and Macy 1995). This bacterial strain also showed potential to utilize nitrate and oxygen as an alternative electron acceptor along with a variety of organic compounds such as acetate, lactate and pyruvate, benzoyl as carbon source and electron donors (Macy et al 1993). Zehr and Oremland (1987) and Tomei et al (1995) reported the reduction of selenate by a sulfur bacteria *Desulfovibrio desulfuricans.* Similarly reduction of selenate has also been reported in specific strains of *Pseudomonas stutzeri* and *Enterobacter cloacae* by Lortie et al (1992) and Losi and Frankenberger (1997) respectively. These strains were capable of reducing sub millimolar concentration of selenate to elemental selenium. Selenate is believed to enter the cell through the sulfate permease system which comprises *cys*A, *cys*U and *cys*W genes (Kredich 1996; LaRossa 1996). Mutations in these genes give rise to selenate resistance (Turner et al 1998). Selenite can also enter the cell through this sulfate transporter. Nonetheless, a different yet uncharacterized carrier likely exists as repression of sulfate permease expression does not totally inhibit selenate uptake (Turner et al 1998). In addition, selenite transport is not shut down by the presence of cysteine (Brown and Shift 1982). It has been suggested that specific membrane transport proteins are expressed in some microorganisms when exposed to selenite. These proteins could be responsible for selenite transport into the cytoplasm. This activity of microorganism to tolerate a high concentration of selenate may be attributed to other reductases that have broad range of substrate specificity. Membrane associated reductases of *E. coli* appear to reduce selenate as indicated by oxidation of reduced methyl viologen (Avazeri et al 1997). A limited amount of work by Altringer et al (1989) and Lortie et al (1992) has suggested that certain facultative anaerobes are capable to reduce SeO_4^{2-} under aerobic or micro-aerophillic conditions. Losi and Frankenberger (1997) isolated *Enterobacter cloacae* from San Luis drain, San Joaquin valley, California having potential for selenium bioremediation process. This facultative anaerobe is capable of using SeO_4^{2-} and NO_3^- as terminal electron acceptor during anaerobic growth. A *Pseudomonas fluorescens* isolate from San Joaquin valley was also studied as a biological means of detoxifying metalloids. Biological reduction of selenium oxyanions to elemental selenium with bacterial strains followed by simultaneous selenide oxidation along with bio-methylation of selenium has been reported by Macy (1989). Recently Hunter and Mantor (2009) isolated a *Pseudomonas* strain from soil with a high tolerance for selenate (up to 64mM) oxyanion.

Most of selenium oxyanion reduction carried out by bacteria results in selenium nanoparticles (NPs) production. A number of reports have been published with reference to the distribution of biologically reduced selenium in and out of the cells. Particles of elemental selenium accumulate in the periplasm (Gerrard et al 1974), in the cytoplasm (Silverberg et al 1976) or outside the cell (Yamada et al 1997), depending upon the species. Oremland et al (2004) reported biogenesis of nano-spheres of elemental selenium after reduction of selenium oxyanions using three physiologically and phylogenetically diverse species of bacteria such as *Sulfurospirillum barnesii*, *Bacillus selenitireducens* and *Selenihalanaerobacter shriftii*. Under anaerobic conditions, selenium oxyanions served as terminal electron acceptors and were reduced into stable, uniform nano-spheres (diameter $\approx$ 300 nm) of Se (0) as extra-cellular granules with monoclinic lattice structures. These selenium nanoparticles were found to be structurally unique when compared with elemental selenium formed by chemical synthesis. Intracellular packets of Se (0) were also observed in these bacterial strains.

2.13 Biomethylation of selenium

Microbial methylation of metalloids like selenium and arsenic is a well-known phenomenon with formation of volatile derivatives such as dimethylselenide or trimethylarsine respectively (Karlson and Frankenberger 1993; Gadd 1993a, b). Methyl derivatives of metalloids have a characteristic garlic-like odour. Bacterial species known to produce methyl derivatives of selenium from SeO_4^{2-} and SeO_3^{2-} include *Aeromonas* sp. (Chau et al 1976), *Bacillus* sp. (Razak et al 1990) and *Pseudomonas* sp. (Chasteen 1993). Dimethylselenide $[(CH_3)_2Se]$ is the most common methylated product. In addition to prokaryotic microflora (Thompson-Eagle et al 1989), some fungal species have also been reported to play a significant role in biomethylation of selenium oxyanions during a prolonged time course studies (Schilling et al 2011; Doran 1982; Fleming and Alexander 1972). The mechanism for selenium methylation appears to involve transfer of methyl groups as carbonium (CH_5^+) ions via the S-adenosyl methionine system. Selenite, selenate and inorganic selenide have been reported to be methylated to trimethyl selenium cation via a pathway involving dimethylselenine (Gadd 1993b).

Challenger (1945) proposed a pathway for microbial methylation of selenium. Subsequently a number of other pathways have been described by other groups (Cook and Bruland 1987; Reamer and Zoller 1980). In general, the formation of DMSe from selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) occurs through successive reduction and methylation steps. Reported methyl donors in the microbial methylation of inorganic selenium are methylcobalamin and S- adenosylmethionine (Doran and Alexander 1977). The organic intermediates in formation of DMSe include dimethylselenone ($(CH_3)_2SeO_2$) (Zhang and Chasteen 1994), selenomethionine (Cook and Bruland 1987) and dimethyl seleno-propionate (DMSeP) (Ansede and Yoch 1997). Doran (1982) purposed that SeO_3^{2-} is reduced via elemental selenium to a selenide form before it is methylated to form methane selenol and finally DMSe. A purposed mechanism of selenium reduction (shown above) speculated the formation of elemental selenium in production of methylated selenium compounds was presented by Doran (1982) and later modified by Chasteen (1993).



2.14 Molecular biology of selenium transformation

The biological mechanisms involved in the reduction of selenate and selenite are still being characterized. Very little is known about the detoxification mechanisms of selenite reduction in aerobic conditions, while more information exists on the respiratory pathways of SeO_3^{2-} in anoxic environments. Antionioli et al (2007) studied the biological mechanisms of aerobic selenite transformation in *S. maltophilia* SeITE02.

From the study it was depicted that the nitrite reductase does not seem to take part in the process of selenite reduction by the bacterial strain SeITE02, although its involvement in this process had been hypothesized in other cases involving anaerobic bacteria. Nitrite strongly interferes with selenite removal when both the oxyanions (NO_2^- and SeO_3^{2-}) are simultaneously present, suggesting that reduction/detoxification pathways of these oxyanions share a common enzymatic step probably at the level of cellular transport. Under *in vitro* conditions, selenite reduction takes place only in the cytoplasm with no enzyme activity in the membrane or periplasmic fractions. A considerable delay in selenite reduction was observed in absence of glutathione.

Thaurea selenatis, an anaerobic bacterial species has been extensively examined for molecular mechanisms underlying Se reduction. This species can obtain energy by respiring with selenate as terminal electron acceptor. Reduction of selenate to selenite is catalysed by a selenate reductase which is a trimeric α-β-γ complex with apparent molecular weight of 180000. The α, β and γ subunits are 96 KDa, 40 KDa and 23 KDa respectively in size. Selenate reductases contain molybdenum, iron, and acid labile sulfur as prosthetic group constituent. The K_m for selenate was 16 μM and V_{max} was 40 μM/min/mg of protein. The enzyme is specific for reduction of selenite; chlorate, nitrite and sulphate were not reduced at detectable rates (Schroder et al 1997). Thorell et al (2003) have isolated chlorate reductase from a chlorate-respiring bacterium *Ideonella dechloratans*, with more than 97% homology with selenate reductase isolated from *Thaurea selenatis*.

Selenium is assimilated in yeast and plants by a sulfur assimilation pathway (De Souza 2000) similar to mechanisms purposed for prokaryotic system (Brown and Shrift 1980). Some studies have suggested specific mechanism for the uptake of selenate and selenite (Brown and Shrift 1982; Brown and Shrift 1981). The reaction of selenite with glutathione produces seleno-di-glutathione and its subsequent reduction to gluta-thio-selenol as an intermediate in the transformation of selenium (Turner 1998). Kessi (2006) investigated the possible reciprocal influence between nitrite dissimilation and Se (IV) reduction in *Rhodospirillum rubrum* and *R. capsulatus*. While in *R. rubrum* the reduction pathway of Se (IV) was eventually completely separated from that of nitrite, in *R. capsulatus* both nitrite and selenite were metabolized simultaneously, with these oxyanions interfering at the level of the transport system rather than at the level of the reductase. However, selenite reductase activity seems to be constitutively expressed in both *R. capsulatus* and *R. rubrum.* Yee et al (2007) investigated the mechanism of Se (VI) reduction by *E. clocae* SLD1a-1. Direct cloning and mutagenesis experiments were used to elucidate the genetic pathway in *E. cloacae* that regulates selenate reductases activity. Their results showed that the global regulatory gene *fnr* (fumarate nitrate reduction regulator [FNR]) confers selenate reductases activity and the ability to precipitate elemental selenium.

Seleno-cysteine and seleno-methionine are two common biological forms of selenium encoded by their own tRNA and provides the selenium - glycine reductase, formate dehydrogenase and NiFeSe hydrogenase (Garcin et al 1999). Regarding the enzymatic systems involved in selenium volatilization, Ranjard et al (2002) isolated a methyl transferase (bTPMT) i.e. bacterial thiopurine methyl transferase that catalyses the S- adenosylmethylation of aromatic and heterocyclic sufhydrlyl compounds like 6-mercaptothiopurine. They reported methylation of organic and inorganic selenium into dimethyl diselenite and dimethyl selenite and demonstrated bTPMT can methylate organic and inorganic selenium to DMDSe and DMSe. Further studies by Oremland and Zehr (1986) indicated presence of demethylation pathway wherein DMSe is degraded to gaseous products by methanogenic and sulfate-reducing bacteria.

2.15 Lacunae in previous studies

In the previous studies, diversity of microorganisms has been exploited for the bioremediation of seleniferous sites using both prokaryotic and eukaryotic microflora along with plants. However no data is available on enzymatic reduction of selenate except a single report on selenate reductase from an anaerobic bacterium *Thaurea selenatis*. Apart from this, there is no report available on the microflora associated with reduction and methylation of selenium oxyanions from the tropical soils of Punjab contaminated with significantly high levels of selenium. Keeping this in mind, the present study was aimed to isolate and characterize the aerobic bacterial microflora with a potential to reduce and biomethylate toxic and bioavailable forms of selenium with following objectives.

- **Physico-chemical characterization of seleniferous soils of Nawashahr district**
- **Isolation and screening of selenium tolerant bacteria in seleniferous soils**
- **Biochemical and genetic studies of selenium mobilization**

3.1 Collection of soil samples

Soil samples were collected from different agricultural fields (predominantly from Jainpur village) of Hoshiarpur- Nawanshahr regions of Punjab in India (75º55 E; 31º56 N) where high concentrations of selenium have been reported. These soil samples were firstly air dried and then sieved through 2mm sieve for further analysis. Composite samples were prepared from the samples collected from site.

3.2 Physicochemical characterization of soil samples

Various physico-chemical characteristics such as pH, electric conductivity (EC), cation exchange capacity (CEC), available N, P, K and S, organic carbon and selenium concentration were determined as follows from the soil samples collected.

3.2.1 pH

Soil pH was measured after preparing the soil suspension using digital pH recorder. 50 g of air dried and sieved soil sample was weighed and taken into a 250 ml beaker. 100 ml of distilled water was added to it and thoroughly stirred for 2-3 min using a glass rod. The suspension was allowed to settle down over 30 min. The pH of sample was measured by immersing the electrode in supernatant solution, after calibrating the pH meter with appropriate buffers.

3.2.2 Electrical conductivity

Electrical conductivity was measured by the method described by Jackson (1967) using soil suspension. 50 g of soil was placed in a 250 ml beaker and 100 ml distilled water was added. The soil-water mixture was shaken thoroughly and allowed to stand undisturbed until the soil settled completely. 0.01 M KCl solution (EC=1413 µS/ cm) was used to calibrate the meter. The electrode was dipped in the supernatant solution and the observation was recorded in replicates.

3.2.3 Organic carbon/matter

Soil organic matter represents a series of products ranging from non- decayed biomass to fairly stable amorphous brown to black decayed material. The organic carbon in the soil was determined by the wet digestion method outlined by Walkley and Black (1934).

Reagents and solutions used

- Potassium dichromate (1N $K_2Cr_2O_7$) solution: 49.04 g of $K_2Cr_2O_7$ (AR grade) was added in distilled water and the volume was made up to 1L.
- Concentrated H_2SO_4 (36N)
- Orthophosphoric acid (85%)
- Diphenylamine indicator: 0.5 g of diphenylamine indicator was added to a mixture of 100 ml concentrated sulphuric acid and 20 ml distilled water. Solution was stored in amber colour bottle.
- Ferrous ammonium sulphate $(NH_4)_2FeSO_4.6H_2O$) solution: 392 g of analytical grade ferrous ammonium sulphate was dissolved in distilled water followed by addition of 15 ml concentrated sulphuric acid. Final volume was made to 2 L with distilled water.

Procedure

Five grams of the soil was collected after sieving through 0.5mm sieve in 500ml conical flask and 25 ml of 1N potassium dichromate solution was added. The contents were shaken gently to mix followed by addition of 50 ml of concentrated sulphuric acid while swirling the flask slowly. The flask was kept till it came to room temperature. 0.5 g of NaF and 100 ml of distilled water was added along with 10 drops of diphenylamine indicator. The reaction mixture was titrated against 0.5N ferrous ammonium sulphate till the colour changed from violet to bright green through blue. A blank titration was also carried out in similar manner.

Calculations

Percentage of organic carbon in soil: (X-Y)/2 x 0.003 x 100/5

Percentage of organic matter in soil: Percentage of organic carbon x 1.724

3.2.4 Cation exchange capacity

Cation exchange capacity of soil was estimated using sodium saturation method as described by Richard (1954) and Rhodes and Polemio (1977) using flame photometer.

Reagents and solutions used

- Reagent A: sodium acetate solution (1 N): 136 g of sodium acetate trihydrate was added in deionized water and the volume was made up to 1L after adjusting the pH to 8.2 by using acetic acid or sodium hydroxide solution.
- Reagent B: Ethanol (95%)
- Reagent C: Ammonium acetate solution (1 N): 57 ml of concentrated acetic acid solution was added to 800 ml deionized water followed by 68 ml of concentrated ammonium hydroxide solution and the final volume was made up to 1L. The pH was adjusted to 7.0 by using acetic acid or ammonium hydroxide solution.
- Standard stock solution (1000 ppm Na): 2.5418 g of dry NaCl was dissolved in de-ionized water to final volume of 1L.
- Working standard Na solution: For working concentrations of standard, 2, 4, 6, 8 and 10 ml of stock solutions was diluted and final volume was made to 100 ml with 1.0 N ammonium acetate solution containing 20, 40, 60, 80 and 100 ppm of Na.

Procedure

Three grams of dried soil was taken in centrifuge tube and 25 ml of 1N sodium acetate trihydrate solution was added and shaked for 5 minutes. The mixture was centrifuged at 3000 rpm until the supernatant became clear and then the supernatant was discarded. The above step was repeated four times with 25 ml of 1N sodium acetate trihydrate solution. Sample was washed thrice with 25 ml of 95% ethanol and the supernatant was discarded each time. Adsorbed Na^+ was replaced from the sample by extraction with the 25 ml of 1N ammonium acetate solution three times. Supernatant from each washing was collected in 100 ml volumetric flask and volume was made up to 100 ml with 1N ammonium acetate solution and mixed well. A calibration curve was prepared after running Na- standard solutions using flame photometer by running the standards and the emission readings were taken at 767 nm. Similarly samples (soil extract) were measured after taking emission readings using flame photometer. The concentration of Na in soil sample was calculated using the calibration curve.

Calculation:

CEC (meq/100g of soil) = meq/L Na (from standard curve) x A/wt x100/1000

Where A= Total volume of extract (ml), wt= weight of dried soil

3.2.5 Available sulphur

The turbidometric method described by Chesnin and Yien (1950) was followed to estimate available sulphur using barium chloride and gum acacia.

Reagents and solutions used

- Extraction solution A (0.15% $CaCl_2$. $2H_2O$ solution): 1.986 g of calcium chloride dihydrate ($CaCl_2$. $2H_2O$) was dissolved in 600 ml of distilled water and final volume was made 1 L.
- Barium chloride ($BaCl_2$)
- Gum acacia solution (0.25%): 0.25 g of gum acacia was dissolved in 80 ml of distilled water and final volume was made 100 ml.
- Standard solution (100 ppm Sulphur): 0.5434 g of the reagent grade potassium sulphate (K_2SO_4) was dissolved in de-ionized water to final volume of 1L.

Procedure

Preparation of standard curve: 0.25, 0.5, 1.0, 2.5 and 5.0 ml of 100 mg/l S solution was pipetted in different 25 ml volumetric flasks followed by addition of 10 ml of 0.15% $CaCl_2$ solution and 1 g of $BaCl_2$ crystals. Contents were swirled for one minute to dissolve crystals. 1 ml of 0.25% gum acacia solution was added and turbidity was measured at 420 nm.

Soil samples: Ten grams of air dried soil sample was taken in 250 ml conical flask followed by the addition of 50 ml 0.15% $CaCl_2$ solution and shaken for 30 min. The suspension was filtered through Whatman 42. 20 ml of filtrate was pipetted out to a 25 ml volumetric flask and processed further as in case of standard curve. A blank was run simultaneously with all the chemicals except soil. Final sulphate concentration was determined from the standard curve.

Calculations

ppm of sulphur in soil: Amount of sulphur in soils from standard curve X dilution factor (6.25) ppm

Kg of S/ ha: ppm X 2.24

3.2.6 Available nitrogen

Available nitrogen was estimated in soil samples as described by Subbiah and Asija (1956).

Reagents and solutions used

- 0.32% $KMnO_4$ solution: 3.2 g of potassium permanganate was added in deionized water and the volume was made up to 1L.
- 2.5% NaOH solution: 25 g of sodium hydroxide was added in deionized water and the volume was made up to 1L.
- 0.02N H_2SO_4 and 0.02N NaOH solution
- Methyl red indicator (0.15%): 0.15 g of methyl red powder was added in absolute alcohol and the volume was made up to 100 ml with absolute alcohol.

Procedure

Five grams of soil sample was taken into a Kjeldahl distillation flask. Sample was moistened with 20 ml distilled water and fixed in the Kjeldahl assembly. 25 ml of 0.32% $KMnO_4$ and 25 ml of 2.5% NaOH were added and the flasks were corked. The delivery tube for the collection of ammonia gas was dipped in conical flask containing 10 ml of 0.02N H_2SO_4 solution containing 3 drops of methyl red indicator. The samples were heated and approximately 30 ml of distillate was collected. Excess of H_2SO_4 was titrated against 0.02 N NaOH till colour changed from pink to yellow.

3.2.7 Available phosphorous

Method outlined by Olsen et al (1954) was used for estimating available phosphorous using sodium bicarbonate.

Reagents and solutions used

- Extraction solution (0.5 M $NaHCO_3$ solution): 84 g of sodium bicarbonate was dissolved in distilled water and the volume was made up to 2 L. The pH was adjusted to 8.5 with 1M HCl or 1N NaOH.
- Reagent A: 12.0 g of ammonium molybdate in 250 ml distilled water and 0.2908 g of antimony potassium tartarate in 100 ml distilled water was added to 1000 ml of 2.5 M H_2SO_4, mixed thoroughly and the volume was made up to 2 L with distilled water.
- Reagent B (freshly prepared): 1.058 g of ascorbic acid was added to 200 ml of reagent A and mixed.
- Standard stock solution (50 ppm P): 0.2917 g potassium di-hydrogen phosphate (KH_2PO_4) was dissolved in water to a final volume of 1L.
- Working standard P solution: 20 ml of (50 ppm P) solution was pipetted and diluted to final volume of 1L.

Procedure

2.5 g soil was placed in a 100 ml Erlenmeyer flask followed by the addition of 50 ml extracting solution. The solution was shaken for 30 mins and filtered through Whatman 42. Ten ml aliquot of the filtrate was transferred to a 100 ml beaker followed by addition of 1 ml of 2.5 M H_2SO_4, 15.5 ml of distilled water, 8 ml of reagent B and another 15.5 ml of distilled water. A blank was prepared devoid of soil. For the standard curve: 0, 2, 5, 10, 15 and 20 ml of standard solution was placed in 50 ml volumetric flasks separately. Ten ml of extracting solution, 1.0 ml of 2.5 M H_2SO_4, 8 ml reagent B was added and the final volume was made up to 50 ml. Final volume prepared corresponds to 0.04, 0.1, 0.2, 0.3 and 0.4 ppm of standard phosphorus respectively. After 10 min., the P concentration was read at 882 nm.

Calculation

P in soil (ppm) = P in extract (ppm) x 20 (the standard soil to solution ratio)

3.2.8 Available potassium (Merwin and Peech 1950)

Reagents and solutions used

- 1N ammonium acetate solution (pH 7): 77.09 g of ammonium acetate was dissolved in distilled water and the volume was made to 1 L. The pH was adjusted to 7 with ammonia solution and acetic acid.
- Standard solution of K (1000ppm): Dissolved 1.91 g of potassium chloride in distilled water and volume was made to 1L with distilled water.

Procedure

Available potassium was estimated using flame photometer after extracting with 1N ammonium acetate (pH-7). 5 g of air dried soil passed through 2 mm sieve was weighed and taken into a 250 ml Erlenmeyer flask. 25 ml of 1N ammonium acetate solution was added and flask was shaken for 30 mins. It was then filtered through Whatman 1 and filtrate was used for measuring the soluble potassium using flame photometer.

Calculation

Soluble potassium (ppm) = ppm potassium (from calibration curve) x A/wt

Where A= Total volume of extract (ml) wt= wt of dried soil

3.2.9 Selenium analysis

For estimation of background selenium concentrations, samples were acid digested (USEPA 1996) followed by quantification using graphite furnace-atomic absorption spectroscopy (Perkin-Elmer AAnalyst 600).

Reagents and solutions used

- Conc. $HClO_4$ and HNO_3
- Palladium and magnesium modifier: Mixed Pd and $Mg(NO_3)_2$ matrix modifier solution was prepared by adding 5 ml of 0.3 % $Mg(NO_3)_2$ solution and 2.5 ml of 10 g/L Pd solution into a volumetric flask of 25 ml and completing to the mark with deionized water. The final concentration of the matrix modifier solution was: 0.06 % $Mg(NO_3)_2$ and 0.1% Pd.

Procedure

Soil samples were oven dried (50ºC) for 2-3 days. Dried soil samples were sieved (0.2 mm mesh). One gram of soil sample was digested in kjeldhal flask by addition of 20 ml mixture of concentrated perchloric acid and nitric acid (1:1). Digested samples were left for cooling and treated with 5 ml of 0.2 % HNO_3, followed by filtration with Whatman-42. Final dilutions were made by using 0.2 % HNO_3. Filtrate was analyzed for Se content by GF-AAS (Perkin-Elmer Analyst 600).

20 µl of acid digested sample was loaded in graphite tube along with 5 µl of a palladium and magnesium modifier to minimize the interferences using AS60 auto-sampler (USEPA 1996). The software, WinLab 32 was used to program the analysis profile, which included drying at 110°C for 30 sec, matrix evaporation at 130°C for 30 sec followed by pyrolysis at 1300°C for 45 sec, in inert conditions provided by a continuous flow of argon. Finally, atomization was carried out under a normal atmosphere at 1900°C for 5 sec and 2400°C for 3 sec for cleaning the remaining residue. Calibration was carried out using analytical grade sodium selenate and sodium selenite.

3.3 Isolation of bacterial strains

Soil samples were supplemented in tryptic soya broth (TSB) enriched with selenium (0.32 mM and 0.64 mM), as selenate or selenite, to isolate selenium tolerant microorganisms. Selenium tolerant strains were isolated by serial dilution technique on tryptic soya agar (TSA) plates supplemented with selenium oxyanions, on the basis of visual inference i.e. formation of red colonies occurring due to reduction of selenium oxyanions to selenium, after incubation in aerobic chambers at 37^{o}C for 24 h. Red colonies, indicating reduction of Se oxyanions, were further re-streaked on TSA plates without Se to confirm that the colour was not due to pigmentation. The pure cultures were maintained in Se spiked plates. Simultaneously culture was grown in TSB plates and an aliquot of the cell pellet was suspended in 50 % (v/v) glycerol and kept frozen at -20°C for storage and maintenance.

3.4 Polyphasic characterization of selected strains

3.4.1 Morphological characters

The isolates were characterized morphologically by examining their appearance. For characterization, parameters that were taken into consideration included configuration, margin, elevation, surface, pigment secretion etc. on nutrient agar plates. In addition to colony morphology, cell morphology was studied in terms of cell wall composition of the bacterial isolates using Gram staining along with cell shape of the isolates.

3.4.2 Biochemical characteristics

These bacterial strains were characterized in terms of their metabolic diversity by testing their utilization of different carbon sources, their production of typical enzymes concerned with biochemical characterization of the isolates (e.g. urease, amylase, proteases), aerobic vs anaerobic capabilities.

3.4.2a Indole production test

The culture was inoculated into tryptone broth (appendix-I) and incubated at 35º C for 48 h. Broth without inoculum was kept as control. After 48 h, one ml of Kovac's reagent (appendix-IIa) was added to each tube including control and shaken after intervals for 10-15 minutes and then the tubes were left un-disturbed for separation of reagent as distinct upper layer. Red colour in upper layer indicated positive result (i.e. indole production from amino acid tryptophan using the enzyme tryptophanase).

3.4.2b Methyl red assay

MR-VP broth (appendix-I) was prepared and sterilized in test tubes followed by its inoculation using broth culture keeping one test tube as control (un-inoculated). These test tubes were incubated at 35ºC for 48 h. After incubation, five drops of methyl red indicator (appendix-IIa) was added to the tubes and then observed for change in colour. Appearance of red colour indicates methyl red positive result with production of organic acids during the metabolism by the strains.

3.4.2c Voges-Proskauer test

MR-VP broth (appendix-I) was prepared and proceeded similarly as for methyl red up to incubation. After incubation, 10-15 drops of reagent VP-I (appendix-IIa) and 2-3 drops of VP-II reagent (appendix-IIa) were added to the tubes along with control and tubes were shaken for 30 sec. Appearance of red colour indicated VP-positive result due to production of acetoin, an intermediate in the production of butylene glycol.

3.4.2d Citrate utilization test

Slants of Simmon's citrate agar (appendix-I) were prepared and inoculated with selected strains by streaking. These test-tubes were incubated at 37ºC for 48 h along with a control (un-inoculated) and observed for the change in colour. Due to production of sodium carbonate and evolution of ammonia with citrate utilization, pH of medium changes to alkaline, hence change in colour from green to blue indicates positive result due to the ability of the strain to utilize citrate as sole carbon source.

3.4.2e Catalase test

To examine whether the bacteria produce catalase or not, each isolated colony was taken on a slide followed by addition of catalase reagent (appendix-IIa). Appearance of bubbles indicated the catalase positive and aerobic nature of the isolate.

3.4.2f Oxidase test

Broth culture was inoculated in TSA (appendix-I) by single streak inoculation followed by its incubation at 37ºC for 48 h. After incubation 2-3 drops of culture was added on the oxidase disc and observed for the change in colouration within 30 seconds. Appearance of blue colour on oxidase disc indicates positive result due to production of cytochrome oxidase, an enzyme associated with electron transport chain.

3.4.2g Carbohydrate fermentation test

Fermentation medium (Appendix-I) containing different sugars was prepared. These test tubes were inoculated with broth culture maintaining un-inoculated test tube as control and incubated at 35ºC for 24-48 h. These test tubes were then observed for the change in colour and appearance of bubble in the durum's tube. Appearance of yellow colour indicates the production of acid with bubbles in durum`s tube for evolution of gas respectively with the utilization of particular sugar.

3.4.2h H_2S production test

Five ml of sulphide- indole-motility (SIM) agar medium (appendix-I) was taken in test tubes and sterilized by autoclaving. Then tubes were inoculated with broth culture by stab inoculation and one kept as control (un-inoculated). The tubes were incubated at 35-37ºC for 48 h and then observed for presence or absence of black colouration. Black colour at the butt indicates the H_2S production due to reduction of sulfur compounds present in the medium.

3.4.2i Gelatin hydrolysis test

Gelatin agar medium (appendix-I) was stab inoculated with selected strains keeping control (un-inoculated). These tubes were kept at 37ºC for 4 to 7 days. After incubation all the tubes were kept at 4ºC for 15 min and then observed for liquefaction of gelatin. Gelatin liquefaction occurs due to production of gelatinases which are proteolytic-like enzymes active in the liquefaction of gelatin.

3.4.2j Starch hydrolysis test

Starch hydrolysis test was performed for the detection of amylase production by test organisms. Starch agar medium (appendix-I) was inoculated by single streak method maintaining one control (un-inoculated). These plates were incubated for 48 h at 37ºC. After incubation, plates were flooded with iodine solution for 30 sec and were observed for the discoloration of blue colour (positive result) along the line of streak due to hydrolysis of starch (amylase production) by the isolates.

3.5 Antibiotic profiling

All the selected strains were characterized in terms of their antibiotic sensitivity against 15 different antibiotics. For optimization of inoculum concentration, an overnight grown culture was compared with Mac-Ferland standard and suitably diluted. Appropriate dilution of the isolates were then spread on Muller-Hinton agar plates prepared for antibiotic sensitivity profiling with standard antibiotics such as: nalidixic acid, penicillin, ceftazidime, streptomycin, gentamicin, chloramphenicol, ciprofloxacin, piperacillin, vancomycin, tetracycline, carbenicillin, kanamycin.

3.6 Multiple metal tolerance studies

These bacterial isolates were further screened for their tolerance and resistance towards different metals. Axenic culture was inoculated in TSB (Appendix-I) supplemented with different concentrations (i.e. 5, 10, 25 and 50 mg/l as an active ingredient) of mercury (Hg), lead (Pb), cadmium (Cd), nickel (Ni), arsenic (As), tin (Sn), zinc (Zn), chromium (Cr) and copper (Cu). Flasks were incubated for overnight at 37°C under shaking conditions (120 rpm). After incubation, flasks were monitored for growth in terms of turbidity.

3.7 Growth kinetics and metal transformation studies

The bacterial isolates were further characterized for their selenium tolerance by studying their growth profiles in presence and absence of selenium. The potential of the selected bacterial isolates to reduce and/or volatilize was estimated by analyzing residual selenium using GF-AAS.

For growth kinetics studies, tryptone Soya broth (TSB–Hi-Media) supplemented with either of the selenium oxyanions (0.32 mM or 0.64 mM) were prepared. These media were inoculated with 2% over-night grown culture and kept at 37°C and 120 rpm. Two controls were simultaneously maintained i.e. one having inoculum but without Se (positive control) and other as negative control having Se but without inoculum. Optical density (λ_{600}) was measured after regular intervals of 4 h. Growth associated sequestration of selenium oxyanions inside the metabolically active biomass was observed resulting immobilization of toxic species of metal ions. To strengthen this observation, samples were collected after regular interval of 8 h for the mass balance of metal in biomass and supernatant and were processed as described in section 3.2.9. In brief, after regular time interval, one ml of culture medium was collected in test tubes and centrifuged at 8000 rpm for 10 minutes to separate biomass and supernatant. Pellet and supernatant containing Se were acid digested with premixed concentrated nitric acid (HNO_3) and perchloric acid ($HClO_4$) in (3:1) ratio. The samples were digested till white fumes of HNO_3 come out indicating the complete digestion of samples. After digestion samples were diluted with 0.2% HNO_3 and were subjected to GF-AAS analysis.

3.8 Volatilization experiments

Out of 30 different isolates obtained, one of the strains, exhibiting potential to reduce both the selenium oxyanions and identified as *Pseudomonas aeruginosa* was further checked for volatilization of selenium oxyanions. Isolate was inoculated in TSB (appendix-I) supplemented with selenium at the concentration of 0.64 mM of either of the selenium oxyanions. The strain was grown in round bottom flasks with the provision of inlet and outlet so as to facilitate the collection of spent air. Fresh sterile air supply was maintained using a steady flow of air with bubbler pumps though pre-sterilized 0.22μ membrane filters (Whatman, USA). Spent air containing volatilized selenium components was trapped in alkaline hydrogen peroxide (Merck, India) (Terry et al 1992). The trapping solution was replaced with fresh solution after 24 h. All sets were maintained in triplicates. Abiotic controls were maintained under similar conditions in tryptic soya broth supplemented with selenium oxyanions. Simultaneously, Se uptake of the isolate was determined after separation of cell pellet from spent cell free supernatant (CFS) followed by acid digestion and subsequent dilution. The residual Se in CFS was also determined after acid digestion and subsequent dilution. Selenium levels in the spent air as trapped in H_2O_2 solution were also quantified after acid digestion using graphite furnace-AAS.

3.9 Characterization of reduced selenium

Biomass of strains along with their control (biomass without selenium oxyanions) was selected for crystallographic characterization of the reduced selenium. For characterization of reduced selenium in the biomass, culture was grown in TSB supplemented with selenate and selenite along with control for 24 h on orbital shaker at

120rpm. Bacterial biomass was separated from the spent medium by centrifugation, washed thrice with phosphate buffer to remove traces of medium, air dried in a conventional oven at 150ºC till no further appreciable change in weight of the biomass. Then dried samples were powdered using pestle mortar. X-ray diffraction patterns of the powder samples were recorded using a Panalytical's X'Pert Pro Powder X-ray diffractometer with Cu K_α radiation ($\lambda = 1.541Å$) keeping step size $0.0017^{o}s^{-1}$ in the 2θ range 30º–80º at generator tension 45 kV and generator current 40 mA. Diffractograms were compared to reference data base obtained from COD-Iorg REV 22182 on the basic of 2θ values along with specific diffraction lines using Match software designed for powdered X-Ray diffraction analysis.

3.10 Molecular characterization

3.10.1 Genomic DNA isolation

Genomic DNA was isolated as described in Molecular Cloning by Sambrook and Russell (2001). Ten previously selected cultures (SG-14, SG-15, SG-16, SG-19, SG-20, SG-21, SG-22, SG-23, SG-26 and SG-29) were grown in Luria Bertani (LB) broth (appendix-I) at 37°C for 24 h at 200 rpm. Cells were harvested from 1.5 ml of stationary phase culture in a sterilize microfuge tube by centrifugation (8000 rpm for 10 min). Medium was decanted off from cell pellets and tubes were kept inverted to remove last traces of media to drain away. Cell pellets were washed using 10mM TE buffer (pH 8.0) (appendix-IIb). The cells were suspended in 0.8ml saline- EDTA (appendix-IIb) buffer thoroughly. To this suspension, 50 µL freshly prepared lysozyme solution (10 mg/ml) was added, mixed well and incubated at 37°C for 20 min. Further, 0.2 ml of 10% SDS solution was added, mixed and incubated in water bath for 15 minutes at 60°C. Nucleic acids were then extracted with saturated phenol (equal volume) and centrifuged for 10 min at 8000 rpm. After centrifugation upper aqueous layer was taken into another microfuge tube and was extracted with a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) followed by transfer of upper layer to another tube. An equal volume of isopropanol was added to precipitate the DNA. The precipitated DNA was dissolved in

TE buffer (500 µl) and mixed by vigorous shaking. Isolated DNA was visualized using gel electrophoresis.

3.10.2 DNA estimation

Quantity of the DNA in the sample was calculated by reading the sample at 260 nm against TE buffer (pH 8.0) as blank. An O.D of 1.0 corresponds to 50 µg/ml of DNA approximately.

3.10.3 PCR for 16S rRNA gene amplification

After DNA isolation, PCR amplification was performed in a total volume of 50 µl, each reaction mixture containing 50 ng of genomic DNA as template, 0.2 mM dNTP each, 3 mM $MgCl_2$, 1µl of each primer and 1U of Taq DNA polymerase. Final volume was prepared with sterile double distilled water. PCR amplification of 16S rRNA gene was carried out by using 27 forward (5-AGA GTTTGATCCTGGCTCAG-3) and 1492 reversed (5-GGTTACCTTGTTACGACTT-3) primers with following cycling parameters.

➢ Initial denaturation	94°C	2 min	
➢ Denaturation	94°C	1 min	37 cycles
➢ Annealing	53°C	2 min	
➢ Elongation	72°C	3 min	
➢ Final elongation	72°C	15 min	
➢ Dwelling temperature	4°C		

After carrying out PCR, 7 µl aliquot of PCR product was used for agarose gel electrophoresis.

3.10.4 Electrophoresis

Agarose gel electrophoresis was carried out with 1% agarose in TBE buffer (appendix-IIb). Agarose was dissolved in 1X TBE buffer (pH 8.0) in a microwave oven. The solution was cooled followed by addition of ethidium bromide solution for visualization of DNA bands under UV-transilluminator. Then the solution was poured into the casting tray with the comb, the teeth of which formed the wells. After the gel was set, comb was removed and the gel was placed in the electrophoresis tank with TBE buffer. The DNA sample was mixed with the loading dye (appendix-IIb) and was loaded into the slot of submerged gel. The gel was run at constant voltage 60 V for the desired time and was visualized under UV-transilluminator followed by gel documentation system.

3.10.5 Ligation

PCR product was ligated with pDrive cloning vector according to the manufacturer's instructions (Qiagen Inc.). The reaction components included 0.3 µg of the vector DNA and 1.2 µg of PCR product DNA, 2 µl of 10X ligation mixture and 1µl of T4 DNA ligase enzyme. Final volume of 20 µl was made up with sterile double distilled water. The reaction was allowed to occur at 4°C overnight. The ligated DNA was used for transformation.

3.10.6 Preparation of competent cells

Competence was generated in *E. coli* DH5α strain by chemical method as described by Sambrook and Russell (2001). A single colony of *E. coli* DH5α was picked from a freshly grown plate and transferred into 20 ml of LB broth in a 250 ml flask. The culture was incubated for 16-20 h at 37^{o}C with vigorous shaking (250 rpm). 200 µl of the above inoculum was transferred into 20 ml of fresh LB broth in a 250 ml flask. The culture was incubated with vigorous shaking at 37^{o}C for 2-3 h. After achieving final OD of ~ 0.5 at 600nm, culture was transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes and was kept in ice bath for 10 min. Cell pellet was separated from spent media by centrifugation at 5000 rpm for 10 min at 4^{o}C. The media was completely removed from the cell pellet. The pellet was re-suspended in 10 ml of ice-cold 0.1 M $CaCl_2$ solution and stored on ice for 10-15 min. Cells were again recovered by centrifugation at 5000 rpm for 10 min at 4^{o}C. The supernatant was decanted and cell pellet was re-suspended in 1 ml of ice-cold 0.1 M $CaCl_2$. At this stage cells were stored on ice for 2 h as $CaCl_2$ treatment for 2 h induces a transient state of "competence" in the cells which can uptake any foreign DNA.

3.10.7 Transformation of competent cells

Competent cells (200 µl) were incubated in ice along with 10 µl of ligated DNA for 30 minutes in microfuge tubes. The mixture was then heat pulsed without agitation at 42°C for 2 min and then placed on ice for 2 min followed by addition of 1ml Luria broth. These microfuge tubes were then incubated for 60 min at 37^oC to allow the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid. 100 µl of transformed cells were taken and streaked on LA + amphicillin (100 µg/ml), 0.4 µg/ml of X-Gal and 1 mM IPTG.

3.10.8 16S rRNA gene sequencing

The cloned genes were then amplified and sequenced using M13 and M14 primers. The sequences were generated by chain termination method (Sanger et al 1977) using an applied biosystems automatic sequencer (DNA sequencing facility, Department of Biochemistry, South campus, Delhi University, New Delhi, India).

3.10.9 Phylogenetic relatedness

Sequences were analyzed by using CHECK-CHIMERA program of the RDP II (Maidak et al 2001), in order to detect the presence of possible chimeric artifacts generated by PCR. The 16S rRNA gene sequences of isolates were compared with those available in GenBank/ EMBL databases using BLAST program (Altschul et al 1997) and at RDP-II (Cole et al 2007). The sequences of closely related strains and uncultured bacteria were retrieved from RDP-II and aligned using multiple alignments CLUSTALW program (Thompson et al 1994). Phylogenetic dendrograms were constructed by neighbour-joining method by the use of MEGA-5 package (Tamura et al 2011).

3.11 Localization of reductase gene

Based on specific reduction pattern, localization of selenate reductase gene was checked in SG-16 and SG-26.

3.11.1 Plasmid DNA isolation

Plasmid DNA was isolated by alkaline lysis as described by Birnboim and Doly (1979). Overnight grown cultures of bacterial isolates were used to inoculate 100 ml Luria broth. The culture was incubated at 37°C with vigorous shaking until the culture reached the OD_{600} of 0.5. One ml culture was centrifuged at 10,000 x g for 10 min, re-suspended in 200 µl of solution-I (appendix-IIb) containing 5 mg/ml lysozyme and was kept at room temperature. After 10 minutes of incubation, 400 µl of solution II (appendix-IIb) was added and the tube was inverted gently to mix the solution. After 15 min of incubation on ice, 300 µl of ice cold solution III (appendix-IIb) was added into it and again placed on ice for 10 min. The sample was then centrifuged at 10,000 g for 20 min at 4°C. The supernatant was transferred into another tube and half the volume of iso-propanol was added to it. After 30 min of incubation at room temperature, DNA was recovered by centrifugation at 10,000g for 20 min. The DNA pellet was dried and dissolved in 100 µl of TE (1x) set at pH 8.0 (appendix-IIb).

3.11.2 Megaplasmid isolation

Isolation of megaplasmid was carried out using modified version of Eckhardt procedure (Eckhardt 1978). Overnight grown cells centrifuged for 5 min at 7,500 x g. Supernatant was decanted and cell pellet was washed twice in 2 ml of TE buffer (appendix-IIb). Finally buffer was removed and the cells were vortexed to re-suspend followed by the addition of 40 µl lysozyme (appendix-IIb). Contents were mixed and loaded immediately into the sample well of an agarose gel (0.6%, w/v). While loading the sample, formation of air bubbles was prevented. The gel was allowed to set for 10 min and then carefully overlayed by SDS mixture (appendix-IIb) such that two layers did not get mixed. The wells were capped with 0.85 % (w/v) molten agarose and were allowed to set. The gel was run in tris-borate buffer (appendix-IIb) in cold, initially at 30

V (5 mA) for 45 min and then at 120 V (~30 mA) until the tracking dye reached the end of the gel.

3.11.3 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out with 1.5 % agarose in TBE buffer as described in section 3.10.4

3.11.4 Primer design

For amplification of selenate reductase gene, primers were designed using primer designing tools available at NCBI using database information on the corresponding gene in *Thaurea selenatis*. *Thaurea selenatis* is the only organism so far has been characterized for selenate reductase gene. Out of four genes coding different sub units of functional selenate reductase enzyme, one gene coding for actual reductase activity (*serA*) was selected. For the amplification of this gene, two set of primers were designed. One set of primer (set-I) was selected from literature on the basis of conserved sequences found in other related/un-related organisms (Thorell et al 2003). Another set of primers was designed by directly uploading gene sequence on NCBI-primer design tool (set-II).

Set-I

F2 5′-GCI GAR GCS CTS ACC GAR ATC-3′
R2 5′-ACS AGG AAI GGS AGI GCS GT-3′

Set-II

F1 5′-CGG CGT CGA CGA GAT GGA GT-3′
R1 5′-CCG CCG GCA GCA CGA TA-3′

3.11.5 PCR for selenate reductase gene amplification

Since no DNA band was observed in agarose gel electrophoresis for plasmid DNA, genomic DNA was used for PCR amplification of selenate reductase gene using above primer sets. PCR was performed in a total volume of 50 µl, each reaction mixture containing 50 ng of genomic DNA as template, 0.2 mM dNTPs each, 3 mM $MgCl_2$, 1 µl of forward and reverse primers and 1 U of Taq DNA polymerase. Final volume was made using sterilized double distilled water. For first set of primers, PCR program was followed as described in previous studies with slight modification in annealing and elongation time only (Thorell et al 2003). The cycling parameters for amplification of selenate reductase gene consisted initial denaturation at 94°C for 2 min followed by 37 cycles of amplification consist of denaturation (94°C for 1 min); annealing (52°C for 1min); elongation (72°C for 2 min); final elongation at 72°C for 15 min. PCR amplification of selenate reductase gene was carried out by using following cycling parameters. For second set of primers (Set-II), annealing temperature was set at 58°C.

3.11.6 Agarose gel electrophoresis

Agarose gel electrophoresis of amplified product was carried out as outlined earlier in section 3.10.4.

3.12 Enzyme studies

3.12.1 Extraction and localization of enzyme

Enzyme studies were also carried out in SG-16 and SG-26. To determine the localization of the enzyme involved in reduction of selenate oxyanions, enzyme activity associated with selenate reduction was examined in different cellular fractions (membrane and cytoplasmic regions) along with the cell free extracts. Periplasmic fraction was prepared as described by Schroder et al (1997). Overnight grown biomass was collected after centrifugation for 10 min at 16000 x g. Washed with 10 mM tris-HCl (pH-8). Cells were re-suspended in 30 mM tris-HCl (pH-8) and 0.75 M sucrose in a ration of 0.45 g wet weight of cells per ml of buffer. The cell suspension was incubated at 0^{o}C for 10 min followed by addition of lysozyme @ 0.4 mg/ml. Incubated for additional 5 min at 0^{o}C. Two volumes of an ice cold solution of 15 mM EDTA was then added slowly over a period of 10 min. The suspension was stirred for another 10 min on ice and incubated at 37^{o}C for 10 min which facilitate the formation of spheroplasts. Spheroplasts were separated by centrifugation at 25,000 x g for 20 min. The supernatant represented periplasmic contents and spheroplasts were further processed for extraction of cytoplasmic contents. Cytoplasmic fraction was prepared by suspending the cell pellet in 1% v/v in saline buffer solution followed by addition of lysozyme (1mg/ml) on ice for 30 min with vortexing at regular interval of 10 min. Finally, the suspension was sonicated and supernatant was collected. These fractions were analysed for presence of enzyme using following protocol.

3.12.2 Enzyme activity

Localization of selenate reductase enzyme was confirmed using the protocol described by Gilliam et al (1993) with some modifications. This protocol was initially described for nitrate reductase. In the present study, nitrate was replaced with selenate. Presence of enzyme was detected in 3 ml reaction mixture prepared by addition of 1.6 ml of 100 mM phosphate buffer; 0.15 ml of 0.1 mM FAD solution; 0.1 ml of 12 mM NADPH solution; 0.3 ml of 100 µM sodium selenate; 0.2 ml of different cellular fractions/ supernatant. Final volume was prepared with double distilled water. Simultaneously blank and control was also maintained by skipping sodium selenate and cellular fractions/ supernatant respectively. Contents were mixed by inversion and incubated at 37^{o}C for 30 min on water bath. A spectrum scan was taken after 30 min between 200 nm to 400 nm. Presence of enzyme was confirmed by oxidation of NADPH with concomitant decrease in optical density at 340 nm.

3.12.3 Ammonium sulphate precipitation

After localization of enzyme in different fractions, ammonium sulphate precipitation was carried out for separation of proteins present in cytoplasmic fraction according to their differential solubility properties in different concentration gradients of ammonium sulphate. Cytoplasmic fraction was subjected to different concentration gradients (i.e. 0-20%; 20-40%; 40-60%; 60-80% and rest) of ammonium sulphate. Precipitated proteins were collected from each fraction and were dissolved in 100 mM phosphate buffer. These samples were dialysed against 25 mM Tris-HCl buffer (pH 8.2) at 4°C. Fraction with maximum enzyme activity was used for determination of Km and Vmax.

3.12.4 Km and Vmax studies

Km and Vmax for selenate reductase was determined using different concentrations of selenate (10-100 µM) keeping all other parameters constant. In other words, velocity of the reaction (V) was determined at different concentrations of substrate (S) followed by calculation of rate of conversion of substrate per unit time. A graph was plotted between enzyme activity and substrate concentration using Graph pad prism and values of K_m and Vmax were determined.

3.12.5 Protein estimation

Protein contents were estimated using Biuret method as described by Steinberg et al (1992). Different fractions were added in test tubes and volume was made to 2 ml using 10 mM sodium phosphate buffer (pH 7.0). To each sample 1ml of Biuret reagent (appendix- IIA) was added. The reaction mixture was incubated for 10 min and concentration of the proteins were calculated from the absorbance values obtained at 540 nm against the standard solution of bovine serum albumin (fraction V).

3.13 Microcosm studies

3.13.1 Soil inoculation

The effect of bioaugmentation of selected strains on selenium removal was studied in selenium contaminated soil samples at microcosm level. Two different strains (SG-16 and SG-26) exhibiting significant Se reducing potential were used for bio-augmentation of soil. These strains were selected due to their specific reduction of different selenium oxyanions (selenate and selenite). These strains were mass cultivated in 10 litre of LB broth and were separately inoculated in soil (@10^8 cfu per gram of soil) amended with 0.32 mM and 0.64 mM of sodium selenite and sodium selenate with positive control in soil without selenium and negative control with soil containing only selenium oxyanions and no inoculum. Pots were kept in natural climatic conditions for a period of 90 days. These pots were irrigated on alternative days with tap water containing Se below detection limits. Soil samples were collected after regular interval of time. First sample was collected on 0 day followed by sample collection on 15, 30, 45, 60, 75 and 90 days.

3.13.2 Estimation of Se content

Soil samples were subjected to oven drying (50ºC) for 2-3 days for removal of moisture. Dried soil samples were sieved (2 mm mesh). One gram of soil sample was digested in kjeldhal flask by addition of 20 ml mixture of concentrated perchloric acid and nitric acid (3:1) (USEPA 1996). Digested samples were left for cooling and treated with 5 ml of 0.2 % HNO_3, followed by filtration with Whatman-42. Final dilutions were made by using 0.2 % HNO_3. Filtrate was analyzed for Se content by GF-AAS (Perkin-Elmer Analyst 600). Concentration of Se in the soil samples was recorded in triplicates and is represented as mg/kg.

3.13.3 Viability studies

Simultaneously taken colony counts of the bioaugmented strains added to soils to check the viability status of augmented culture with selected antibiotic as a marker. One gram of soil sample was serially diluted in saline and three successive dilutions were plated on nutrient agar plates supplemented with antibiotic. For soil samples augmented with SG-16, plating was carried out on nutrient agar plates supplemented with carbenicillin (50 mg/l). Similarly soil samples inoculated with SG-26, plating was done on nutrient agar plates supplemented with chloramphenicol (50 mg/l). Simultaneously control soil was also serially diluted and plated on nutrient agar plates supplemented separately with these antibiotics. Colonies were counted on the plates with 30-300 colonies and viable cells were calculated in terms of colony forming units (cfu) after multiplying with dilution factor.

3.14 Statistical analysis

Growth and transformation experiments were carried out in triplicate and results are presented as mean values along with standard error in the respective figures. Statistical analysis of data where-ever applicable was carried out using GraphPad Prism 4.0 and MS excel.

4.0 Results

From last two decades, presence of excess of selenium in seleniferous soils present in Nawanshar-Hoshiarpur region of Punjab posed a threat to the inhabitants living there along with livestock. Present study was carried out to evaluate the status of these seleniferous soils present in this region of Punjab in terms of their physico-chemical properties vis-à-vis an attempt to explore indigenous bacteria present in soils towards efficient biotransformation of bio-available selenium.

4.1 Physico-chemical profile of seleniferous soils

Soil is a complex medium of gaseous, liquid and solid phases, between which contaminants get partitioned. The liquid phase may include both water and organic liquids while the solid phase may contain minerals, native organic matter and recalcitrant organic residues, which can influence the fate and behaviour of its constituents. The characteristics of the soils of the Nawanshahr region were dominantly alkaline constituting the nature of the typical agricultural soils present in Punjab. Along with other physico-chemical characteristics, selenium was also quantified in these soil samples.

Twenty three samples (rhizospheric soil) were collected from agricultural soils from seleniferous sites of the Nawanshahr-Hoshiarpur district of Punjab, India. These soil samples were examined for various physico-chemical characteristics to understand the nature of contaminated soils (Table 4.1). With an exception of a few soil samples, most samples were observed alkaline with a moderate electrical conductivity. These soil samples were also tested for various other chemical parameters like cation exchange capacity (CEC), organic carbon, available nitrogen, available potassium, and available phosphorous and available sulfur. Data obtained for these parameters represented typical characteristic features of Punjab soils.

The pH of soil samples ranged typically towards alkaline with a minimum of 8.26 to maximum of 8.95 in different soil samples collected from seleniferous region. A wide variation and comparatively high EC values were found in different samples collected from seleniferous soils. EC values determined using conductivity meter from the soil samples collected from Simbli (S1-S4) ranged from 368 μS/cm which is close to minimum EC and to unexpectedly highest EC values of upto 1665 μS/cm. Similar results were obtained for the soil samples collected from Jainpur village (S16-S19) with an EC value ranged from 425 to 1554 μS/cm. In all other soil samples collected from Barwa (S20-S23), Mehindpur (S5-S10) and Rakhra villages (S11-S15) comparatively lower EC values with a narrow range between 345- 984 μS/cm was found.

Cation-exchange capacity (CEC) is defined as the degree to which a soil can adsorb and exchange cations. A concomitant increase in CEC of soil has been observed with an increase in organic contents, availability of different cations and slight uphold by change in pH towards alkalinity. In Jainpur soils, the CEC varied from 254 to 489 meq/100g of soil. Similarly CEC of soil samples collected from Simbli village ranged between 185 to 368 meq/100g of soil. In all other villages, CEC values lies between these values as indicated in Table 4.1.

Soil organic matter constitutes a whole series of products which range from undecayed plant and animal tissues to fairly stable amorphous brown to black material bearing no traces of the anatomical structure from the living material it was derived. Microbial metabolites and products are also important constituents of organic matter. Maximum organic matter (0.547%) was observed in S-16 soil sample collected from Jainpur village followed S-13 of Rakhra village with 0.532% organic matter. A substantially low amount of organic matter was determined in S-2 soil sample collected from Simbli village. A wide variation in organic matter was observed in different soil samples collected from same village as represented in Table 4.1.

A wide variation in overall potassium contents of soil samples collected from different villages and soil samples collected from same village has been observed in the study. A minimum of 258 kg/ha and maximum of 661 kg/ha available potassium has been observed in soil samples collected from Jainpur and Mehindpur respectively. In soil samples collected from different fields of Jainpur, a substantial difference in lowest and highest amount of available potassium (254 and 594 kg/ha) was measured. In contrary, potassium content of soil samples collected from other villages shows less variability.

Phosphorus availability to plants is strongly influenced by soil pH, and its availability is maximized when pH lies in alkaline conditions. Besides the pH, availability of inorganic phosphorous depends upon the types of anionic legends to which phosphorous is bound. Available phosphorous contents were typically low in all the soil samples collected from seleniferous soils with reference to the recommended optimum requirement of available P for agricultural crops (Bajwa et al 1997)**.** Minimum bio-available form of phosphorus was observed in the soil samples collected from Simbli village with a maximum value for Barwa village.

Available nitrogen is a measure of fraction of total nitrogen that is easily used by the plants and comprises ammonical, nitrate and the easily oxidizable organic nitrogen. A marginal variation in the organic nitrogen contents was observed in the soil samples collected from one village as compared to the available nitrogen contents in soil samples collected from different villages. In soil samples collected from Simbli village (S1-S4), first three samples were observed to contain same amount of nitrogen (188.16 kg/ha) with a marginally less amount in S-4 sample with 175 kg/ha nitrogen contents. Nitrogen contents in soils of Simbli village were typically lower than the soil samples collected from other villages (Table 4.1). Significantly high levels of nitrogen contents (351 kg/ha and 301 kg/ ha) were observed in soil samples collected from Jainpur village (S-18) followed by Barwa (S-13 and S-15) respectively.

Available sulphur is the inorganic sulphur present in the soil samples in the form of sulphate which is readily absorbed by the plants. Maximum level of available sulphur was observed in the soil samples (S-12 and S-13) collected from Rakhra and Simbli village respectively (Table 4.1a and 4.1d). Substantial low amount of available sulphur was observed in soil samples collected from Simbli and Barwa villages.

Table 4.1: Profile of physico-chemical parametes of seleniferous soils collected from various locations of the sampling site

Name of Village	No. Of Samples	pH	Electric Conductivity (µS/cm)	Cation Exchange Capacity (meq/100g)	Organic Carbon (%age)	Available Nitrogen (kg/ha)	Availble P (kg/ha)	Available K (kg/ha)	Available S (kg/ha)
Simbli	4	8.7±0.08	841±564.9	278±79.30	0.34±0.06	185.0±6.3	0.35±0.08	401.5±94.50	14.57±9.3
Mehindpur	6	8.80±0.09	750±193.7	270.5±59.35	0.41±0.05	181.9±20.6	0.41±0.08	504.4±123.9	17±4.3
Rakhra	5	8.41±0.12	594.4±183.2	307.4±54.23	0.45±0.07	288.5±23.47	0.52±0.23	379.2±76.53	13.72±8.4
Jainpur	4	8.38±0.15	822.8±502.2	308.5±120.6	0.42±0.10	288.5±46.94	0.72±0.10	432.3±179.4	11.59±6.3
Barwa	4	8.43±0.10	464.5±192.4	282.3±51.98	0.39±0.04	222.7±27.81	0.39±0.14	343.8±85.75	10.81±4.3

In general, all the parameters of the soil examined in the study reflected the pattern similar to the agricultural soil of the region and rest of the province. These parameters were studied to examine the profile of characteristics pertinent to typical agricultural soils of the region, as this area is under intensive agricultural activity.

4.2 Estimation of Se in various soils

Soil samples collected from Hoshiarpur -Nawanshar region were also analysed for selenium content. An elevated concentration of selenium was observed in almost all the soil samples collected from the study sites of this region as compared to the soil samples collected from Patiala area. Selenium contents were significantly lower in Patiala soil (0.14 mg/kg), a non-seleniferous region. Maximum selenium contents were observed in S-22 and S-23 soil samples collected from Barwa village followed by S-9 soil sample of Mehindpur. Selenium contents in soil samples collected from above two villages along with other three villages varied from 0.761 to 1.892 mg/kg (Fig 4.1).

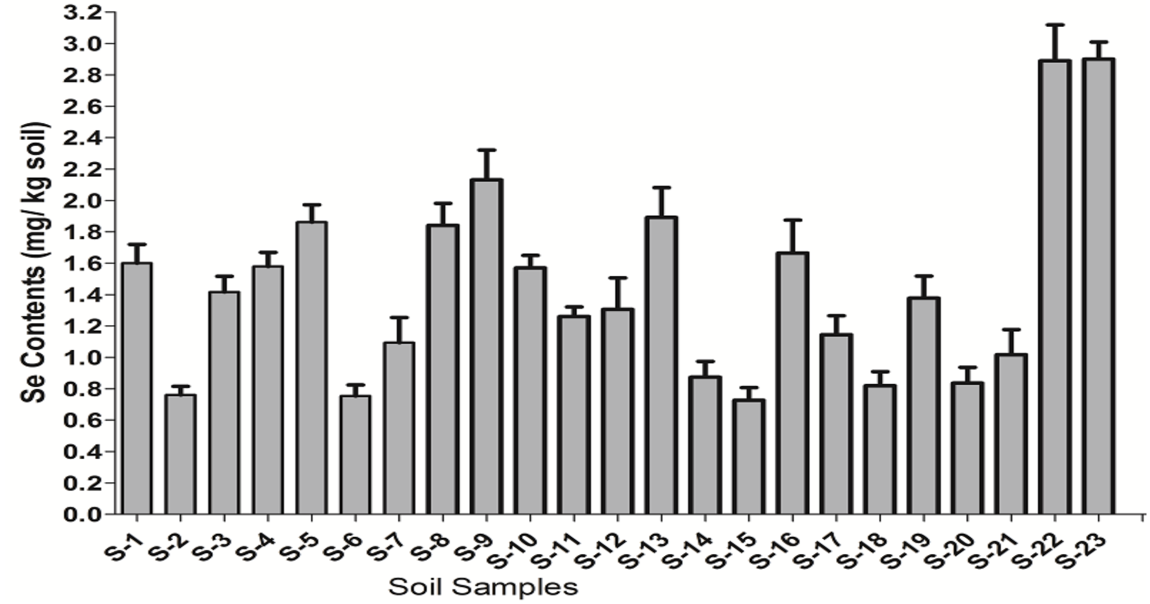


Fig 4.1: Selenium contents in soil samples collected from Hoshiarpur-Nawanshahr region

4.3 Biological profile of the soil samples

4.3.1 Isolation and screening of Se- tolerant bacteria

The soil samples, used for physico-chemical estimations, were then screened for culturable bacterial diversity exhibiting potential to transform/tolerate elevated concentrations of Se-oxyanions. Enrichment of selenium oxyanion tolerant bacteria was carried out after repeated sub-culturing in tryptone soya broth (TSB) supplemented with selenium oxyanions. Bacterial strains were isolated on tryptic soy agar (TSA) supplemented with Se as Na_2SeO_3 and Na_2SeO_4 accounting for 0.64 mM of Se in the growth medium at 37ºC. Axenic nature of these isolates was confirmed after repeated streaking of the individual isolates in tryptic soy agar plates. Thirty bacterial isolates were selected, based on their morphology and potential to tolerate selenium oxyanions supplemented as Na_2SeO_4 and Na_2SeO_3 in tryptic soya broth.

4.3.2 Selection of potential strains

A sufficient elevated concentration of selenium oxyanions in medium was used to ensure isolation of strains through which 30 strains exhibiting (SG-1 to SG-30) potential to uptake and reduce Se was further examined. Dominantly, three different

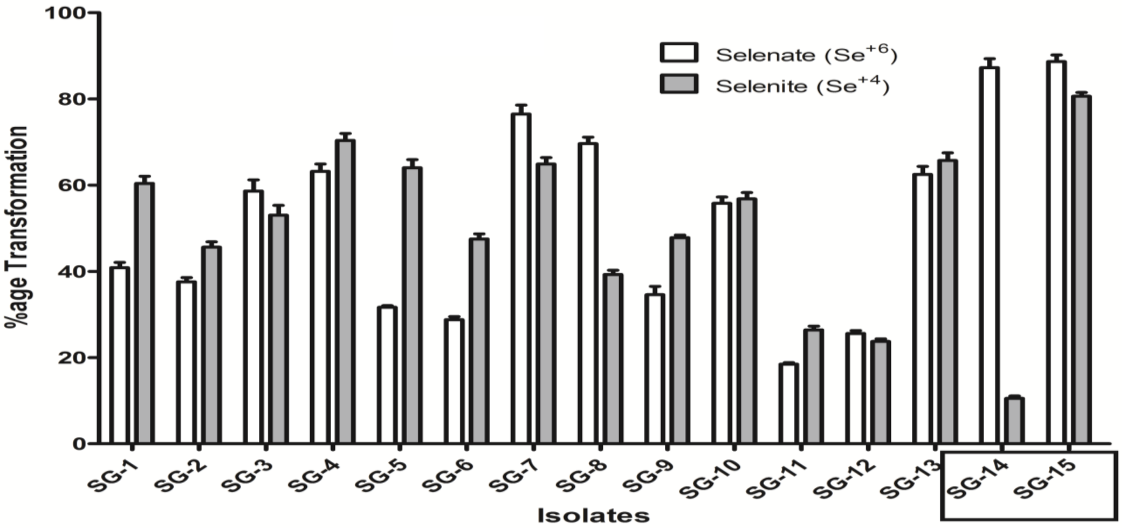


Fig. 4.2a: Selenium removal potential of isolates exposed to different selenium oxyanions

types of isolates were identified with one group observed to reduce only selenate; the second reducing only selenite and the third reducing both the selenium oxyanions as indicated in Fig 4.2. Along with above criterion for the selection of at least one specific strain of every type, maximum uptake of selenium oxyanions was also taken into account while strains were selected for further studies (Fig. 4.2). Hence, out of these thirty isolates, only ten isolates were selected for further studies on the basis of the relatively significant reduction of selenium oxyanions.

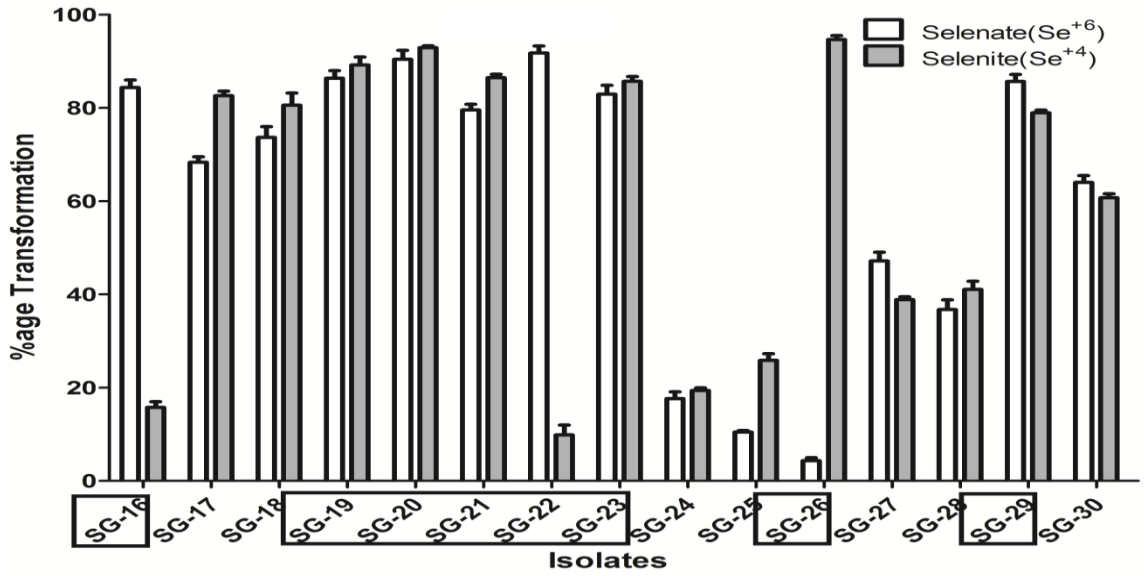


Fig.4.2b: Selenium removal potential of isolates exposed to different selenium oxyanions

After 24 h of incubation in TSB flasks supplemented with selenium oxyanions under shake flask conditions, more than 80% of either of the selenium oxyanions or both oxyanions were removed by the biomass of SG-15, SG-19, SG-20, SG-21, SG-23 and SG-29. Similar data has been obtained with SG-14, SG-16, SG-22 and SG-26 with selenate and selenite respectively (Fig. 4.2). The potential of the obtained isolates to uptake and reduce selenite is a significant phenomenon, as limited reports are available in literature on the reduction of this selenium oxyanion by aerobic bacteria.

4.3.3 Polyphasic characterization of the isolates

The above mentioned ten isolates were then characterized using different tools including morphological, physiological and biochemical properties along with their antibiotic sensitivity profiling to explore their unique identity and response on exposure to different environmental, nutritional and metabolic stress. Detailed profile of the characteristics of these isolates is presented in appendix-II. These bacterial strains were also characterized in terms of their metabolic diversity towards utilization of different carbon sources, production of certain enzymes concerned with biochemical characterization of the isolates like urease, amylase, proteases etc. along with the ecological niche in terms of their aerobic and anaerobic characteristics. Although, these strains differed greatly in their 'colony' morphologies and patterns of metabolism, the isolates observed were mostly rod shaped with a minute difference in cell size. Morphological and biochemical characteristics were examined for isolates following methods outlined in methodology section. Morphological and biochemical characteristics of the mentioned strains are also presented in the appendix-II.

Out of these ten isolates, three isolates namely SG-14, SG-23 and SG-26 were found Gram negative. Rest seven isolates (SG-15, SG-16, SG-19, SG-20, SG-21, SG-22 and SG-29) retained primary stain of crystal violet during Gram staining, hence were assigned as Gram positive. Two (SG-14 and SG-26) of the three Gram negative strains showed biochemical properties consistent with that of typical facultative anaerobic bacteria. Negative nitrate test indicates strong aerobic nature of SG-23. Except SG-14, two other Gram negative (SG-23 and SG-26) were found indole negative (in-ability to degrade tryptophan into indole), methyl red negative (acid production) and nitrate positive i.e. capable of anaerobic respiration. Morphologically, all the Gram negative strains were motile and non-spore formers. SG-23 and SG-26 were found secreting a mucilaginous polysaccharide which covered the exterior of the individual cell and gave a smooth and slimey appearance to the colony. SG-26 also secreted a water soluble green fluorescent pigment. These strains were observed to be oxidase and catalase positive indicating their ability to grow under aerobic conditions. These strains also found to hydrolyse lipids and use citrate as a sole carbon source. Physiologically, SG-23 and SG-26 were observed to tolerate elevated temperature of 42°C and 45°C while SG-14 did not. These three isolates were also observed to tolerate highly alkaline condition and grew well upto pH 11 except SG-14 which was viable up to pH 9 only. A high salt tolerance (up to 7% NaCl) was observed in SG-26 as compared to SG-14 and SG-23 which tolerated only 5% NaCl.

All Gram positive strains were invariably catalase positive indicating their strong aerobic character. SG-16, SG-20 and SG-29 were found positive for nitrate reduction which specifies the ability of these strains to grow under anaerobic conditions. Except SG-20 and SG-22, all other Gram positive strains (SG-15, SG-16, SG-19, SG-21 and SG-29) were motile. SG-15, SG-19 and SG-20 secrete extracellular slimey polysaccharide identified as capsule which gave a smooth appearance to the colonies on nutrient agar plates. No water soluble pigment was secreted by any of the Gram positive strain as indicated by creamish white colonies and no fluorescence under UV light. Physiologically, these three isolates were found to tolerate an elevated temperature of 42^{o}C with SG-19 being viable up to 45^{o}C. SG-15, SG-19 and SG-22 were found able to grow when supplemented with 7% NaCl while SG-20 tolerated salt upto 5% only. SG-16, SG-19, SG-20 and SG-29 were exceptionally tolerant to high alkaline condition and showed growth even at pH-11. All the strains lack the ability to metabolize tryptophan (indole negative) and do not produced acid as indicated by negative methyl red results except SG-14, SG-16 and SG-29 respectively. A wide variation in the data obtained with different isolates for different parameters indicated the metabolic diversity and distinct identity of the isolates (appendix- III).

4.3.4 Antibiotic profiling

All these selected bacterial strains were screened for their antibiotic sensitivity profile against 15 different antibiotics. A wide variation in the antibiotic sensitivity profile of these isolates was observed to add on the speculation of being different on the basis of their morphological, physiological and biochemical characteristics. Antibiotic

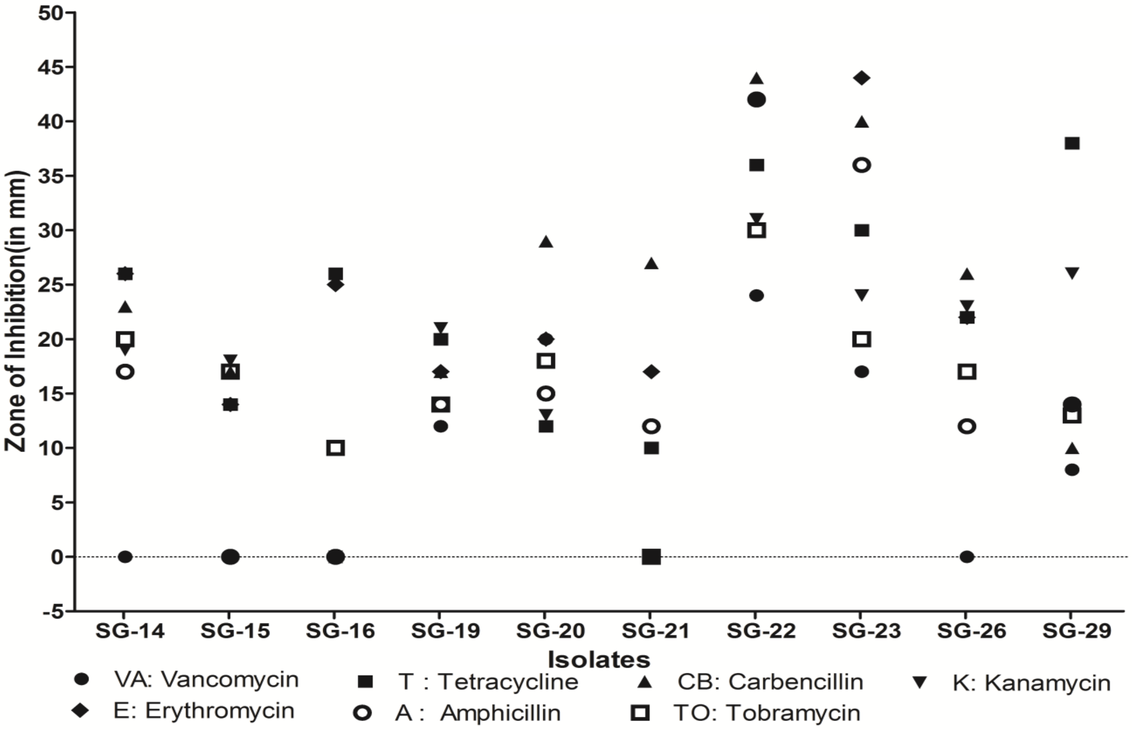


Fig. 4.3a: Antibiotic sensitivity profile of selected strains against different antibiotics

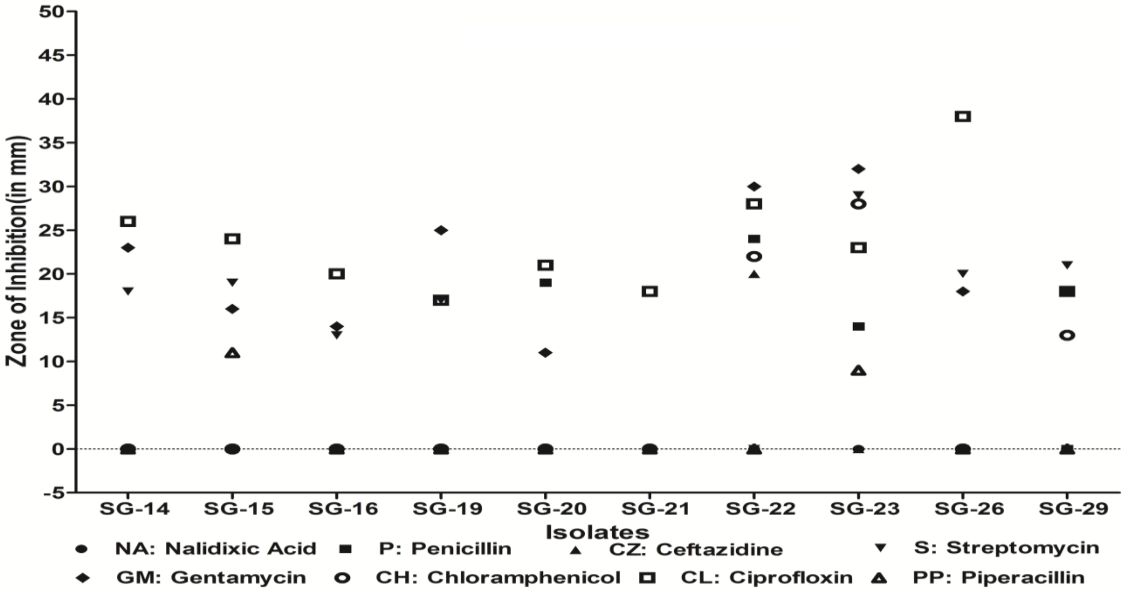


Fig. 4.3b: Antibiotic sensitivity profile of selected strains against different antibiotics

susceptibility of each isolate was determined using disc diffusion method with individual antibiotic impregnated discs. Observations on the antibiotic profiling gave a comparative account of the resistance of the test strains towards various antibiotics. Size of the zone of inhibition were measured and compared with the standard table. Based on the zone of inhibition observed for different antibiotics, isolates were categorized in terms of percent resistance to various antibiotics. Almost all the strains harbour resistance against one or another antibiotic. For certain antibiotics like nalidixic acid and ceitazidine, all the cultures were invariably resistant except SG-22. Growth of cultures was inhibited to a variable extent with different antibiotics. Except SG-20, SG-22 and SG-23, all the strains were observed typically resistant to penicillin. A wide variation in zone of inhibition was observed for other antibiotics with these strains (Fig 4.3a and 4.3b). All the strains were resistant to streptomycin except SG-20, SG-21 while only SG-21 was resistant against gentamycin. SG-21 showed resistance against maximum antibiotics (10) while in all other strains (SG-19, SG-20, SG-22 and SG-23) a variable sensitivity towards different antibiotics was observed. For these strains, size of zone of inhibition varied for different antibiotics (Fig 4.3a and Fig. 4.3b).

Resistance to a group of selective antibiotics can effectively be used as a screening factor to track the survival rate of isolates bio-augmented into soils, amongst the native bacteria (Lawrence 2000).

4.3.5 Multiple metal tolerance analysis

A wide variation in minimum inhibitory concentration (MIC) of different metals was observed for different isolates. All the isolates except SG-20, SG-21 and SG-23 invariably tolerated lead up to 50 mg/l. SG-15 and SG-16 also showed resistance upto 50 mg/l for cadmium and nickel while SG-19, SG-22 and SG-29 showed higher resistance for nickel only (Table 4.3).

Minimum inhibitory concentration (MIC) of other metals for these isolates were observed in an intermittent range (Table 4.3). SG-20, SG-21 and SG-23 showed least

Table 4.3: Heavy metal tolerance/MIC (mg/l) profile of different isolates

Metals	Isolates (SG #)									
	14	**15**	**16**	**19**	**20**	**21**	**22**	**23**	**26**	**29**
Lead (Pb)	50	50	50	50	25	25	50	10	50	25
Mercury (Hg)	25	50	10	25	25	10	25	00	25	10
Cadmium (Cd)	25	50	50	25	10	25	25	00	50	25
Nickel (Ni)	25	50	50	50	10	25	50	10	25	50
Arsenic (As)	25	25	10	10	00	25	10	00	25	10
Selenium (Se)	50	50	50	50	50	50	50	50	50	50
Zinc (Zn)	25	50	10	25	10	10	10	10	50	10
Chromium (Cr)	10	50	25	25	10	10	10	10	10	10
Copper (Cu)	05	50	10	10	05	05	10	05	10	05

* In TSB medium

tolerance for most of the metals with maximum resistance for selenium (50 mg/l) only. On the basis of tolerance for different metals, these strains were also exploited for biosequestration and transformation of other metals.

4.4 Growth kinetics

Selected bacterial isolates were tested for growth in the presence of different concentrations (0.32 mM and 0.64 mM) of selenium as selenate (SeO_4^{-2}) or selenite (SeO_3^{-2}). Although the control culture (0 mM) showed relatively faster growth rate, similar levels of growth were observed with all the strains in the presence of selenate and selenite (Fig 4.4a, 4.4b, 4.4c. 4.4d and Fig 4.4e) over a period of 12 h. The cultures grown in the presence of two different concentrations (0.32 mM and 0.64 mM) of sodium selenate and sodium selenite turned red as growth progressed indicating the reduction of selenium oxyanions to elemental red selenium (Buchanan et al 1995). The

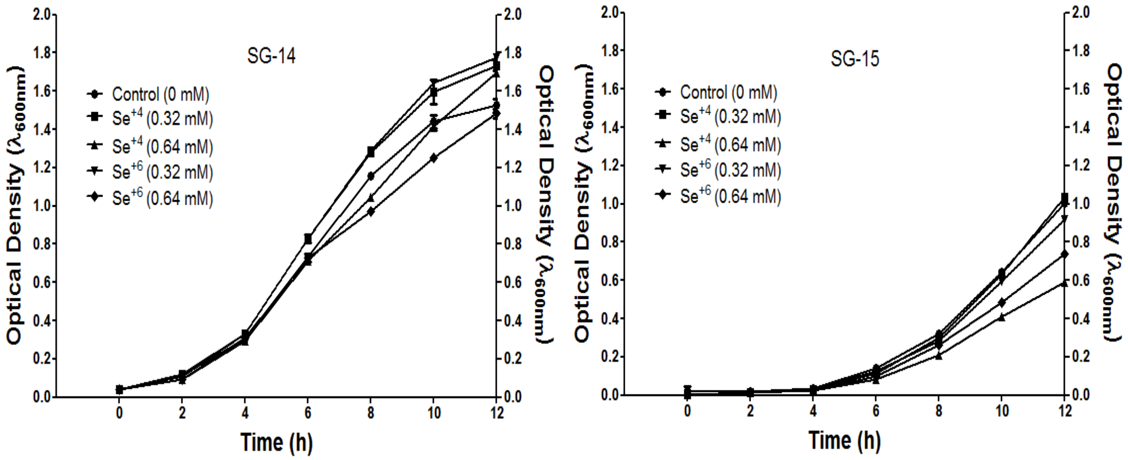


Fig. 4.4a: Growth profile of SG-14 and SG-15 in presence of Se^{+4} (0.32 mM and 0.64 mM) and Se^{+6} (0.32 mM and 0.64 mM)

growth profiles were similar in control vis-à-vis the culture grown in selenium supplemented medium, as determined by optical density measurements (Fig 4.4a and Fig. 4.4b).

Overnight grown cells of the ten isolates were inoculated in growth medium containing (0.32 mM and 0.64 mM) of both selenium oxyanions. Although a wide variation was observed in growth profile of most of the isolates, SG-14, SG-16, SG-19,

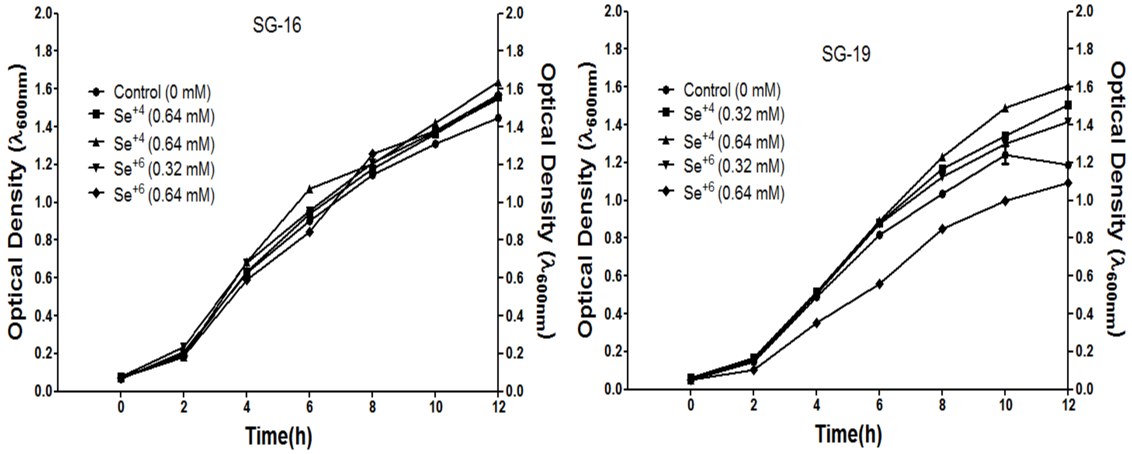


Fig. 4.4b: Growth profile of SG-16 and SG-19 in presence of Se^{+4} (0.32 mM and 0.64 mM) and Se^{+6} (0.32 mM and 0.64 mM)

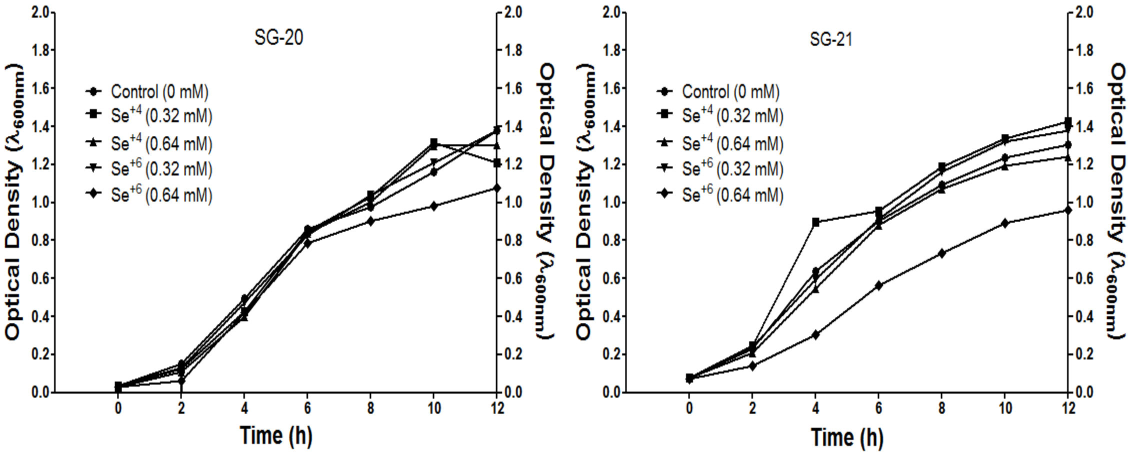


Fig. 4.4c: Growth profile of SG-20 and SG-21 in presence of Se^{+4} (0.32 mM and 0.64 mM) and Se^{+6} (0.32 mM and 0.64 mM)

SG-20, SG-21 and SG-29 showed comparative growth profile in the presence of both selenium oxyanions along with control. SG-15, SG-22 and SG-23 exhibited a prolonged lag phase during growth in selenate and selenite supplemented broth with reference to their respective controls (without selenium). Whereas, the growth profile of SG-26 was similar to control when exposed to selenite. However, limited growth was observed in selenate supplemented medium even after prolonged incubation. A visible inference was also made on the basis of intensity of the red colour developed in the broth due to

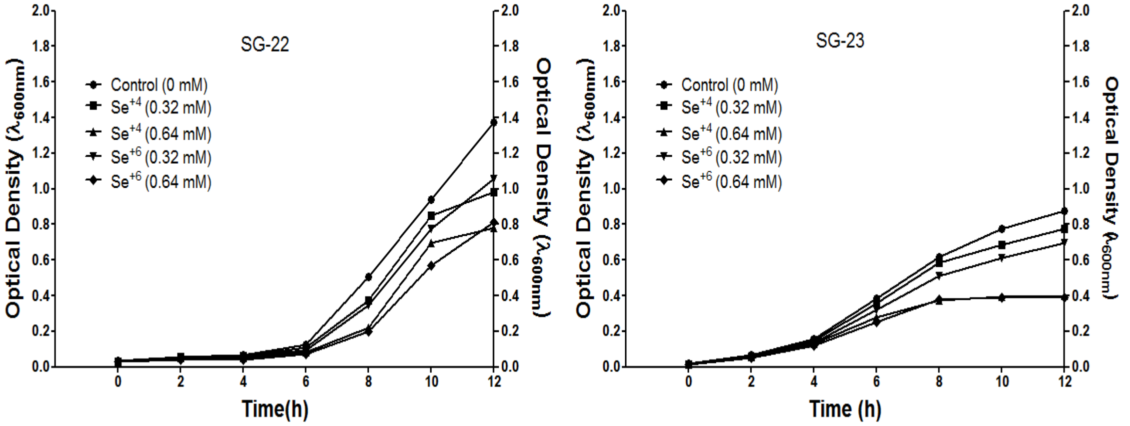


Fig. 4.4d: Growth profile of SG-22 and SG-23 in presence of Se^{+4} (0.32 mM and 0.64 mM) and Se^{+6} (0.32 mM and 0.64 mM)

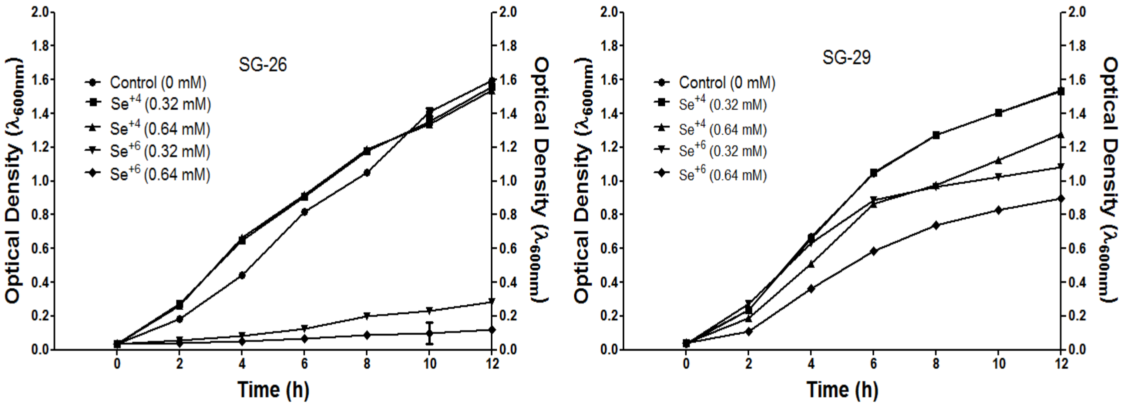


Fig. 4.4e: Growth profile of SG-26 and SG-29 in presence of Se^{+4} (0.32 mM and 0.64 mM) and Se^{+6} (0.32 mM and 0.64 mM)

conversion of selenium oxyanions into elemental selenium. Even after sufficient growth of SG-26 in selenate supplemented broth, intensity of red colour which is an indication of Se reduction, was marginal when compared to cultures grown in selenite indicating that transformation of selenate might bypass the normal reduction pathway leading to formation of elemental selenium reported by previous researchers (Yadav et al 2008; Higashi et al 2005). Growth kinetics of the isolates was studied for a limited time period of 12 h as further extension of the incubation resulted in a concomitant development of red colouration was observed with increase in biomass. The strain, SG-16, was an exception, where although significant growth in biomass was observed in selenite supplemented broth, no indication of Se reduction was observed.

4.5 Uptake of selenium oxyanions

The uptake of selenium oxyanions was monitored for 24 h at a regular interval of 8 h. A wide variation in uptake of selenium by bacterial strains exposed to either of the selenium oxyanions was observed (Fig. 4.5a-4.5j). A substantial difference in uptake of selenium oxyanions was evident at different concentrations of these oxyanions. After first 8 h of incubation, SG-14, SG-15, SG-20 and SG-22 accumulated almost 40% and 55% of selenite inside the biomass from the media supplemented with 0.32 mM and 0.64 mM selenite respectively (Fig. 4.5a; 4.5b; 4.5e; 4.5g). While approximately 70 %

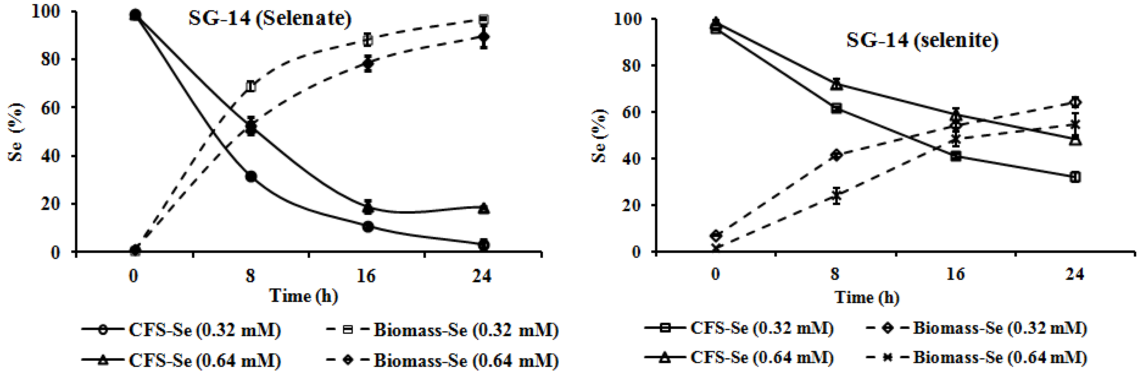


Fig. 4.5a: % selenium contents in different fractions supplemented with 0.32 mM and 0.64 mM selenium oxyanions (Se $^{+4}$ and Se^{+6}) using SG-14

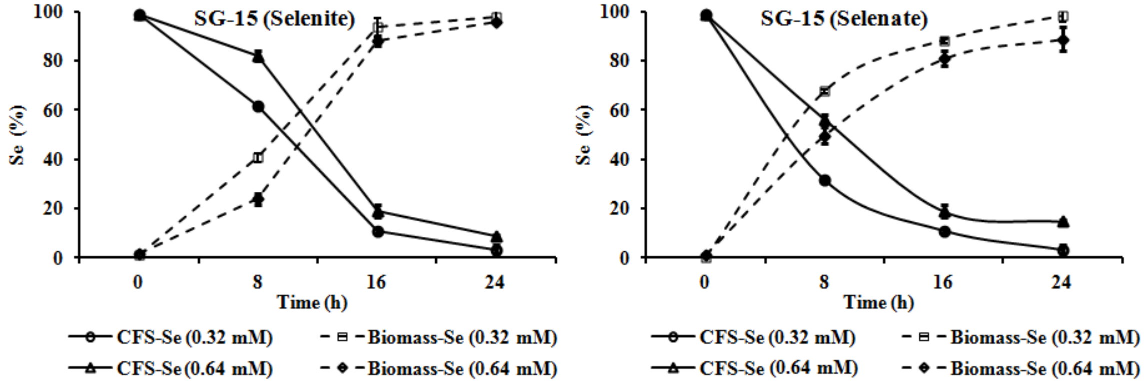


Fig. 4.5b: % selenium contents in different fractions supplemented with 0.32 mM and 0.64 mM selenium oxyanions (Se $^{+4}$ and Se^{+6}) using SG-15

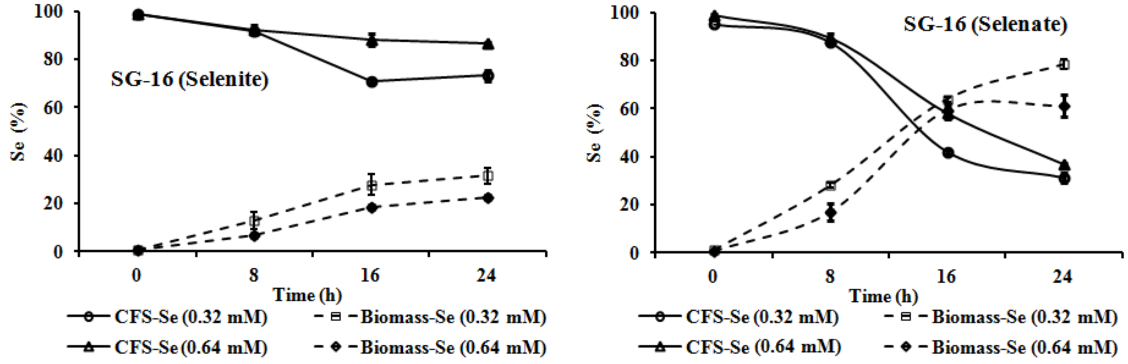


Fig. 4.5c: % selenium contents in different fractions supplemented with 0.32 mM and 0.64 mM selenium oxyanions (Se $^{+4}$ and Se^{+6}) using SG-16

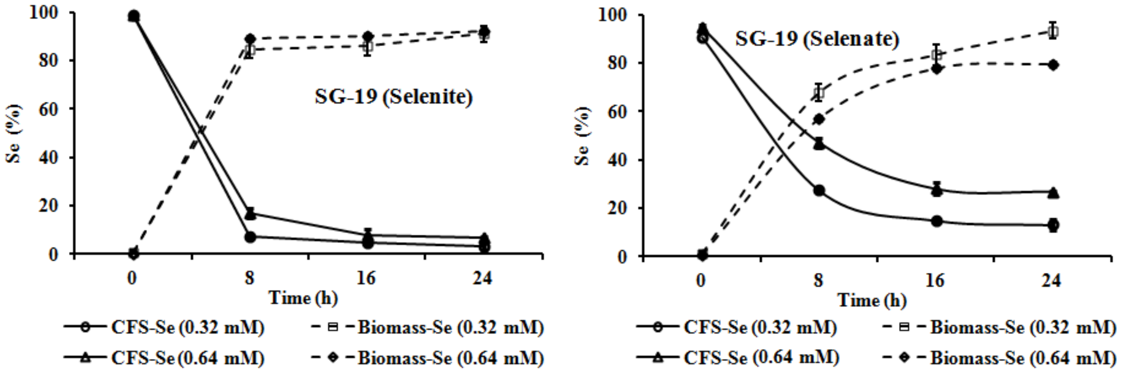


Fig. 4.5d: % selenium contents in different fractions supplemented with 0.32 mM and 0.64 mM selenium oxyanions (Se $^{+4}$ and Se^{+6}) using SG-19

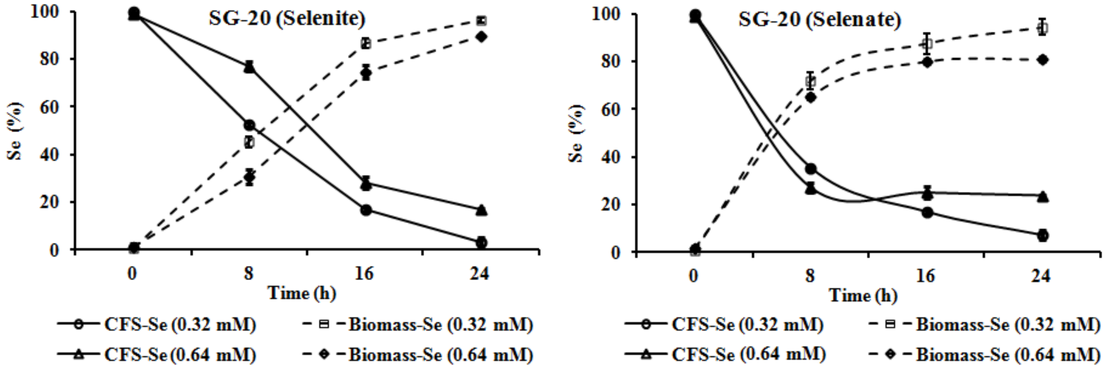


Fig. 4.5e: % selenium contents in different fractions supplemented with 0.32 mM and 0.64 mM selenium oxyanions (Se $^{+4}$ and Se^{+6}) using SG-20

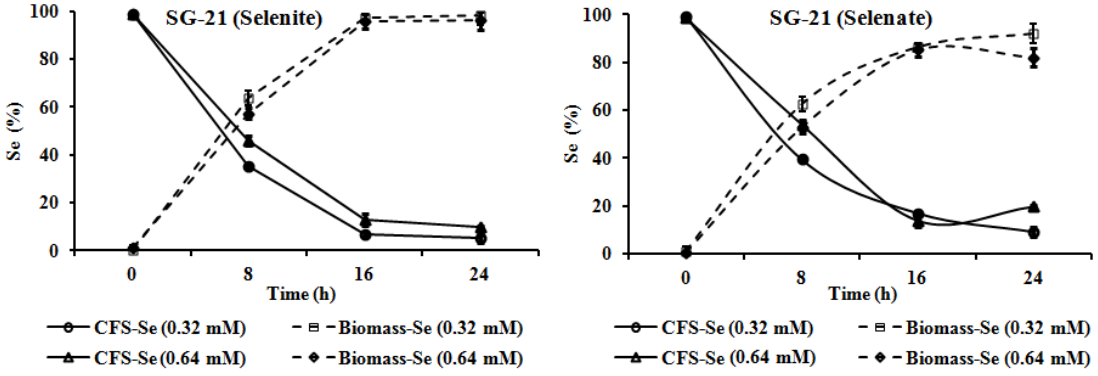


Fig. 4.5f: % selenium contents in different fractions supplemented with 0.32 mM and 0.64 mM selenium oxyanions (Se $^{+4}$ and Se^{+6}) using SG-21

and 55 % of selenate was found in the biomass when corresponding levels of selenate were supplemented in the broth.

After 16 h of incubation, there was no appreciable increase in the accumulation of selenium in strain SG-14 and SG-22 supplemented with selenite (Fig. 4.4a and 4.5g). Almost 80- 90 % of supplemented Se^{+6} was found in the biomass of these two strains (SG-14 and SG-22) irrespective of the concentration of selenium oxyanions supplemented. Other two strains (SG-15, SG-20) showed a similar increase in accumulation of selenium in the biomass supplemented with either selenium oxyanions (Fig. 4.5b and 4.5e). The isolates SG-15 and SG-20 showed an appreciable increase in uptake of Se as Se^{+4} as compared to SG-14 and SG-22. This trend in accumulation continued even after 24 h of incubation. SG-21 and SG-29 followed the nearly similar accumulation pattern with selenite and selenate over a time period of 24 h (Fig. 4.5f and Fig. 4.5j). In constrast, isolate SG-19 showed just an opposite pattern to the above isolates as almost 100% of supplemented selenite was reduced accumulated within the biomass after 8 h of incubation while comparatively lesser uptake (60-70%) of selenate was observed after 8 h (Fig. 4.5d).

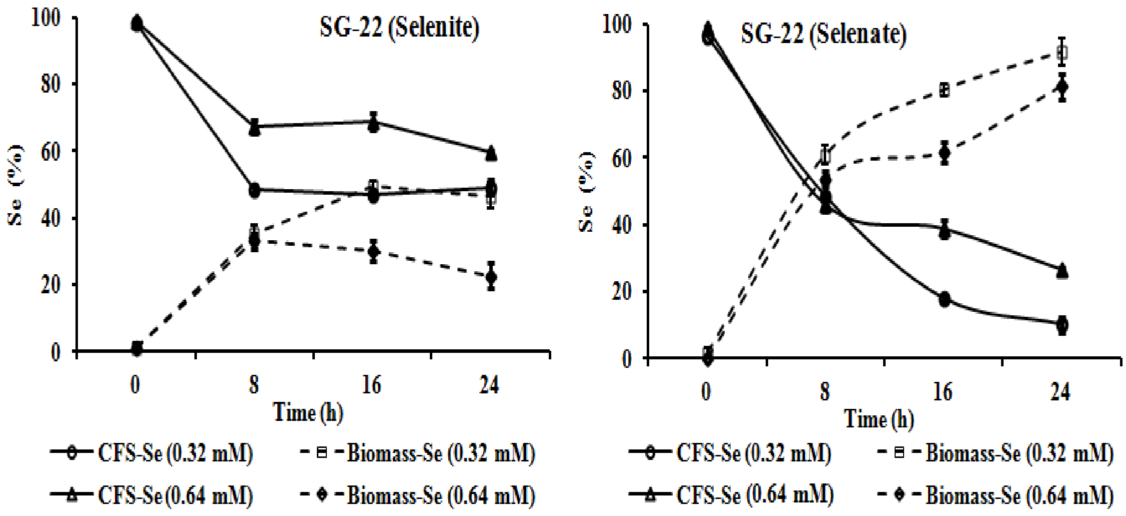


Fig. 4.5g: % selenium contents in different fractions supplemented with 0.32 mM and 0.64 mM selenium oxyanions (Se $^{+4}$ and Se^{+6}) using SG-22

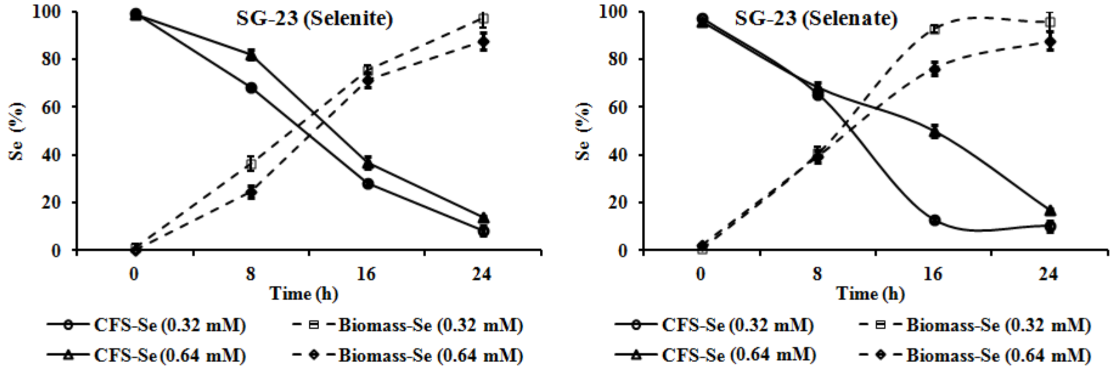


Fig. 4.5h: % selenium contents in different fractions supplemented with 0.32 mM and 0.64 mM selenium oxyanions (Se $^{+4}$ and Se^{+6}) using SG-23

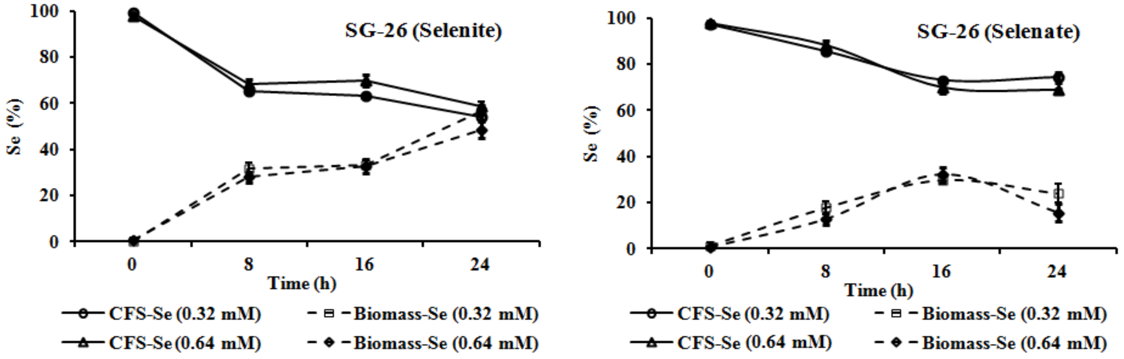


Fig. 4.5i: % selenium contents in different fractions supplemented with 0.32 mM and 0.64 mM selenium oxyanions (Se $^{+4}$ and Se^{+6}) using SG-26

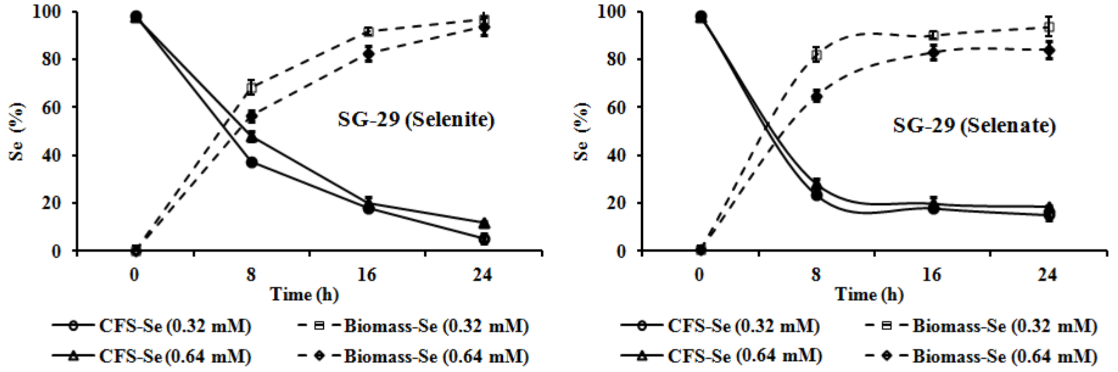


Fig. 4.5j: % selenium contents in different fractions supplemented with 0.32 mM and 0.64 mM selenium oxyanions (Se $^{+4}$ and Se^{+6}) using SG-29

SG-16 and SG-26 followed a unique and opposite reduction pattern with selenite and selenate oxyanions of selenium (Fig. 4.5c and Fig. 4.5i). SG-16 appreciably reduced and accumulated selenate over a time period of 24 h with an initial marginal reduction over a period of 8 h. While no accumulation and reduction of selenite occurred in biomass grown in selenite supplemented broth. Almost 70 % and 80 % reduction of 0.32 mM and 0.64 mM supplemented selenate was observed respectively as compared to maximum of 30% reduction in case of 0.32 mM supplemented selenite. In contrary to above reduction profile of SG-16, an efficient reduction of selenite oxyanions occurred in case of SG-26 over a period of 24 h with little reduction of selenate oxyanion in the biomass. Almost 60% of selenite was transformed and found accumulated and reduced in the biomass as compared to 20% in case of selenate supplemented biomass.

4.6 Effect of pH on growth of organism and selenium reduction

The studies on influence of pH on growth vis-à-vis selenium reduction were carried to examine the selenium reduction potential of the isolates at different pH conditions (pH 4, 6, 8 and 10). The isolates were observed to reduce both oxyanions of Se in slightly acidic to alkaline range of pH although growth of the strains and reduction of oxyanions was more prominent in lower alkaline range (pH 6-8). At pH 10, all other strains except SG-14, SG-16, SG-19, SG-20, SG-23 and SG-29 showed reduced growth at early exponential phase. However, a variable recovery of the growth was noted at late exponential phase with each oxyanions as well as type of strain except SG-26. Above mentioned strains showed a comparable growth to the neutral pH at different pH conditions. A marginal period of acclimatization was followed up by rapid growth and reduction of selenite at extreme alkaline pH. While a prolonged lag phase was observed in growth profiles under selenate supplemented conditions, at extreme alkaline pH used in the present study. In contrast, SG-14 and SG-16 showed complete recovery similar to control when exposed to both selenite and selenate oxyanions of selenium. Even at neutral pH, some strains (SG-23, SG-26 and SG-29) did not recover completely after prolonged incubation when exposed to 0.64 mM selenate. Selenite showed no effect on growth profile at pH 10 in case of SG-16 and SG-29 where as the inhibitory effect of selenite, at pH 10, was prominent till early exponential phase followed by complete recovery at late exponential phase in all other strains. The effect of selenite and selenate on SG-23 strain, at pH 10, was similar to that of SG-16 and SG-26 respectively for these oxyanions with growth inhibition at early exponential phase followed by marginal recovery at late exponential phase. The isolates did not show any growth under strong acidic conditions indicating the pH optima for growth and selenium reduction by these isolates typically occurs in dominantly alkaline conditions.

4.7 Effect of temperature on growth of organism and selenium reduction

All microorganisms have a particular temperature range and cardinal points at which they can survive. These cardinal points may be distinctly wide for certain specific type of bacteria. Bacteria isolated from rhizospheric soils and phyllospheres often grow best at 20°C-30°C. The present study was carried out to examine the growth profile of these selected selenium tolerant isolates (SG-14, SG-15, SG-16, SG-19, SG-20, SG-21, SG-22, SG-23, SG-26 and SG-29) with reference to different temperature levels along with an exposure to a particular concentration of either of the selenium oxyanions. General observations on all strains indicated a delayed growth and reduction of selenium oxyanions i.e. after 10 h, in terms of colour change at 20°C which may be due to prolonged generation time at low temperature. At 30°C and 40°C, normal growth concomitant with Se- oxyanions reduction was observed in all the strains. While at 50°C, both the growth profile and the reduction of Se were observed to be distinctly inhibited. With reference to the effect of a wide range of temperature on the growth profile of each strain, these isolates exhibited varied profiles at specific temperature. In all strains except SG-16, SG-20, SG-21 and SG-23, extended lag phase was observed at 20ºC, where as prominent inhibition of growth was observed with Se^{+6} with a similar growth profile to control in the presence of Se^{+4} at that particular temperature. At 30ºC, which is optimal for these organisms, presence of selenate resulted in extended log phase in SG-19 and SG-21, with recovery of growth after 8 h. No recovery was observed in SG-26 exposed to selenate. The growth profiles of some strains (SG-14, SG-16, SG-19, SG-26 and SG-29) at 40ºC were significantly rapid than the normal optimum temperature with concomitant reduction of selenium oxyanions. At 50ºC, there was no growth observed either in control and selenium oxyanions supplemented broth with no reduction of selenium oxyanions in SG-15, SG-20, SG-21, SG-22 and SG-23. The strain SG-14, SG-16, SG-19, SG-26 and SG-29 exhibited different growth profiles as compared to other strains when exposed to selenium oxyanions at 50ºC temperature. The strains were observably not influenced by either temperature or the presence of selenium oxyanions up to 40ºC, whereas at 50ºC, both temperature and presence of the selenium oxyanions cumulatively have been observed to inhibit the growth whereas the growth in control under the influence of increased temperature remained same or marginally affected as in other growth temperatures.

4.8 16S rDNA based characterization and identification

All bacterial isolates were subjected to 16S rDNA amplification using universal primers, and about 1.5 Kb amplicon was observed in all the isolates (Fig. 4.8a, 4.8b and 4.8c). The 16S rDNA products from were then sequenced using Applied Biosystems automatic sequencer. Sequencing reactions were performed with the primers T7 and SP6. The sequences were analyzed by multiple sequence alignment to check the similarities among the isolates. Sequences were compared for the similarity in the GenBank DNA database BlastN (NCBI) (Altschul et al 1997), which revealed that 16S rDNA of bacterial isolates have 96% to 99% similarity with the sequences of NCBI database. The 16S rRNA gene sequences determined for isolates under study were deposited in GenBank of NCBI data library. BLAST homology search for 16S rDNA gene sequences of isolates indicated 98-99% similarity with their closest matches.

Analysis of the obtained 16S rDNA sequences with BLAST revealed the identity of culture related to the two distinct phyla firmicutes and proteobacteria associated with the genera *Bacillus* and *Pseudomonas* with 99% homology. Classification of these strains to different genera was confirmed using RDP II Classifier (Wang et al 2007). For

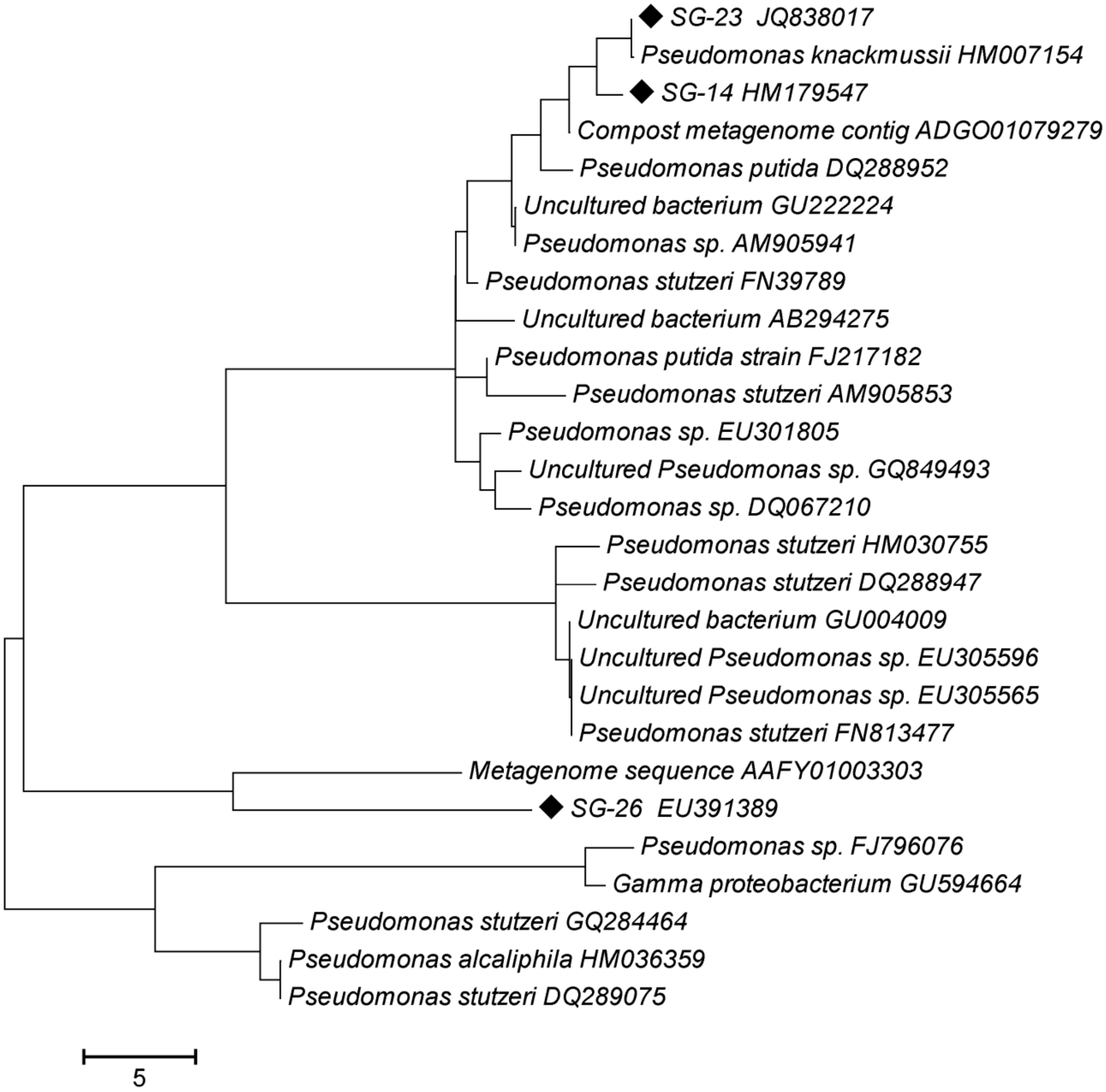


Figure 4.8a: Molecular phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-21779.0806) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al 2011).

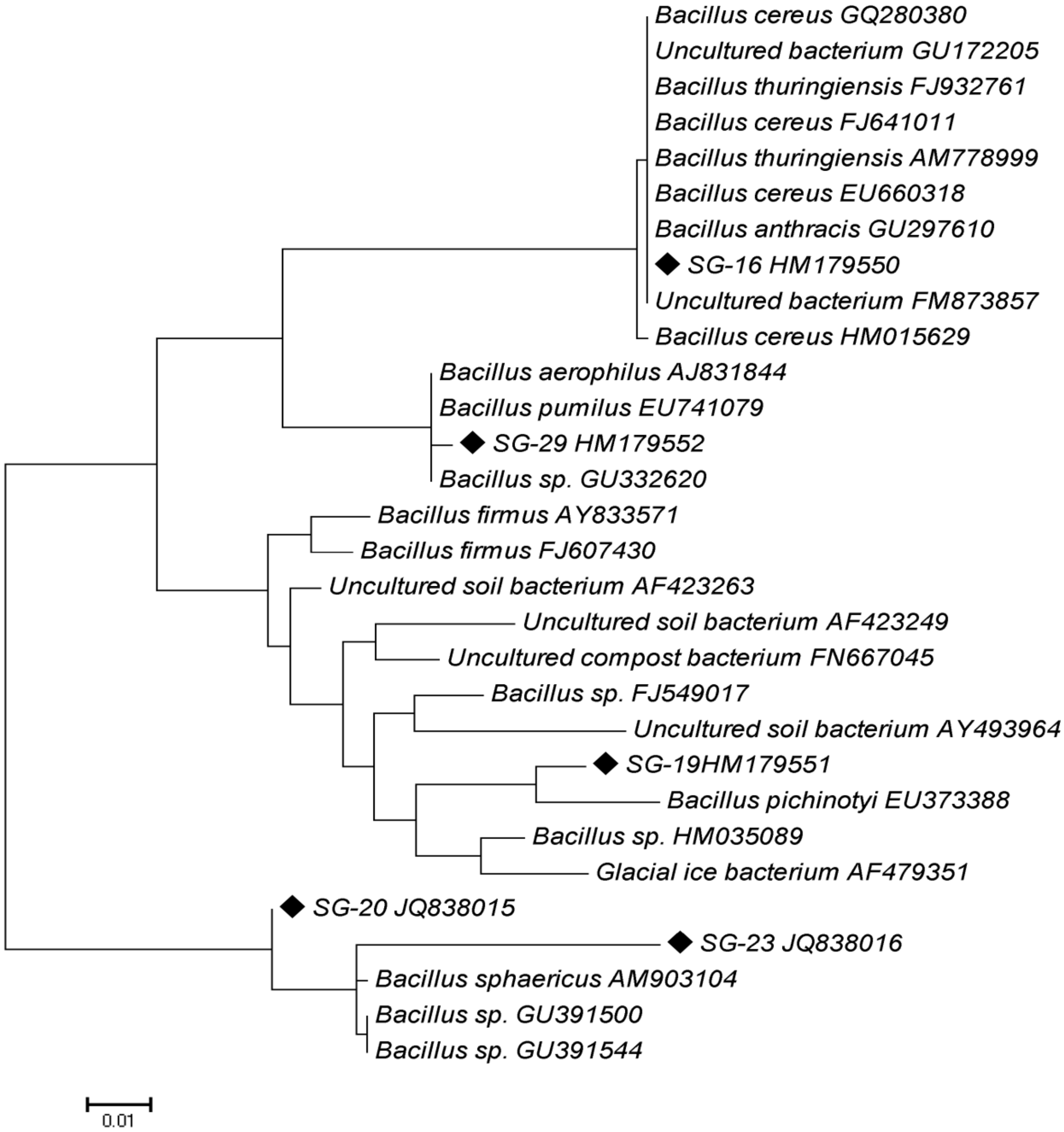


Figure 4.8b: Molecular phylogenetic analysis by Maximum Likelihood method
The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-2400.3964) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al 2011).

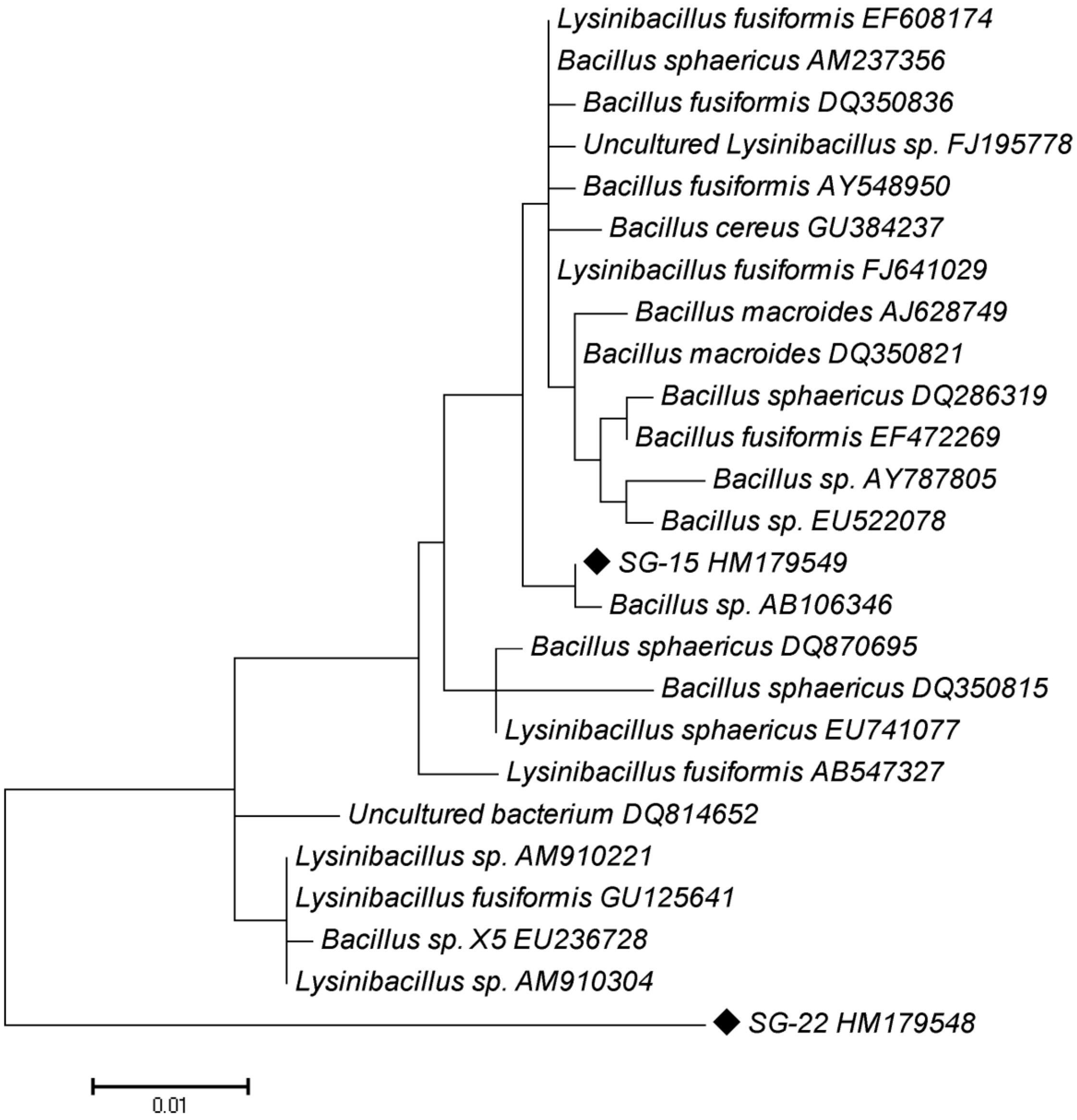


Figure 4.8c: Molecular phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-1343.9008) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al 2011).

phylogenetic profiling, additional sequences were obtained from GenBank and Ribosomal Database Project (RDP II - release 9.58) (Cole et al 2007; Benson et al 2006). Sequences were aligned using Clustal W (Thomson et al 1997) and the resulting multiple alignments were optimized visually. The evolutionary distance was calculated by Kimura 2 parameter and phylogenetic dendograms were constructed by maximum likelihood method using MEGA 5.0.1 (Dudley et al 2007).

Phylogenetic analysis of these strains showed 98% homology to *Pseudomonas* sp. (SG-14, SG-23 and SG-26), *Bacillus sp.* (SG-16, SG-19, SG-20, SG-21 and SG-29) and *Lysinibacillus* sp. (SG-15 and SG-22).

4.9 Volatilization of Se- oxyanions with selected strain

Further studies on potential of SG-26 identified as *P. aeruginosa* to volatilize selenium were carried out to estimate the extent of selenium volatilization by this strain.

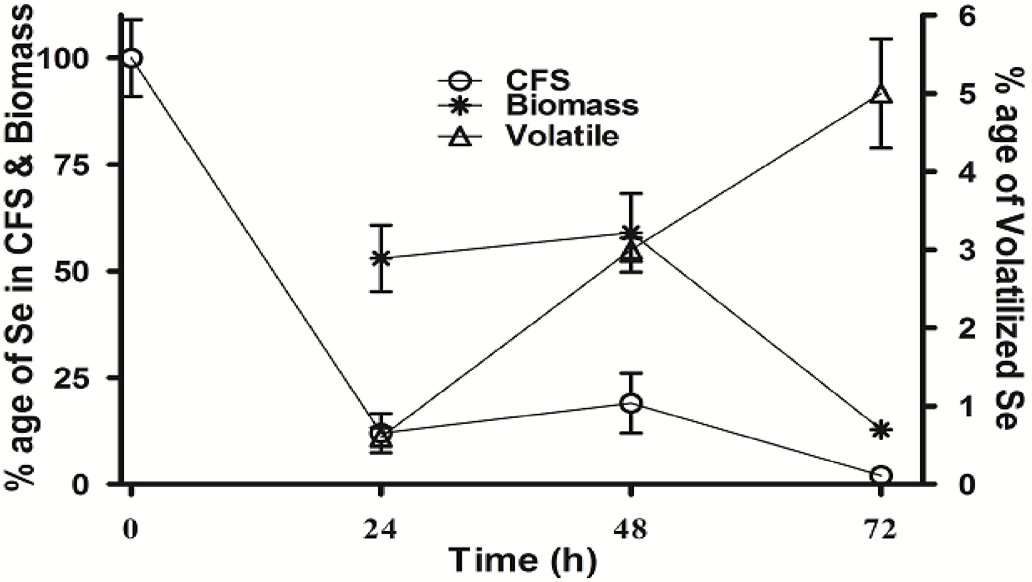


Fig. 4.9a: Concentration of total selenium in biomass, cell free supernatant and trapping solution analyzed after exposure of SG-26 to 1.28 mM selenite oxyanion

More than 50 % of the selenium was found to transformed/reduced by the isolate, when subjected to 1.28 mM of selenium as selenite after 24 h, as compared to only ~10 % in

48 h when the isolate was exposed to same concentration of selenium as selenate. However, it has been observed that overall volatilization of Se in selenite and selenate exposed cultures seems to follow a similar trend after reaching stationary phase as a substantial increased volatilization has been observed (4.7 % in selenite and 5.1 % in selenate-spiked medium) in trapping solution in the time course of 72 h.

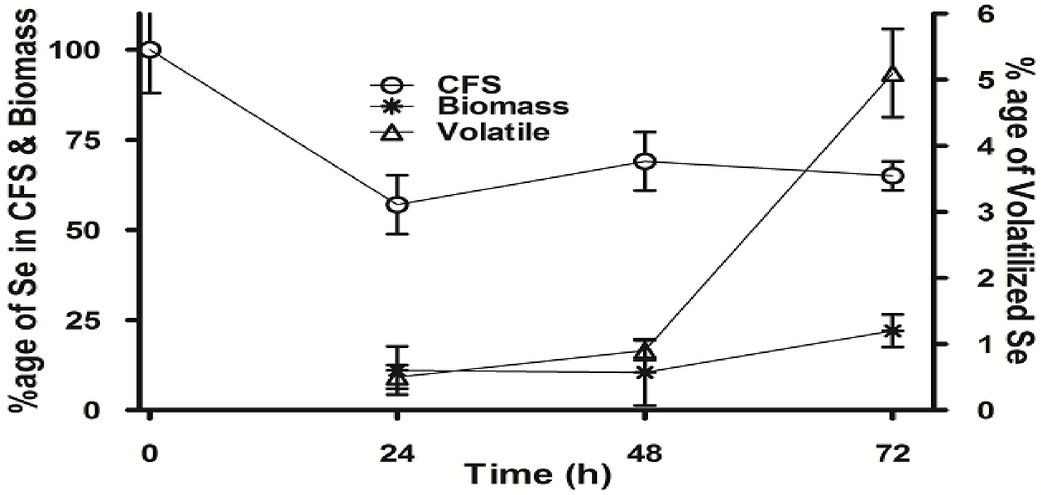


Fig. 4.9b: Concentration of total selenium in biomass, cell free supernatant and trapping solution analyzed after exposure of SG-26 to 1.28 mM selenate oxyanions

More than half of the selenium supplemented as selenite was accumulated in cell pellet in first 24 h as compared to only one-tenth in case of selenate. At the end, approximately 5-7 % of selenium was found volatilized in both selenate and selenite spiked media (Fig. 4.9). A complete mass balance of the total selenium could not be achieved due to adherence of the biomass to containers and loss of Se during the extraction and AAS estimation.

4.10 Characterization of sequestered and transformed selenium

Sequestration followed by transformation of heavy metals and metalloids through oxidation-reduction reactions play a significant role in modifying the mobility and toxicity of these elements. In the recorded XRD pattern for the culture exposed to selenite (Fig 4.10a), six major peaks assigned as 1 to 6 in diffractrogarm at 2θ values:

23.55º, 29.74º, 43.67º, 51.73º, 56.18º and 61.22º respectively were identified. All these major peaks were identical with reference data base available for elemental selenium (File no. 96-901-1649) in the literature (Keller et al 1977). A significant matching of other parameters associated with X-ray diffractogram like d-spacing (Å) and relative intensity (%) was observed in reference data sheet and present study (Table 4.10).

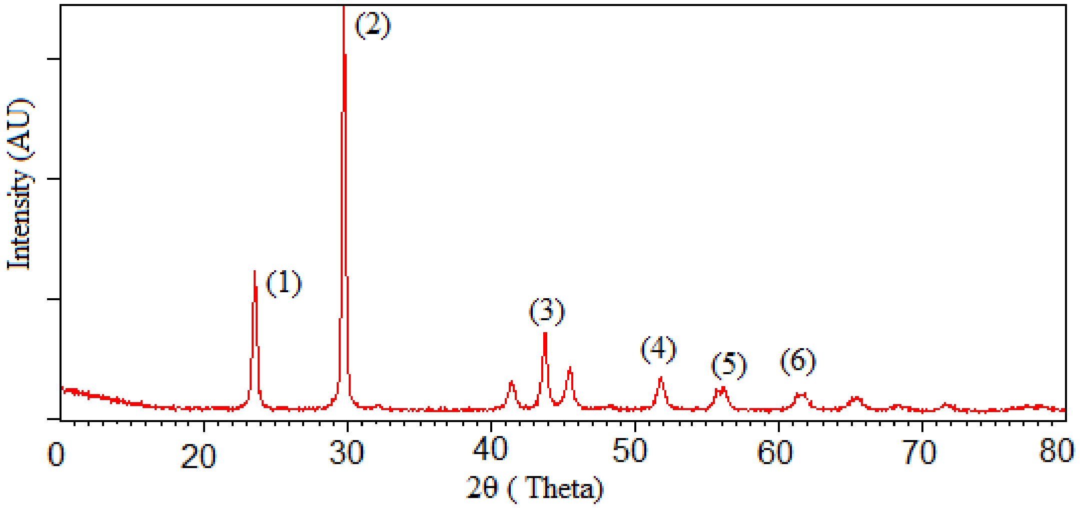


Fig. 4.10a: XRD pattern of sequestered and transformed selenium with SG-26 exposed to selenite

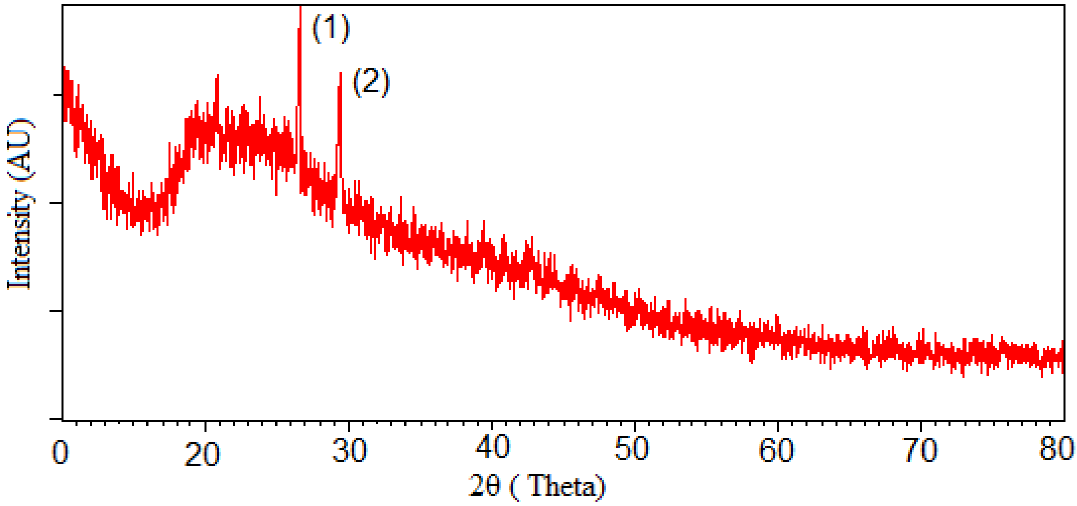


Fig. 4.10b: XRD pattern of sequestered and transformed selenium with SG-26 exposed to selenate

Table 4.10: XRD comparsion of biomass (exposed to selenite) and reference sample

Sr. No.	Biomass sample			Reference sample*		
	Position (2θ)	d-spacing (Å)	Rel. Int. (%)	Position (2θ)	d-spacing (Å)	Rel. Int. (%)
1.	23.5592	3.78	33.59	23.5592	4.14	39.50
2.	29.7492	3.00	100.00	29.7492	3.00	90.40
3.	43.6752	2.08	18.60	43.6752	2.08	29.00
4.	51.7319	1.77	7.55	51.7319	1.77	18.80
5.	56.1829	1.64	4.97	56.1829	1.64	10.10
6.	61.2269	1.51	3.79	61.2269	1.51	4.50

*Keller et al (1977)

On the basis of reference data, reduction of selenite into elemental selenium was confirmed with hexagonal crystal lattice. While a substantial difference in recorded XRD pattern was observed for the selenate exposed culture (Fig. 4.10b). No significant peak was observed in XRD. Out of the two peaks assigned as 1 and 2 with corresponding 2θ values of 26.35 and 29.38 respectively, only 29.38 showed some matching with the reference material (Table 4.10).

4.11 Se removal/volatilization experiments

Soil- microbe interactions play an important role in remediation of contaminated sites as a number of physical and chemical factors are responsible for optimum activity of indigenous microflora. Potential strains (SG-16 and SG-26) isolated from seleniferous soils were further explored for mobilization of selenium oxyanions in soils supplemented with different selenium oxyanions. The conditions of the pot experiment such as moisture content in soil and inoculum size were standardized and efficacy of individual strain (SG-16 and SG-26) for transformation of Se oxyanions in soil was then tested. These two strains were selected due to their differential response towards the

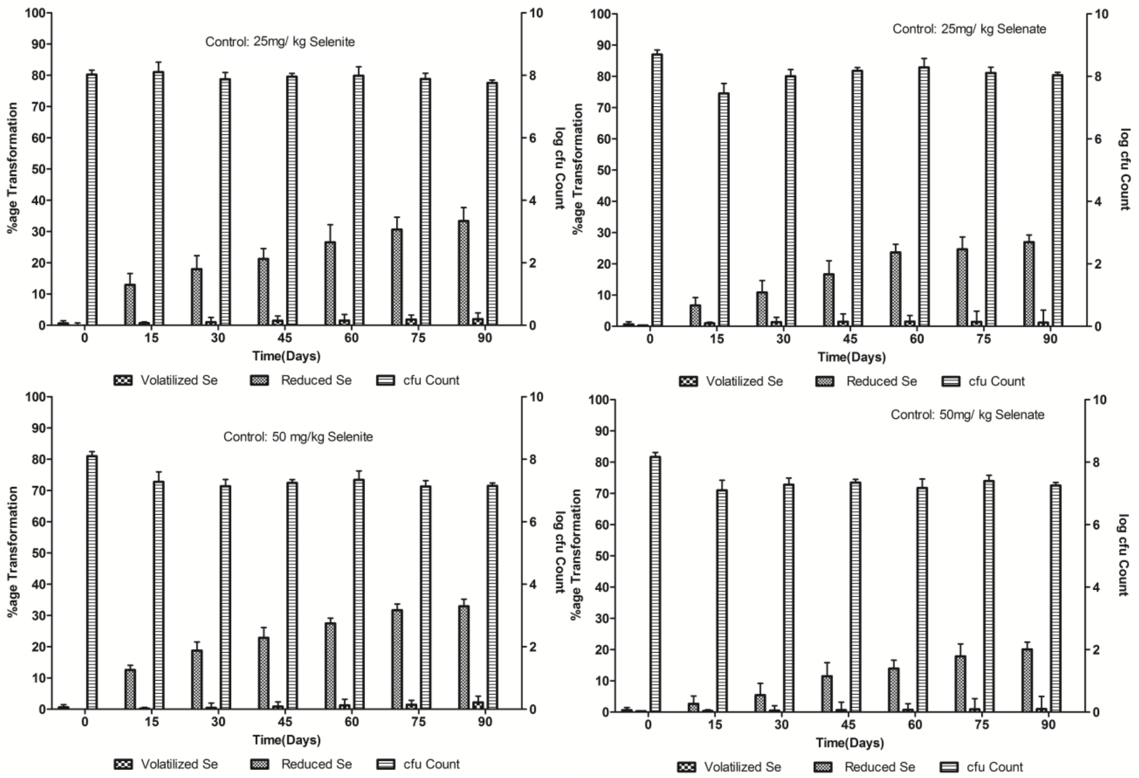


Figure 4.11a: Biotransformed (uptake and volatilization) selenium contents in soil studies upto 90 days with control

mitigation of two pronounced selenium oxyanions/species i.e. selenate and selenite present in the soils. Soil sample was analysed for various physico-chemical characteristics (pH 8.1; EC- 728 µS/cm; Organic carbon- 0.365%; Available phosphorus-0.739 kg/ha; Available nitrogen- 219 kg/ha and Available sulphur- 5.937 kg/ha). Carbenicillin and chloramphenicol resistance of the SG-16 and SG-26 respectively were taken as indicator for survival of inoculated strains and the rate of transformation was estimated based on difference between the initial concentrations of augmented Se to that of water soluble and total residual concentration of selenium in soil at different intervals in percentage.

In Se mobilization experiments, population survival that was based on antibiotics resistance profile, showed variable survival pattern dependent upon the selenium oxyanions used. An increase in colony forming units (CFU) in all selenite treatments

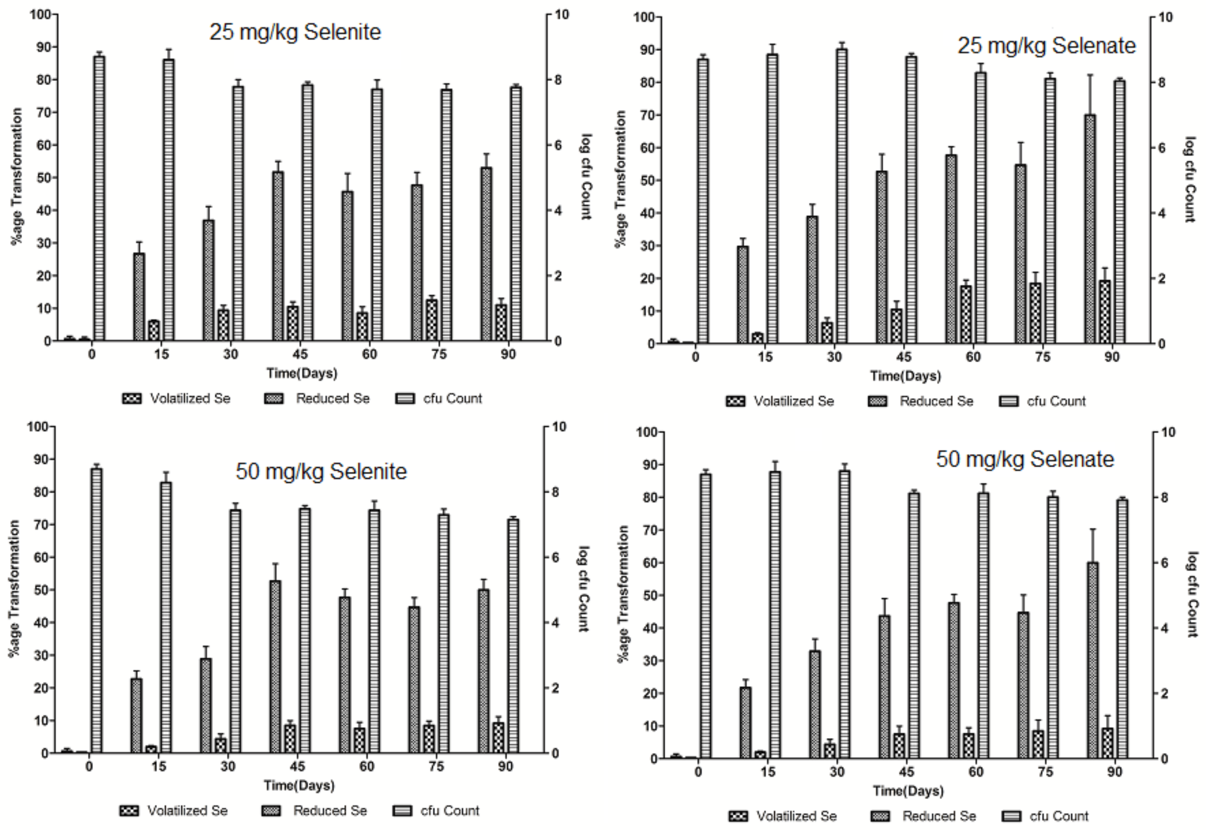


Figure 4.11b: Biotransformed (reduced and volatilization) selenium contents in soil studies upto 90 days with SG-16

was observed for SG-26 upto 45 days followed by near constant or decreased viable count in the subsequent period from 60 to 90 days studies (Fig 4.11c). A pronounced stimulatory affect of selenite supplementation at the rate of 0.32 mM was observed. While in pots containing 0.64 mM of selenite a combined both stimulatory and inhibitory effect of selenite was observed.

A significant but less increase in viable population was recorded with reference to 0.32 mM supplemented selenite. For selenate, a tenfold decrease in cfu count was observed in first 30 days followed by a marginal variation in cfu count from 45 to 90 days. In the pots inoculated with SG-16 a similar but less significant and opposite survival profile was obtained with reference to selenium oxyanions. An initial increase of almost ten

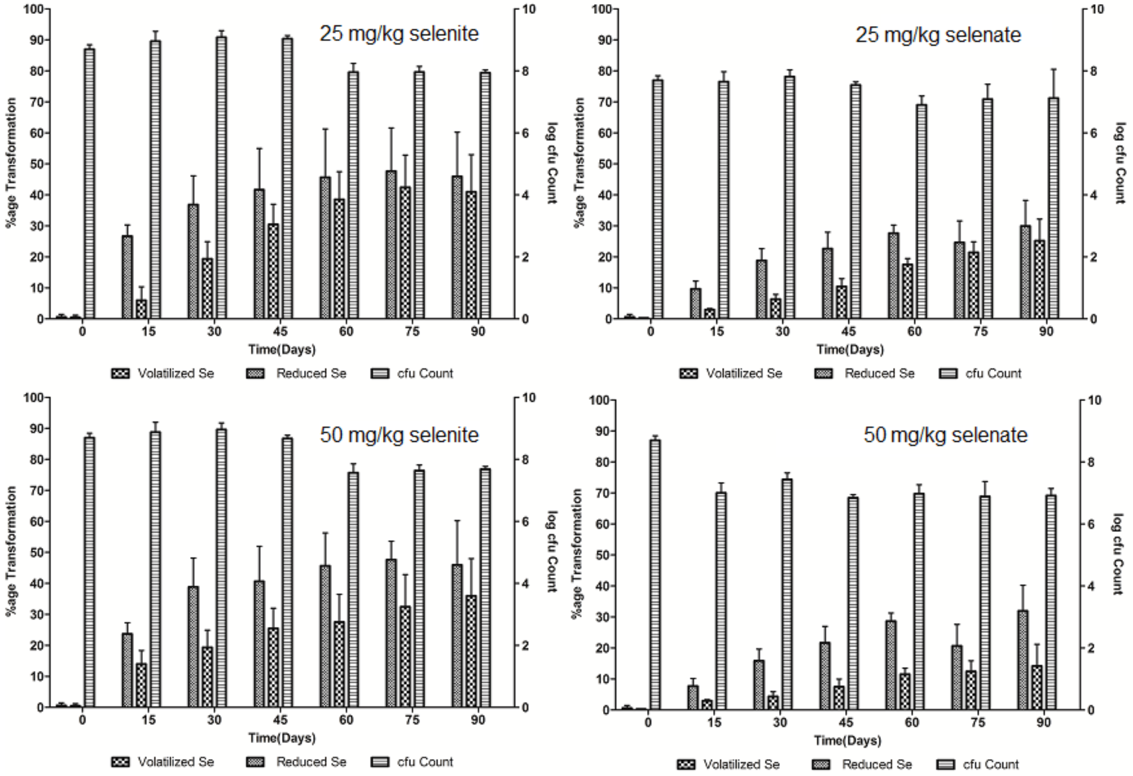


Figure 4.11c: Biotransformed (reduced and volatilization) selenium contents in pot studies upto 90 days with SG-26

times in viable count was observed in first 30 days in the pots supplemented with 0.32 mM of selenium.

In contrary to above observation, a marginal increase in viable count was observed in 0.64 mM selenate supplemented pots. A marginal variation in count of SG-16 strain was observed for selenite supplemented pots with an initial 2-3 fold decrease followed by almost a constant count. The corresponding trends in mobilization of selenium over time, on exposure to 0.32 mM and 0.64 mM of both selenium oxyanions, are also represented in Fig 4.11b. Uptake of selenium oxyanions by indigenous microbiota present in control soils supplemented with 0.32 mM selenite and selenate revealed 35% and 28% reduction respectively to water insoluble forms. A significant decrease in reduction of selenate (21%) was observed while similar reduction pattern for selenite (35%) was observed in control soils supplemented with 0.64 mM of selenite and selenate (Fig. 4.11a).

4.12 Activity assay of selenate reductase

An attempt towards the extraction and partial purification of selenate reductase enzyme from SG-26 was made as a part of this study. Different cell fractions (periplasmic and cytoplasmic) were prepared using protocol as described in methodology. No evidence of selenate reductase was obtained in periplasmic fraction while a prominent selenate reductase activity was observed in cytoplasmic fraction with reduction of selenate oxyanion. Proteins present in the cytoplasmic fractions were further separated following ammonium sulphate precipitation. Maximum reductase activity was obtained in the proteins collected after precipitation with a 60-80% concentration gradient of ammonium sulphate. Simultaneously protein contents were determined to establish partial purification of enzyme. An enzyme assay was also performed using different substrate concentrations to determine Km and Vmax. Separation of proteins in cytoplasmic and periplasmic content and subsequent enzyme assay confirmed localization of enzyme in the cytoplasmic fraction. Due to presence of large fraction of proteins in cytoplasmic fraction, appreciable purification of selenate reductase enzyme could not achieved at this juncture (Table 4.12a).

Table 4.12a: Protein contents and selenate reductase activity in different fractions

Fractions	Protein contents (mg)	Enzyme activity (Units)	Specific activity (Units/mg)	Purification (Folds)
Cell extract	984	1680	1.70	0
Cytoplasmic fraction	587	1037	1.76	1.03
$(NH_4)_2SO_4$ precipitates (60-80%)	146	362	2.48	1.45

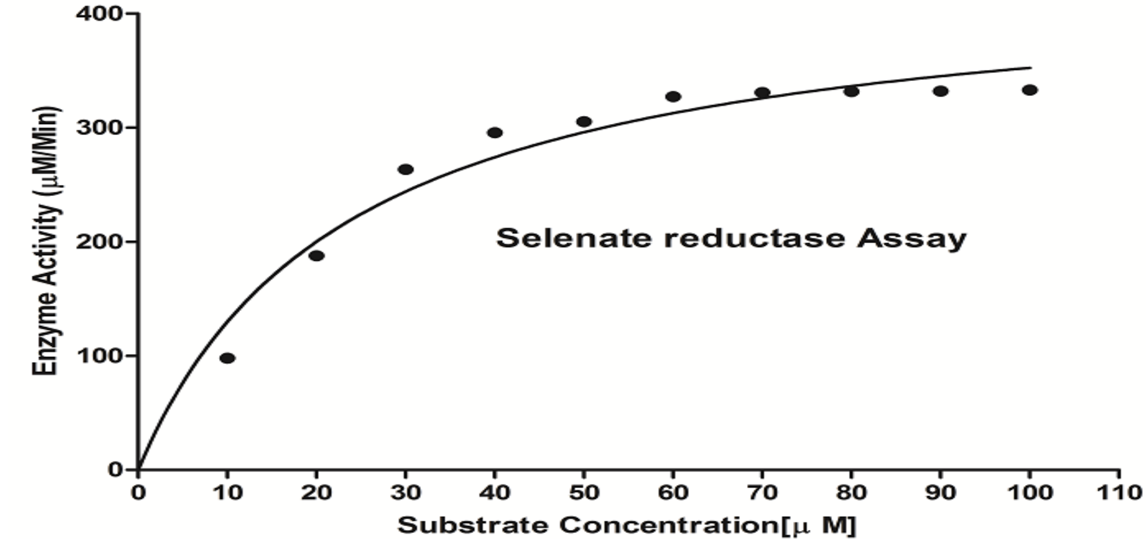


Figure 4.12: Enzyme activity at different concentrations of selenate

Further fractionation of cytoplasmic proteins with ammonium sulphate resulted in substantial purification of enzyme as indicated by the enzyme activity and protein contents in Table 4.11a. Maximum enzyme activity was observed in 60-80% concentration gradient ammonium sulphate. From the Michelis menton graph (Fig. 4.12), Km and Vmax for reductase was found to 23.55 µM and 435 µM/ min (Table 4.12b).

Table 4.12b: Km and Vmax for selenate reductase

Parameter	Values
Best-fit values	
Vmax	435 (±13.8)
Km	23.55(± 2.4)

4.13 Isolation of plasmid involved in selenate reduction

A quest for the localization of selenate reductase gene continued our attempts towards differential isolation of plasmid. But no plasmid was isolated with either protocol for isolation of micro and mega plasmid. Hence it was assumed that gene responsible for selenate reductase is present on genomic DNA.

4.14 PCR amplification of selenate reductase gene

PCR amphilification of the gene was carried out using the degenerate primers reported for chlorate reductase from *Ideonella dechloratans* with 97% gene homology with *selA* present on *Thauera selenatis* (Thorell et al 2003). Two set of primers were designed but no amplification was observed with either primer set under different set of conditions. No amplification with these primers indicated the divergence of gene coded by the strains used in the present study. The preliminary results indicate that the selenate reductase gene in SG-26 could possibly be different from the previously reported *SelA* in *T.selenatis* opening an avenue for future studies on the molecular mechanisms associated with aerobic reduction of selenate/selenite.

5.0 DISCUSSION

5.1 Characterization of seleniferous soils and Se tolerant bacteria

5.1.1 Characterization of soils

Twenty-three rhizospheric soil samples were collected from agricultural fields of selenium contaminated regions of Nawanshahr-Hoshiarpur districts of Punjab, India. These soil samples were processed and subjected to physico-chemical characterization to study the influence of these characters on the binding of selenium onto soils and sediments. In the studies reported earlier, selenium content was reported to depend on many factors e.g. pH, EC, Se speciation, competing anions, iron oxides and type of clay minerals (Dhillon and Dhillon 2003a; Seby et al 1997). Soil pH directly influences the availability of different micro-nutrients required by plants and changes in the pH of soil solutions alter the relative proportion of available and unavailable form of these minerals. Oxidation state of these nutrients in soil is strongly affected by soil pH which in turn influences their availability. The pH of soil samples collected from Barwa-Jainpur villages of seleniferous belt was found 8.6 (±0.044) (Gupta et al 2010). The oxidation state of Se present in soils depends upon pH, oxidation–reduction potential, complexing ability of soluble and solid ligands and biological interactions (McNeal and Balisterieri 1989; Van-Dorst and Peterson 1984). The pH of soil samples collected from the seleniferous sites was invariably alkaline. In poorly aerated acidic soils, inorganic Se predominates as the relatively insoluble selenide and elemental forms.

Se as selenate is highly soluble and weakly adsorbed to soil particles and therefore, mobile at most pH values (Ahlrichs and Hossner 1987). Selenate is the predominant form in alkaline soils, which makes selenium more available to plants. For instance, at pH 6; only 47% selenium was transferred from soil to ryegrass leaves and on increasing the pH to 7 selenium assimilation was found to be increased up to 70 % (Haygarth et al 1995). Present study also resulted in similar observations noted in terms of relationship between Se uptake by the microorganisms and pH of the media.

Comparatively, selenium accumulated at a higher rate and in higher concentrations in the alkaline conditions than those of acidic and near neutral pH. A positive relationship was observed in *Allium cepa* towards uptake of selenium oxyanion when grown in these soils under controlled conditions (Yadav et al 2007).

Electrical conductivity (EC) measures the salt concentration in a field as well as that of the field's constituents such as amount of sand, clay, and organic matter. In soil samples collected from different sites of selenium contaminated region, a wide variation in EC values of different soil samples was observed ranged between 345-1665 μS/cm. Clay minerals and organic matter play an important role in chemical processes controlling the mobility of Se in soils (Dhillon and Dhillon 2003b). Christensen et al (1989) reported that (64–65%) Se present in the form of selenite was fixed to clay particles followed by silt (45–61%) and sand (>5%). These observations are further supported by the hypothesis of Bar-Yosef and Meek (1987) suggesting adsorption of selenium occurring at the edges of clay particles. While assessing solid phase speciation and geochemical transformations of soil Se, Sharmasarkar and Vance (1995) observed a positive relationship between total Se and clay content ($r = 0.81$) and a negative relationship of selenium with sand content ($r = - 0.69$) in the soils, thereby indicating an association of Se with clay sized particles in some range and mine soils.

Soil particles and organic matter have negative charges on their surfaces. Mineral cations can adsorb to the negative surface charges or the inorganic and organic soil particle. Once adsorbed, these minerals are not easily lost when the soil is leached by water and they also provide a nutrient reserve available to plant roots. These minerals can then be replaced or exchanged by other cations (i.e. cation exchange). CEC is highly dependent upon soil texture and organic matter content. In general, higher CEC is always observed in soils with more clay and organic matter. Clay content is important because these small particles have a high ratio of surface area to volume. In general, the CEC of most soils increases with an increase in soil pH. Plant roots also possess cation exchange capacity. Hydrogen ions from the root hairs and microorganisms may replace nutrient cations from the exchange complex on soil colloids. The nutrient cations are then released into the soil solution where they can be taken up by the adsorptive surfaces of roots and soil organisms. Additionally, high levels of one nutrient may influence uptake of another (antagonistic relationship). For example, potassium uptake by plants is limited by high levels of Ca in some soils. High levels of potassium can in turn, limit magnesium (Mg) uptake even if magnesium levels in soil are high. Gypsum addition reduced pH hence reduced the cation exchange capacity accompanied by low potassium content in soils. In Simbli soils, the CEC values varied almost two fold (185 meq/100g and 365 meq/100g) with increase in pH of the respective soil samples of the region. Similar variations were noted in soil samples collected from Jainpur village in terms of CEC values which were found maximum in the region (489 meq/100g) as compared to gypsum treated soils of same village where CEC varied between 237 meq/100g to 254 meq/100g. The potassium levels in samples collected from Simbli region was observed to be in range of 304.6 - 660.8 kg/ha. In untreated soils of Jainpur, the concentration was 593.6 and in gypsum treated soils of the Jainpur region. The potassium level was found to be lower (256.48 kg/ha) as compared to untreated soils of other villages like Mehindpur, Rakhra and Barwa with a lowest potassium contents (264.32 kg/ha) to 521.92 kg/ha of Barwa and Mehindpur respectively. Nature of Se adsorption in soil solution also depends on amount of anions and cations as they also compete for the available binding sites. Selenate adsorption was observed to increase on amorphous iron oxide with the addition of cations, such as Cd, Cu, Co and Zn (Benjamin 1983).

Micronutrients are retained by the cation-exchange sites of soil particles, which can make some tightly bound micronutrients unavailable to crops. Micronutrients also undergo a number of other chemical and physical transformations affecting plant availability. Micronutrients are attracted and held tightly to calcium carbonate minerals, and soils with high pH have limited micronutrient availability since their reaction with these minerals increases with increasing pH. As a result, plants growing in soils containing high levels of calcium carbonate often have micronutrient deficiencies. In contrast, micronutrient availability can increase with the addition of organic matter due to the increase in exchangeable and soluble micronutrients, with the micronutrients bound to dissolve organic matter being slowly released and made available to crops. However, not all micronutrients behave the same in organic and mineral soils. The amount of phosphorus available to plants is also governed by pH of respective soil sample in which plant was grown. In Simbli region, soil phosphorus content was found to be in range of 0.228 kg/ha – 0.557 kg/ha representing the lowest in the all soil samples collected from various villages of the region. Direct influence of pH on the phosphorous contents has been observed showing that phosphorus levels decrease with increase in pH. In untreated soils of Barwa, phosphorus content was more than gypsum treated soils and was not pH dependent. Use of phosphate fertilizer, in general, has impact on the Se status of soils as selenite can be desorbed by phosphate over a large pH range and desorbed Se could be easily oxidized to selenate that in turn could easily leach out due to the higher solubility of selenate. Probably this phenomenon will be weaker in low pH soils as phosphate competes more effectively at pH above 7 (Hingston et al 1971).

In the present study, the estimation of available sulphur was also important in terms of its role in altering the sorption /accumulation of selenium oxyanions into plants. Sulphur and selenium share many physical and chemical properties along with structural similarities of their compounds. Hence, the presence of higher amount of available sulphur will compete to selenium oxyanions for their uptake by plants (White et al 2004). A wide variation in the sulphur contents of various soil samples was observed with a range between 5.06 kg/ha to 28.15 kg/ ha of Simbli and Rakhra village. These results are significantly in corroboration with the observations of estimated selenium content in the same soil samples collected from these villages. With increase in sulphur content in the soil, an increase in the selenium content of soil sample indicated the competitive uptake of these two anions by the plants.

The selenium concentration in the surface and sub surface layers of soils were 2.12 ± 1.13 mg/kg and 1.16 ± 0.51 mg/kg, respectively (Dhillon and Dhillon 2003b). However, as indicated earlier, on-going studies with different analytical techniques indicated a wide range of selenium (1.4- 6.5 mg/kg) in soil samples collected from this region (Sharma et al 2009; Gupta et al 2010). It seems that the deposition of seleniferous materials transported by seasonal rivulets from higher reaches of the Shiwalik hills and, particularly, the use of groundwater (with >290 mg Se per liter) for frequent irrigation of crops like lowland rice in this relatively arid region, leads to the development of pockets of particularly high selenium (Dhillon and Dhillon 2003a; Sharma et al 2009). Parent material of the soils is derived from Upper Shiwalik rocks comprised mainly of polymictic conglomerates of variable composition containing fragments of granite, basalt and limestone that can produce a range of weathering products (Dhillon and Dhillon 2003b). These lithologies can have a range of Se concentrations but do not normally have high concentrations unless they are sulfide-rich. These consequences could be attributed to these biogeochemical processes resulted in the mobilization of selenium into aquifers. It is clear that some process, probably associated with sub-surface water-rock-microbe interactions and the subsequent cycle of irrigation and evaporation, is responsible for continuing Se-enrichment. Hence resultant selenium accumulation in food and fodder has caused a deleterious effect on the environment with the consequences for health and socioeconomic conditions in this region (Hira et al 2004).

5.1.2 Isolation of Se tolerant bacteria

Ten isolates prominent in Se oxyanions reduction were selected for further studies out of total 30 isolates obtained from rhizospheric soils of selenium contaminated regions of Nawanshahar and Hoshiarpur districts. Their selection was based on their unique and significant pattern of transformation of selenium as selenate (Na_2SeO_4) and selenite (Na_2SeO_3). Dominantly, three different types of isolates have been identified with one group observed to reduce only selenate; the second transforming only selenite and the third reducing both the selenium oxyanions. Along with above criterion for the selection of at least one specific strain of every type, maximum uptake of selenium oxyanions was also taken in account while strains were selected for further studies. These strains were designated as SG-14, SG-15, SG-16, SG-19, SG-20, SG-21, SG-22, SG-23, SG-26 and SG-29. The potential of the obtained isolates to reduce/transform selenite is a significant phenomenon, as limited reports are available in literature on the reduction of selenite by aerobic bacteria. Studies on immobilization of selenium by facultative bacteria have been reported for a diverse variety of species such as *Rhodobacter sphaeroides* (Fleet-Stalder et al 2000), *Enterobacter cloacae* (Losi and Frankenberger 1997; Dungan and Frankenberger 2000), *Pseudomonas fluorescens* (Garibasu et al 1996; Hapuarachchi et al 2004) and *Bacillus subtilis* (Garibasu et al 1996). Recently our group also reported the isolation of bacterial strains with a potential to reduce both selenate and selenite oxyanions of selenium (Prakash et al 2009).

5.1.2.1 Biochemical characterization

Biochemical and morphological tests that were carried out indicated differences in the strains and exhibited varying sets of biochemical responses. Selected strains were grouped into two major categories of bacteria on the basis of Gram staining (A differential staining technique). Seven isolates (SG-15, SG-16, SG-19, SG-20, SG-21, SG-22 and SG-29) retained primary stain of crystal violet during Gram staining, hence were assigned as Gram positive while three isolates namely SG-14, SG-23 and SG-26 uptake red colour of counter-stain, hence were Gram negative. Two (SG-14 and SG-26) of the three gram negative strains showed biochemical properties consistent with that of typical facultative anaerobic bacteria with positive catalase and nitrate reduction tests. However, negative nitrate test indicates strong aerobic nature of SG-23. All Gram positive strains were invariably catalase positive indicating their strong aerobic character. SG-16, SG-20 and SG-29 were found positive for nitrate reduction which specifies the ability of these strains to grow under anaerobic conditions. However, in general, the results indicated similarity with that of the characteristics reported by other authors on the nature of *Bacillus* sp. (Olivera et al 2005). All above mentioned results of biochemical characterization except observations of indole test were also according to the observations made by Foldes et al (2000) in which they had considered the isolate obtained by the group, belonging to genus *Bacillus.* Morphological and biochemical features of bacterial isolates established the different metabolic pathways and special physiological conditions for a particular strain (Prakash et al 2010). In the present study this feature was observed particularly with reference to tolerance and reduction of selenium oxyanions. All these isolates were assigned to only three genera on the basis of 16S rRNA gene sequence (described later). Different isolates belonging to same genera showed variable Se reduction profile for different selenium oxyanions.

5.1.3 Antibiotic profiling

The antibiotic profiling studies were carried out for all isolates using 15 different antibiotics which gave a profile of resistance to these antibiotics. Amongst the studies carried out with different antibiotics, isolates showed sensitivity towards some antibiotics at very lower concentration, which was accompanied by formation of zone of inhibition on exposure to those antibiotics. Erthromycin, tetracycline and cloxacillin antibiotics were the antibiotics effective against all isolates. Certain other antibiotics like carbenicillin, kanamycin, ampicillin and tobramycin showed a significant antimicrobial activity on most of the isolates tested for their sensitivity towards these antibiotics. All isolates under study showed sensitivity towards carbenicillin even at low concentration (10 mcg/disc) except SG-16 which showed no zone of inhibition when exposed to this antibiotic. Hence, resistance towards carbenicillin was used as index for monitoring the fate of SG-16 augmented in the pot experiments while chloramphenicol was used for monitoring viability and persistence of SG-26 in pot trials.

Antibiotic profiling can be used to screen the inoculated population from the non-resistant indigenous population. However, horizontal gene transfer is a common phenomenon in soil and indigenous soil bacteria may be conferred with multi-drug resistance by the process thus getting selected on the antibiotic plate. Therefore, this method may be used as an initial marker for selection but molecular approach may be a better way to have a realistic estimation of the survival of inoculated population under natural conditions.

Many investigators have reported the association between the heavy metal and antibiotic resistance. The studies were based on the concept that in metal stress environment, bacterial cells acquire resistance either by mutation or by transfer of resistant genes between bacteria (Verma et al 2000; Lawrence 2000; Bhattacherjee et al 1988). Even within a single species, strains can exhibit different degrees of antibiotic resistance. Bacterial isolates belonging to the genera *Bacillus, Corynebacterium, Aeromonas,* and *Enterobacter* isolated from a municipal waste landfill in Durham, NC were generally found to be sensitive to tetracycline and chloramphenicol (13 of 14 isolates) and generally resistant to ampicillin (9 of 9), erythromycin (10 of 14), streptomycin (8 of 14), with 3 of 14 isolates having multiple resistance to the last three antibiotics (Nwosu and Ladapo 1999). Isolates belonging to *Bacillus* species screened for different antibiotics showed resistance of 35-40 μg in case of ampicillin, 15-20 μg against tetracycline and 10-30 μg in streptomycin and kanamycin (Kannan and Lee 2008). Similarly, in our study of antibiotic profiling as depicted by resistance of isolates against carbenicillin, a relation between heavy metal and antibiotic resistance is anticipated due to significant tolerance of the test strains towards diverse variety of antibiotics tested.

5.1.4 Multiple metal tolerance studies

Multiple heavy metal tolerance of microbial isolates could be potentially used in bioremediation of heavy metal-contaminated environment (Mohamed and Abo-Amer 2012). A wide variation in minimum inhibitory concentration of different metals was observed for the all the isolates. All of these strains were invariably tolerant to high concentration of selenium oxyanions. Besides selenium, some strains showed substantial tolerance level for other heavy metals including lead and mercury. SG-15 has already been reported for mercury sequestration and transformation (Gupta et al 2012) while other strains were also explored for bio-sequestration of lead (un-published data). Burton et al (1987) have reported the MICs of selenite, selenate, arsenite, arsenate, tellurite and tellurate in different bacterial species isolated from selenium polluted site. A significant level of tolerance was observed in almost all the isolates for oxyanions of other metalloids. Recently Mohamed and Abo-Amer (2012) isolated bacterial and fungal strains from soil and phylloplane with multiple metal tolerance and variable minimum inhibitory concentration of these metals for different isolates. Similarly bacterial isolates obtained in the present study from seleniferous soils with multiple metal tolerances may be exploited in managing the heavy metal pollution through bio-sequestration of these metals along with lead within biomass.

5.1.5 16S rRNA gene analysis

PCR provides a sensitive and specific method of identifying a known DNA sequence. To further confirm, the genetic variability in the representative isolates, 16S rDNA of nine isolates was amplified and sequenced. All the nine isolates showed 96-99% similar with closely related matches as shown in Fig. 4.8a, 4.8b and 4.8c. The biochemical characteristics and 16S rDNA results were in concordance and showed variability at each level. Phylogenetic analysis revealed that the selenium-tolerant microorganisms in present study belong to *Pseudomonas* and *Bacillus* group. Members of the genus *Bacillus* are in general well known for conferring resistance to various other metals and metalloids (Prakash et al 2010; Stolz et al 2006; Nies 1999) as was shown by isolates under study towards Se oxyanions upto concentration of 1.28 mM. Similar studies were reported by Ghosh et al (2008) for selenium tolerant microorganism and stated that these were common soil bacteria belonging to the classes of *β*-Proteobacteria and *Bacilli* and thus offer an insight into the metal tolerant bacterial population present in tropical selenium contaminated sites. Phylogenetic analysis of a Gram negative bacterial isolate from seleniferous soils from India revealed its identity to *Pseudomonas aeruginosa* (Gupta et al 2010). A number of reports appeared in last decade with similar observations on Gram negative, aerobic bacterial strains with efficient tolerance and reduction potential for selenium oxyanions (Hunter and Manter 2009; Hunter and Manter 2008). A strict aerobic bacteria *Pseudomonas sp*. strain CA5 with a potential to reduce selenite to elemental red selenium was characterized and identified using 16S rRNA gene sequencing and BLAST after amplification with universal primers. Above reported strain selectively tolerated and reduced selenite when exposed to even 150 mM concentration (Hunter and Manter 2009). Strain KMT was isolated with unique metabolic abilities to respire selenate as the electron acceptor using acetate as the carbon substrate. 16S rRNA gene phylogeny revealed the identity of this isolate as *Pelobacter seleniigenes* sp. *nov.* (Narasingarao and Haggblom 2007). An earlier report also described an anaerobic betaproteobacterium *Thauera selenatis* with capability of respiring selenium oxyanions anaerobically (Rech and Macy 1992). Similarly Dhanjal and Cameotra (2010) isolated a Gram positive bacterial strain from coalmine soils of west Bangal, India with a potential to reduce even 0.5 mM selenite concentration with formation of elemental selenium. Later on this strain was characterized and identified as *Bacillus cereus* using 16S rRNA gene homology analysis. Fisher and Hollibaugh (2008) also isolated and identified a *Bacillus* strain having low G+C contents from Mono Lake, California, using 16S rRNA gene sequence. These observations are in corroboration with the present report when selenium oxyanions reducing feature has been observed only in these two representative genera in the soils.

5.2 Growth and selenium sequestration studies

The bacterial isolates were tested for growth in the presence of different concentrations (0.32 mM and 0.64 mM) of Na_2SeO_4 and Na_2SeO_3 respectively. Although the control culture (0 mM) showed relatively faster growth rate, similar levels of growth were observed with all the strains in the presence of selenate and selenite over a period of 12 h. The cultures grown in the presence of sodium selenate and sodium selenite turned red as growth progressed indicating the reduction of selenium oxyanions to elemental red selenium. These observations are in corroboration with the previous reports on reduction of selenium oxyanions with formation elemental selenium (Hunter and Manter 2009; Buchanan et al 1995). Out of total 10 strains, six strains (SG-14, SG-16, SG-19, SG-20, SG-21 and SG-29) showed comparative growth profile in the presence of both selenium oxyanions (0.32 mM and 0.64 mM) along with control. A comparative growth profile of a *Bacillus* strain was also reported by Dhanjal and Cameotra (2010) on exposure to different concentration of selenite (0.5 mM-10 mM) with respect to control. Three strains (SG-15, SG-22 and SG-23) exhibited a prolonged lag phase during growth in selenate and selenite supplemented broth with reference to their respective controls (without selenium). Hunter and Manter (2009) reported a substantial decrease in growth on exposure of selected strain to 50, 100, and 150 mM selenite by 28, 57, and 66%, respectively. While same group reported a bacterial strain *Tetrathiobacter kashmirensis* that tolerated and converted upto 64 mM selenite efficiently without any observable effect on growth (Hunter and Manter 2008). Inhibition of selenite reduction was observed in *Anaeromyxobacter dehalogenans* on exposure to selenite at elevated concentrations (He and Yao 2010). Growth profile of SG-26 was similar to control when exposed to selenite while a limited growth was observed in selenate supplemented medium even after prolonged incubation. The reduction of selenate into elemental selenium is a two- step reaction, in which selenate is reduced to selenite and then possibly to Se (II) and/or red amorphous elemental selenium (Altringer et al 1989). In the present study, two isolates (SG-16 and SG-26) followed a unique and opposite reduction pattern with selenite and selenate oxyanions of selenium. SG-16 appreciably reduced and accumulated selenate over a time period of 24 h with an initial marginal reduction over a period of 8 h with limited accumulation and reduction of selenite occurring in biomass grown in selenite supplemented broth. An efficient reduction of selenite oxyanions occurred in case of SG-26 over a period of 24 h with little reduction of selenate oxyanion in the biomass. Almost 60% of selenite was transformed and found to be accumulated and reduced in the biomass as compared to 20% in case of selenate supplemented biomass. The intensity of red colour which was used as a measure of Se reduction, was marginal compared to selenite. We reported the production of a persistent red colour on exposure to selenite with no red coloration using SNT-SG1 (Gupta et al 2010). Marginal growth was observed in broth supplemented with selenate while exposure to selenite did not affect the growth of the organism at any of the Se concentrations tested.This is presumably due to the bypassing of selenate transformation through the normal reduction pathway leading to formation of elemental selenium reported in our previous reports (Yadav et al 2008). Similarly, Higashi et al (2005) reported the reduction of selenate into selenite and organo-selenium compound after selenium speciation. This data also supported our observation on selenate reduction by SG-26 as selenite and organo-selenium compounds are also colourless.

5.3 Effect of pH on growth of organism and selenium reduction

In formulation of bioremediation approaches, it is important to understand the behaviour of soil bacteria towards the presence of Se in prevailing soil and water conditions. Behaviour of bacteria also varies in presence of sulphates, phosphates, chlorides, etc., which would also influence the pH directly or indirectly, under different temperature conditions, thus altering the growth and Se biotransformation potential of *in situ* bacteria. The isolates in the present study indicated the potential to reduce both oxyanions in neutral to alkaline range although growth of the strains and subsequent reduction was more prominent in lower alkaline range (pH 6- pH 8). Similar observation of pH range of 7-10 was found in case of *Bacillus patagoniensis,* isolated from alkaline soils of north eastern Patagonia, Argentina (Olivera et al 2005). All other strains except SG-14, SG-16, SG-19, SG-20, SG-23 and SG-29 showed reduced growth at early exponential phase. However, a variable recovery of the growth profiles was noted at late exponential phase with each oxyanions as well as type of strain except SG-26 at pH 10. A marginal period of acclimatization was followed up by rapid growth and reduction of selenite at extreme alkaline pH. A prolonged lag phase was observed in growth profiles under selenate supplemented conditions, at the extreme alkaline pH values used in the present study. In contrast, SG-14 and SG-16 showed a recovery similar to the control when exposed to both selenite and selenate oxyanions. Even at neutral pH, some strains (SG-23, SG-26 and SG-29) did not recover completely after prolonged incubation when exposed to 0.64 mM selenate. Selenite showed no effect on growth profile at pH 10 in case of SG-16 and SG-29 where as in case of other strains, an inhibitory effect was prominent till early exponential phase followed by complete recovery at late exponential phase in all other strains. On exposure to acidic conditions (pH 4), no reduction was observed by any isolate. Reports in other parts of the world, mainly from seleniferous soils and sediments of Kesterson reservoir, California (Wu 2004) in addition to those from soil of China (Weng and Huang 2004) and Taiwan (Wang and Chen 2003) showed presence of selenium tolerant bacteria dominantly in alkaline soils. The range of pH permitting growth of *Bacillus cereus* in laboratory media has been reported to be pH 4.9 to 9.3 (Goepfert et al 1972). The effects of pH on growth of *Bacillus cereus* vary with strain and growth medium used. However, Goepfert et al (1972) indicated that extreme pH on either acidic or alkaline range values are expected to prevent the growth of *B. cereus*. In case of *Enterobacter taylorae*, which can reduce selenate as elemental selenium, the activity was observed to be significant in high salt and high pH contaminated agricultural drainage water (Zahir et al 2003). *In-vitro* studies on *Stenotrophomonas maltophilia* SeITE02, a new bacterial strain used for bioremediation of selenite contaminated environmental matrices also revealed the inhibitory action of acidic pH on uptake and reduction of selenium (Antonioli et al 2007). In relation to the reported pH profiles of various bacterial species, the current observations indicate that the isolates of the tropical alkaline soils can sustain growth at higher alkaline levels (pH 10) than the reported species and also tolerate impact of Se in ambient environments.

5.4 Effect of temperature on growth of organism and selenium reduction

In order to understand the mechanism of the bacterial bio-transformations and their application in field, it is pre-requisite to examine their potential to exist and various physico-chemical conditions such as temperature. On exposure to different temperature conditions, isolates, obtained in the present study, showed variable behaviour in terms of selenium reduction. In all strains except SG-16, SG-20, SG-21 and SG-23, extended lag phase was observed at 20ºC in control, where as prominent inhibition of growth was observed with Se^{+6}. While growth profile in the presence of either of the Se^{+4} was similar to control at that particular temperature. At 30ºC, which is optimal for these organisms, presence of selenate resulted in extended lag phase in SG-19 and SG-21, with recovery of growth after 8 h. No recovery was observed in SG-26 exposed to selenate. The growth profiles of some strains at 40ºC were significantly faster than the optimum temperature (37°C) with concomitant reduction of selenium oxyanions. At 50ºC, there was no growth observed either in control or under selenium oxyanions supplementation. Similar observations were also reported by Fermanian et al (1994), where no *Bacillus* strain was observed to grow at 50°C; and maximal growth-permissive temperatures were in the range 46-50°C.

The above observations are of significance during design of selenium bioremediation protocols using *in situ* soil bacteria, as different bacteria exhibit varied behaviours in similar soil conditions. With tropical soil temperature, reaching higher than 35ºC in certain areas of this seleniferous belt, the persistence and selenium mobilizing activity by these organisms would invariably be influenced by the ambient temperature. To date there are no reports on temperature-dependent modulation of growth of Se tolerant microorganisms with only limited reports on anaerobic isolates obtained from temperate soils (Zahir et al 2003). Based on the present observations, the growth profile of bacterial strains isolated from selenium impacted soils was demonstrated, indicating a maximum tolerance of temperature up to 45°C by certain strains (Gupta et al 2010). Hence growth associated Se-transformation is strongly influenced by temperature.

5.5 Selenium volatilization

Certain metals in the biological system are converted to volatile methylated forms. Two dominant species i.e. dimethylselenide and dimethyldiselenide have been reported in case of selenium. These two compounds are volatile and the bacteria may get rid of toxic concentrations of Se through channelizing excess of this element to its volatile forms followed by its removal from the micro/macro ecosystem. In the present study, SG-26 was examined for its volatilization potential. Almost 5 % of both the selenium oxyanions were volatilized by the isolate after 72 h of incubation (Fig. 4.9a and 4.9b). Volatilized selenium content as analyzed by GF-AAS, was found to be 5.1 %

and 4.7 % during selenate and selenite exposure at 1.28 mM (Gupta et al 2010). Volatilization of selenium in the form of dimethyl selenide has been observed as a result biological processes and is thought to be a protective mechanism used by microorganisms to detoxify their surrounding environment (Frankenberger and Karlson 1994; Frankenberger et al 2004). The constructed wetland effectively removed Se from selenite-contaminated oil refinery wastewater (Hansen et al 1998). Almost 89 % of the Se removal with 10−30 % by biological volatilization was observed. Dimethylselenide (DMSe) has been identified as the major biological metabolite of Se methylation (Dungan and Frankenberger 1999). Volatilization of mercuric chloride by a bacterial strain isolated from selenium impacted soils further strengthen the speculation of dispersal of these toxic metals in far away regions (Gupta et al 2012).

The biotransformation of Se to volatile Se compounds is considered a major process in the movement of Se in the environment (Haygarth 1994). Although the biological significance of Se methylation is yet to be elucidated, once volatile Se compounds are released to the atmosphere, Se loses its hazardous potential until it precipitates through rain. Therefore, microbial transformations of Se to less toxic volatile forms may ultimately prove to be an effective approach to remediate seleniferous environments. Rhizospheric bacteria associated with Indian mustard plants were observed to enhance selenium reduction and volatilization by several folds (de Souza 1999). Climatic conditions such as precipitation and temperature significantly affects the rate of selenium volatilization in constructed wetlands. Volatilization within a surface flow wetland has been observed to vary from approximately 9% in the winter to over 50% in the summer months in constructed wetland in California (Johnson et al 2009; Terry 1992).

At molecular level over-expression of bacterial thiopurine methyl transferease (bTPMT) gene conferred resistance in *E. coli* towards selenium oxyanions along with methylation of inorganic and organic selenium into dimethylselenide (DMSe) and dimethyldiselenide (DMDSe) (Ranjard et al 2002). A novel genes encoding COQ5 methyltransferase (BoCOQ5-2) in ubiquinone biosynthetic pathway has been isolated and found to specifically promote selenium volatilization but not sulfur in both bacteria and plants. Transgenic bacteria expressing *BoCOQ5-2* produced over 160-fold volatile selenium compounds when they were exposed to selenate in addition to enhanced tolerance to selenate and selenite (Zhou and Li 2010).

5.6 Characterization of intracellular selenium

Significant work has been documented on different mechanisms of transformation of selenium oxyanions by different bacterial strains (Sarathchandra and Watkinson 1981; Doran and Alexander 1977; Barkes and Fleming 1974). Reduction of soluble selenium forms to insoluble forms such as elemental selenium (Se^0) or assimilation into organic components of bio-molecules such as seleno-amino acids has been reported in various microorganisms (Oremland 1994; Thompson- Eagle and Frankenberger 1992). SG-26 was exploited for characterization of intracellular selenium sequestered by the biomass on exposure to different selenium oxyanions. With selenite, formation of elemental selenium was confirmed from the XRD pattern of recorded diffractogram. Kessi et al (1999) also reported formation of elemental selenium by phototrophic bacterium *Rhodospirillum rubrum* on its exposure to selenite. Losi and Frankenberger (1997) also confirmed the formation of elemental selenium by XRD with a possibility of additional reduced products of selenium such as selenoamino acids present in the biomass. No significant peaks were obtained for elemental selenium in recorded diffractrogram of biomass harvested from selenate exposed culture. de Souza et al (2001) reported accumulation of a mixture of selenate and selenomethionine using Se K-edge X-ray absorption spectroscopy of *Halomonas* sp. exposed to selenate oxyanions. This observation was consistent with a speculation that selenate was assimilated via sulphur assimilation pathway. Significant difference in the bioacccumulated species of selenium was observed in purple nonsulphur bacterium, *Rhodobacter sphaeroides* on its exposure to different selenium oxyanions (Fleet-Stalder et al 2000). Se K-edge X-ray absorption spectroscopy showed accumulation of approximately 97% of supplemented selenite in form of elemental selenium with only

13-15% of Se (0) in selenate supplemented culture. In case of selenate, maximum selenium was accumulated as dimethyl selenide and selenomethionine. These results are in corroboration with the present observation which indicated a unique transformation pattern of the isolate for different selenium oxyanions.

5.7 Removal of selenium from soils bioaugmented with bacterial strains

Out of the ten isolates obtained in this study, two potential bacterial strains (SG-16 and SG-26) with a unique reduction pattern were examined for their viability and Se mobilizing potential in Se enriched soils in competition with the native microbiota. Microcosm studies were carried out *in situ* in pots supplemented with two different concentrations (0.32 mM and 0.64 mM) of selenate and selenite. The results indicated that under natural conditions of soil, effective mobilization of Se was obtained in bioaugmented soils where these isolates were inoculated. Transformation of selenium oxyanions by different microbiota in soils supplemented with selenite and selenate oxyanion revealed different pattern. Volatilization of selenium oxyanions was conferred indirectly on the basis of an appreciable decrease in total selenium estimated in these controlled microcosm studies in comparison with control. In SG-16 supplemented pots, almost 80% and 50% removal of selenate and selenite respectively at both concentration (0.32 mM and 0.64 mM) over 90 days. In case of SG-26, almost 40% of selenium was volatilized in the soils inoculated with SG-26 irrespective of the selenium oxyanions and their concentrations. Mobilization of selenate was comparatively less than the selenite in SG-26 supplemented soil samples. Martin et al (2009) reported *in situ* microbial reduction treatment at full scale at Homestake Mining company`s Grants New Mexico reclamation project. More than tenfold decrease (50 to 100 µg/l to less than 5 µg/l) in bio-available forms of selenium was observed by adding nutrient solutions (Sobolewski 2005). Removal of selenium by *in situ* microflora from initial concentration of 460 µg/l to less than 10 µg/l was observed to be largely influenced by the addition of nutrients. Addition of nutrients especially phosphate leads to algal blooms that serve as source for organic carbon facilitating bacterial reduction (Martin et al 2009).

Further, the viable population of these cultures was in the range of 3 – 8 (log value) after 120 days. Plots showing higher transformation of Se oxyanions also showed more microbial presence. This is in concurrence with literature, as it has always been seen that rhizospheric soil harbours microorganisms capable of enhanced xenobiotic degradation abilities. Rhizospheric bacteria has been observed to enhance selenate uptake in different plant species two to threefold faster than in plants with inhibited rhizospheric activity (de Souza et al 1999; 2000). Almost complete removal of selenium after 120 days was observed in soils supplemented with 2.5 mg/ kg and 5.0 mg/kg of selenite with a comparative low removal rate on exposure to same concentration of selenate (Prakash et al 2009). The survival of these inoculated organisms has been studied by culturing them in media supplemented with antibiotics. Antibiotic plating is the most common method used for monitoring of augmented microflora in the contaminated site (Yang et al 2005; Mallick et al 1999). Based on survival of carbenicillin and chloramphenicol resistant population, maximum survival rate was observed in soil supplemented with 25 mg/kg of selenate and selenite during 90 day period with SG-26 and SG-16 respectively. Mishra et al (2004) monitored the survivability of strains of *A. baumannii* using dilution plating method with selective antibiotics and reported three fold decrease in microbial population over 90 days, confirming the stability of modified organism in soil. Hirkala and Germida (2004) observed the survival of an antibiotic-resistant *Pseudomonas putida* in field and microcosm soils over 49 days upto 7 weeks with a significant residual survival in microcosm soils as compared to field soils while higher cell densities in the rhizosphere was found when inoculated with seed coating. Multiple antibiotic resistances were also observed for *Pseudomonas aeruginosa* (Okoh 2003) and a number of other bacterial isolates from soil. Microorganisms can take up the elements and accumulate them in their biomass via intracellular sequestration or precipitation, or adsorb them onto cell walls and exopolymers released into their surroundings (Zaidi and Musarrat 2004). Similarly, the fate of selenium in contaminated soils is linked to the activity of soil bacteria, which is the primary method by which soluble and biologically active forms of selenium can be effectively assimilated and removed from ambient environment (Stolz et al 2002), as observed in the present study. The role of rhizosphere processes in removal of inorganic contaminants is, largely unexplored and needs to be addressed before the potential of this approach can be properly evaluated. Soil microorganisms are known to convert some metals and metalloids (i.e. arsenic, boron, antimony, selenium, tin, tellurium, lead, mercury) to their volatile species. In the case of selenium, the volatile methylated species are less toxic than inorganic forms (Wilber 1980). This microbial conversion is usually considered as a detoxification mechanism by which the microorganisms decrease the toxicity of the surrounding microenvironment. However, the use of micro-organisms for bioremediation requires an understanding of all physiological, microbiological, ecological, biochemical and molecular aspects involved in pollutant transformation (Iranzo et al 2001).

5.8 Localization of selenate reductase enzyme

Biological reduction of selenium oxyanions is an important strategy for the remediation of seleniferous sites. Although significant studies have been carried out towards the isolation and characterization of bacterial strains responsible for the immobilization of these selenium oxyanions in soils and sediments, limited data is available on the enzyme(s) associated with reduction of these oxyanions in aerobic species. Unlike insoluble elemental selenium, selenate is difficult to be removed from contaminated water due to its high solubility (Schroder et al 1997). The biological reduction of selenate to elemental selenium is therefore of interest as a potential strategy for bioremediation. Little is known about the biochemistry of selenate reduction by bacteria. Selenate may be reduced by a specific selenate reductase or, alternatively, by an enzyme of the nitrate reduction pathway. SG-26 was examined for extraction and ammonium sulphate fractionation of selenate reductase and kinetics studies were carried out. Crude form of selenate reductase was extracted from cytoplasmic fraction confirming the intracellular localization of enzyme. Schroder et al (1997) has purified selenate reductase from periplasmic space of *Thauera selenatis* under aerobic conditions. The enzyme was observed a complex enzyme with three heterologous subunits and apparent molecular weight of 180,000. The α, β, and γ subunits were 96 kDa, 40 kDa, and 23 kDa, respectively, in size. Selenate reductase isolated from *Thauera selenatis* also contains molybdenum, iron, and acid-labile sulfur as prosthetic group constituents along with one cytochrome *b* per αβγ complex. The K_m and $V_{\max}$ for selenate was observed 16 μM, and 40 μmol/min/mg of protein respectively. In the present study, approximately two fold increase in enzyme activity was observed in 60-80% ammonium sulphate fraction. Km and Vmax of the enzyme were found in the range of 407 to 463 and 18.62 to 28.48 respectively at 95% level of confidence.

5.9 Localization of selenate reductase gene

Further elucidation of selenate reduction pathway was continued in terms of its localization on plasmid or genomic DNA. No plasmid DNA was obtained in selected isolates hence gene was present on genomic DNA. PCR amplification of the gene was carried out using the degenerate primers reported for chlorate reductase from *Ideonella dechloratans* with 97% gene homology with *serA* present on *Thauera selenatis* (Thorell et al 2003). No amplification with these primers indicated the divergence of gene coded by the strains used in the present study. Selenate reductase gene so far has been characterized only in *Thauera selenatis* with highly conserved sequence with limited homology with aerobic strains.

To summarize, the present study focussed on use of microorganisms towards the mobilization of selenium oxyanions present predominately in selenium contaminated sites. As a part of this study, physico-chemical characterization of seleniferous soils of Punjab was carried out along with biological transformation of bio-available selenium species in to inert forms. Different bacterial strains belonging to three genera (Pseudomonas, Bacillus and Lysniformis) have been characterized in terms of their morphology, physiology, biochemical properties along with antibiotic

sensitivity profile. Further, selected microorganisms were exploited for growth associated reduction and volatilization of selenium oxyanions. These strains were categorised on the basis of their unique behaviour towards these oxyanions. These isolates showed a wide tolerance to pH, temperature and salt concentrations, hence may serve excellent tools for remediation of selenium contaminated sites. Survival of these organisms in microcosm and efficient transformation of selenium oxyanions further strengthened these findings. An intracellular enzyme in cytoplasmic fraction has been characterized with a potential to convert selenate into selenite. Further work may be pursued in the complete purification and structural characterization of this enzyme. Limitation of the present study lies in validation of experimental findings at field trials which has not been carried out.

In summary, the work aimed at examining the following objectives

- **Physico-chemical characterization of seleniferous soils of Nawashahr district**
- **Isolation and screening of selenium tolerant bacteria in seleniferous soils**
- **Biochemical and genetic studies of selenium mobilization**

The salient findings in the study are as follows:

1. The soil samples (23) examined from the Se-impacted pockets of agricultural lands under study were found to be alkaline in the range of 7.00-8.08, electrical conductivity (EC): 345-1660 μS, cation exchange capacity: 185- 489, available N: 150-351 kg/ha; available P: 0.22-0.91 kg/ha; available sulphur: 6-28 kg/ha and potassium levels were found 256-660 kg/ha respectively.
2. The soil samples obtained from seleniferous region contain 0.7-2.9 mg/kg of selenium in different soil samples. The selenium levels observed in the soil and ground water in this region are significantly high.
3. Thirty bacterial strains were isolated from these soil samples and were tested for biotransformation potential for different selenium oxyanions. On the basis of tolerance and unique reduction pattern of these isolates, ten strains were selected for growth and sequestration studies.
4. Similar growth trend was observed in SG-14, SG-16, SG-19, SG-20, SG-21 and SG-29 test strains in the presence of either of the selenium oxyanions viz., selenate and selenite over a period of 12 h as determined by optical density measurement.
5. SG-15, SG-22 and SG-23 exhibited a prolonged lag phase during growth in selenate and selenite supplemented broth with reference to their respective controls (without selenium oxyanion).
6. Even after sufficient growth of SG-26 in selenate supplemented broth, intensity of red colour which is an indication of Se reduction, was marginal when compared to cultures grown in selenite indicating that transformation of selenate might bypass the normal reduction pathway leading to formation of elemental selenium.

7. All the test strains except SG-16 and SG-26 showed more than 90% sequestration and reduction of selenium oxyanions in the biomass irrespective of the discrimination of selenium oxyanion used.

8. Morphological and gram characteristics of isolates revealed that isolates belongs to both Gram+ve and Gram-ve groups with same rod shaped cell morphology.

9. Almost all the selected isolates gave at least one or more different results for the biochemical test performed. This indicates all the strains are in variably different and might be belonging to same genera but different species.

10. All the isolates were resistant to more than one antibiotic with a variable resistance pattern with respect to different antibiotic. Antibiotic profiles of isolates further strengthen our above speculation that all the isolates are not same.

11. Bacterial isolates obtained in present study showed resistance for other heavy metals. Hence, these isolates may be exploited for the remediation of multiple heavy metal contaminated sites.

12. The isolates in the present study reduced both oxyanions in neutral to alkaline range of pH although growth and reduction was more prominent in lower alkaline range

13. In the present study, isolates showed variable behaviour in terms of reduction potential on exposure to different temperatures. Slow growth and delayed reduction was noted at lower temperature range (20°C) and at higher temperature (50°C), decline in growth was observed along with absence of Se reduction.

14. The molecular identification based on 16S rDNA analysis lead to characterization of isolates. All isolates belong to *Pseudomonas*, *Bacillus* and *Lysnibacillus* species based on homology and phylogenetic analysis.

15. Almost 5% of both the selenium oxyanions were volatilized by the SG-26 after 72 h of incubation. The methylation of Se is a biological process and is thought to be a protective mechanism used by microorganisms to detoxify their surrounding environment.

16. XRD studies indicated the different route followed by the isolated strain (SG-26) for mitigation of toxic selenium oxyanions.

16. Complete transformation of selenium oxyanions by a particular strain was observed in soils supplemented with 25 and 50 mg/kg of selenium oxyanions across 90 days. With SG-16, almost 80% and 50% sequestration and transformation of selenate and selenite respectively was observed with little volatilization. An appreciable fraction (40%) of transformed selenium oxyanions was volatilized by SG-26 with more than 90% overall sequestration and transformation. Antibiotic based survival rate revealed highest survival of SG-16 in 0.32 mM selenate and lowest in 0.64 mM selenite augmented soils after 90 days. An increase in colony forming units (CFU) in all selenite treatments was observed for SG-26 upto 45 days followed by almost constant and decreased viable count in the subsequent period from 60 to 90 days studies.

17. Km and Vmax of the selenate reductase enzyme were found in the range of 407 to 463 and 18.62 to 28.48 respectively after ammonium sulphate precipitation.

18. Absence of plasmid in the isolates ensured presence of gene responsible for selenate reductase on genomic DNA. But no amplification was observed with either primer set designed using previous data base reported for selenate reductase gene in an anaerobic strain Thaurea selenatis further leads to speculation that a different gene is responsible for selenate reductase in this study.

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APPENDIX-I

Preparation of culture media

All the media prepared were sterilized in autoclave for 15 minutes at 15 lbs and 121°C.

Tryptone soya agar

Pancreatic digest of casein	15gm
Papaic digest of soyabean meal	5.0 gm
Sodium chloride	5.0 gm
Agar	15.0 gm
Distilled water	1000 ml

Tryptone soya broth

Pancreatic digest of casein	15 gm
Papaic digest of soyabean meal	5.0 gm
Sodium chloride	5.0 gm
Distilled water	1000 ml

Luria broth

Tryptone	10.0 gm
Yeast extract	5.0 gm
Sodium chloride	10.0 gm
Distilled water	1000 ml

Tryptone broth

Tryptone	10.0 gm
Sodium chloride	5.0 gm
Calcium chloride	1.0 gm
Distilled water	1000 ml

MR-VP broth

Peptone	7.0 gm
Dextrose/ Glucose	5.0 gm
Potassium phosphate	5.0 gm
Distilled water	1000 ml

Simmons's Citrate Agar

$NH_4(H)_2PO_4$	1.0 gm
Di-potassium phosphate	1.0 gm
Sodium chloride	5.0 gm
Sodium citrate	2.0 gm
Magnesium sulphate	0.2 gm
Agar	15.0 gm
Bromothymol blue	0.8 gm
Distilled water	1000 ml

SIM agar media

Peptone	30.0 gm
Beef extract	3.0 gm
Ferrous ammonium sulphate	0.2 gm
Sodium thiosulphate	0.025 gm
Agar	3.0 gm
Distilled water	1000 ml

Fermentation medium

Peptone	10.0 gm
Carbohydrate*	5.0 gm
Sodium chloride	15.0 gm
Phenol red	0.018 gm
Distilled water	1000 ml
pH	7.3

*specific carbohydrate such as glucose, sucrose, lactose, maltose and mannose are added

APPENDIX-IIa

Preparation of reagents for biochemical tests

Kovac's reagent (for Detection of indole)

P- Dimethylaminobenzaldehyde	5.0 gm
Amyl alcohol	75.0 ml
Hydrochloric acid (conc.)	25.0 ml

Alpha (α) Naphthol solution (Voges-Proskauer test reagent-I)

α-naphthol	5.0 gm
Ethyl alcohol (absolute)	95.0 ml

Dissolve the alpha naphthol in ethyl alcohol with constant stirring.

40% Potassium Hydroxide (Voges-Proskauer test reagent-II)

Dissolve 50 gm of potassium hydroxide in 95 ml of ethyl alcohol with constant stirring.

Methyl red indicator

Methyl red	0.1 gm
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

Phenol red indicator

Phenol red	0.2 gm
Ethyl alcohol	500 ml
Distilled water	500 ml

Crystal violet

Solution A

Crystal violet (90% dye)	2.0 gm
Ethyl alcohol (95%)	20.0 ml

Solution B

Ammonium oxalate	0.8 gm
Distilled water	80 ml

Dissolve crystal violet in ethyl alcohol and ammonium oxalate in distilled water. Mix solution A and B.

Gram's iodine

Iodine	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300 ml

Dissolve iodine and potassium iodide in distilled water.

Ethyl alcohol (95%)

Ethyl alcohol (100%)	95 ml
Distilled water	5 ml

Safranin

Safranin (2.5% solution in 95% ethyl alcohol)	10.0 ml
Distilled water	100 ml

APPENDIX-II B

Preparation of solutions and buffers

Modified Universal buffer (5X)

Tris (hydroxyl methyl) amino methane	3.025 gm
Maleic acid	2.90 gm
Citric acid	3.50 gm
Boric acid	1.57 gm
Sodium hydroxide (1N)	122 ml
Distilled water	250 ml
pH	5.5

Phosphate buffer 1M (pH-7.2)

Stock solution A

Monobasic sodium phosphate monohydrate (2M)	276 g/L

Stock solution B

Dibasic sodium phosphate (2M)	284 g/L

28.0 ml Stock solution A and 72.0 ml of stock solution B were mixed and final volume was prepared to 200 ml.

Solutions for Plasmid extraction

Solution I (10X)

Tris-HCl(pH 8.0)	25 mM
Glucose	50 mM
Saline EDTA	10mM

Solution II

Sodium hydroxide	5M
SDS	10%

Solution III (pH 4.5)

Potassium acetate	5.0 M

Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

Ligation reaction of amplicon in pTZ57R/T

Plasmid pTZ57R/T (50ng/µl)	3µl
Insert (75ng/µl)	4µl
Buffer (10X)	3µl
T4 Ligase	1µl
H_2O	19µl

Saline EDTA (Ethylene diamine tetraacetic acid)

Solution A

EDTA	3.72 gm
Distilled water	100.0 ml

Solution B

Sodium chloride	0.87 gm
Distilled water	100.0 ml

Mix solution A and B.

50mM Tris buffer

Tris base	1.214 gm
Distilled water	200.0 ml

1.0M Tris-HCl

Tris base	24.24 gm

HCl	8.4 ml
Distilled water	100.0 ml

TE buffer

Solution A

Tris base	0.121 gm
HCL	0.042 ml
Distilled water	100.0 ml

Solution B

EDTA	0.037 gm
Distilled water	100.0 ml

Mix solution A and B.

Lysozyme solution

Lysozyme	10.0 mg
Distilled water	1.0 ml

Sodium Dodecyl Sulphate (SDS)

SDS	10.0 gm
Distilled water	100.0 ml

Gel loading dye (6X)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Ficoll-type (400,000)	15 g
Distilled Water	100 ml

The prepared solution was stored at room temperature

Tris Borate EDTA (TBE) buffer (10X)

Boric acid	55.0 g
Tris base	107.0 g
Na_2EDTA	3.3 g

The pH of the buffer was maintained at pH 8.3

Biuret reagent

NaOH	30 g
CuSO4	0.12 g
Total volume	100 ml

Sodium hydroxide was dissolved in ice-bath and cupric sulfate was added slowly with continuous stirring

Morphological, physiological and biochemical characteristics of selected isolates

Characters	**SG-14**	**SG-15**	**SG-16**
Morphological			
Cell Shape	Rod shape	Rod shape	Rod shape
Gram Stain	Gram negative	Gram positive	Gram positive
Motility	+ve	+ve	+ve
Capsule	+ve	+ve	-ve
Colony Morphology	Dry, wrinkled rough	white opaque	Large, Undulate margin Flat elevation
Pigmentation	yellow/ white	No pigmentation	No pigmentation
Physiological			
Growth at 4°C	-ve	-ve	-ve
Growth at 42°C	+ve	+ve	-ve
Growth at 45°C	+ve	-ve	-ve
Growth with 7% NaCl	+ve	+ve	-ve
Growth with 10% NaCl	-ve	-ve	-ve
Growth at pH-2	-ve	-ve	-ve
Growth at pH-5	-ve	-ve	-ve
Growth at pH-8	+ve	+ve	+ve
Growth at pH-11	+ve	-ve	+ve
Biochemical Characteristics			
Indole Utilization	-ve	-ve	-ve
Methyl Red	-ve	-ve	+ve
Voges Proskeur	+ve	-ve	+ve
Citrate Utilization	+ve	+ve	-ve
Nitrate Reduction	+ve	-ve	+ve
H_2S Production	-ve	-ve	-ve
Urease	-ve	-ve	-ve
Starch Hydrolysis	+ve	-ve	-ve
Casein Hydrolysis	-ve	-ve	+ve
Lipid Hydrolysis	+ve	-ve	+ve
Gelatin Liquefaction	-ve	+ve	+ve
Oxidase	+ve	+ve	+ve
Catalase	+ve	+ve	+ve
Carbohydrate Fermentation Test			
Sucrose	-ve	-ve	+ve
Glucose	+ve	+ve	-ve
Lactose	-ve	-ve	+ve

NA- Nutrient agar

Contd…

Characters	SG-19	SG-20	SG-21
Morphological			
Cell Shape	Rod shape	Rod shape	Rod shape
Gram Stain	Gram negative	Gram positive	Gram positive
Motility	+ve	-ve	+ve
Capsule	+ve	+ve	-ve
Colony Morphology	Undulate margin smooth	flat, raised irregular margin	Large, flat,opaque irregular margin
Pigmentation	yellow/ white	grey white	No pigmentation
Physiological			
Growth at 4°C	-ve	-ve	+ve
Growth at 42°C	+ve	+ve	-ve
Growth at 45°C	+ve	-ve	-ve
Growth with 7% NaCl	+ve	-ve	-ve
Growth with 10% NaCl	-ve	-ve	+ve
Growth at pH-2	-ve	-ve	-ve
Growth at pH-5	+ve	+ve	+ve
Growth at pH-8	+ve	+ve	+ve
Growth at pH-11	+ve	+ve	-ve
Biochemical Characteristics			
Indole Utilization	-ve	+ve	-ve
Methyl Red	-ve	-ve	-ve
Voges Proskeur	+ve	+ve	+ve
Citrate Utilization	+ve	+ve	+ve
Nitrate Reduction	-ve	+ve	-ve
H_2S Production	-ve	+ve	-ve
Urease	-ve	+ve	-ve
Starch Hydrolysis	-ve	+ve	+ve
Casein Hydrolysis	+ve	+ve	+ve
Lipid Hydrolysis	+ve	+ve	+ve
Gelatin Liquefaction	+ve	+ve	+ve
Oxidase	+ve	+ve	-ve
Catalase	+ve	+ve	+ve
Carbohydrate Fermentation Test			
Sucrose	+ve	+ve	-ve
Glucose	+ve	+ve	+ve
Lactose	±ve	+ve	-ve

NA- Nutrient agar

Contd…

Characters	**SG-22**	**SG-23**	**SG-26**
Morphological			
Cell Shape	Rod shape	Rod shape	Rod shape
Gram Stain	Gram positive	Gram negative	Gram negative
Motility	-ve	+ve	+ve
Capsule	-ve	-ve	+ve
Colony Morphology	flat, white, opaque circular, raised	flat, irregular undulate margin	flat, irregular opaque
Pigmentation	No pigmentation	florescent	blue green
Physiological			
Growth at 4°C	-ve	+ve	-ve
Growth at 42°C	+ve	-ve	+ve
Growth at 45°C	-ve	-ve	+ve
Growth with 7% NaCl	+ve	-ve	-ve
Growth with 10% NaCl	-ve	-ve	-ve
Growth at pH-2	-ve	-ve	-ve
Growth at pH-5	-ve	-ve	-ve
Growth at pH-8	+ve	+ve	+ve
Growth at pH-11	-ve	+ve	-ve
Biochemical Characteristics			
Indole Utilization	-ve	-ve	-ve
Methyl Red	-ve	-ve	-ve
Voges Proskeur	-ve	+ve	-ve
Citrate Utilization	-ve	+ve	+ve
Nitrate Reduction	-ve	-ve	+ve
H_2S Production	-ve	-ve	-ve
Urease	-ve	-ve	-ve
Starch Hydrolysis	-ve	-ve	-ve
Casein Hydrolysis	-ve	-ve	+ve
Lipid Hydrolysis	-ve	+ve	+ve
Gelatin Liquefaction	-ve	-ve	+ve
Oxidase	-ve	+ve	+ve
Catalase	+ve	+ve	+ve
Carbohydrate Fermentation Test			
Sucrose	-ve	-ve	-ve
Glucose	-ve	-ve	+ve
Lactose	-ve	-ve	-ve

NA- Nutrient agar