Soil Microbe Interaction and its Impact on Selenium uptake by *Allium* species

A thesis Submitted in fulfilment of the requirement for the award of the degree of

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BIOTECHNOLOGY

by

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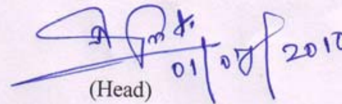

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Candidate's Declaration

I, hereby declare that the work presented in the thesis entitled "Soil-Microbe Interaction and its Impact on Selenium uptake by *Allium* species" 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. N.Tejo Prakash, Assistant Professor, Department of Biotechnology & Environmental Sciences, 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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“ With the Grace of All Mighty seed of thought flourished into sapling
In deed God and Guide work hand in hand ”

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Neetu Sharma

h	Hours
d	Days
rpm	Revolution per minute
g	Gram
L	Litre
V	Volume of the solution
µS cm^{-1}	Microsiemens per cm
mL	Millilitre
µL	Microlitre
µg ml^{-1}	Microgram per millilitre
mg kg^{-1}	milligram per kilogram
mg l^{-1}	Milligrams per liter
µg g^{-1}	Microgram per gram
cfu g^{-1}	Colony forming units per gram
mM	milli Molar
N	Normality (normal)
bp	Base pair
Kb	Kilobase
dNTP	2'-deoxynucleoside-5'-triphosphate
%	Percentage
min	Minute
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
rDNA	Ribososmal deoxyribonucleic acid
RNA	Ribonucleic acid
RDP	Ribosomal Database Project
Se (IV)	Sodium selenite
Se (VI)	Sodium selenate

INAA	Instrumental Neutron Activation Analysis
GF-AAS	Graphite-furnace Atomic Absorption Spectroscopy
XRD	X-Ray Diffraction
ESEM	Environmental Scanning Electron Microscope
TEM	Transmission Electron Microscope
EDX	Energy dispersive X-Ray Spectroscopy
e.g.	For example
H_2SO_4	Sulfuric acid
EDTA	Ethylene diamine tetraacetic acid
HCl	Hydrochloric acid
LiCl	Lithium Chloride
Co^{60}	Cobalt-60
LB	Luria Broth
TBE	Tris Borate EDTA
SDS	Sodium dodecyl sulphate
Tris	Tris (hydroxymethyl) amino methane
TE	Tris EDTA
nm	Nanometer
OD	Optical density

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Dedicated to my Family

1.0 Introduction

Selenium (Se) was discovered in 1817 by a Swedish chemist named Jons Jakob Berzelius. It is one of the rarest elements, being about 70th in abundance among the 88 naturally occurring in the earth's crust. Berzelius discovered the presence of an unknown substance with properties very much like those of tellurium, as a reddish deposit, obtained in burning pyrites, and covering the floor of a lead chamber in the manufacture of sulfuric acid. When examining this deposit, since 'tellus' is the name of our planet, he named the new element: selenium, after the Greek Goddess of the moon: 'Selene'. Although Berzelius is said to be the discoverer of Se, it was Marco Polo (13th century) who first observed the toxicity of Se, when he described the effect of a poisonous plant. When feeding on such plants, hoofs of the livestock was observed to drop off reducing the mobility of animals. The first reports on Se induced carcinogenicity appeared in 1943; resulting in a negative perception on this element (Nelson et al., 1943). The evidence of Se as an essential element gathered over time through observations on the nutritional importance of Se when the replacement of vitamin E by Se in experimental animal diets did not cause adverse effects (Whanger, 2002; Combs, 1990; Schwarz and Foltz, 1957).

Se took its place in Mendeleev's table in 1869. Selenium has an atomic weight of 78.96 and its atomic number is 34. It belongs to Group 16/VIA with oxygen, sulfur, tellurium and polonium, with bromine and arsenic as its neighbors. It shares similar chemical properties significantly with sulfur and to a lesser extent with tellurium. Selenium exists in four oxidation states: 0 (elemental selenium), 2- (e.g., Na_2Se - sodium selenide; $(NH_2CH(COOH)CH_2CH_2SeCH_3$ - selenomethionine, 4+ (e.g., Na_2SeO_3, sodium selenite; H_2SeO_3, selenious acid) and 6+ (e.g., Na_2SeO_4, sodium selenate; H_2SeO_4, selenic acid). Selenium has six naturally occurring isotopes, five of which are stable: ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, and ^{80}Se. The last three also occur as fission products, along with ^{79}Se which has a half-life of 295,000 years. The final naturally occurring isotope, ^{82}Se, has a very long half-life (~1020 yr, decaying via double beta decay to ^{82}Kr), which, for practical purposes, can be considered to be stable. Twenty-three other unstable isotopes have been characterized (Table 1.1).

Table 1.1

Isotope	(n,γ) Product nuclei	Half-life	γ-Energy in keV
^{74}Se	^{75}Se	121 d	121,136,265, 280, 401
^{76}Se	^{77m}Se	17.5 s	161
^{77}Se	-	-	-
^{78}Se	^{79m}Se	3.91 m	96
^{80}Se	^{81m}Se	18.6 m	280
^{82}Se	^{83m}Se	70 s	350,650,1010,2020

1.1 Distribution of selenium in environment

Selenium concentration in soils is closely related to its geological parent materials. Selenium is originally produced from volcanic activity and is found naturally in igneous (volcanic) rocks, granite, sandstone, limestone, coal and some crude oil deposits (Van Metre and Calan, 2001). Through the weathering of Se-rich rocks, water and wind erosion and sedimentation processes, selenium particles are deposited into top soil. Soil Se can exist in 4 oxidation states (-II, 0, +IV, +VI) and these forms differ widely in their solubility and short-term bioavailability. Selenium in environmental and biological systems exists as inorganic, organic, methylated and low molecular mass species (Table 1.2). 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, artificial fertilization, the rate of rainfall and human activities (industrial emissions, the use of fertilizers, sewage sludge, fly ash) (Seby et al., 1997). Under acidic conditions the formation of selenite (SeO_3^{2-}), which adsorbs to clays, is favored. This form has an extremely low solubility and remains unavailable for plants. 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 Kothany, 1973). The seleniferious soils in areas where some plants accumulate high amounts of Se are typically alkaline and cope with low rainfall. As elemental Se is formed under reducing conditions from SeO_3^{2-} and SeO_4^{2-}, Se can become unavailable for plants. The availability of Se to plants is affected by soil moisture: the element is most available to plants where there are not much precipitation and low soil leaching (Gissel-

Nielsen, 1998). Selenate is the most prevalent form and the most bioavailable form of soluble Se for plants. Speciation of saturation extracts from selected seleniferous San Joaquin Valley soils revealed that 98% of soluble Se was present as selenate (4– 640 µg l^{-1}) followed by selenite (1–4 µg l^{-1}). The hydrophobic organic Se (less than 1–4 µg l^{-1}) associated with the humic acid part of the dissolved organic carbon (Fio and Fujji, 1990). Organic forms of Se such as seleno-amino acids represent an important source of plant available Se and selenomethionine is more bioavailable than selenocystine. Selenite and selenate are produced by bacteria and fungi from less soluble forms of selenium (Sarathchandra and Watikinson, 1981).

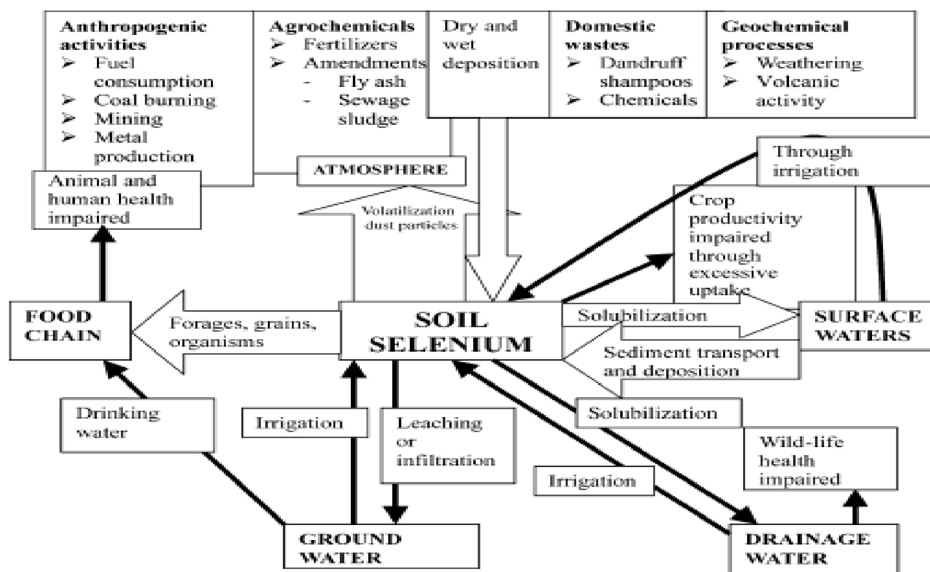
Table 1.2: Selenium species present in environmental and biological systems (D'Ulivo, 1997)

Type	Species
Inorganic species	Selenite, Selenate, Se^0,Se^{-2}
Simple organic and methylated species	MeSeH, Me_2Se,Me_2Se_2,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)

In natural waters, the predominant species of selenium are the oxyanions, selenite (Se^{4+}) and selenate (Se^{6+}) with limited evidence of dissolved organo-selenium forms is present in some aquatic ecosystems (Cutter and Cutter, 1998). Some Dissolved selenium oxyanions are primarily absorbed by bacteria and microphytes and biotransformed into organo-selenium forms and elemental selenium. Furthermore, selenium can be transferred through different aquatic consumers, such as insect larvae, zooplankton and larval fish, into the top predators including predatory fish and waterfowl. Selenium biomagnification and further chemical transformation can occur as selenium passes through the food chain.

The maximum admissible limit of selenium in drinking water as per the guidelines of WHO is 10 μg l^{-1} (WHO, 1971). Generally, groundwater contains higher selenium concentrations than surface water, mainly due to the time dependent mobilization of Se into the water table presumably through sub-surface bio-geo-chemical activity. Sewage sludge, gold mining, petroleum refineries and agricultural drain water are the major anthropogenic sources of selenium contamination to the water system (Hamilton, 2004).

Figure 1.1. Diagrammatic representation of sources of Selenium in soil (Dhillon and Dhillon, 2003)



1.2 Essentiality

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. Selenium is an essential element for anti-oxidant reactions in humans and animals and it is involved as selenocysteine (SeCys) in functioning at the catalytic center of several selenoproteins such as glutathione peroxidase (GSHPx), thioredoxin reductase, and iodothyronine deiodinasees. In recent years, the role of selenium in the prevention of a number of

degenerative conditions including cancer, inflammatory diseases, thyroid function, cardiovascular disease, neurological diseases, ageing, infertility, and infections, has been established by laboratory experiments, clinical trials and epidemiological data. Most of the effects in these conditions are related to the function of selenium in anti-oxidant enzyme systems. Plants are the major dietary sources of selenium in most countries around the world. A major influence on the selenium content in food depends on the soil where these plants are grown. Selenium deficiency can occur in certain areas where the soil is poor in selenium, such as certain regions of China, Russia and Scandanavian countries.

1.3 Se-related human diseases and their prevention

Selenium is critical to the health of living organisms. It has been postulated that the vast majority of the world's population has suboptimal Se intakes, and hence is at increased risk of several diseases such as cancer, heart disease, viral diseases and other conditions that involve increased levels of oxidative stress (Combs, 2001). World Health Organization recommended Se intake of 40 µg day^{-1} as an average intake level needed to ensure meeting normative requirements of healthy adults (Combs, 2001). Recommended intakes of selenium for various age groups from three different publications are summarized in Table 1.3.

As an essential trace element, selenium has the narrowest range between dietary deficiency (<40 µg day^{-1}) and toxic levels (>400 µg day^{-1}). Selenium deficiency is more widespread than selenosis. Two endemic diseases related to insufficient intake of Se have been elucidated in China in a region where the soil is depleted of Se and hence vegetables are low in Se concentration. Further investigation showed that soil in this region was low in phytoaccessible selenium. Keshan disease is a cardiovascular disease, which mainly affects children and young women. The disease includes chronic cardiac insufficiency, heart enlargement and rhythm disorder. Several studies were devoted to this disease and supplementation with Se diminishes the incidence of Keshan disease (Chen et al., 1980).

Another major ailment associated with Se deficiency is Kashin-Beck disease, which is characterized by chronic disabling degenerative osteoarthritis reported to affect about millions of children in China, North Korea and several parts of Russia (Peng et al.,

1992). The diseases might occur due to the combination of several factors: low Se-intake and high oxidative stress.

Table 1.3: Reference values for intakes of selenium (μg/day)[a] (Bates, 2005)

Population group	UK LRNI	UK RNI	US AI/ RDA[b]	WHO/ FAO RNI
0–6 months	4–5	10–13	AI:15	6
7–12 months	5–6	10	AI:20	10
1–3 years	7	15	RDA:20	17
4–6 years	10	20	30	22
7–10 years	16	30	30-40	21-26
11–18 years, male	25-40	45-70	40-55	32
11–18 years, female	25-40	45-60	40-55	26
19–65 years, male	40	70	55	34
19–65 years, female	40	60	55	26
65 years, male	40	70	55	33
65 years, female	40	60	55	25
Pregnant	40	60	60	26-30
Lactating	55	75	70	35-42

a-Where a range of values is given, the population group described in this table overlapped across more than one population group in the source table.
b-The first two age groups are AI; the remainder are RDA. LRNI, Lower Reference Nutrient Intake; RNI, Reference Nutrient Intake; AI, Adequate Intake; RDA, Recommended Dietary Allowance.

Sources:
UK: Department of Health (1991) Dietary Reference Values for Food Energy and Nutrients for the United Kingdom, Report on Health and Social Subjects No. 41. London: HMSO.

USA: Food and Nutrition Board, Institute of Medicine (2000) Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium and Carotenoids. Washington, DC: National Academy Press.

WHO/FAO: WHO/FAO (2002) Human Vitamin and Mineral Requirements. Report of a Joint FAO/WHO Expert Consultation, Bangkok, Thailand. Rome: WHO/FAO.

A too low Se intake leads to several diseases, but an excessive intake of the element results in intoxication or selenosis. Selenosis occurs in areas where the soil contains high amounts of Se and the population heavily relies upon local produce for their food. The symptoms observed in the case of selenosis are: hair loss, damaged nails, and abnormalities of the nervous system, skin rash, gastrointestinal problems and a garlic breath odour (WHO, 1987).

1.4 Bioavailability of Selenium in body

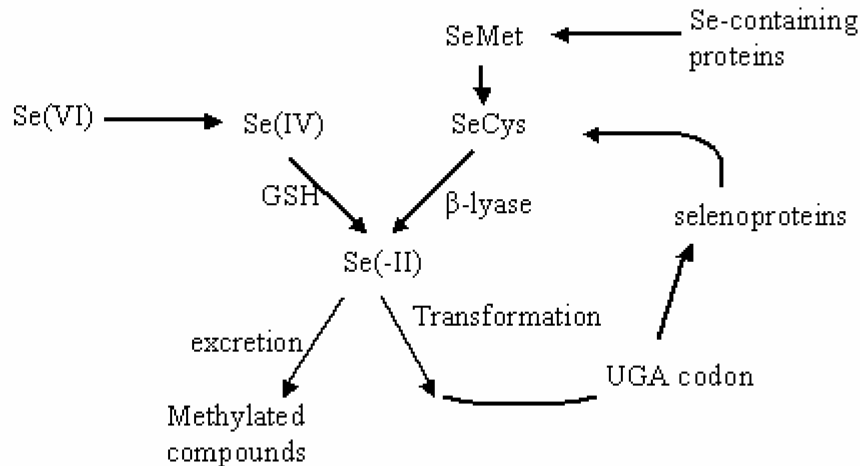
Animal and human studies have established that the chemical form of selenium in foods and supplements influences not only the bioavailability of this element but also its distribution in the body. Bioavailability can be defined as a quantitative measure of the utilization of a nutrient in a food or meal, to support normal structural and physiological processes occurring in the body. Selenium compounds are generally very efficiently absorbed by humans, and selenium absorption does not appear to be under homeostatic control (WHO, 1987). For example, absorption of the selenite form of selenium is greater than 80 percent, whereas that of selenium as selenomethionine or as selenate may be greater than 90 percent (Patterson et al., 1993; WHO, 1987). Therefore, the rate limiting step determining the overall availability of dietary selenium is not likely to be its absorption but rather its conversion within tissues to its metabolically active forms (e.g., its incorporation into GSHPx or 5'-deiodinase) (Contempre et al., 1996).

On assimilation in the organism, different Se containing compounds are reported to follow different pathways for absorption. Se-Met is thought to follow the methionine pathway and is absorbed from the gastrointestinal tract through the Na^+-dependent neutral amino acid transport system. SeO_3^{2-} seems to be absorbed via passive diffusion in the small intestine. SeO_4^{2-} is thought to follow the same pathway as sulfate. Both inorganic (selenite and selenate) and organic (seleno-methionine and selenocysteine) forms of selenium may be utilized by the body, with the selenoamino acids showing greatest bioavailability. They are reduced to selenide as the assumed intermediate and then selenium is incorporated into selenoproteins after being transformed stepwise to selenophosphate and selenocysteinyl tRNA. Thus, the reaction for selenium in the body is presumed to follow steps that comprise: 1) reduction of inorganic species by glutathione (GSH) to selenide; 2) conversion of SeMet and SeCys into selenide; 3) direct non specific

incorporation of SeMet into proteins; 4) cleavage reaction of organic species by the ß-lyase leading to methylselenol, CH_3SeH; 5) utilization as SeCys according to the UGA codon leading to synthesis of selenoproteins and 6) excretion after being metabolized to methylated species and selenosugars (Rayman et al., 2008; Birringer et al., 2002).

The bioavailability of Se gets further enhanced through the presence of antioxidants (vitamin C and E), nutritionally adequate levels of methionine, total protein intake and restricted intake of food (Levander, 1991). The bioavailability of Se as Se-Met and Se-Cys compounds in plant sources is high (85-100%), while the bioavailability from animal sources is rather low to moderate (15%). Although the highest Se concentration is measured in seafood, the bioavailability of Se from this food source (20-50%) is considerably lower than from vegetables. Dairy products have the lowest bioavailability ranging from less than 2% to 7% (Diaz-Alarcón et al., 1996; Shen et al., 1993; Levander et al., 1983; Cantor et al., 1975). In wheat, which is a dominant staple food across the world, is also most efficient Se accumulator among cereals and main source of Se for humans (Cubadda et al., 2010; Rayman 2008).

Figure 1.2: Metabolic pathways for selenium in the body (Pyrzynska, 2002)



1.5 Plants

1.5.1 Se uptake in plants

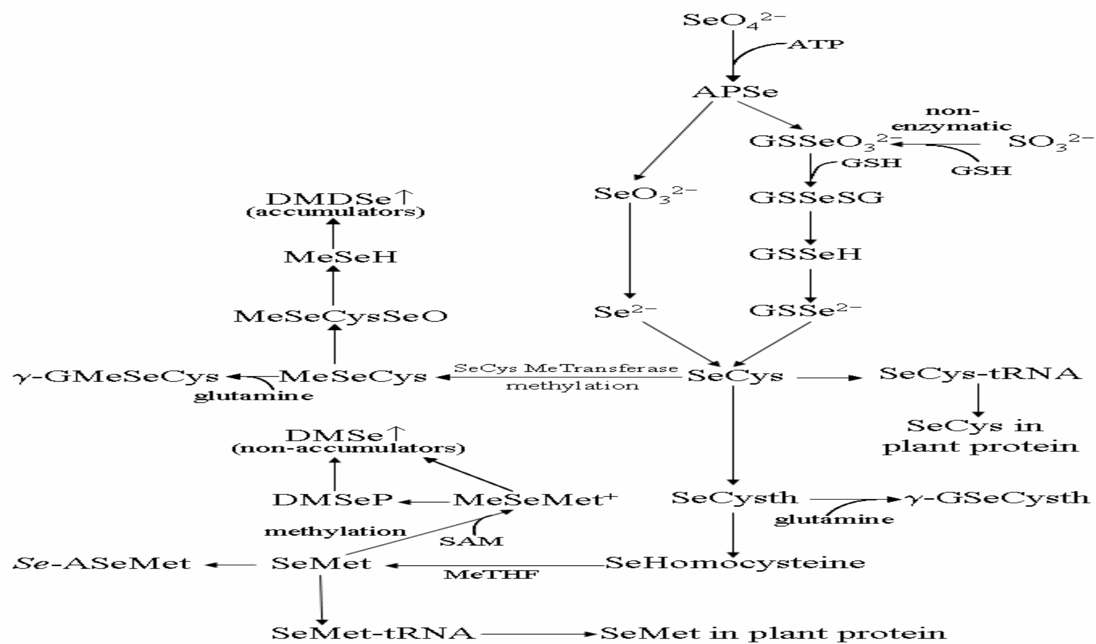
Plants, nature's best manufacturing system, have been providing the sole source of food, feed and fiber to society for many centuries. Selenium has not been scientifically demonstrated to be essential for the growth of many plants, through which humans obtain their nutritional needs. Plants differ greatly in their ability to accumulate selenium from soils and therefore could be divided into three major groups: (1) selenium accumulators, plants that are able to accumulate extraordinary high levels of selenium in stems and leaves when grown in selenium-rich soil (Terry et al., 2000) (2) secondary selenium accumulators, which can accumulate high selenium contents from soil that contains medium or low of the element (Terry et al., 2000) (3) non-accumulators, this category includes most forage, cereal grains, and oil meal crop plants. In nonaccumulators, the threshold Se concentration in the shoot tissue resulting in a 10 % reduction in yield varied from 2 mg Se kg^{-1} in white clover (Mikkelsen et al., 1989). Se accumulators, on the other hand, may contain Se concentrations in excess of 4000 mg Se kg^{-1} without exhibiting any negative effects on growth (Shrift, 1969). The selenium content in plants varies greatly with Se availability in soils. After absorption, the distribution of selenium in various tissues mainly depends on the species, the stage of growth, the chemical form of selenium absorbed, and physiological conditions.

1.5.2 Metabolic pathway of Se in plants

Since man and animals rely on plants as their main dietary source, knowledge on the Se compounds of plants is crucial. Se enters the food chain through incorporation into plant proteins, mostly as Se-Methionine, and to lesser extent as Se-Cysteine, at normal levels. Se-Met accounts to more than 70% of the total Se in wheat grain irrespective of the total Se content (Cubadda et al., 2010). In nonaccumulator plants, Se-Met can be further metabolized to: Se-adenosyl-Se-Met or undergo methylation leading to Se volatilization as dimethylselenide, a known mechanism for elimination of excess Se. In accumulator plants, the non protein selenoamino acids Se-methyl-selenocysteine (MeSeCys) and γ-glutamyl-Se-Methyl-selenocysteine (γ-Glu-MeSeCys) are biosynthesized from SeCys, thereby preventing the damaging effects on plant functions

resulting from incorporations of SeCys and SeMet in proteins. Elimination of excess Se in accumulators occurs as dimethyl-diselenide (Rayman et al., 2008). The Se-forms, present in the proteins will finally be used by animals in the synthesis of their own proteins, facilitating their accumulation (Simonoff, 1991). The formation of various volatile Se compounds by plants is important since certain plants can take up a huge amount of Se from the soil, and transform it through several biochemical steps into volatile species. This is called phytovolatilization and may be part of the mechanism governing phytoremediation, important for its potential in cleaning up highly polluted sites (Meija et al., 2002).

Figure 1.3: Biosynthetic pathways of Se assimilation in plants (Rayman et al., 2008)



1.6 Selenium Phytoremediation and Biofortification

Modern agriculture has had reasonable success in meeting the energy needs of developing countries. In the past 40 years, agricultural research in developing countries has met Malthus' challenge by placing increased cereal production at its center. However, agriculture is now focusing on a new paradigm that will not only produce more food, but

bring better quality food as well. By producing staple foods whose edible portions are denser in bioavailable minerals and vitamins, a process referred to here as "biofortification," scientists are attempting to provide farmers with crop varieties that naturally reduce anemia, cognitive impairment, and other nutritionally related health problems in hundreds of millions of people.

The first and perhaps the leading example of the use of plants to take out excess concentration of an element in soils came about because of environmental problems, popularly exemplified by presence of unusually high concentrations of selenium of geochemical origin on the west side of the California San Joaquin Valley. A plant based cleanup technology, defined as Phytoremediation, and involves all plant – influenced biological, chemical and physical processes that aid in the uptake, sequestration, degradation and metabolism of contaminants, either by plants or by free-living organisms that constitutes the plants rhizosphere. The phytoremediation of Se and using it as a means for nutraceutical is still in the early stages of exploration (Cubadda et al., 2010). Despite clinical findings demonstrating the chemopreventive attributes of organoselenium and organosulfur compounds, limited investigations are reported on the efficiency of Se uptake, assimilation, and possible competitive inhibition in plants. This assimilation is more so important, due to its chemical relationship with sulfur and replacement of sulfur in S rich plants such as *Alliums.*

1.7 Status of Selenifereous environment in India

For the past few years the attention of several workers has been engaged in the study of the role of Se in Indian soil and plant and its impact on animal health. Preliminary reports on chronic Se toxicity as a cause of hoof and horn disease in livestock in seleniferous region of Punjab were given by Gupta et al. (1982). The most characteristic symptoms and significant lesions were seen in the hooves and horns in the form of cracks which became deeper in due course of time. Consequently animal became weak and depressed. Also failure of conception and abortion in pregnant animals were frequent observations of this disease irrespective of species. It was observed that the average Se content in water sample from seleniferous areas was nine times more than that from the non-seleniferous areas. The toxic sites are located at the dead end of several seasonal rivulets flowing from upper ranges of Siwalik Hills, which are used for

continuous cropping by farmers. It is likely that materials rich in Se might have been transported down along with the floodwater and deposited in the low-lying areas. When these sites were brought under cultivation, the topsoil might have been enriched through recycling of selenium by continuous cropping. When plants are exposed to high concentrations of Se in their root medium, they may exhibit symptoms of injury including stunted growth, chlorosis, withering and drying of leaves, decreased protein at the initial phases of growth. Selenium content of water and milk in the endemic area was 9.4 ±1.0 and 5.92 ± 1.0 mg/100ml, compared with 0.6± 1.0 and 1.6 ± 0.5mg/100 ml, respectively, in the non-endemic area. Concentration of selenium in wheat chapatti was 74.2 ± 19.0 mg/100 g in the endemic area while it was less than 0.01 ± 1.0 mg/100 g in non-endemic area. Similarly, most legumes and vegetables in the endemic area were also found to contain high selenium content as compared with the non-endemic area. Selenium contribution from milk was 3 and 3.3 % for men and women in the endemic area, compared with 12.4 and 12.0 %, respectively, in the non-endemic area. With reference to Se uptake by human population, the average selenium intake by men and women was found to be 632 ± 31 and 475 ± 53 µg day^{-1} whereas in non-seleniferous, areas were 65 ± 2 and 52 ± 1 µg day^{-1}, respectively (Hira et al., 2003).

Interest in Selenium pollution and remediation technology has escalated during the past two-decades. Se toxicity is strictly related to the phytoavailability of its chemical forms; with the oxyanion Se (VI) being more mobile and plant available than Se (IV). Se (VI) predominates in oxidative conditions, especially in well aerated soils. The bioavailability and toxicity of Se to plants depends highly on the Se species present in the soil solution and the concentration to which the plants are exposed. More knowledge about the selective determination of a particular chemical species of selenium would be very useful to understand the biochemical and biogeochemical cycle of selenium from both biological and environmental points of view. Characterization of this relationship should be critical for the production of Se-enriched *Allium* for pharmacological investigation. Microbial mobilization of elements is accomplished with the help of diverse group of microorganisms, particularly the indigenous bacteria present in the soil. Also, limited reports are available on selenium reduction potential of microbial strains and consortia along with plants from selenifereous tropical soils which shows that ample work is required for understanding the role of microbes inhabiting these soils. Thus

keeping this point in view, plant microbe interactions has also been studied in present work as plant-microbe interaction in rhizosphere can provide an economical method for enhancing the microbial degradation of contaminant sites and these interactions will be important in modeling the full impact of phytoremediation in the restoration of derelict lands. Furthermore, the remediation of heavy metal contaminated sites using rhizobacteria is an exciting area of research, since these organisms can easily and inexpensively be mass-produced.

2.0 Review of Literature

2.1 Selenium in the environment

Selenium exists in different oxidation states namely selenide (-2), elemental selenium (0), selenite (+4), selenate (+6) exhibiting a different chemical behavior in each of the states. The concentration, speciation and association of selenium in a given environment are dependent upon redox condition, solubility of its salt, and the complexing ability of soluble forms, biological interactions and reaction kinetics. Selenide and elemental selenium occur in acidic, reducing and organic rich environment. The oxidized forms such as SeO_4^{2-} and SeO_3^{2-} are more soluble and more easily absorbed by plants, whereas Se^{2-} and Se^0 are less available (Banuelos et al., 2002; Fishbein, 1991). Volatile compounds such as dimethylselenide (DMSe) and dimethyldiselenide (DMDSe), and the trimethylated compound trimethylselenonium ion (TMSe), are less toxic methylated species (Pyrzynska, 1998; Lemly, 1997 a).

The process of distribution of Se through the environment involves a variety of physical, chemical and biological activities including volcanic activity, combustion of fossil fuels, weathering of rocks and soils, soil leaching, groundwater transport, plant and animal uptake and release, adsorption and desorption, chemical and biological reduction and oxidation reaction, mineral formation and disposal of sewage sludge (Fordyce, 2005). The use of fertilizers and irrigation water containing Se also contributes to soil Se inputs in certain areas. The selenium content in soils ranges from 0.01 to 2 mg Se kg^{-1} while the mean value is 0.4 mg Se kg^{-1} worldwide, although concentrations of $\geq$1200 mg Se kg^{-1} can occur in seleniferous soils. Soils rich in selenium are referred to as being “seleniferous” (Fordyce, 2005). Some parts of the world like Denmark, Finland, New Zealand, Eastern and Central Siberia, parts of China, Central Africa, Northern Korea, Nepal and Tibet, are known to have a low Se-soil content ($<0.1 \mu g\ g^{-1}$) (Oldfield, 1999).

Significantly high levels of Se was first documented in wheat grains from different countries of the world including North and South America, South Africa, Algeria, Morocco, Spain, Australia, New Zealand, France and Germany. (Robinson, 1936). In India, the Se toxic sites identified include parts of Northwestern region of Punjab (Dhillon and Dhillon, 1991); Haryana (Arora et al., 1975); Assam and Meghalaya

(Dey et al., 1999) and West Bengal (Ghosh et al., 1993). Amongst all the locations identified, 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, 2003). The affected villages, covering over 1000 acres are Barwa, Bhano Majra, Sikandarpur, Rakker, Simbli, Jainpur, Mahenpur and Nazarpur (Dhillon and Dhillon, 1997). Selenium toxic areas were identified on the basis of selenium content of plant and soil samples. With reference to Se uptake by human population, the average selenium intake of both men and women was more than nine times then that of the non-endemic area and exceeded the maximum tolerable limit in more than 60% of men. Mean selenium content of the hair, nails and urine of both men and women was ten times higher than in the non-endemic area. Based on the study results, steps need to be taken to educate the public in the endemic area to avoid selenium toxicity (Hira et al., 2003).

In Haryana state, a few acres of land producing toxic fodders were identified in the village Chamarkhera near Karnal. In the sub- Himalayan West Bengal, soils growing Se toxic pastures are located in the Jalpaiguri district. The affected areas remain flooded for 3–4 months during the year. In North-eastern India, investigations revealed that wild animal species from various locations of Assam and Meghalaya, viz. the Reserve Forest near Umkiang, Jaintia hills, Meghalaya; the Lailad Reserve forest, Ribhoi, Meghalaya and Rani Reserve Forest, Assam are suffering from known toxic effects of Se (Dey et al., 1999).

2.2 Biological profile of selenium

An element is considered essential to an organism when reduction of its exposure below certain limit results consistently in a reduction in a physiologically important function, or when the element is an integral part of an organic structure performing a vital function in the organism (WHO/FAO/IAEA, 1996). The two faces of selenium, as nutrient and as a potent toxin, make it a particularly important trace element in the health of both animals and human. The narrow margin between beneficial and harmful levels has important implications for human activities that increase the amount of selenium in the environment. Various substances such as arsenic, cysteine, metals, sulfate and vitamins A and E, can alter the toxicity of Se (Wilber, 1980). On the other hand, Se modifies the toxic effects of a variety of metals, e.g. As, Pb, Hg, Zn (Fan et al., 1988). Selenium easily

forms compounds with metals and occurs in about 50 minerals, which occur in very low concentrations in soils. The most common are: clockmannite—CuSe; ferroselite—FeSe; clausthalite—PbSe; naumannite— Ag_2Se; tiemannite—HgSe and Zinc selenide- ZnSe (rare form). Mercury selectively binds with selenium to form insoluble mercury selenides (Moller and Danscher, 1991). The first report on the protective effect of selenite against mercury toxicity appeared in 1967 (Parizek and Ostadalova, 1967). Since then, numerous studies have shown selenium supplementation counteracts the negative impacts of exposure to mercury, particularly in regard to neurotoxicity, fetotoxicity, and developmental toxicity. The ability of selenium compounds to decrease the toxic action of mercury has been established in all investigated species of mammals, birds, and fish (Culvin-Aralar and Furness, 1991). Selenium and arsenic antagonism may occur by several pathways. The selenium and arsenic species may bind and form an insoluble complex, such as orpiment (As_2Se_3), resulting in a biologically unavailable selenium and arsenic species (Scott et al., 2009).

Selenium has three levels of biological activity. (Hamilton, 2004): (i) trace concentrations are required for normal growth and development; (ii) moderate concentrations can be stored to maintain homeostatic functions; and (iii) elevated concentrations can result in toxic effects. The World Health Organization states that a Se intake of 40 μg day^{-1} is the average intake level needed to ensure meeting normative requirements of healthy adults (Combs, 2001). Toxicity of Se, similar to other trace elements, is not only dependent on the total concentration of the element, but also on the species to which an individual is exposed.

Se deficiency is associated with Keshan disease, a cardiomyopathy affecting mainly children and women of child-bearing age. The disease occurs in areas where grain crops contained <0.04 μg Se g^{-1} and daily intake is between 10 and 15 μg Se day^{-1}. Selenium deficiency may lower th body's defences against cancer (Diwadkar-Navsariwala et al., 2006). A dietary selenium supplement 100-200 μg day^{-1} results in decreased incidence of cancers as Lung and prostate (Clark et al., 1996; Ip and Ganther, 1992). Methyl selenocystine (Finley et al., 2000) plays an important role, a form of selenium preventing cancer which is purported to cause apoptosis, cell-cycle arrest, inhibition of tumor cell invasion angiogenesis (Zeng & Combs, 2008). Selenium fortifies

resistance against viral infections, such as HIV (Arthur, 2003; Broome et al., 2002). Replenishing selenium in deficiency conditions appears to have immune-stimulating effects, particularly in patients undergoing chemotherapy. However, increasing the levels of selenoprotein anti-oxidant enzymes (glutathione peroxidase, thioredoxin reductase, etc.) appears to be only one of many ways in which selenium-based metabolites contribute to normal cellular growth and function. Animal data, epidemiological data, and intervention trials have shown a clear role of selenium compounds in both prevention of specific cancers and antitumorigenic effects. Selenium has been linked with a reduced risk of cataract and activates the antioxidant enzyme glutathione peroxidase, protecting cell membranes from oxidative damage (Birringer et al., 2002). An intake of Se ≥55 μg day^{-1} gives optimal plasma glutathione peroxidase (GPx) activity and is the basis of current dietary recommendations.

Excessive selenium taken up by the body in the diet is excreted into urine and breath in the form of methylated selenides. Excretion of selenium is either from H_2Se through a methylated selenosugar (1β-methylseleno-Nacetyl-D-galactosamine) in urine or by further methylation of CH_3SH to dimethyl selenide ($(CH_3)_2Se$) which is exhaled in breath, and trimethyl selenonium ion ($(CH_3)_3Se^+$) excreted in urine. Though 1β-methylseleno-N-acetyl-D-galactosamine is the most significant urinary metabolite in most individuals, $(CH_3)_3Se^+$ is a major product from Se-methylselenocysteine (Rayman et al., 2008). Methylated selenium [$(CH_3)_3Se^+$] accounts for very little percent of selenium in urine in normal subjects but becomes predominant if the nutritional intake of selenium increases. $(CH_3)_2Se$ is exhaled in breath only in the case of a large excessive dose (Hira et al., 2003; Pyrzynska, 2002).

Se is strongly bioaccumulated by aquatic organisms and even slight increases in waterborne concentrations can quickly result in toxic effects such as deformed embryos and reproductive failure in wildlife (Lemly, 1997 b).

2.3 Selenium in foods

People receive the majority of their dietary selenium from food. The Se level in food depends on several geological, geochemical and climatic factors. Selenium enters the food chain through plants, and the Se concentration of plants varies according to available soil Se concentration, its bioavailability for uptake into plant roots and species

of plants (Dhillon and Dhillon, 1997). The amount of selenium concentrated in plant-based food is determined by the content in soils, which typically vary from 0.01 to 2.0 µg g^{-1} across the world. In meat, fish and eggs the value fluctuates between 0.01 and 0.36 µg g^{-1} fresh weight. The Se-content in milk and dairy products can be lower than 0.001 µg $l^{-1}g^{-1}$ and can rise up to 0.17 µg $l^{-1}g^{-1}$. The Se content of vegetables and fruits is the lowest with values between 0.001 and 0.022 µg g^{-1} fresh weights (Reilly and Aust, 1993).

Fortification using selenised fertilizers was successfully adopted in 1984 by Finland as agronomic practice, formerly a low-Se country, which resulted in boost in Se levels in crop produce (Makela et al., 1993). Similar practice in many Se deficient European countries would facilitate to achieve desired Se-intake levels in populations as per the RDA. (Rayman, 2008)

Selenomethionine (SeMet) is the major selenium-containing species in cereal grains and soybeans, and generally constitutes > 70% of total selenium (Cubadda et al., 2010). SeMet was also identified in most extracts of Indian mustard, sunflower, and white lupine (Ximenez-Embun et al., 2004). In selenium-enriched plants such as broccoli, onions, sprouts wild leeks and wheat, Se-methylselenocysteine was identified as the major organo-selenium compound (Cubadda et al., 2010; Whanger, 2002). Consumption of food rich in vitamin E, like nuts and seeds can increase the effectiveness of selenium. Products of wheat are important selenium sources for human population especially in European societies. Different types of bread contain varying amounts of selenium depending on the amount of the bioaccessible selenium in the soil where bread wheat was grown. A study showed that selenium contents in US and Canadian brown bread contained 0.41 to 0.68 µg g^{-1} and 0.06 to 0.71 µg g^{-1} of selenium respectively (Murphy and Cashman, 2001). Cereals other than wheat are important dietary selenium sources elsewhere. In many countries, such as China, Thailand, and Japan, rice is the most common source of dietary selenium. The range of mean selenium levels in rice has been reported as 0.02 µg g^{-1} in China (Wang et al., 1997), 0.05 µg g^{-1} in Thailand (Shirichakwal et al., 2005), 0.10 µg g^{-1} in the UK (Barclay et al., 1995).

The increased research in human cancer prevention shows great interest in the feasibility of using selenium supplementation to lower cancer risks. Selenium supplementation will be beneficial for people with lower dietary selenium intake and for people with iodine deficiency (Corvilain et al., 1993; Arthur, 1991). Various nutritional

supplements based on selenium, mainly in the form of selenite or Se-enriched yeast containing predominantly selenomethionine, are available. Since selenomethionine cannot be synthesized in humans, and most selenium is in the form of L-selenomethionine in enriched wheat, corn and grains (Yang et al., 1997), this organo-selenium compound is considered the most appropriate form for selenium supplementation. Yeast is considered as an economical source of organo-selenium as a supplement due to ease of management and the yield of the product. The selenomethionine concentration ranged from 60% to 84% of selenium in ten selenium enriched yeast samples (Rayman, 2004). Another study reported that selenomethionine consisted of 85% of total selenium in enriched yeasts (Ip et al., 2000).

2.4 Selenium hyperaccumulation in plants: Mobilization and remediation

Selenium has not been classified as an essential element for plants, although its role has been considered to be beneficial in plants capable of accumulating large amounts of the element (Shanker, 2006). In cultivated plants, the selenium content has been increased using various techniques of enrichment. Plants that have been examined for Se fortification through soil or foliar application include Indian mustard, onion, garlic, soybean and cereals (Sugihara et al., 2004; Ximenez-Embun et al., 2004; Wrobel et al., 2004; Kahakachchi et al., 2004; Stadlober et al., 2001; Kotrebai et al., 1999, Gupta and MacLeod, 1994; Sima and Gissel-Nielsen, 1985). Se-enriched vegetables may be a better delivery source for organoselenium analogs than the commonly used selenite or selenomethionine. A survey on the research concerning to the Se uptake and its allocation in plants tissues shows that the studies have been extensively carried out in various species and reviewed (Cartes et al., 2006; Terry et al., 2000). With reference to the crops cultivated in seleniferous soils of India, Dhillon and Dhillon (2009a) recently reported the usefulness of Brassica-based cropping systems for phytoremediation of Se-contaminated soils. Irrespective of harvesting stage, total Se removed in the above-ground biomass of rapeseed-based cropping sequences was significantly higher than wheat-rice cultivation pattern commonly applied in this region.

Plants are thought to take up and metabolize Se via the sulfur assimilation pathway, resulting in Se accumulation in plant tissue and production of (low-toxic) volatile methylated selenium compounds (Pilon-Smits et al., 1999; Lewis et al., 1966).

Hyperaccumulators are efficient selenium extractors however their phytoremediation potential is often limited by their slow growth rate and low biomass production (Germ et al., 2007). Selenium hyperaccumulator species prominently show high levels of Se volatilization (Terry et al., 2000). Plants have the ability to absorb and sequester selenium and to convert inorganic selenium to volatile forms of organic compounds that are released harmlessly into the atmosphere (Banuelos et al., 2002). Once absorbed by plant roots, Se is translocated to the shoot where it may be harvested and removed from the site (Wu et al., 2003). Indicator plants such as the *Astragalus* species can accumulate extremely large amounts of selenium, ranging from 1000 to 10,000 μg selenium g^{-1}, because they synthesize mostly non-protein seleno-amino acids (Brown and Shrift, 1981). The Se-accumulators can take up high concentration soil selenium without exhibiting any negative effects due to presence of non-proteinogenic Se-amino acids such as Se-methylselenocysteine, selenocystathionine, Se-methylselenomethionine, γ-glutamyl-Se-methylselenocysteine, selenohomocysteine and selenopeptides.(Whanger, 2002; Terry et al., 2000). The major form of selenium is Se-methylselenocysteine in *Astragalus* and selenium enriched garlic (Ip et al., 2000; Kotrebai et al., 1999; Uden et al., 1998; Bird et al., 1997; Cai et al., 1995), onions (Uden et al., 1998; Cai et al., 1995), broccoli florets (Cai et al., 1995) and sprouts (Finley et al., 2001), and wild leeks (Whanger et al., 2000). At low selenium concentrations, Se-methylselenocysteine is the major form present but at elevated concentrations γ-glutamyl-Se-methylselenocysteine is the predominant one, and it has been hypothesized that it serves primarily as a carrier of Se-methylselenocysteine (Dong et al., 2001).

Wheat (*Triticum aestivum*) is known to be a good source for bioavailable selenium. Wheat and alfalfa were less resistant to excess Se and have been found to enrich it in sprouts up to concentrations of 100 and 150 mg of Se kg^{-1} of dry mass, respectively (Lintschinger et al., 2000).The highest selenium concentrations ever recorded in wheat was 185 µg g^{-1}, which was found in grain collected in the Nawanshahr-Hoshiarpur Region of Punjab, India (Cubadda et al., 2010). In another study, plants of broccoli (*Brassica oleracea* var. *botrytis L.*), Indian mustard (*Brassica juncea L.*), sugarbeet (*Beta vulgaris L.*) and rice (*Oryza sativa L.*) were grown hydroponically in growth chambers and treated for 1 week with 20 mM Se as Na_2SeO_4, Na_2SeO_3 or *L*-selenomethionine (SeMet) and increasing sulfate levels. The data show that shoots of

SeO_4^{2-} supplied plants accumulated the highest amount of Se, followed by those supplied with SeMet then SeO_3^{2-}. In roots, the highest Se concentrations were attained when SeMet was supplied, followed by SeO_3^{2-}, then SeO_4^{2-}. The rate of Se volatilization by plants followed the same pattern as that of Se accumulation in roots, but the differences were greater. Speciation analysis (X-ray absorption spectroscopy) showed that most of the Se taken up by SeO_4^{2-} supplied plants remained unchanged, whereas plants supplied with SeO_3^{2-} or SeMet contained only SeMet like species. Increasing the sulfate level from 0.25 mM to 10 mM inhibited SeO_3^{2-} and SeMet uptake by 33% and 15-25%, respectively, as compared to an inhibition of 90% of SeO_4^{2-} uptake (Zayed et al., 1998). Shane et al. (1988) examined the uptake of selenium in agricultural soils amended with various percentages of coal fly ash. Broccoli, lettuce, onions, spinach, tomatoes, and perennial ryegrass were cultured in pots or pillow packs containing growth media that had been amended with increasing percentages of soft coal fly ash. The crops absorbed selenium in proportion to the percentage of fly ash in the growth media. Broccoli, onions, and lettuce absorbed the highest concentrations of selenium.

Inorganic selenium uptake and its transformation in different species were also evaluated in Indian mustard (*Brassica juncea*), sunflower (*Helianthus annus*), white lupine (*Lupinus albus*) garlic (Kotrebai et al., 2000; McSheehy et al., 2000) and white clover (Emteborg et al., 1998). More than 1.2 g kg^{-1} (dry matter) of Se was found in the aerial part of Indian mustard when growing on 1 mg L^{-1} of Se as Na_2SeO_4, and approximately half this amount was determined in the leaves of the lupine. Selenomethionine was the main selenium-containing amino acid identified in most of the extracts by HPLC-ICP-MS. The higher values were 6.8 and 14.5 mg kg^{-1} (expressed as Se in dry matter) in the leaves of lupine and sunflower, respectively. Se concentrations in broccoli floret <5 mg kg^{-1} were considered safe for human consumption (Von-Vleet, 1992). Selenium application could increase selenium content from 0.025 μg g^{-1} in regular polished rice to 0.471-0.640 μg g^{-1} in Se-enriched polished rice (Chen et al., 2002). This is of great importance because some authors have considered the combination of this enriched material with non-enriched food as a source of selenium supplementation (Ximenez-Embun et al., 2004).

In terms of characterization of the forms of selenium in the hyperaccumulating crops, selenium-enriched plants, such as hyperaccumulative phytoremediation plants

(*Astragalus praleongus*, 517 μg g^{-1} Se, and *Brassica juncea*, 138 μg g^{-1} Se in dry sample), yeast (1200, 1922 and 2100 μg g^{-1} Se in dry sample), ramp (*Allium tricoccum*, 48, 77, 230, 252, 405 and 524 μg g^{-1} Se in dry sample), onion (*Allium cepa*, 96 and 140 mg g^{-1} Se in dry sample) and garlic (*Allium sativum*, 68, 112, 135, 296, 1355 μg g^{-1} Se in dry sample) were analyzed by HPLC-ICP-MS for their selenium content and speciation. The samples analyzed fell into three distinct categories. In the first category, selenomethionine was the principal component of the different yeast samples. The second category includes the *Allium* samples (ramp, garlic and onion) containing mainly Se-methylselenocysteine or related compounds. Phytoremediation samples form category three, with very poor inorganic to organic conversion in *Brassica juncea* and with the formation of *Se*-cystathionine in *Astragalus praleongus* (Kotrebai et al., 2000).

Selenium (Se) is also observed to accumulate in kale (*Brassica oleracea L. var. acephala*), which has high levels of lutein and *β*-carotene. Selenium, lutein, and *β*-carotene have important human health benefits and possess strong antioxidant properties of Kale. Kale was grown in hydroponic nutrient solution supplemented with sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3). Increase in either selenate or selenite resulted in decrease in kale leaf tissue biomass. Neither of the Se treatments had an effect on the accumulation of lutein or *β*-carotene in leaf tissues. Growing kale in the presence of SeO_4^{-2} resulted in the accumulation of high levels of tissue Se without affecting carotenoid concentrations (Lefsrud et al., 2006)

Similar, studies have also been carried out with other edible crops such as Chicory (*Cichorium intybus*) (Germ et al., 2007); pea (*Pisum sativum*) (Smrkolj et al., 2006); potato (*Solanum tubersum*) (Turakainen et al., 2006); radish (*Raphanus sativus*) (Pedrero et al., 2006) and Pursh brassica (*Stanley pinnata*) (Feist and Parker, 2001) by various researchers.

Glutathione peroxidase (GSH-Px) like superoxide dismutase is of fundamental importance to the life of the cell, and its activity is not readily reduced by deficiencies in dietary intake of these nutrients. Nevertheless, one selenium intervention study reported remarkably lower risks of several cancers after 4.5 years of selenium at 200 μg day^{-1} (Clark et al., 1996). The effects were so strong on total cancer mortality that the study was stopped prematurely. Biologically selenium acts as an antioxidant by preventing the

reduction of tocopherol concentrations, inhibiting lipid peroxidation and by enhancing superoxide dismutase (SOD) and GPx activity in ryegrass (Xue et al., 2001, Hartikainen et al., 2000).

Selenium has not been recognized as an essential element in plants, but several studies demonstrate that it has antioxidant effects (Djanaguiram et al., 2005; Xue et al., 2001). Some vegetal species grown in selenium enriched media are shown to enhance the photooxidative stress tolerance of the plant (Breznik et al., 2005; Sepanen et al., 2003). In plants, low concentration of selenium inhibits lipid peroxidation in *Lolium perenne* and this decrease coincides with an enhancement of growth (Hartikainen et al., 2000). At high concentration, it acts as pro-oxidant and leads to significant reduction in yield. Selenium can enhance the total antioxidant capacity in "Seoul" lettuce (Lee et al., 2001) cultivated in hydroponic condition without any nutritional loss. Rios et al. (2008) reported that the Se augumented LOX activity, foliar concentration of MDA, and protein oxidation measured with carbonyl groups was higher in Se (IV) as compared to Se (VI). Inverse relationship was observed between the TBARS and GSH-Px levels of control and experimental plants indicating the influence of Se exposure on the modulation of both pro and anti-oxidant activity. The high Se dosage, in general, was observed to boost the GSH-Px activity to neutralize the Se induced oxidative stress recorded as a distinct increase in TBARS (Thiobarbituric acid reactive substances) values. Such variations during the growth were reported by Hartikainen et al. (2000) in rye-grass supplemented with selenium. It plays a significant role as antioxidant in higher plants.

2.5 Observations on Se accumulation and therapeutic activity in *Alliums*

For most of the world's cultures, onion consumption exceeds garlic consumption. Onion also produces greater edible bulb biomass than garlic, making it an additional and perhaps important target for Se-enriched vegetables for human consumption. Although bulbs of vegetables of the *Allium* genus are commonly studied, in some geographical areas the green tops of the onions (so called green onions (*Allium cepa*)) are consumed.

Preliminary reports indicate the hyperaccumulating potential of *Allium* species, but these crops have never been envisaged to be phytoremediating crops (Whanger et al., 2000; Ip and Lisk, 1994a). Although Ip and Lisk (1995) and other researchers showed the implications of using Se hyperaccumulating crops like *Allium* for prevention of cancer,

the concept of using metal hyperaccumulating crops for nutraceutical applications is still a recent concept. Garlic and onion accumulate higher concentration of selenium due to presence of chemically similar sulfur and their derivatives (Klapec et al., 2004; Ellis and Salt, 2003; Ip et al., 1992). Amongst the *Alliums*, it is reported that garlic cultivated with selenium fertilization was superior to regular garlic in mammary cancer prevention in the rat. Supplementation of 1 or 2 μg g^{-1} Se in the diet from the high-selenium garlic produced a 56% or 75% reduction respectively in the total tumor yield. Two distinct differences were noted with the high-selenium onion regarding its capacity to accumulate selenium and its efficacy in cancer prevention. First, the selenium concentration in onion was considerably lower (28 μg g^{-1} Se dry wt) as compared to that found in garlic (110-150 μg g^{-1} Se). Second, given the same levels of selenium supplementation, the high-selenium onion was apparently not as powerful as the high-selenium garlic in mammary cancer inhibition. Thus different plants, even those of the same genus, were observed to respond in their unique way towards the biological benefits of selenium enrichment depending on the species (Ip et al., 2000; Ip and Lisk, 1994b). Ramps (*Allium tricoccum*) were grown either in a mixture of vermiculite and peat moss or hydroponically with various concentrations of selenium as sodium selenate. The concentrations used were from 30 to 300 mg Se kg^{-1} of vermiculite-peat moss or from 10 to 120 mg L^{-1} in the hydroponic solutions. Concentrations as high as 784 mg Se kg^{-1} were obtained in the ramp bulbs when grown with high levels of selenium in the vermiculite-peat moss. In case of cultivation in hydroponic conditions, accumulation of up to 600 mg of selenium kg^{-1} was obtained. The predominant form of selenium in the ramp bulbs at all concentrations of selenium was Se-methylselenocysteine, with lower amounts of selenate, Se-cystathionine and glutamyl-Se-methylselenocysteine. There was an approximately 43% reduction in chemically induced mammary tumors when rats were fed a diet with Se-enriched ramps. Dietary Se-enriched ramps for rats did not result in excessive tissue selenium accumulation or undesirable side effects. Bioavailability studies with rats indicated that selenium in ramps was 15-28% more available for regeneration of glutathione peroxidase activity than inorganic selenium as selenite. Therefore, Se enriched ramps appear to have potential for the reduction of cancer in humans (Whanger et al., 2000).

Further to the above observations, selenium accumulation has been documented in onion (Gutenmann and Lisk, 1996; Ip and Lisk, 1994). A suite of unique organosulfur compounds present in the various vegetable *Allium* species, including onion (*A. cepa*) and garlic (*A. sativum*), possess medicinal properties, among them chemopreventative (Belman et al., 1987), cardiopreventative (Goldman et al., 1996; Goldman et al., 1995), and other health-related effects. Se is thought to play a role in reducing the growth of cancerous tumors in animal systems (Axley et al., 1991) and lung, colorectal, and prostate cancers in humans (Clark et al., 1996). In studies using Se-enriched garlic, Ip and Lisk (1995) demonstrated that organoselenium compounds are more active than S analogs in chemoprevention. It was further observed that Se-methyl selenocysteine and γ-glutamyl-Se-methyl selenocysteine, the two major Se-compounds in garlic and onion are anti-cancer agents with similar action mechanism (Dong et al., 2001). The highest chemopreventive activity was observed for Se-methyl selenocysteine and Se-allyl selenocysteine (Arnault and Auger, 2006; Block et al., 2001). Feeding Se-enriched garlic and onion to cancer-induced rats reduced total tumor yield but did not cause excessive Se accumulation in animal tissues (Ip and Lisk, 1994), suggesting that Se-enriched vegetables may be a better delivery source for organoselenium analogs than the commonly used selenite or selenomethionine.

Onions contain a wide variety of microconstituents such as flavonoids, mainly quercetin, and sulfur compounds, like sulfides and polysulfides, which may have protective effects against cancer. The administration of such components in pure form to animals was able to influence the carcinogenicity of environmental chemicals (Suschetet et al., 1998; Smith at al., 1994). Allyl sulfides and alkyl sulfides have been shown to increase phase II enzymes such as glutathione S-transferase (GSI) and UDPglucuronosyltransferase (UGT) implicated in mechanisms of cancer prevention (Teyssier et al., 2001; Guyonnet, et al., 1999) and/or modulate cytochromes P450 (CYP) activities responsible for carcinogen activation (Siess et al., 1997; Reicks and Crankshaw, 1996). By controlling the intensity and frequency of the crop fertilization with water-soluble selenite salts it is possible to cultivate Se-enriched garlic with 100-1355 $\mu g\ g^{-1}$ of Se (Ip and Lisk, 1995). As a point of reference, natural garlic sold in grocery stores contains $<0.05\ \mu g\ g^{-1}$ Se.

The chemistry and biochemistry of selenium is more easily expressed in terms of the better-known sulfur chemistry because of the great similarities in chemical properties that selenium and sulfur share by virtue of being adjacent group VIA elements, Antagonistic sulfate/selenate interactions in uptake have been reported in single cells, excised roots, and whole plants (Lauchli, 1993). To understand the sulphur-selenium interactions, 'Granex 33' onions were grown in nutrient solutions with one concentration of S and increasing Se concentrations. Selenium was applied as sodium selenate (Na_2SeO_4) at concentrations of 0, 0.5, 1.0, 1.5, and 2.0 mg L^{-1}. Selenium depletion from the nutrient solution increased linearly with increasing treatment concentrations of Na_2SeO_4. Sulfur depletion increased and then decreased with increasing Na_2SeO_4 treatment concentrations. Selenium and S accumulation were highest in leaf tissues, less in root tissues, and lowest in bulb tissues at plant maturity (Kopsell and RandIe, 1997).

2.6 Phytoremediation and biofortification

As many of the metals that can be hyperaccumulated are also essential nutrients, it is easy to say that food fortification and phytoremediation are two sides of the same coin (Guerinot and Salt, 2001). Plants that accumulate elements such as selenium may be used as a natural source of mineral supplements for both animals and human beings, especially in areas that are mineral deficient (Moreno et al., 2001; Elles et al., 2000). The field of plant mineral nutrition has been around for a long time, but the idea of fortifying foods pre-harvest with the essential minerals required for healthy diet is relatively new (Guerinot and Salt, 2001; Grusak and Della-Penna, 1999). Fortification within the edible plant parts enhances nutritional quality for all types of food materials. A new paradigm for agriculture in the 21st century was proposed (Welch and Graham, 2000) that views agriculture as an instrument for public health and focuses attention on the role of agriculture in delivering nutrients to humans and animals in balanced amounts that can sustain maximal physical and mental activity of the humans. The Biofortification Concept seeks to bring the full potential of agricultural and nutrition science to bear on the persistent problem of micronutrient malnutrition (Guerinot and Salt, 2001). Some vegetables also contains various organic forms of Se such as Se-methyl-SeCys present in *Allium,* wheat and *Brassica* crops which can be converted directly into methyl selenol, a bioactive substance that may protect against cancer, thus making these crops as potential

targets for biofortification programme (Cubadda et al., 2010; Broadley et al., 2006; Whanger, 2004; Lu et al., 1995). Se-methyl-SeCys was found to be very minor compound in high Se wheat.

2.7 Phyto/Rhizoremediation

An important contribution to the transformation of soil contaminants is ascribed to microbes present in the rhizosphere of plants present in contaminated site. This contribution of the rhizospheric population is referred to as rhizoremediation (Schwab and Banks 1994; Anderson et al., 1993). The use of plants in combination with microbes has the advantage of causing an increase in microbial population numbers and metabolic activity in rhizosphere. It also can establish an increase in contact between the microbes associated with the roots and the contaminants in the soil (Kuiper et al., 2001; Nichols et al., 1997; Schwab et al., 1995; Kingsley et al., 1994; April and Sims 1990). Very few studies report the direct introduction of a microbial strain for xenobiotic degrading activities on plant seed, which subsequently is able to efficiently colonize the root and sustain on the root system (Sriprang et al., 2002; Kuiper et al., 2001; Ronchel and Ramos 2001).

Metal tolerant growth promoting rhizobacteria have shown a substantial protection to plants against metal toxicity, and consequently improved the growth, symbiosis and seed yield of plants (Wani et al., 2008; Wani et al., 2007a; Chaudri et al., 2000). Rhizosphere bacteria seem to be also responsible for enhancing plant-mediated selenium volatilisation and the accumulation of selenium in plants (De Souza et al. 1999a; 1999b). Inoculation of rhizobacterial strains obtained from the rhizosphere of *A. murale* grown on a serpentine site proved to enhance nickel extractability from soil and to increase its uptake in *A. murale* and similar reports were also observed in terms of uptake of zinc in *Thlaspi caerulescens.* (Abou- Shanab et al., 2006; Whiting et al., 2001).

Besides their role in protecting the plants from metal toxicity, the plant growth promoting rhizobacteria are also well known for their role in enhancing the soil fertility and promoting crop productivity by providing essential nutrients (Zaidi and Khan 2006; Zaidi et al., 2004; Gupta et al., 2004) and growth regulators (Wani et al., 2007b).

2.8 Tracing microbial treatment

The success of introduced rhizosphere microbial treatment in the soils can be monitored by using different strategies such as most probable number (MPN), antibiotic resistance pattern, selection on specific growth substrate, PCR amplification of specific genes involved in degradation process etc. All these techniques have one or more limitation. Versalovic and co-workers (1991) described a method for fingerprinting bacterial genomes by examining strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes. Two main sets of repetitive elements are used for typing purposes. The repetitive extragenic palindromic (REP) elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem (Stern et al., 1984). The enterobacterial repetitive intergenic consensus (ERIC) sequences are a second set of DNA sequences which have been successfully used for DNA typing.

The REP or ERIC amplification can be performed with a single primer, a single set of primers, or multiple sets of primers. Application of both REP and ERIC-PCR to samples to be typed increases the discriminatory power over that of either technique used alone. Rep-PCR is fast becoming the most widely used method of DNA typing. Rep-PCR with primers based on REP and ERIC sequences has been successfully used to differentiate strains of *Bartonella* (Rodriguez et al.,1995), *Bacillus subtilis* (Versalovic et al.,1991), *Citrobacter diversus* (Harvey et al.,1995; Woods et al.,1992) *Enterobacter aerogenes* (Georghiou et al.,1995), *Rhizobium meliloti* (De Bruijn, 1992), methicillin-resistant *Staphylococcus aureus* (Del Vecchio et al., 1995), *Streptococcus pneumoniae* (Zoe-Jordans et al.,1995; Versalovic et al.,1993), *Acinetobacter baumanii* (Dijkshoorn et al.,1996; Reboli et al.,1994), *Burkholderia cepacia* (Hamill et al.,1995), *Legionella pneumophilia* (Georghiou et al.,1994), *Helicobacter pylori* (Kwon et al.,1998), *Neisseria gonorrhoeae* (Poh et al.,1996), and *Neisseria meningitidis* (Woods et al.,1996). The technique is easy to perform and can be applied to large or small numbers of isolates. Rep-PCR shows broader species applicability and better discriminatory power than either plasmid profiling or genomic fingerprinting (Georghiou et al., 1995).

2.9 Analytical approaches for detection of selenium

The range of analytical techniques in the field of selenium is very broad. A variety of analytical methods can be used to determine trace concentrations (ng g^{-1}) of selenium in biological tissues. The analytical methods generally fall into two groups: (1) those that do not require the destruction of organic materials in the sample and (2) those that require the elimination of interfering matter before the selenium content can be measured. X-ray fluorescence and some of the neutron activation analysis techniques do not require sample destruction, whereas spectrophotometry (Huang et al., 1996; Hoste and Gillis, 1955; Ramachandran et al., 1993), GC-ECD (Singh et al., 1997; Poole et al., 1977), atomic absorption spectrometry (Oster and Prellwitz, 1982), ICP-MS (Pedersen and Larsen, 1997; Goessler et al.,1997; Ge et al., 1996 ;Yang and Jiang, 1995), fluorometry (Bellanger, 1995), and other neutron activation analysis techniques require some degree of sample destruction. Fluorometry and atomic absorption spectrometry are the most frequently used methods. Inductively coupled plasma (ICP) emission spectrometry (ICP-AES) can be used to measure selenium concentrations in biological materials if the hydride generation (HG) technique is used to improve the detection power. ICP techniques offer multielement capabilities, but instrumentation is costly and the analysis is influenced by background interference. ICP-MS has been used to determine the concentration of selenium in cloud water at detection limits of 100 and 25 pg ml^{-1} using pneumatic and ultrasonic nebulization, respectively (Richter et al., 1998).

AAS techniques are commonly used for the determination of selenium in environmental samples. Hydride generation AAS is more sensitive than flame or graphite furnace AAS for the determination of selenium in materials of variable composition. Water samples, including freshwater, river water, sea water, and surface waters, and industrial wastes, muds, sediments, and soil samples have been analyzed by AAS techniques to detect selenium at parts-per-trillion levels (Bem, 1981). Selenium (VI) and selenium (IV) can be distinguished in water samples with GFAAS by selective extraction procedures. Graphite furnace atomic absorption spectroscopy (GFAAS) offers high sensitivity ($5x10^{-11}$ g selenium g^{-1} sample), but interference from the matrix can cause significant difficulties (Lewis 1988). One advantage of GFAAS techniques is that material in the graphite sample cell can be chemically treated *insitu* to reduce chemical interferences. HGAAS can also be used to distinguish between selenium (VI) and

selenium (IV) in environmental samples because selenium (VI) does not readily form the hydride without reduction (Koirtyohann and Morris, 1986). HGAAS offers reduced chemical interferences but requires larger sample volumes than GFAAS techniques.

NAA has been used to determine selenium levels in environmental samples. For determining selenium levels in soil, radiochemical variants of NAA have been commonly employed (Bem, 1981). Chemical separation in conjuction with INAA is used for speciation studies of selenium. NAA techniques provide lower detection limits for selenium (between 10^{-8} and 10^{-9} g selenium g^{-1} of sample), but there are few reactors at which NAA facilities and expertise are available (Koirtyohann and Morris, 1986). The most common NAA procedure for selenium determination is to produce the long lived ^{75}Se radionuclide (half-life of 119 days) and count the samples after a 50–100-hour irradiation period and a 2–10-week cooling period. A faster NAA technique utilizes metastable ^{77m}Se, which has a much shorter half-life (17.4 seconds), so that counting can be initiated after an irradiation and cooling period of <1 minute (Koirtyohann and Morris, 1986).

Numerous analytical methods are available for the determination of selenium levels in environmental media (Bem, 1981; Koirtyohann and Morris, 1986). For the determination of total selenium only, fluorometry, chromatography, or spectrometry is the preferred techniques. However most of these require modifiers, detectors and pre-treatment steps.When conducting a multielement analysis or when analyzing a complex matrix, more sophisticated methods are required. The advantages of NAA over other techniques are its non-destructive, low detection limits and multielement capability (Molokhia et al., 1979).

The accurate determination of selenium is of great importance in food stuffs (Camara et al., 1995; Ip and Lisk, 1994a). Studies on the selenium levels in vegetables and food grains have been carried out using INAA and allied methods by various research groups. Some of the prominent reports on Se levels in vegetables of genus allium, in meat and cereals and in Portuguese meat and vegetables and crops estimated using NAA include Ventura et al. (2007); Naughton and Marks, (2002); Combs, (2001); Diaz-Alarcon et al. (1996) and Noda et al. (1983) respectively.

2.10 Lacunae

A review of reports across the literature indicated the following:

(a) There are limited studies on Se tolerant bacteria from tropical seleniferous soils especially of that Indian sub-continent and their Se mobilization potential

(b) Studies on the role of microbes in modulating the Se uptake by plants from soil are still scarce.

(c) Although, various reports show hyperaccumulating potential of *Allium* species (Ip and Lisk, 1995), information on use of these crops for mobilization of Se from natural soils is limited

Keeping in view (a) the need to understand the Se uptake and its biological activity in plants, and (b) an aim to introduce *Allium* as a Se hyperaccumulating and fortifying crop, the present study had the following objectives:

(a) To characterize seleniferous soils and isolate selenium tolerant bacteria

(b) To determine the uptake of soil selenium by *Allium* species with rhizospheric bacteria

(c) To examine the anti-oxidant and related properties in plants grown in seleniferous soils

3.0 Materials and Methods

3.1 Sample Collection

Selenium contaminated soil samples were collected from a seleniferous site bordering the Nawanshahr-Hoshiarpur region in North-west India (75º55 E; 31º56 N). Soil samples were collected from agricultural fields of this belt from rhizospheric zone of crop plants, dominantly wheat, in sterile polybags and stored at 4 ^{o}C until their further use. For further analysis, composite samples were prepared from each sampling site. The samples were tested for various parameters as outlined in section 3.2.

3.2 Soil Analysis

3.2.1 Determination of pH (Jackson, 1967)

I. 10 g of air dried soil was weighed and passed through 2 mm sieve into a 100 ml beaker.
II. 20 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.
III. The pH of sample was measured by immersing the electrode in supernatant solution, after calibrating the pH meter with appropriate buffers.
IV. The pH value was recorded on obtaining stabilized reading.

3.2.2 Electrical conductivity (Jackson, 1967)

I. Electrical conductivity was measured in μS cm^{-1}.
II. 10 g of soil was placed in a 100 ml beaker and 20 ml distilled water was added.
III. The soil-water mixture was allowed to stand undisturbed until the soil settled completely. 0.01 M KCl solution (EC=1413 μS cm^{-1}) was used to calibrate the meter.
IV. The electrode was dipped in the supernatant solution and the observation was recorded.

3.2.3 Cation Exchange Capacity (Rhoades and Polemio, 1977)

Reagents

I. 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 1.0 l after adjusting the pH to 8.2 by using acetic acid or sodium hydroxide solution.

II. Reagent B: Ethanol (95%)

III. 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 upto 1.0 l. The pH was adjusted to 7.0 by using acetic acid or ammonium hydroxide solution.

IV. Standard Stock solution (1000 ppm Na): 2.5418 g of dry NaCl was dissolved in deionized water to final volume of 1l.

V. Working Standard Na solution: For standard, 2, 4, 6, 8, 10, 15, 20 ml of stock solutions were pipetted in volumetric flask and final volume was made to 100 ml with 1.0 N Ammonium Acetate solution and 25 ml LiCl solution. These solutions contained 20, 40, 60, 80, 100, 150 and 200 ppm of Na along with equal concentrations of LiCl (25 ppm).

***Procedure*:**

I. 4 g of dried soil was taken in centrifuge tube and 33 ml of 1.0 N ammonium acetate 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.

II. The above step was repeated for four times with 33 ml of 1.0 N ammonium acetate solution

III. The sample was washed with 33 ml of 95% ethanol three times and the supernatant liquid was discarded each time.

IV. Adsorbed Na^+ was replaced from the sample by extraction with the 33 ml of 1.0 N 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 1.0 N Ammonium acetate solution.

V. Calibration curve was drawn using flame photometer by running the standards and the emission readings were taken at 767 nm.

VI. The concentration of Na in soil sample was calculated using 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= wt of dried soil

3.2.4 Estimation of soluble potassium

I. This fraction was measure of amount of potassium extracted from soil by water.

Reagents

II. Standard Stock solution (1000 ppm K): 1.907 g of dried KCl was dissolved in deionized water to final volume of 1.0 l.

III. Standard K solution: 2, 4, 6, 8, 10, 15 and 20 ml were pipetted out of stock solution in different volumetric flasks and finally made the volume to 100 ml with deionized water. The potassium concentrations of these solutions were 20, 40, 60, 80, 100, 150 and 200 ppm respectively.

Procedure

IV. 5 g of air dried soil was weighed and passed through 2 mm sieve into a 250 ml Erlenmeyer flask.

V. 100 ml of deionized water was added to the soil and mixture was shaken for 1 h.

VI. It was filtered using Whatman-42 filter paper 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

3.2.5 Estimation of available phosphorus (Olsen et al., 1954)

Reagents

I. 0.5 M sodium bicarbonate ($NaHCO_3$) extracting 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.

II. 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 volume was made up to 2 l with distilled water.

III. Reagent B (freshly prepared): 1.058 g of ascorbic acid was added to 200 ml of reagent A and mixed.

IV. Sulphuric acid (2.5 M): 140 ml of concentrated H_2SO_4 was taken and diluted to 1 l.

V. 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 1.0 l.

VI. Working Standard P solution (1 ppm): 20 ml of (50 ppm P) solution was pipetted and diluted to final volume of 1.0 l.

Procedure

I. 2.5 g soil/fly ash was placed in a 100 ml Erlenmeyer flask followed by the addition of 50 ml extracting solution.

II. The solution was kept on a shaker for 30 minutes and filtered through Whatman No. 42 filter paper.

III. 10 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.

IV. A blank was prepared as above. 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. The P concentrations of these solutions were 0.04, 0.1, 0.2, 0.3 and 0.4 ppm respectively. After 10 minutes, 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).

3.2.6 Elemental Analysis using Neutron Activation Analysis

I. The NAA technique is based on irradiation of a sample with neutrons, available from different neutron sources, and subsequent measurement of the induced radioactivity (β, γ) for determination of the concentration of an element. The decay characteristics permit the unambiguous identification and measurement of the radionuclides in the irradiated sample. Selenium has six stable isotopes. The

data of interest in connection with activation in a reactor with thermal neutrons are summarized in Table 1.1. Of these isotopes Se-75 has been used in several cases because the long half-life allows a chemical purification before measurement of the activity. On the other hand a long irradiation time is necessary to obtain a good sensitivity.

Procedure

II. The plant and soil samples were dried at 40ºC for 2-3 days for removal of moisture.
III. Dried soil samples were crushed and sieved using 0.2 mm mesh. Dried plant parts were crushed and homogenized using mortar and pestle after ethanol cleaning of the grinding apparatus.
IV. Samples were air dried followed by oven at 40°C for 48 h before irradiation.
V. Reference materials, IAEA SL-1 and NRCC CRM DOLT-1 were also used.
VI. Samples and reference materials weighing about 100 mg were packed in thin aluminum foils.
VII. The samples, the reference materials and blank silica were introduced into Harwell cans and irradiated in self-server position of CIRUS reactor (Bhabha Atomic Research Centre, Mumbai, India) for 7 h duration at a neutron flux of $\sim 10^{13}$ $cm^{-2}s.^{-1}$
VIII. Selenium estimation was done by producing long lived ^{75}Se radionuclide (half-life of 119 days).
IX. The samples were allowed to cool for about ten days before radioactive assay and the samples were counted for 1-10 h depending on selenium concentration levels.
X. The gamma-ray spectrometric measurements were carried out using a Compton suppressed spectrometer consisting of HPGe-BGO detector systems coupled to PC based 8k MCA card (PHAST-BARC-India).
XI. The resolution of detector was 2.0 keV at 1332 keV of ^{60}Co.
XII. The efficiency calibration of the detector was carried out by using standard gamma ray sources of ^{152}Eu.
XIII. The peak areas were determined using peak-fit software PHAST.
XIV. Relative method of NAA was used to calculate Se concentrations in the sample (Lin et al., 1997). In this method, elemental standard was co-irradiated with the sample and the activities from both sample and standard were measured in identical

geometry with respect to the detector. The mass of an element in standard ($m_{x,std}$) and count rates of standard ($cps_{x,std}$) and sample ($cps_{x,sample}$), the mass of the element in the sample ($m_{x,sample}$) is determined by the following equation. D_{std} and D_{sample} were the decay time of standard and sample respectively. The $m_{x,sample}$ (μg) is converted to concentration (e.g., $\mu g.g^{-1}$) b dividing with sample mass(g).

$$m_{x,sample} = m_{x,std} \cdot cps_{x,sample} / cps_{x,std} \cdot D_{std}/D_{sample}$$

3.3 Isolation and characterization of bacterial strains

3.3.1 Isolation of strains

I. For isolation and enumeration of cultivable bacteria, soil samples were diluted in saline (0.85%) and plated on tryptic soya agar agar (TSA) (Appendix).

II. Single bacterial isolates were selected and plated in tryptic soy agar (TSA) supplemented with Se as Na_2SeO_3 and Na_2SeO_4 accounting for 50 mg L^{-1} of Se in the growth medium at 37ºC.

III. Red colonies, indicating reduction of Se oxyanions, were re-streaked on TSA without Se to confirm that the color was not due to pigmentation.

IV. The pure cultures were maintained on Se supplemented plates.

V. Four strains were selected, based on their potential to tolerate up to 100 mg L^{-1} of selenium as sodium selenite (Na_2SeO_4) (Merck chemicals) and sodium selenate (Na_2SeO_3) (CDH chemicals) in tryptone soy broth (TSB) (Appendix) and minimal salt medium (Appendix).

3.3.2 Morphological and biochemical studies of Se tolerant isolates

3.3.2.1 Gram staining

I. Bacterial smear from actively growing cells were spread on a glass slide and heat fixed.

II. The slide was flooded with filtered crystal violet for 10 sec followed by washing briefly in water to remove excess crystal violet.

III. Further, the slide was flooded by Gram's iodine for 10 sec and washed briefly in water.

IV. The slide was decolourised with acetone until the moving dye front has passed the lower edge of the section and washed it immediately in tap water

V. Safranine was used to counter stain for 15 sec and washed with water to remove the excessive stain.
VI. The slides were visualized under microscope at different magnifications.

3.3.2.2 Catalase test

I. A small volume of bacterial cells were placed onto a clean microscope slide.
II. Few drops of H_2O_2 (3%) were added on the smear.
III. Rapid evolution of O_2 as evidenced by bubbling was considered as positive result and no bubbles were considered as negative result.

3.3.2.3 Oxidase test

I. A colony of the organism was picked from an agar slant with a sterile swab.
II. A drop of reagent (N, N, N', N'-tetramethyl phenylenediamine dihydrochloride) was put on the swab containing the culture.
III. Change of color from violet to purple within 30 seconds was taken as positive result. Delayed reactions were ignored.

3.3.2.4 Nitrate reduction test

I. Nitrate media was used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. Nitrate broth contained nutrients and potassium nitrate as a source of nitrate.
II. After incubating the culture in nitrate broth at 37°C for 48 h, 2-3 drops of sulfanilic acid and α-naphthylamine were added. If the organism had reduced nitrate to nitrite, the nitrites in the medium would form nitrous acid.
III. Further, sulfanilic acid was added; which reacts with the nitrous acid to produce diazotized sulfanilic acid. This reacted with the α-naphthylamine to form a red-colored compound. Therefore, if the medium turned red after the addition of the nitrate reagents, it was considered a positive result for nitrate reduction.

3.3.2.5 Starch Hydrolysis test

I. In this test, starch agar was inoculated with the bacterial culture.

II. After incubation at an appropriate temperature for 48 h, iodine was added to the surface of the agar.
III. Iodine turned blue-black in the presence of starch. Absence of the blue-black color indicated that starch was no longer present in the medium.
IV. Bacteria which showed a clear zone around the growth produced the exoenzyme amylase and are indicative of starch hydrolyzers.

3.3.2.6 Indole test

I. The test organism was inoculated into tryptone broth, a rich source of the amino acid tryptophan.
II. Indole positive bacteria produced tryptophanase, an enzyme that cleaved tryptophan, producing indole and other products.
III. When Kovac's reagent (p-dimethylaminobenzaldehyde) was added to a broth with indole in it and incubated at 37° C for 18 to 24 h, a dark pink color that developed was observed visually.

3.3.2.7 Methyl red test

I. The methyl red test was used to identify enteric bacteria based on their pattern of glucose metabolism. All enterics initially produced pyruvic acid from glucose metabolism. Some enteric subsequently used the mixed acid pathway to metabolize pyruvic acid to other acids, such as lactic, acetic, and formic acids. These bacteria were called methyl-red positive .Other enterics subsequently used the butylene glycol pathway to metabolize pyruvic acid to neutral end-products. These bacteria were called methyl-red-negative.
II. Each isolate was inoculated into a tube containing buffered glucose broth with a sterile transfer loop.
III. The tube was incubated at 37°C for 2-5 days. After incubation, few drops of the pH indicator methyl red were added to this tube.
IV. The tube was gently rolled between the palms of the hands to disperse the methyl red.

V. Organism that subsequently metabolized pyruvic acid to other acids lowered the pH of the medium to 4.2. At this pH, methyl red turned red which indicates positive nature of the test.

VI. Organism that subsequently metabolized pyruvic acid to neutral end-products lowered the pH of the medium to only 6.0 and in this neutral pH the growth of the bacteria was not inhibited. At this pH, methyl red was yellow. A yellow color represented a negative test.

3.4. Test of growth in variable pH and temperature

I. To determine the growth of the bacterial cells, growth experiments were carried out with 0.5ml inoculum in 50 ml TSB medium fortified with 25 mg L^{-1} of selenium as selenate and selenite individually, under various pH conditions ranging from pH 4 to pH 10.

II. The pH of the medium was adjusted using 1N HCl and 1N NaOH before inoculation.

III. The initial optical density was taken at 0 h followed by OD examination at 3 h interval for 12 h.

IV. The experiment was carried out at 37°C and 120 rpm.

V. Controls with selenate and selenite at neutral pH were maintained during the experiment. pH was recorded in the medium after 12 h exposure of organisms.

VI. Similarly, to examine the growth profile of the organisms at various temperatures, the cultures were inoculated and maintained in conditions of neutral pH but varying temperature conditions ranging from 20°C to 50°C.

VII. The observations were taken at intervals as mentioned above.

VIII. Control in this particular case was maintained at 37°C without selenate and selenite, during the experiment.

3.5 Determination of Growth in Se spiked medium

I. The growth curve of selenium tolerant bacterial isolates SNTP1, NS2, NS3 and NS4 was studied by plotting the absorbance of cultures at hourly intervals as a function of time (Cappuccino, 1987).

II. The overnight grown bacterial culture each of SNTP1, NS2, NS3, and NS4 (in duplicates) was checked for absorbance at 600 nm and a zero h reading was taken.
III. The culture was inoculated in the flasks containing TSB - 0, 5, 15 and 25 mg L^{-1} of selenium added in form of sodium selenate and sodium selenite and the flasks were placed on the shaker at 37°C and 120 rpm.
IV. The absorbance was measured after every 2 h and the process continued up to 12 h using UV-visible spectrophotometer at optical density of 600nm (OD_{600}).
V. Simultaneously, counts of the colony forming units were taken by plating the culture along with observation of OD_{600}.
VI. Observations beyond 12 h were avoided due to interference of red color of elemental selenium with the optical density.
VII. Viability was assessed through serial dilutions of culture samples, followed by plating on medium lacking selenate and selenite to determine the number of viable cells.
VIII. The growth of isolates was measured as a function of time by plotting the absorbance against each hour.

3.6 Antibiotic profiling of bacterial isolates

I. Antibiotic profiling of bacterial isolates SNTP1, NS2, NS3, and NS4 was carried out by using standard antibiotics (Cappuccino, 1987). Susceptibility of the isolate was assessed by multi-disc diffusion method (NCCLS guidelines M23-A2 and M37-A2).
II. Isolated colonies of the bacterial cultures were selected from agar plate culture and transferred into nutrient broth.
III. After incubation for overnight at 37°C under aerobic conditions, a sterile cotton swab was dipped into the suspension and streaked onto the entire surface of a Mueller-Hinton agar plate.
IV. Working solutions of antibiotics were made and filter-sterilized in the laminar flow cabin. Other range of concentrations of different antibiotics, not available commercially, were prepared with appropriate concentration in triplicate and used to assess the antibiotic resistance behavior of bacterial species as per the NCCLS standard as mentioned earlier.

V. Four to six antibiotic disks (Hi-Media) were placed onto each plate using sterile tweezers.

VI. The plates were left undisturbed for an h followed by incubation at 37ºC for 24-48 h.

VII. The diameter of clear zones (zones of inhibition) around the discs were noted and measured with callipers.

3.7 FAME Analysis

I. Fatty acid methyl esters (FAMEs) were characterized in test strains (SNTP1, NS2, NS3, and NS4) at the Microbial Type Culture Collection (MTCC-IMTECH), Chandigarh, India

II. Fatty acid methyl esters (FAMEs) were obtained from cells grown on tryptic soy agar (TSA; HiMedia, India) for 24 h at 30ºC followed by saponification, methylation and extraction (Pandey et al., 2002).

III. The methyl ester mixtures were separated by gas chromatography and analyzed by Sherlock Microbial Identification Systems software (MIDI, USA).

3.8 Molecular Methods

3.8.1 Extraction of DNA

3.8.1.1 Isolation of genomic DNA

I. A single colony of bacterial isolate was picked from a freshly grown plate and transferred it into 20 ml of alkaline nutrient broth in a 250 ml of flask. The culture was incubated for 16-24 h at 37 ^{o}C with vigorous shaking (120 cycles/minute in a rotary shaker).

II. Fully grown cells were harvested in 2.0 ml sterile microfuge tube. The media was decanted completely from the cell pellets. The residual cell pellet may be washed here using 10 mM Tris-HCl *p*H 8.0).

III. Each cell pellet was re-suspended in approximately 0.8 ml saline-EDTA buffer thoroughly. 50 µl freshly prepared lysozyme solution was added and mixed well. The reaction mixture was incubated at 37 ^{o}C for 20 min.

IV. Further, 0.2 ml 10% sodium dodecyl sulphate (SDS) was added and mixed well before incubating the same in the water bath at 60 ^{o}C for 15 min.

V. Cell suspension was extracted with organic solvents to remove proteinsand cell debris: first with phenol: chloroform: isoamyl alcohol (25:24:1) solution and centrifuged at 12000 rpm for ten min and the upper aqueous phase was transferred to a sterile microfuge tube. The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1).

VI. Nucleic acids were precipitated by adding equal volume of isopropanol to the aqueous phase, followed by 10 min centrifugation at 12,000 rpm.

VII. After centrifugation, the supernatant was discarded and the pellet was air dried.

VIII. The DNA pellets were washed with 750 µl EtOH (70%) and microfuged another 10 min.

IX. DNA was dissolved in in 40 µl TE buffer/milliQ water and stored at 4°C for further use.

3.8.1.2 Electrophoresis of DNA on agarose gels

I. DNA was loaded on agarose gels prepared in 1x TAE, pH 8.0 using a 6x loading dye (Appendix).

II. Agarose concentration of 0.7 % (w/v), 1.2% (w/v) and 1.5% (w/v) were used for genomic DNA, amplified PCR product and for RFLP patterns respectively. Ethidium bromide (0.5 -1 µg ml^{-1}) was added to stain the gel prior to pouring. The nucleic acids were then electrophoresed at 50 volts for 45-60 min and visualized on a U.V. transilluminator.

III. The DNA was purified by elution through the Wizard DNA Clean up system (Promega) according to manufacturer's instructions in order to remove contaminants, which can hamper in manipulation of DNA.

3.8.1.3 Spectrophotometric quantification of DNA

I. The concentration of extracted DNA in suspension was estimated by spectrophotometric measurement at A_{260}. For double-stranded DNA suspensions, an OD of 1.0 at a wavelength of 260 nm and using a cuvette with 1 cm light path is equal to a concentration of 50 µg ml^{-1}.

II. The quality of the DNA was evaluated by measurement of the A_{260} and A_{280} and the A_{230}/A_{260} ratios with ideal ratio expected to be 1.8-2.0.

3.8.1.4 Ethidium bromide (EtBr) fluorescent DNA quantification

I. DNA was migrated electrophoretically in an agarose gel containing EtBr (0.5 µg ml^{-1}).

II. The quantity of DNA was visually determined with reference to a known DNA concentration of lambda phage (SD 0011, MBI fermentas Canada) by comparing the intensity of fluorescence.

III. The molecule, thus obtained, was linear double stranded undigested DNA.

3.8.2 ERIC PCR based DNA fingerprinting

I. Reaction mixture for the ERIC PCR contained 1X PCR buffer (Invitrogen, USA), each deoxynucleotide triphosphate at a concentation of 200µm, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 µm and 2.5U of Taq DNA polymerase (Invitrogen, USA) in a final volume of 100 µl.

II. DNA amplification was performed with Genamp PCR system (Applied Biosystem, USA) by using the following program; initial denaturation 95 ^{o}C for 2 min, 35 cycles of 92 ^{o}C for 30 sec, 50 ^{o}C for 80 sec and 68 ^{o}C for 200 sec, final extension at 68 ^{o}C for 8 min and final soak at 4 ^{o}C.

III. The ERIC PCR primers were

a. ERIC – I R 5’– ATG TAA GCT CCT GGG GAA TCA C – 3’

b. ERIC – 2 5’– AAG TAA GTG ACT GGG GTG AGC G – 3’

3.8.3 Amplification of 16S rDNA and Purification of PCR products

I. For amplification of 16S rRNA gene, the primers used were:
Forward primer 5’-AGAGTTTGATCCTGGCTCAG-3’ and
Reverse primer 5’-ACGGGCGGTGTGTTC-3’ (Weisberg et al., 1991).

II. DNA amplification was performed with Genamp PCR system (Applied Biosystem, USA).

III. Reaction mixture for the PCR contained 1X PCR buffer (InVitrogen Inc., USA), each dNTPs at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a

concentration of 0.1 μM and 2.5 U of Taq DNA polymerase in a final volume of 100 μl.

IV. PCR conditions were as follows: Preheating at 92 °C for 2 min, 36 cycles of 92 °C for 1 min, 48 °C for 30 sec and 72 °C for 2 sec and final extension 72 °C for 6 min 10 sec.

V. Amplified DNA was verified by electrophoresis of aliquots of PCR product (5 μl) on a 1.0% agarose gel in 1 X TAE buffer. 16S rDNA amplicon was gel eluted using QIAquick columns (Quiagen Inc., USA).

VI. PCR products were purified by agarose gel (0.8%) electrophoresis prior to cloning.

VII. After staining with EtBr, a defined band was visualized under UV irradiation and excised.

VIII. Besides removing surplus primers, nucleotides, and salts, this method possessed the advantage that incomplete (shorter) amplification fragments are also removed prior to cloning.

IX. The PCR product was eluted and purified using Gel elution kit (Sigma).

3.8.4 Ligation in T-vectors

I. The 16S rDNA amplicon was ligated into pTZ57R/T or pGEM-Teasy vector.

II. The final reaction volume for ligation was 30μl and 10μl (Appendix) and incubated at 22 °C and 4 °C for pTZ57R/T and pGEM-Teasy vector respectively.

III. The reaction mixture was kept overnight and analyzed on 0.7 % agarose gel.

3.8.5 Genetic Transformation of *E. coli*

I. 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°C with vigorous shaking (200-250 cycles/min in a rotary shaker).

II. 200 μl of the above-saturated culture was septically transferred into 20 ml of fresh LB broth in a 250 ml flask. The culture was incubated with vigorous shaking at 37°C for 2-3 h. To monitor the growth of the culture, the OD_{600} was recorded on hourly basis to achieve final OD of ~ 0.5.

III. The above culture was transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. The culture was cooled to 0^oC by storing the tubes on ice for 10 min

IV. The cells were recovered by centrifugation at 5000 rpm for 10 min at 4^oC. The media was completely removed from the cell pellet. Pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and store on ice for 10-15 min.

V. Cells were recovered by centrifugation at 5000 rpm for 10 min at 4^oC. The fluid was again decanted from the cell pellets completely.

VI. The cell pellet was resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$. The cells in this stage were stored on ice for 2-2.5 h. $CaCl_2$ treatment for 2 h induces considerably a transient state of "competence" in the *E. coli* cells.

VII. 100 µl of the suspension of competent cells was transferred to a sterile and pre-chilled 1.5 ml microfuge tube. Plasmid DNA sample (~100 ng in a volume of 5 µl or less) was added to each tube, with a control tube devoid of plasmid DNA. The content of the tubes were mixed gently and stored in ice for 30 min.

VIII. The tubes were then incubated in a circulating water bath preheated to 42^oC for exactly 2 min without shaking.

IX. The contents of the tubes were rapidly transferred to fresh tubes kept in ice bath and the cells were chilled for 1-2 min.

X. The tubes with cells were transferred to room temperature.

XI. 1 ml of LB broth was added to each tube followed by incubation for 45-60 min at 37^oC to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.

XII. 100 µl of transformed cells were taken and streaked on 90 mm LA-antibiotic-X-GAL and IPTG plates and incubated at 37^oC. Appearance of transformed colonies was recorded after 12-16 h.

3.8.6 Screening for recombinant plasmids

I. After transformation of the ligated product, the E. coli DH5α (LacZ-) bacterial host cells were plated on Luria Agar (Appendix) medium containing 50 µg ml^{-1} ampicillin, for selection of transformants.

II. X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid. The cloning site in the pTZ57R/T or pGEM-Teasy vector is located in the multiple cloning sites (MCS) of the plasmid's lacZαgene; if insert was present, non-functional β-galactosidase is produced, and the transformed bacterial colony is white.

III. White colonies were picked and grown in 2 ml LB containing ampicillin (50 μg ml^{-1}) and simultaneously patch of these cultures were done on LA containing ampicillin.

IV. Plasmid was isolated (described in the preceding section) and re-amplification of the insert was done using vector's promoter specific sequences.

3.8.7 Isolation and purification of plasmid DNA from bacteria by alkaline lysis method

I. A single colony was picked from plate containing transformed *E. coli* colonies, into 2 ml of LB medium containing appropriate antibiotic viz., ampicillin used in a final concentration of 50 μg ml^{-1}, in a capped 15-ml tube. The culture was incubated overnight at 37°C with vigorous shaking.

II. 1.5-2.0 ml of the above-saturated culture was poured into an eppendorf tube and centrifuged at 8000 rpm for 5 min in a microfuge.

III. The medium was drained and the pellet was resuspended in 200 μl of ice-cold Solution I (Appendix) after vigorously vortexing. Similar steps were followed by adding 200 μl of freshly prepared Solution II (Appendix) and 300 μl of ice-cold Solution III (Appendix) was added. Finally, the content was mixed by gentle shaking and stored in ice for 5-10 min.

IV. The content was further centrifuged at 12,000 rpm for 10 min at 4°C in a microfuge and the supernatant was rransferred carefully to a fresh tube.

V. DNA was precipitated with equal volume of isopropanol, and the content was mixed well. The mixture was allowed to stand at room temperature for 5-10 min followed by centrifugation at 10,000 rpm for 10 min at 4°C.

VI. The supernatant was removed and the pellet was air dried before dissolving in 500 μl of TE buffer (*p*H 8.0).

VII. DNase free RNase solution was added to the above solution, to a final concentration of 20 µg ml^{-1}, and incubated at 37 ^oC for 30 min, with occasional shaking.

VIII. DNA was once extracted with equal volume of phenol: chloroform followed by one extraction by equal equal volume of chloroform.

IX. The upper aqueous layer was transferred to a fresh eppendorf tube, and one-tenth volume of 3.0 M sodium acetate (*p*H 4.5) was added followed by further addition of 2 volumes of ethanol.

X. The content was mixed well and kept at –20 ^oC for 1 h followed by centrifugation at 10,000 rpm for 10 min at 4^oC.

XI. The supernatant was removed and the pellet was rinsed with 1 ml of 70% ethanol at 4 ^oC.

XII. The pellet was allowed to dry in air for 5-10 min and redissolved in 50 µl of TE (*p*H 8.0).

XIII. The DNA was stored at –20^oC for further use.

3.8.8 Restriction analysis of DNA samples by agarose gel electrophoresis

I. The DNA solution was taken in a sterile microfuge tube and sterile water was added to make up a volume of 17 µl (600 ng).

II. 2 µl of the appropriate 10x restriction enzyme assay buffer (Promega) was added, mixed thoroughly by tapping the tube.

III. 1 µl (2-5 units) of the restriction enzyme was further added mixed by tapping the tube.

IV. The mixture was incubated at 37^oC for 1-2 h.

V. 4-5 µl gel-loading buffer was added to stop the reaction and content was mixed by vortexing briefly.

3.8.9 Sequencing

I. The 16S rDNA inserts were sequenced for both strands using T7 and SP6 for pGEM-T easy vector and M13 forward and reverse for pTZ57R/T vectors.

II. 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).

3.8.10 Analysis of sequence data

I. 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 artefacts generated by PCR.

II. Similarities were calculated for nearly complete 16S rDNA sequences using only unambiguously determined nucleotide positions.

III. The 16S rDNA 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., 2003).

IV. The sequences of closely related strains and uncultured bacteria were retrieved from RDP-II and aligned using multiple alignments CLUSTALW program (Thompson et al., 1997).

V. The evolutionary distance was calculated by Kimura 2 parameter, phylogenetic dendrograms were constructed by neighbor-joining method by the use of MEGA 2 package (Tamura et al., 2007).

VI. For analysis, 1500 bootstrap replicates were performed to assess the statistical support for the tree.

3.9 Selenium reduction by bacteria & biogenesis of nanoparticles

I. The isolates were further examined for their facultative nature by determining potential to reduce selenium in aerobic and anaerobic conditions. One isolate, characterized as a species related to *Bacillus* sp. (NS-3) was selected for the study.

II. The reduced form of selenium was subjected to different experimental conditions to observe the forms of selenium formed during biological reduction and further tranformations.

3.9.1 Growth profile and Se (IV) reduction under aerobic and anaerobic conditions

I. Erlenmeyer flasks and serum bottles, containing sterile TSB supplemented with 1 mM Se, were inoculated with a culture grown to log phase so as to examine the growth profile in aerobic and anaerobic conditions. Additional supplementation of 7.5 g l^{-1} sodium lactate was provided as an electron donor to the anaerobic cultures.

II. Hydrogen gas was also passed through the medium in the serum bottles for 5 min as an additional electron donor.

III. Positive (TSB with inoculum and without selenium) and negative (TSB with selenium and without inoculum) controls were maintained both for aerobic and anaerobic conditions.

IV. The negative control was used to assess the potential for chemical reduction of Se by the media. Growth of the cultures was observed by measuring the change in optical density (OD_{600}) over a 24 hour period.

V. The removal of selenite was determined by measuring the concentration of the oxyanion in the cell free supernatant (CFS) using ion chromatography (Dionex DX 600) with 9mM sodium carbonate as the mobile phase and an AS 9-HC column at 2454 psi back pressure.

VI. The samples were diluted appropriately and introduced through a GP50 gradient pump to a CD 20 conductivity detector.

3.9.2 Characterization of reduced selenium

I. The solid fraction of the inocula (reduced selenium phases and biomass) were separated by centrifugation at 4000 rpm/15 m.

II. These samples were then imaged using an environmental scanning electron microscope (ESEM, Phillips XL) employing a GSE detector at 0.4-0.7 Torr.

III. Energy dispersive X-ray (EDX) spectroscopy was performed at 16-20 kV, using spot size of 200 nm and a counting time of 100 sec to provide qualitative chemical characterization of the phases produced.

IV. The biomass with the Se-phases was then subjected to sequential washing and centrifugation (4000 rpm/5 m) steps in 70%, 80%, 90% and 99% ethanol/water (90:1,v/v), followed by a final washing step in chloroform/methanol (1:1,v/v).

V. The products were also examined using ESEM/EDX and further examined using transmission electron microscopy (TEM, Philips, CM-200). X-ray diffraction analysis using a Bruker D8 Advance X-ray diffractometer (Bruker AXS Ltd., Coventry, UK) with a Cu $K\alpha1$ source was used to determine the crystal structure of the selenium phases produced.

3.10 Selenium transformation by bacteria and plants

3.10.1 Bioaugumentation of isolates in Se supplemented soils

I. Plots (2 X 2 m) were amended with selenium in form of sodium selenate and sodium selenite to achieve final concentration of 2.5, 5.0 and 7.5 mg kg^{-1} soil.

II. All plots were maintained in duplicate.

III. Consortium was grown in Luria broth and harvested at log phase of growth.

IV. Bacterial cells, washed and resuspended in mineral salt medium were added to the soils, to a concentration of 3.9×10^{9} cells g^{-1} soil.

V. The concentration of the cells was determined by plating on Luria Agar (LA), containing carbenicillin (40 μg ml^{-1}) as marker after suitable serial dilution.

VI. Two plots were maintained as controls, one without inoculum and selenium and the other inoculated with consortia, but devoid of selenium.

VII. Soil samples (5g/plot) were withdrawn at regular time intervals in triplicates and analyzed for total residual selenium using neutron activation analysis with Lake Sediment Material (IAEA-SL-1) as reference material. Survival of populations of inoculated strains was assessed by antibiotic plating using carbenicillin as marker and Rep PCR.

3.10.1.1 Molecular methods for mapping of isolate survivability

3.10.1.2 Extraction of Genomic DNA for molecular fingerprinting

I. Bacterial colonies were resuspended in 100 μl double distilled (autoclaved) water.

II. This mixture was boiled at 95ºC for 10 min. and chilled immediately to 4ºC using PCR machine.
III. Cell debris was pelleted by centrifugation at 10,000 x g for 5 min (Biofuge, Haereus).
IV. The supernatant was diluted appropriately and used directly for PCR amplifications.

3.10.1.3 Development of molecular markers using PCR

I. Reaction mixture for the REP PCR contained 1X PCR buffer (Invitrogen, USA), each deoxynucleotide triphosphate at a concentration of 200 μm, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 μm and 2.5 U of Taq DNA polymerase (Invitrogen, USA) in a final volume of 100 μl.
II. DNA amplification was performed with Genamp PCR system (Applied Biosystem, USA) by using the following program; initial denaturation 95°C for 2 min, 35 cycles of 92°C for 30 sec, 38°C for 80 sec and 60°C for 200 sec, final extension at 68°C for 8 min and final soak at 4°C.
III. The REP PCR primers were:
REP F 5' - III ICG ICG ICA TCI GGC -3' and
REP R 5'-ICG ICT TAT CIG GCC TAC-3'.

3.10.1.4 Visualization of PCR products

I. DNA was loaded on agarose gels prepared in 1x TAE, *p*H 8.0 (Appendix) using a 6x loading dye (Appendix).
II. For amplified PCR product 1.2% (w/v) of agarose concentrations were used in gel and electrophoresed at 50 volts for 45-60 min and visualized on a U.V. transilluminator.

3.10.2 Microcosm Studies – Pot Cultivation of plants in Se supplemented soils

I. Onion plantlets (variety Nasik red) were taken from seed store Patiala. The test soil was taken from agricultural land within Thapar campus.
II. Trays with capacity of holding 11 kg soil were used for the study.

III. The plantlets were grown in Se enriched soil using sodium selenite and sodium selenate as selenium source in trays under natural climatic conditions for a period of 120 days.

IV. Selenium concentrations applied to soil were - 5 mg kg^{-1} of Se as selenate and selenite in solution.

V. The numbers of trays were- 0 mg L^{-1} (Without selenium) - 1 tray as Control, 5 mg L^{-1} (Sodium Selenite)-3 trays, 5 mg L^{-1} (Sodium Selenate)- 3 trays (with each tray having 6 plantlets).

VI. The trays were irrigated on alternative days with tap water.

VII. Sampling of plants and respective soil samples were performed (harvesting plants in duplicates for each concentration) on 0 day and after 30, 60, 90 and 120 days.

VIII. Plantlets were harvested carefully and soil samples were taken from their respective root rhizosphere.

IX. Plant samples were covered in foil, and both labeled plants and soil samples were subjected to oven drying (50ºC) for 2-3 days for removal of moisture.

X. Dried soil samples were sieved (0.2 mm mesh).

XI. Dry weight of plant samples was recorded.

XII. 1 g soil sample and in case of plant, entire dried plant sample was taken in kjeldhal flask followed by addition of 20 ml mixture of concentrated analytical grade perchloric acid (98% purity – SDFine, India) and 0.2% nitric acid (1:1) (69% purity – SDFine, India) was added gradually (USEPA, 1996).

XIII. Samples were subjected to heating in kjeldhal heating assembly set at approximately 95 ºC until 1 ml of acid with yellowish white precipitates in case of soil and no precipitates in case of plants was left.

XIV. Digested samples were left for cooling and treated with 5 ml of 0.2% HNO_3, followed by filtration with Whatman-42.

XV. Final dilutions were made by using 0.2% HNO_3.

XVI. Filtrate was analyzed for Se content by GF-AAS (Perkin-Elmer Analyst 600 - detection limit of Se-0.004 mg L^{-1}). Analytical programming with GF-AAS was performed using Win lab 3.0 software with furnace programmed set at – drying 110 ºC and 130 ºC, pyrolysis 1300 ºC, and atomization/ read step at temperature 1900 ºC, cleanout temperature 2400 ºC. A 0.3% (m/v) magnesium nitrate solution

was prepared by dissolving an appropriate amount of Mg $(NO_3)_2.6H_2O$ in the deionized water. The 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^{-1} 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.

XVII. Calibration was carried out using analytical grade sodium selenate and sodium selenite as mentioned earlier.

XVIII. Concentration of Se in the samples was recorded in triplicate.

XIX. The concentration of selenium augmented in soil was represented as mg kg^{-1} and selenium accumulated in plants were represented as μg $g.^{-1}$

3.10.3 Microcosm Studies – Field cultivation of plants in Se supplemented soils

3.10.3.1 Plot preparation

I. Field plots were prepared with size 2.0 x 2.0 x 1.0 feet for cultivating *Allium cepa* in soils with and without supplementation of selenium oxyanions.

II. Individual plots were treated with selenate and selenite (2.5, 5.0 and 7.5 mg kg^{-1} soil).

To study the role of test rhizosphere bacterial isolates on mobilization of selenium from soil to plants:

III. One set of plots were bioaugumented with inoculum of isolate consortium added at approximately 4.0 X 10^9 cells g^{-1} soil.

IV. Another set of plots were maintained without any bacterial treatment.

V. Control plots were maintained with plants (with and without inoculum) grown in soils devoid of supplemented selenium.

3.10.3.2 Plant growth studies

I. Plantlets of *Allium cepa* were cultivated in Se-supplemented (2.5, 5.0 and 7.5 mg kg^{-1} as selenite (Na_2SeO_3) and selenate (Na_2SeO_4)) soils to observe dose and time dependent uptake in presence/absence of rhizospheric bacteria.

II. Further, the effect of different concentrations of Se oxyanions on growth and accumulation profile in plants was studied.
III. Randomized block design pattern (Zar, 2004) was followed in laying of plots and sowing of plantlets.
IV. The plantlets were irrigated on alternative days with tap water (Se – Below detection limit with NAA). Plants were harvested (in triplicates for each concentration) along with rhizospheric soil at intervals of 40, 80 and 120 days.
V. The fresh weight of the plant samples were recorded for the whole plant after washing the extraneous soil under tap water and drying of plant using water sorbing paper.
VI. Plant parts namely leaf, bulb and root were taken separately for elemental and biochemical studies from plants harvested after 80 and 120 days. However, for plants harvested after 40 days, whole plant was taken for analysis as there was no clear distinction between bulb/root sections from that of over-ground tissues.

3.10.3.3 Elemental Analysis

I. The plant and soil samples were packed and dried at 40ºC for 2-3 days for removal of moisture.
II. Dried soil samples were crushed and sieved using 0.2 mm mesh.
III. Dry weight of plant samples was recorded. The whole plant was taken for analysis in case of 40 days, while different plants parts viz. root, bulb and leaf were analyzed in case of 80 and 120 days.
IV. Dried plant and soil samples were analyzed for total selenium concentration as described above in section 3.2.6.
V. The concentration of selenium augmented in soil was represented as mg kg^{-1} and selenium accumulated in plants were represented as μg $g.^{-1}$

3.10.3.4 Fingerprinting of isolates using molecular markers i.e. REP-PCR (PCR based on Repetitive Extragenic Palindromic sequences)

Population survival of inoculated bacterial strains was determined as outlined in section 3.10.1.1.

3.11 Analysis of pro-/anti-oxidant properties

Biochemical analysis to study the modulations in TBARS and GSH-Px with corresponding levels of selenium in whole plant were carried out for the plants grown in pots (as outlined in section 3.10.2) as well as in field conditions (as outlined in section 3.10.3).

3.11.1 Thiobarbituric acid reactive substances (TBARS method)

I. The determination of TBARS, as an assay for content of malondialdehyde, was carried out using the method outlined by Miller and Aust, 1989.

II. The parts of the plant harvested viz. leaves, bulb and roots were homogenized in 0.15M potassium chloride (SD Fine),0.25M tris HCl buffer(Hi-Media), 2 mM ADP(Hi-Media) and 10 µM ferrous sulphate (SD Fine).

III. Then 700µl volume from homogenate mixture was incubated at 37°C for 5 min.

IV. After 5 min, the reaction was initiated by adding 0.1 mM ascorbic acid. The final volume of reaction mixture was 1 ml.

V. The reaction was terminated after 30 mins by adding 2 ml of thiobarbituric acid reagent (0.375%TBA (HiMedia) + 15% Trichloroacetic acid (SDFine) + 0.2N HCl (Merck) Solution).

VI. The aldehydes formed were estimated at 535 nm using UV-Visible spectrophotometer (Hitachi U2800).

VII. The molar extinction coefficient was calculated 1.56 x10^{5} M^{-1} cm^{-1}.

VIII. Appropriate blank was taken without tissue homogenate. The result is represented as concentration of malondialdehyde (MDA) in M mg^{-1}.

3.11.2 Glutathione peroxidase (GSH-Px)

I. GSH-Px (EC 1.11.1.9) activity was measured by a modification of the method of Flohe and Gunzler (1984) by using 1.3 mM H_2O_2 as substrate.

II. The enzyme was extracted by the method described by Hartikainen et al. (2000) and modified by inclusion of 1 mM EDTA and 1% polyvinylpyrrolidone as protease inhibitors.

III. The enzyme activity was calculated as a level of GSH measured at 412 nm using UV-visible spectrophotometer (Hitachi U2900) and shown as nmol GSH 100 mg^{-1} FW.

Reagents

I. 1 mM DTNB (5, 5'-Dithio-bis (2-nitrobenzoic acid, M.W=396.3): 0.04 g of DTNB was dissolved in 100 ml absolute ethanol.

II. Stock solution A: 13.8 g $NaH_2PO_4.H_2O$ was dissolved in 500 ml MQ water

III. Stock solution B: 19.0 g $NaH_2PO_4.2H_2O$ was dissolved in 500 ml MQ water

IV. 100 mM phosphate buffer solution (pH: 7.0) Mixed reagent of 195.0 ml of solution A and 305.0 ml of solution B, pH was adjusted to 7.0 and final volume was made to 1 l.

V. 1.0 mM Na_2 EDTA: 9.306 g of Na_2 EDTA was dissolved in 50 ml MQ water. The pH was adjusted in range of 8-9 before adjusting the final volume.

VI. Phosphate buffer containing EDTA: 200 µl of Na_2 EDTA stock solution was pippeted in 100 ml of 100 mM phosphate buffer.

VII. 1.3 mM H_2O_2 : Pipette 102 µl of 30% H_2O_2 /10 ml of MQ water = 0.1 M H_2O_2. Pipette from this solution 130 µl /10 ml MQ water = 1.3 mM H_2O_2.

VIII. 1 % TCA (Trichloroacetic acid): 1.0 g of TCA was dissolved in 100 ml of MQ water.

IX. 1.0 mM GSH (Glutathione): 0.0615 g GSH was dissolved in 200 ml of MQ water.

Procedure

I. 60-90 mg of plant tissues, frozen in liquid nitrogen, was weighed and sample was ground to a mortar with 1.5 ml of buffer (100 mM phosphate buffer solution, pH 7.0) having 1.0 mM Na_2 EDTA and 1% PVP. Mortar was rinsed twice with the 0.75 ml of buffer and poured to the test tube (3 ml of buffer in total).

II. 2 ml of sample was pipetted to eppendorf and centrifuged at 4ºC for 20 min at 10,000 rpm.

III. 0.2 ml of supernatant was taken and 0.2 ml of buffer containing EDTA and 0.4 ml of 1.0 mM GSH was added. In control tubes 0.2 ml of buffer containing EDTA and 0.2 ml of buffer without EDTA was added.

IV. All the test tubes were kept in water bath at 25 ºC for 5 min.

V. The tubes were removed and the enzyme reaction was initiated by adding 0.2 ml of 1.3 mM H_2O_2 at 10 sec intervals.
VI. After 10 min. of the first pipetting, reaction was terminated by adding 1 ml of 1% TCA solution.
VII. The reaction mixture was kept in ice bath for 30 min.
VIII. In fresh test tubes 2.2 ml of 100 mM phosphate buffer, 0.3 ml of DTNB was taken and 0.5 ml of sample solution was added.
IX. The mixture was mixed properly and the absorbance was measured at 412 nm.
X. The enzyme activity was calculated as a level of GSH measured at 412 nm using UV-visible spectrophotometer.

4.0 Results

Section A: Characterization of seleniferous soils and isolation of selenium tolerant bacteria

4.1 Characterization of soils

Soil is a complex medium of gaseous, liquid and solid phases, between which contaminants can partition. 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 behavior of its constituents.

Soil samples (rhizospheric soil) were collected from agricultural soils from seleniferous site of Nawanshahr-Hoshiarpur districts of Punjab, India and non-seleniferous soil of Patiala. The soil was characterized for selected chemical parameters like pH, conductivity (EC), potassium, phosphorous and cation exchange capacity. The samples were also subjected to elemental analysis using the neutron activation analysis (NAA) method.

The pH of soil samples ranged from acidic to alkaline, i.e 6.2 in gypsum treated soil to 8.1 in seleniferous soil, whereas in case of soil of Patiala, pH is observably towards the alkaline range. In untreated soils of Barwa region, pH was observed to be lied in the neutral range with pH of 7 and in Jainpur the range was observed from neutral (7.1) to be alkaline (8.1). Application of different ameliorants such as gypsum to seleniferous soils to reduce the uptake of selenium in plants was noted to reduce the pH to 6.2. The reduction of pH by gypsum is mainly attributed to the acidity of gypsum.

EC values were found to be lower in gypsum treated soils in Barwa region where value was found to be 115 µS cm^{-1} and in un-treated soil of Barwa this values was 209 µS cm^{-1}. In Jainpur soils, EC value ranged from 221- 393 µS cm^{-1}.and in Patiala it was 190 µS cm^{-1}.

Table 4.1: Physico-chemical characteristics of seleniferous and non-seleniferous soils

S.No	Sample	pH	EC $\mu S\ cm^{-1}$.	Cation exchange capacity (meq/100 gm)	Phosphorous ($mg.kg^{-1}$)	Potassium ($mg\ kg^{-1}$)
1	Jainpur (severly affected)	7.1± 0.14	221 ± 0.70	333 ± 3.53	4.65 ± 0.07	40.5 ± 0.98
2	Jainpur (affected)	7.3± 0.42	272 ± 2.82	386 ± 2.12	4.39 ± 0.18	44.6 ± 1.13
3	Jainpur mustard	8.1± 0.21	393 ± 1.41	510 ± 1.41	1.60 ± 0.42	38.1 ± 2.61
4	Barwa	7.0± 0.07	209 ± 2.12	299 ± 2.10	4.86 ± 0.14	42.6 ± 2.61
5	Gypsum treated Barwa soil	6.2± 0.28	115 ± 4.24	199 ± 2.82	3.89 ± 0.08	32.3 ± 3.18
6	Patiala	7.7± 0.21	2.12 ± 1.70	250 ± 0.70	2.6 ± 0.26	14.2 ± 1.34

Cation-exchange capacity (CEC) is defined as the degree to which a soil can adsorb and exchange cations. Cation exchange capacity of soil increased with increase in pH as given in Table 4.1. In Jainpur soils, the CEC varied from 333 to 510 meq/100g of Jainpur soil where mustard was sown. Similarly in gypsum treated soils of Barwa, CEC decreased to 199 meq/100g as compared to 299 meq/100g in untreated soils. CEC of Patiala soils was found to be 250 meq/100g. The potassium content of Jainpur soil was observed to be 38.1 $mg\ kg^{-1}$ in Jainpur mustard soil, 40.1 in Jainpur affected soil and highest with value of 44.6 $mg\ kg^{-1}$ in Jainpur severly affected seleniferous soils. In untreated soils of Barwa, the concentration was 42.6 and in Gypsum treated soils of the same area, the concentration decreased to 32.3 $mg\ kg^{-1}$. In soils of Patiala potassium content was found to be lowest with value of 14.20 $mg\ kg^{-1}$.

Phosphorus availability to plants is strongly influenced by soil pH, and its availability is maximized when pH is between 5.5 and 7.5. In Gypsum amended soils of Barwa, Phosphorus levels were found to be 3.89 and in untreated soils were 4.86 $mg\ kg^{-1}$. Concentration of phosphorus was noted to decline towards alkaline range in Patiala soil with value of 2.60 and in Jainpur mustard soils with concentration of 1.6 $mg\ kg^{-1}$ accompanied by highest pH of 8.1. In severely affected soils of Jainpur, phosphorus levels were found to be 4.65 and in affected soils were 4.39 $mg\ kg^{-1}$ respectively.

4.2 Selenium content in crops cultivated in seleniferous soils

The selenium levels observed in the crop products (15-670 mg kg^{-1}) in this region are significantly higher than the global data on Se in food crops. The selenium concentrations determined in two reference materials, IAEA RM SL-1 (Lake Sediment) and NRCC CRM DOLT-1 (Dogfish Liver) are 2.82±0.08 mg kg^{-1} and 7.43±0.18 mg kg^{-1} respectively as against the reported values of 2.9 and 7.34±0.42 mg kg^{-1} respectively. The percent deviations from certified/information values are within ±3%. The selenium concentrations obtained in soil and crop products from two locations (villages) are given in Table 4.2. The uncertainties quoted in Table 4.2 are the standard deviations at ±1s confidence limits obtained from four independent sample analyses and the percent relative standard deviations are in the range of 1.5-5%.

The selenium levels in the wheat grain (115.1±2.4 mg kg^{-1}) and husks (115.2±1.8 mg kg^{-1}) were nearly same whereas in the case of mustard, the Se uptake in the seeds (670±18 mg kg^{-1}) was twice that of mustard pods (278±5 mg kg^{-1}). The relatively high level of Se in mustard when compared to wheat is presumably due to its similar chemistry with sulphur (S), which is expected to be high in mustard. Selenium levels in rice indicated a different pattern with concentration in the husk (21.7±1.1 mg kg^{-1}), which is greater than the level observed in grains (16.21±0.5 mg kg^{-1}). Relatively low uptake of selenium by rice crop was assumed to be due to volatilization of selenium during the water logging process, reducing the availability of selenium to plants. Observations on the crop products from moderately affected area indicated a similar trend of higher levels in grains/seeds of both mustard (594±14 mg kg^{-1}) and wheat (41.1±1.0 mg kg^{-1}). The soil samples examined from the Se-impacted pockets of agricultural lands from the two villages were found to be alkaline and contain 6.5 and 2.7 mg kg^{-1} of selenium respectively (Table 4.2).

4.3 Isolation and characterization of bacteria

Rhizospheric soil samples were collected from selenifereous sites of Nawanshahr and Hoshiarpur region and the bacteria were isolated. Bacterial strains were isolated

using repeated enrichment technique at 37ºC in tryptic soy agar (TSA, Hi-Media, Mumbai, India) supplemented with Se as Na_2SeO_3 (Merck, Bangalore, India) and Na_2SeO_4 (SD Fine, Mumbai, India) accounting for 50 mg L^{-1} of Se in the growth medium. Four strains were selected, based on their morphology and potential to tolerate up to 100 mg L^{-1} of selenium as Na_2SeO_4 and Na_2SeO_3 in TSB and minimal salt medium (Appendix). Four isolates prominent in Se oxyanions reduction were selected and designated as SNTP-1, NS-2, NS-3 and NS-4.

Table 4.2. Selenium content in crop produce and soil collected from two sites in the seleniferous region

Crop Product	Selenium levels (mg kg^{-1})	
	Village Jainpur	Village Barwa
Wheat Grain	115.1 ± 2.4	41.1 ± 1.0
Husk	115.2 ± 1.8	14.9 ±0.5
Maize	13.0 ± 0.5	ND
Mustard	670 ± 18	594 ± 14
Mustard Pods	278 ± 5	263.8 ± 8.9
Rice	16.2 ± 0.5	ND
Rice Husk	21.7 ± 1.1	ND
Soil	6.5 ± 0.3	2.7 ± 0.1

ND – not determined

The bacterial isolates (SNTP-1, NS-2, NS-3 and NS-4) were tested for growth in the presence of different concentrations of selenate (Na_2SeO_4) and selenite (Na_2SeO_3). Although the control culture (0 mg L^{-1}) showed relatively faster growth rate, similar levels of growth were reached in all the strains in the presence of selenate (Fig.4.1) and selenite (Fig.4.2) over a period of 24 h. The cultures grown in the presence of 5 mg L^{-1}, 15 mg $L^{-1}$and 25 mg L^{-1} sodium selenate and sodium selenite turned red as growth progressed indicating the reduction of selenium oxyanions to elemental red selenium (Buchanan et al., 1995). Similar growth profiles were observed in control vis-à-vis the culture grown in selenium supplemented medium, as determined by total viable counts (Fig. 4.1 and Fig. 4.2). The rate of selenium reduction in terms of growth kinetics and

colony forming units were also carried out using consortia of above mentioned four isolates at above mentioned conditions i.e. at different concentrations of selenate and selenite to estimate the efficiency of consortia. Consortia showed faster growth in terms of selenium reduction in presence of both selenium oxyanions and color change in media occurred at fifth hour in both cases.

Figure 4.1 Growth profile of four bacterial isolates exposed to different concentrations of selenate

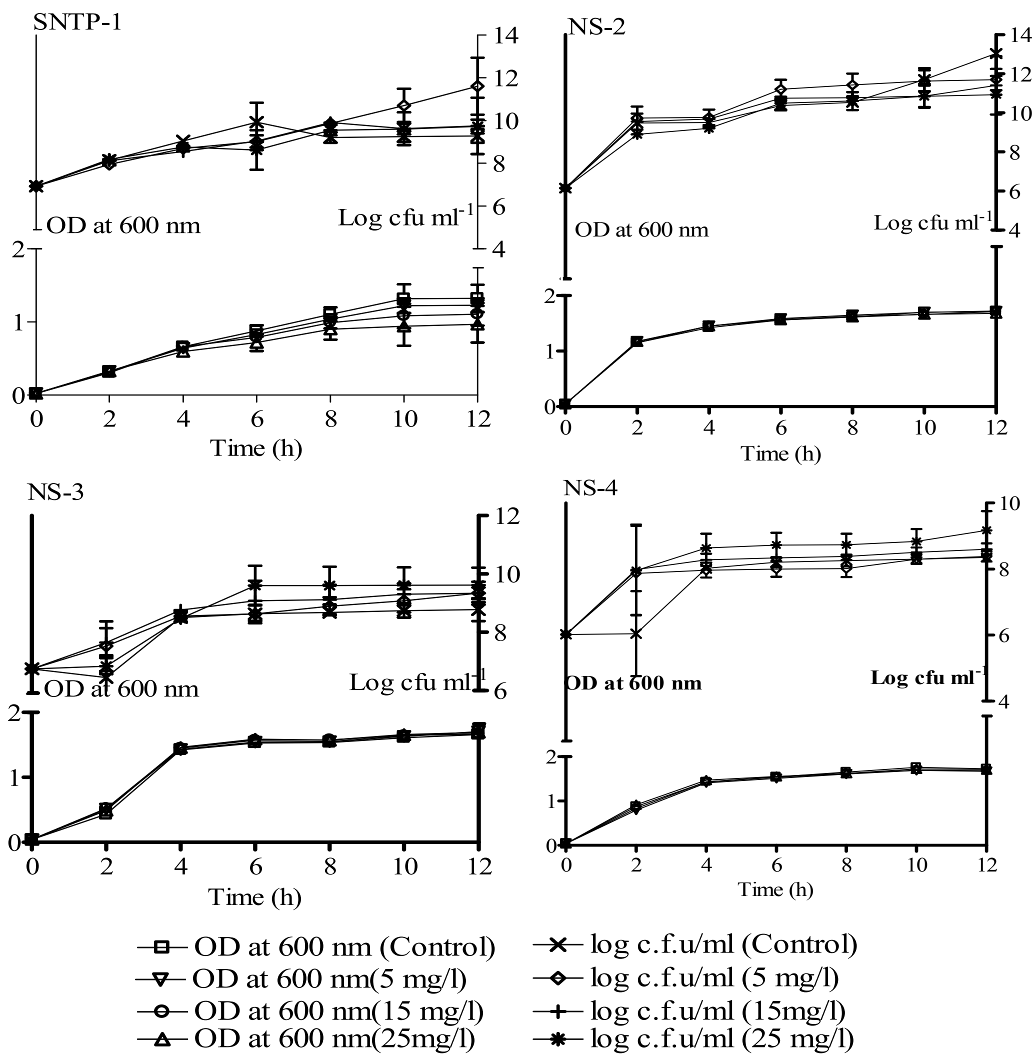
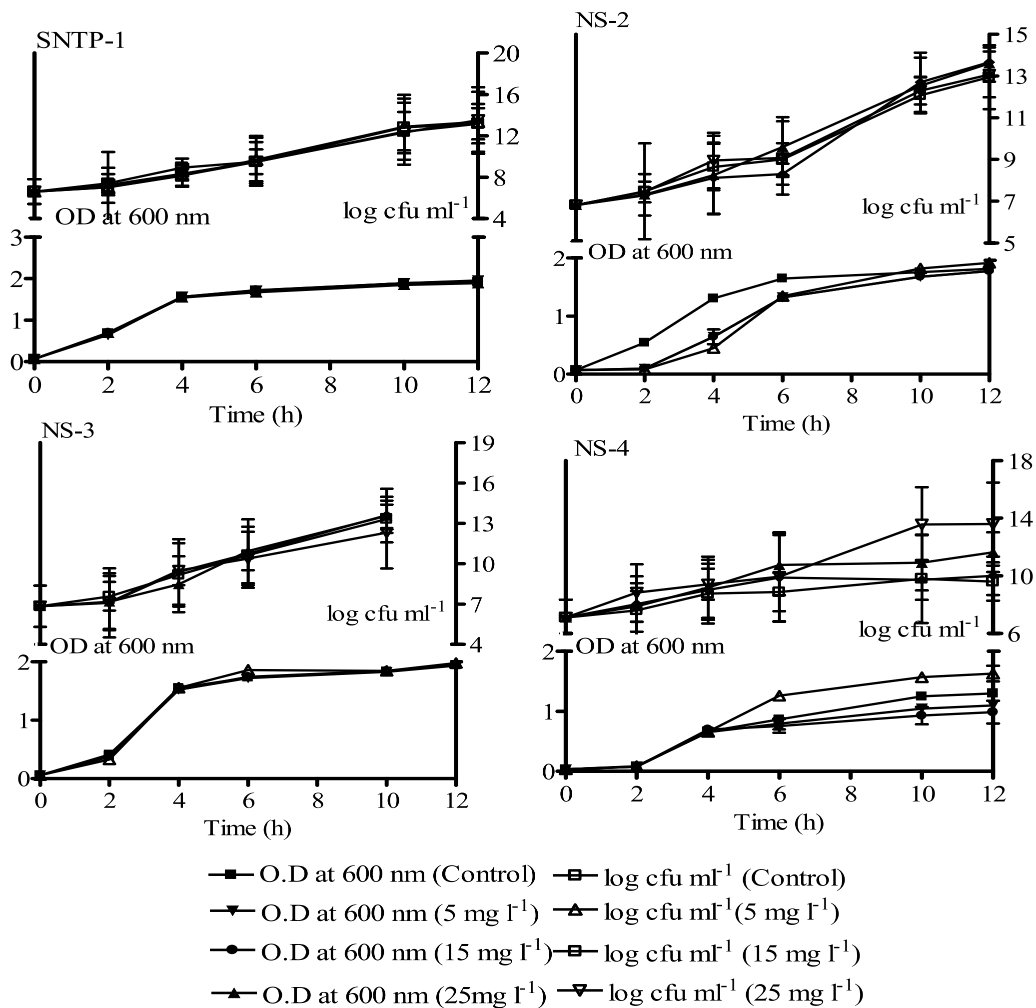


Figure 4.2 Growth profile of four bacterial isolates exposed to different concentrations of selenite



4.4 Biochemical Characterization

Bacterial colonies differ greatly in their metabolism and morphologies. Biochemical tests help to identify various metabolic properties of different bacterial species and can be used to differentiate even closely related organisms.

Morphological and biochemical characteristics were examined for isolates following methods outlined in section 3.3.2. Table 4.3 presents the morphological and biochemical characteristics of the mentioned strains.

Table 4.3 Biochemical/morphological characteristics of test strains

Biochemical Tests	SNTP-1	NS-2	NS-3	NS-4
Gram's reaction	+ ve	+ve	+ ve	+ ve
Colony morphology	Rod shaped	Rod shaped	Rod shaped	Rod shaped
Oxidase test	+ ve	+ ve	+ ve	+ ve
Catalase test	+ ve	- ve	+ ve	+ ve
Indole test	+ ve	+ ve	+ ve	+ ve
Methyl red test	- ve	- ve	- ve	- ve
Nitrate test	+ ve	- ve	+ ve	+ ve

All isolates showed gram positive reaction and rod shaped colony morphology. Three of the four strains were consistent with that of typical facultative anaerobic bacteria. However, the absence of positive test in catalase and nitrate tests indicate strong aerobic nature of NS-2. Isolates were also found to be indole positive (ability to degrade tryptophan into indole), methyl red negative (acid production) and nitrate positive i.e. capable of anaerobic respiration.

4.5 Antibiotic Profiling

Antibiotic susceptibility of each isolate was determined by multi - disc diffusion method with individual and modular antibiotic impregnated discs. Observations on the antibiotic profiling gave a comparative account of the resistance of the test strains towards various antibiotics. Between strains, the resistance was found to be similar towards most of the antibiotics tested (Fig. 4.3 and Fig. 4.4).

Amongst the 28 antibiotics tested, carbenicillin at concentration of upto 300 mcg did not inhibit the growth of bacteria indicating resistance of the isolates to these antibiotics, except NS-3 which showed resistance upto 40 mcg. Additionally, NS-3 and NS-4 also indicated resistance upto 90 mcg of piperacillin. The isolates were susceptible to erythromycin, kanamycin, tetracycline, oxacillin, cloxacillin and certain other antibiotics even at 1 mcg concentration as shown in Figs. 4.3 and 4.4. Resistance to a group of selective antibiotics can effectively be used as a screening factor to track the survival rate of isolates bioaugmented into soils, amongst the native bacteria (Lawrence, 2000).

Fig. 4.3. Antibiotic profiling of SNTP-1 and NS-2 in presence of different antibiotics. Arrow and bar indicate the resistance of the strains to carbenicillin over their response to other antibiotics

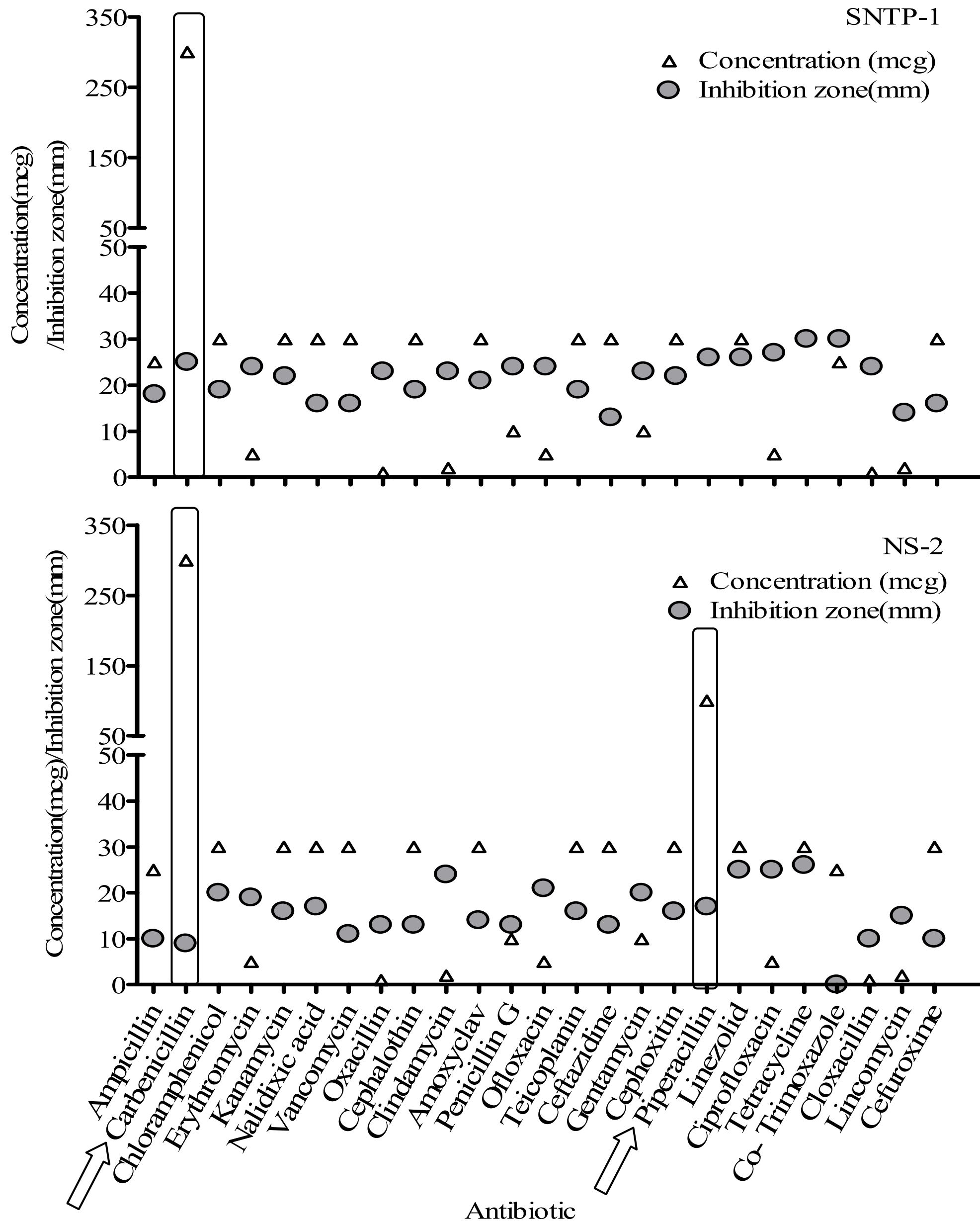
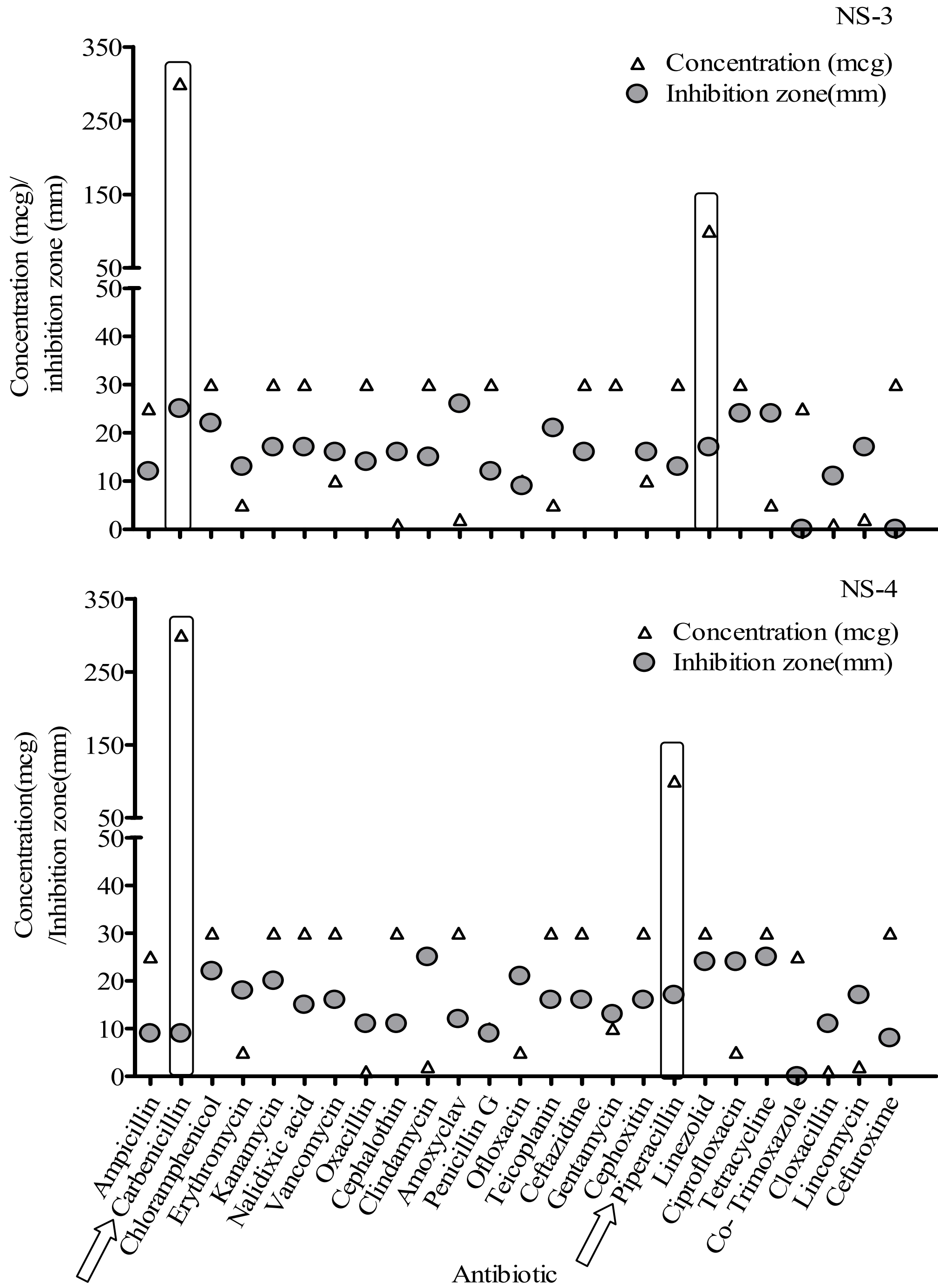


Fig. 4.4. Antibiotic profiling of NS-3 and NS-4 in presence of different antibiotics. Arrow and bar indicate the the resistance of the strains to carbenicillin and piperacillin over their response to other antibiotics



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 neutral to alkaline range of pH although growth of the strains reduction was more prominent in lower alkaline range (pH 6- pH 8). At pH 10, all strains except SNTP-1 showed reduced growth at early exponential phase. However, variable recovery of the growth profiles was noted at late exponential phase in case of each oxyanion as well as type of strain. With reference to NS-2, complete recovery similar to control was obtained when exposed to selenate but recovery was not complete in case of selenite. Selenate had no effect on growth profile at pH 10 in case of NS-3 where as the inhibitory effect of selenite, at pH 10, was prominent till early exponential phase followed by complete recovery at late exponential phase. The effect of selenate and selenite on NS-4 strain, at pH 10, was similar to that of NS-3 with growth inhibition at early exponential phase followed by marginal recovery at late exponential phase. However none of the isolates could reduce selenium in acidic conditions (pH 4) indicating that selenium reduction typically occurs in alkaline conditions (Fig. 4.5 and Fig. 4.6).

4.7 Effect of temperature on growth of organism and selenium reduction

All bacteria have a particular temperature range at which they can survive. For a specific type of bacteria, the optimum range can sometimes be distinctly narrow. Bacteria isolated from soil and plants 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 (SNTP-1, NS-2 – NS-4), with reference to different temperature levels, during exposure at a particular concentration of either of the selenium oxyanions.

General observations on all strains indicate that at 20°C, delayed reduction was observed i.e. after 9 h, in terms of colour change. At 30°C and 40°C, normal growth concomitant with Se reduction was observed as shown in Figure 4.7. While at 50°C, both the growth profile and the reduction of Se were observed to be distinctly inhibited. With reference to the growth profile of each strain to variations in temperature of growth medium, all the isolates exhibited varied profiles at specific temperature. In case of SNTP-1 (Fig. 4.7 A), extended log phase was observed at 20°C in control, where in

growth inhibition was observed in the presence of either of the Se oxyanions at that particular temperature.

Figure 4.5. Growth profile of SNTP-1 and NS-2 at pH 4, 6, 8 and 10 in presence of selenate and selenite (25 mg L^{-1})

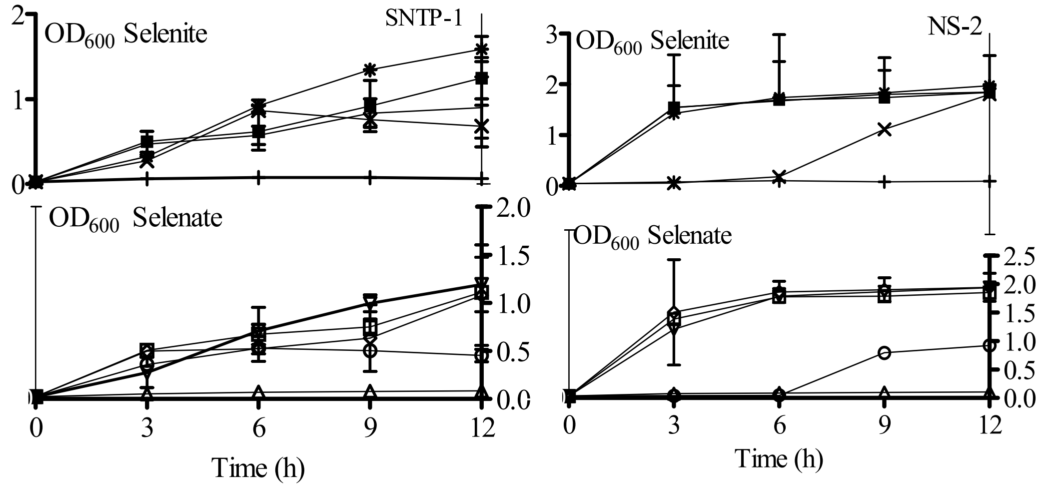
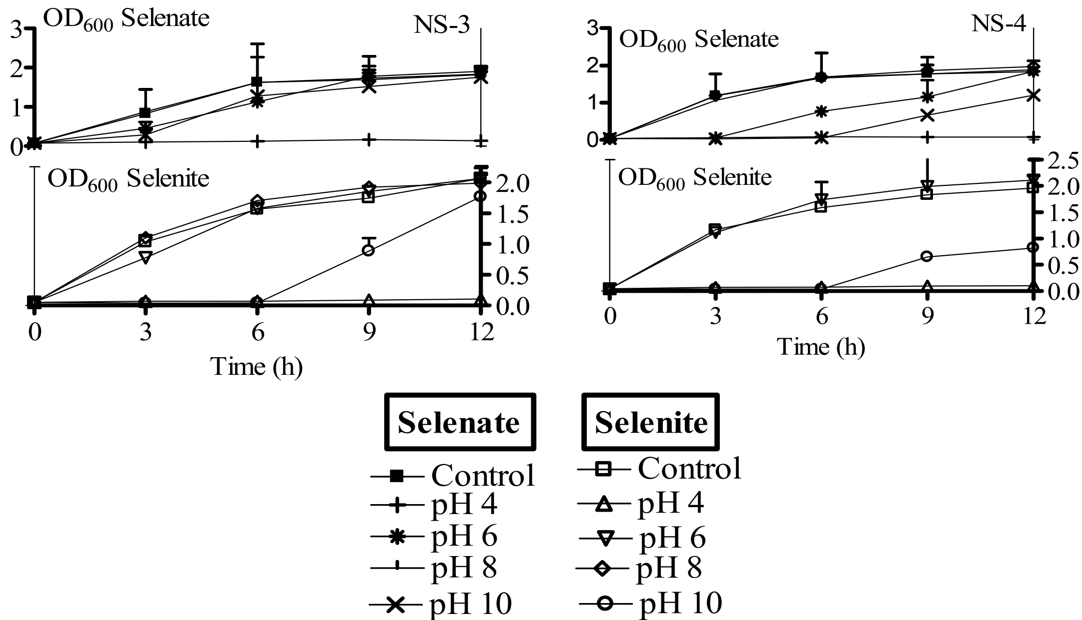


Figure 4.6. Growth profile of NS-3 and NS-4 at pH 4, 6, 8 and 10 in presence of selenate and selenite (25 mg L^{-1})



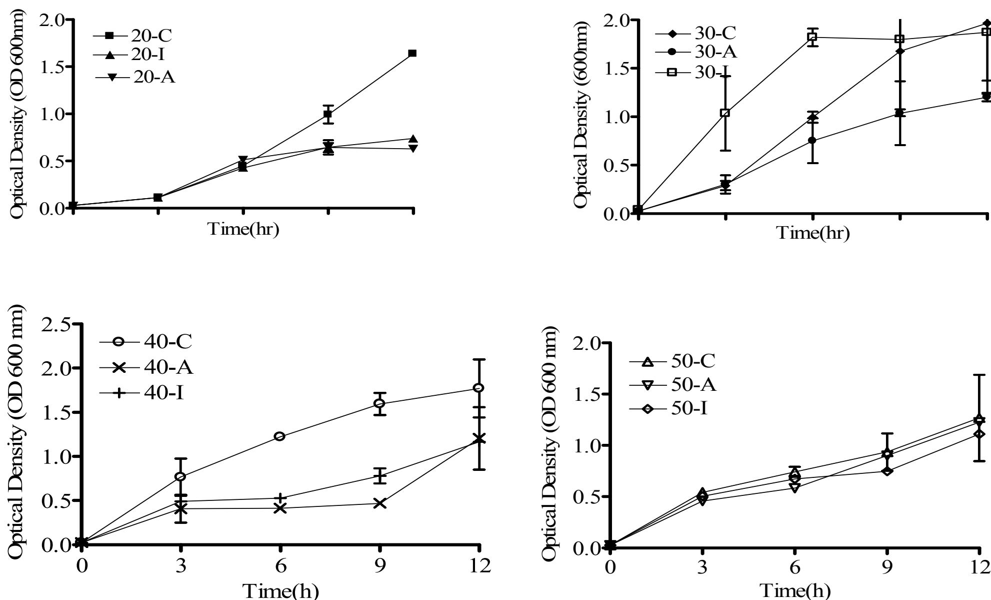
At 30°C, which is optimal for these organisms, presence of selenate resulted in extended log phase with recovery of growth after 8 h. However, exposure to selenite was observed to inhibit the growth even at this optimal temperature. The growth profile, at 40°C and 50°C, was observed to be significantly affected in presence or absence of selenium.

The strain NS-2, exhibited significantly different growth profile when compared to SNTP-1, when exposed to selenium oxyanions at different temperatures (Fig. 4.7 B). The strain is observably not influenced by either temperature or the presence of selenium upto 40°C, where as at 50°C, both the selenium oxyanions have been observed to inhibit the growth whereas the profile at control remained the same as in other growth temperatures.

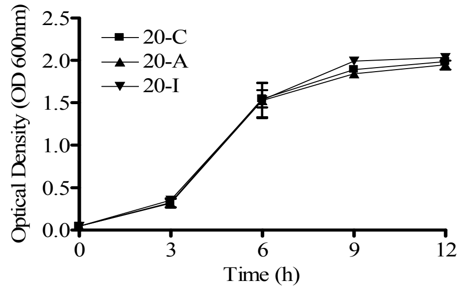
The growth profile of NS-4 as represented in Fig. 4.7 D, is observably not influenced by either temperature or the presence of selenium upto 40°C, where as at 50°C, both the selenium oxyanions inhibited the growth.

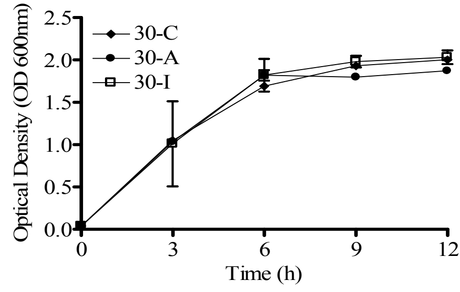
Figure 4.7. Growth profile of test strains (A-D) at 20°C to 50°C in presence of selenate (A) and selenite (I) (25 mg L^{-1}) with reference to control (C)

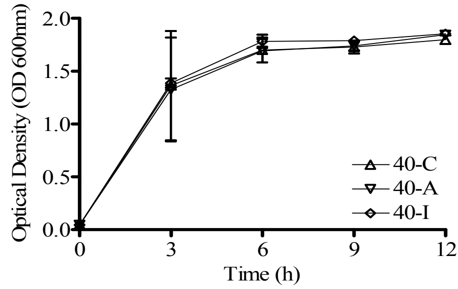
A. SNTP-1

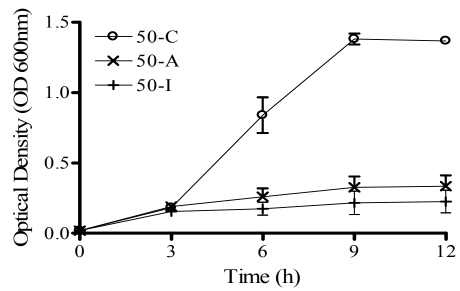


B. NS-2

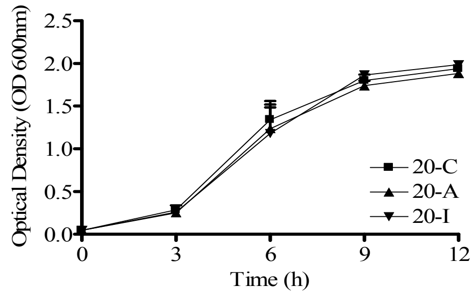


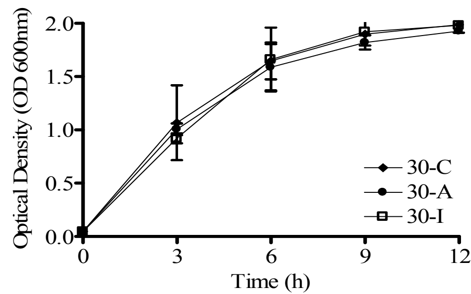


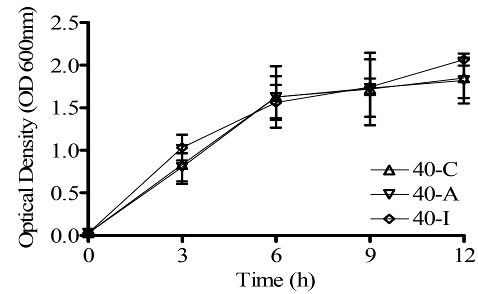


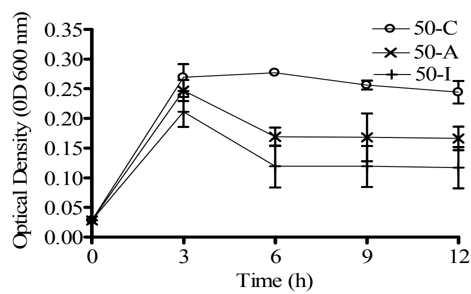


C. NS-3

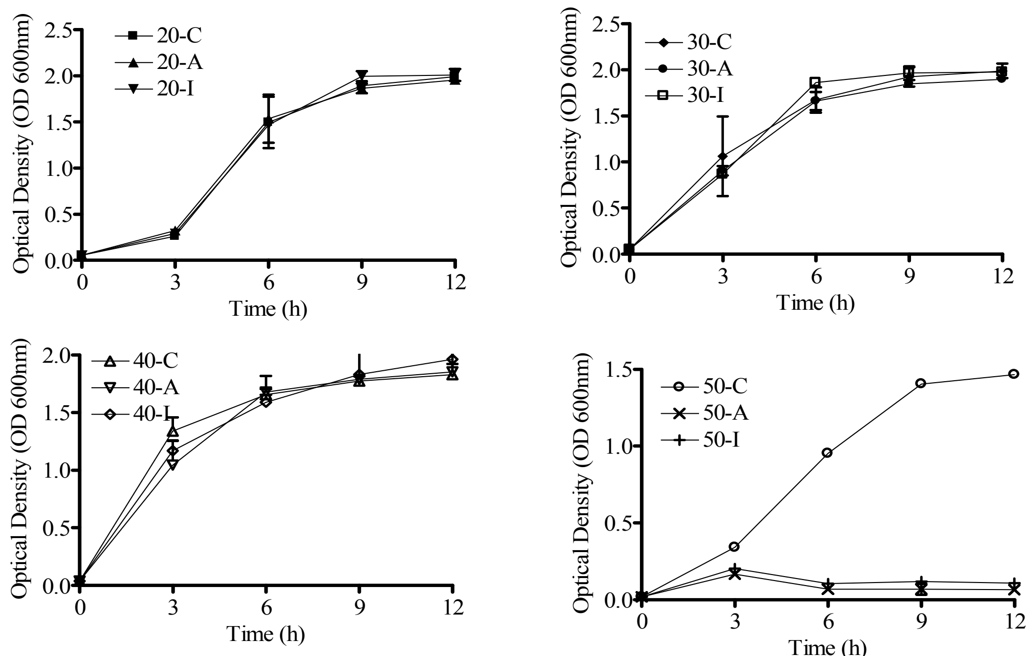








D. NS-4



4.8 FAME based identification

Fatty acid methyl ester analysis revealed that the dominant cellular fatty acids present in four isolates were 13-methyltetradacanoin (iso-C15:O) - 44.32% (SNTP-1), 26.38% (NS-2), 20.12% (NS-3), 30.52% (NS-4) followed by 12-Methyltetradecanoic (anteiso-C_{15}) -35.07% (SNTP-1), 4.57% (NS-2), 5.91% (NS-3). Strains NS-2, NS-3 and NS-4 belong to Kaneda group E (*B. anthracis, B. cereus, B. thuringiensis*) in which iso-$C_{15:O}$ (19-31%) is the most abundant fatty acid with small proportion of unsaturated fatty acids (7-12%). The microorganisms were also identified based on the comparison of total fatty acid profile of individual isolates based on MIDI similarity indices (TSBA library match) as well as literature. The Sherlock Microbial Identification System employed by MIDI Labs Inc. (Newark, DE) uses a database containing over 100,000 profiles of bacterial species. (Sasser, 2001).The Similarity Index of Sherlock Microbial Identification System (MIS) is a numerical value, which expresses how closely the fatty acid composition of an unknown compares with the mean fatty acid composition of the strains used to create library entry or entries listed as its match. This index value is the computer generated calculation of the distance, in multidimensional space, between the profile of the unknown and the mean profile of the most similar library entry. The fatty

acid methyl ester profiles were identified and clustered using the Microbial Identification System (MIS) software and database (TSBA 50 library). (Table 4.4).

Table 4.4. Similarity profile of the test strains based on fatty acid methyl ester (FAME) analysis

Strain	FAME analysis (Similarity index)	Group	Strain match
SNTP-1	0.440	Bacilli	*Bacillus pumillus*
NS-2	0.290	Bacilli	*Bacillus sp.*
NS-3	0.493	Bacilli	*Bacillus cereus*
NS-4	0.237	Bacilli	*Bacillus sp.*

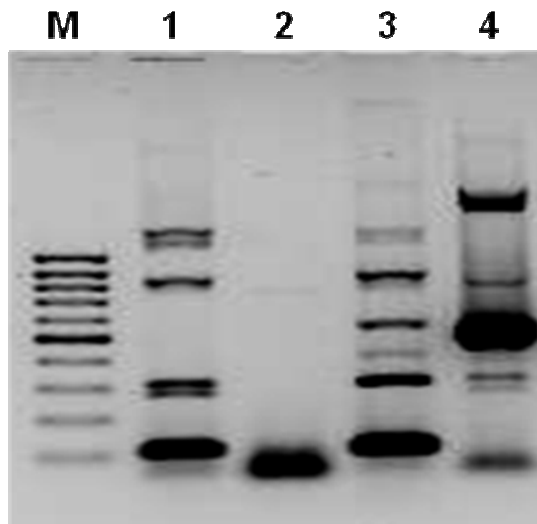
4.9 ERIC PCR based fingerprinting

After physiological studies, genetic diversity of selenium tolerant isolates was studied by PCR based molecular typing. For molecular characterization of isolates, amplification of the regions between adjacent enterobacterial repetitive intergenic elements (ERIC PCR) of isolate-specific DNA fingerprints was generated. ERIC PCR for all bacterial isolates yielded a complex genomic fingerprint consisting of ~12 Kbp to 100 bp amplicons of varying intensity (Fig. 4.8). Maximum number of amplicons was generated by NS-3. Visual observation of the DNA fingerprints clearly showed that all isolates are genetically distinct. These results also showed complex banding patterns, which reflected a high degree of inter or intra specific genetic unrelatedness among isolates (Fig. 4.8).

4.10 Identification of the bacterial isolates

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. 4.9). 16S rDNA PCR products were cloned into pGEM-T Easy Vector (Promega Inc., USA) System.

Figure 4.8. Differentiation of bacterial isolates based on ERIC PCR.
Lane 1-4: SNTP-1, NS-2, NS-3 and NS-4; and Lane M: 100 bp marker



The plasmid DNA was extracted from different clones and amplified with T7 and SP6 primers. The amplified products of different clones were subjected for Restriction Fragment Length Polymorphism (RFLP) analysis to see the variation in the 16S rDNA region (Fig. 4.10). The 16S rDNA products from selected clones were then sequenced using Applied Biosystems automatic sequencer. Sequencing reactions were performed with the primers T7 and SP6. The 16S rDNA sequences of isolates ranged from 1486 to 1576 bp. 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 rDNA gene sequences determined for isolates under study were deposited in GenBank of NCBI data library under accession numbers SNTP-1 (EU532490), NS-2 (EU622629), NS-3 (EU573774) and NS-4 (EU622630). BLAST homology search for 16S rDNA gene sequences of isolates indicated 98-99% similarity with their closest matches. The related sequences showing similarity in BLAST were retrieved from GenBank and RDPII and aligned using the program CLUSTALW (Thompson et al., 1997). The resulting multiple alignments were optimized visually and

the evolutionary distance were calculated by Kimura 2 parameter, phylogenetic dendograms were constructed by neighbor-joining method using MEGA 3 package (Kumar et al., 2004). Gaps were treated as missing data. Only unambiguous alignments were used in phylogenetic analyses. Phylogenetic analysis revealed that all isolates were related to the phylum Firmicutes, family Bacillaceae and associated with the genera *Bacillus*. The 16S rDNA gene sequene of SNTP-1 showed 100% similarity with *Bacillus pumilus*, thus clustered along with other *B. pumilus* sequences. The sequences of strains NS-2, NS-3 and NS-4 showed broad identity *B. cereus* and *B. thuringiensis* (Fig. 4.11).

Figure 4.9. 16S rDNA amplification of bacterial isolates. Lane 1-4: SNTP-1, NS-2, NS-3 and NS-4; Lane 5: Control and Lane M: 1 Kb marker

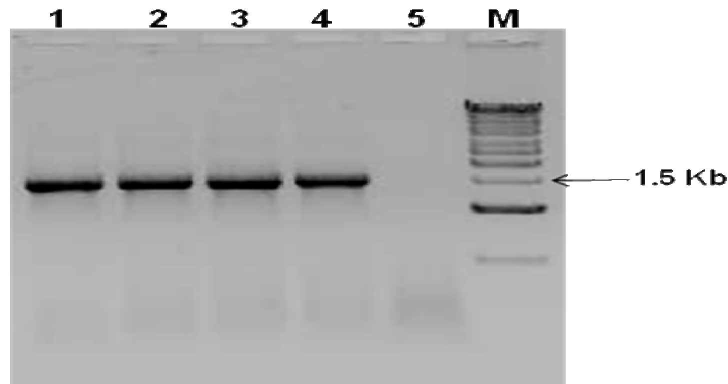


Figure 4.10. Restriction digestion patterns of bacterial isolates.
Lane M: 1 Kb marker (Fermentas); Lane 1-4: SNTP-1, NS-2, NS-3 and NS-4 digested with *Taq* I; Lane 5-8: SNTP-1, NS-2, NS-3 and NS-4 digested with *Alu* I; and Lane 9-12: SNTP-1, NS-2, NS-3 and NS-4 digested with *Hinf* I

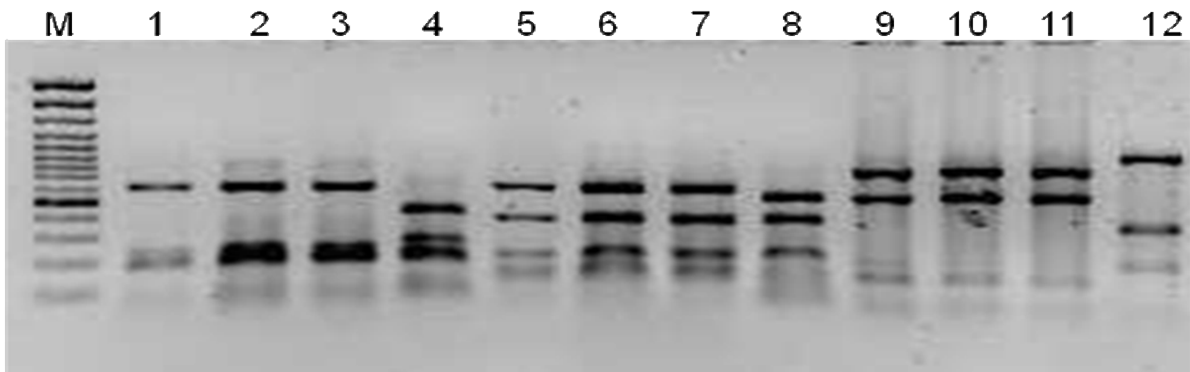
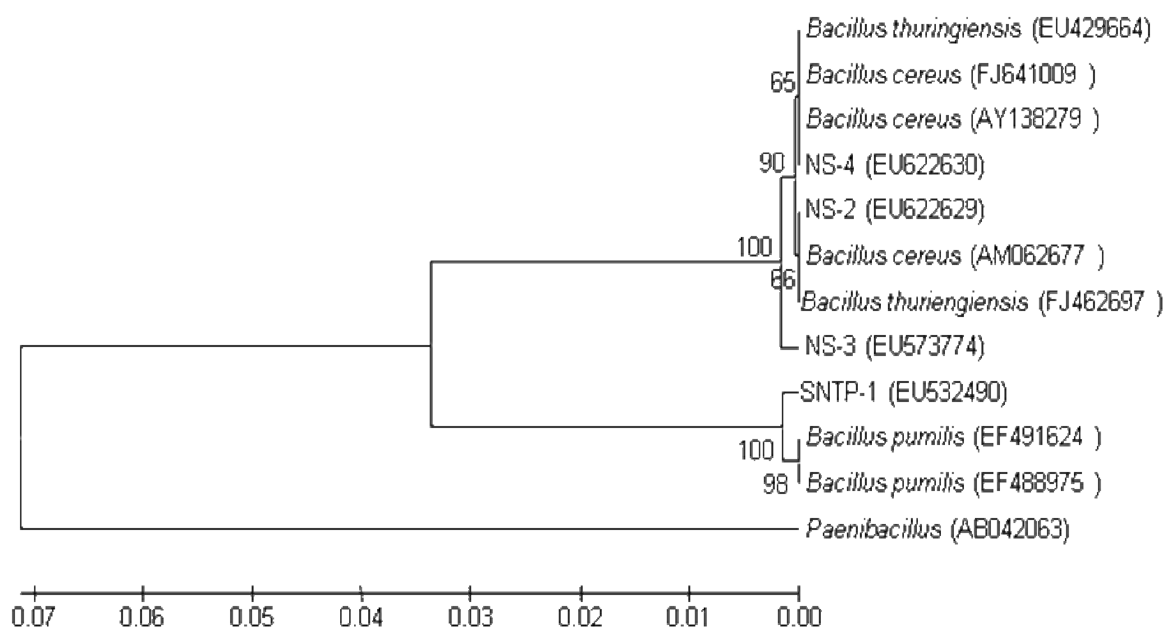


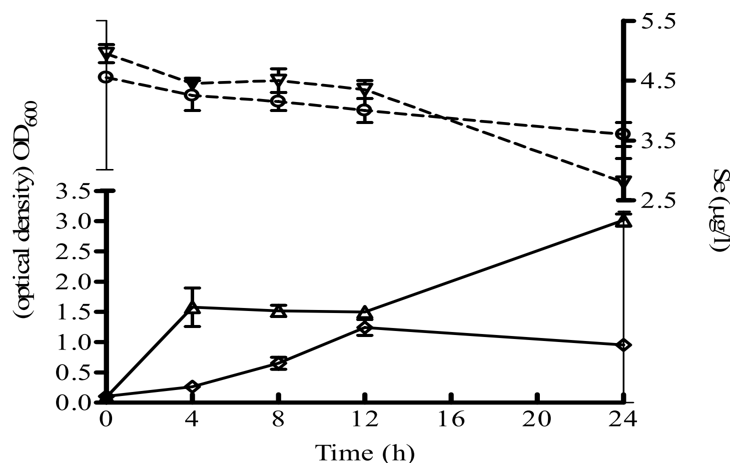
Fig.4.11. Neighbour joining tree based on 16S rDNA gene sequences of strains SNTP-1, NS-2, NS-3, NS-4 of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1000 replicates.



4.11 Selenite reduction under aerobic and anaerobic conditions

The potential of one of the isolates, a strain of *Bacillus* sp. was examined for its potential to transform selenium oxyanions (viz., selenite) was assessed by challenging both aerobic and anaerobic cultures with sodium selenite (1 mM). IC data showed a decrease in Se (IV) concentration in the cell free supernatant, corresponding with growth of the culture under aerobic conditions, as shown by an increase in OD_{600nm} (Fig. 4.12). During growth and Se (IV) reduction, production of a red precipitate was observed in the aerobic cultures. Growth and Se (IV) reduction was limited under anaerobic conditions. A marginal decrease in Se (IV) concentration (~10%) as a result of chemical reduction by the TSB media was observed in the no-cell controls under anaerobic conditions but not under aerobic conditions.

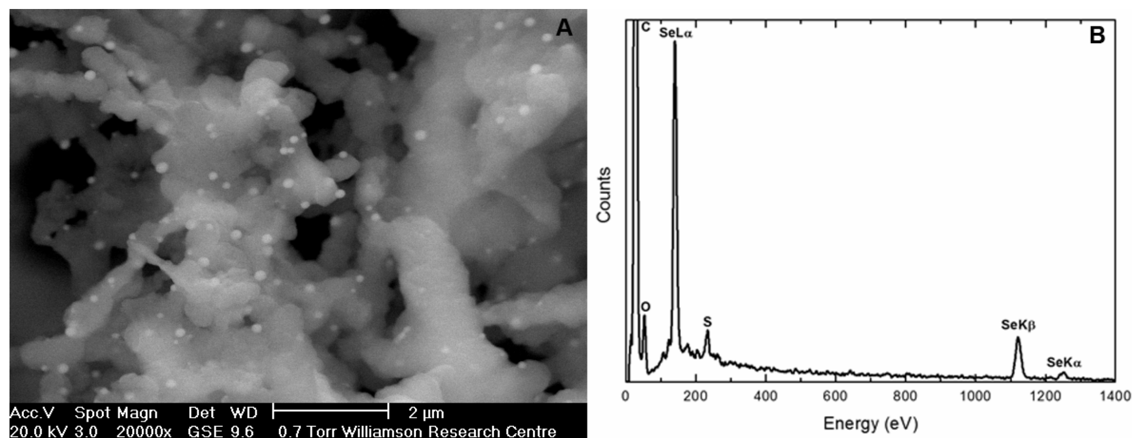
Figure 4.12 Change in selenite concentration under aerobic (∇) or anaerobic (o) conditions in cell free supernatant and growth profile under aerobic (Δ) or anaerobic conditions (◊) during 24 h exposure of *Bacillus* sp. to 1mM selenite



4.12 Characterization of selenium nanostructures

ESEM examination of the red cell pellet from the Se-supplemented medium inoculated with the *Bacillus* species revealed 100-200 nm nanospheres associated with the biomass (Fig. 4.13A). EDX analysis revealed the nanospheres to be made exclusively of Se (Fig. 4.13B) indicating the formation of Se (0).

Figure 4.13 ESEM image (A) and EDX (B) of selenium nanospheres associated with the *Bacillus* sp. biomass.



The post-preparative treatment process, involving H_2O-ethanol and chloroform-methanol solvent systems, was employed to isolate the Se nanospheres from the biomass. However, this treatment resulted in the gradual change in the precipitate colour from red to black. ESEM images show that this color change was concurrent with a morphological change from the relatively small (10-200 nm) nanospheres (Fig. 4.13A) via hexagonal, faceted and increasingly platy nano-structures (Fig. 4.14A) to larger (up to 10 µm) nano-rosettes comprising several crystalline nano-rods (Fig. 4.15A). EDX analysis indicated that the intermediate nano-structures and the nano-rosettes were also composed solely of selenium (Figs. 4.14B and 4.15B).

Figure 4.14 ESEM image (A) and EDX (B) of intermediate selenium nano-structures showing hexagonal facet development. The inset shows the platy nano-structures on 1µm scale.

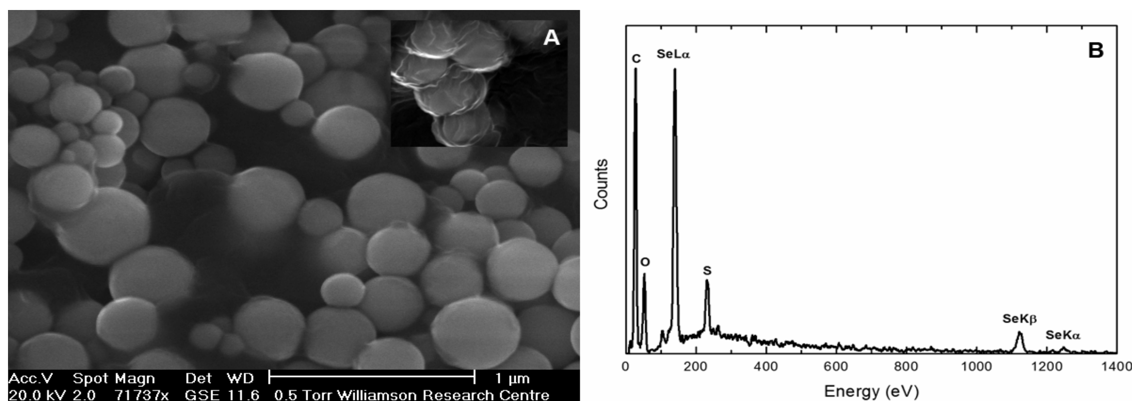
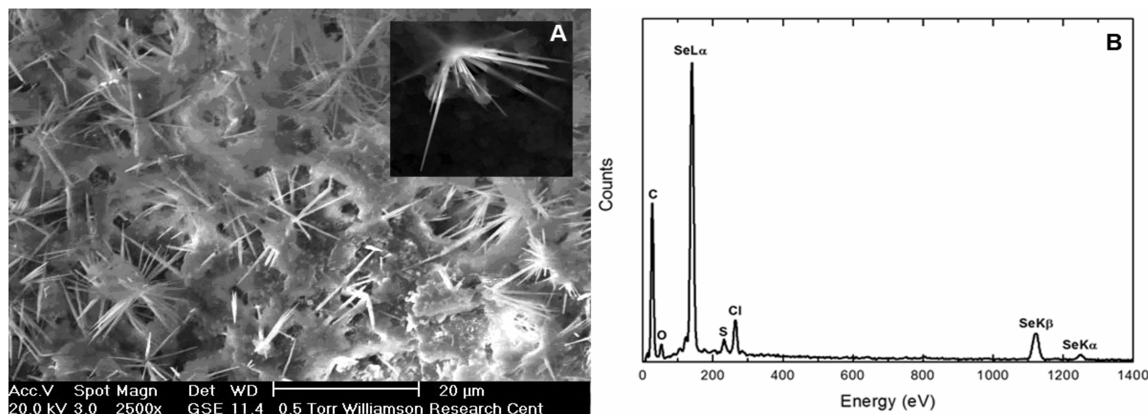


Figure 4.15 ESEM image (A) and EDX (B) of nanowire/rods formed on biomass in the form of rosettes. Inset shows a closer view of a rosette on 5µm scale.



TEM examination revealed each nano-rod in the rosette to be ~5-10 μm length and 0.5-1 μm wide (Fig. 4.16 A and B). XRD analysis revealed that the red nano-spheres were amorphous Se(0) (Fig. 4.17A) The diffraction pattern of the black Se nano-rosettes was the same as crystalline synthetic hexagonal Se (powder diffraction file 060362, International Centre for Diffraction Data) with characteristic peaks (2θ) at 23.42, 29.64 and 43.58 (Fig. 4.17B). The abnormal intensity of the (100) peak (as compared to that of synthetic Se) indicates that the Se nano-rosettes are preferentially orientated along the c axis, the [001] direction.

Figure 4.16. TEM images of Se nanorods formed as rosettes from the biomass-associated Se nanospheres, showing (A) some precursor nanospheres, and (B) a detail of a nanorod.

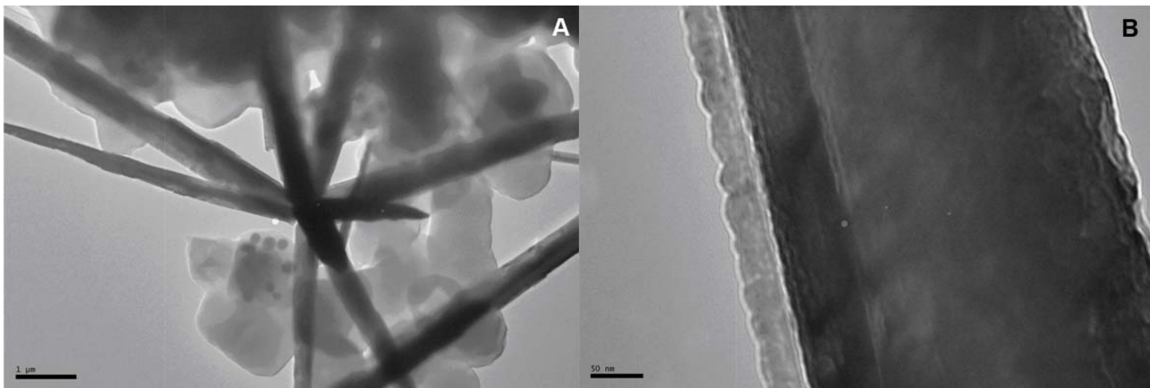
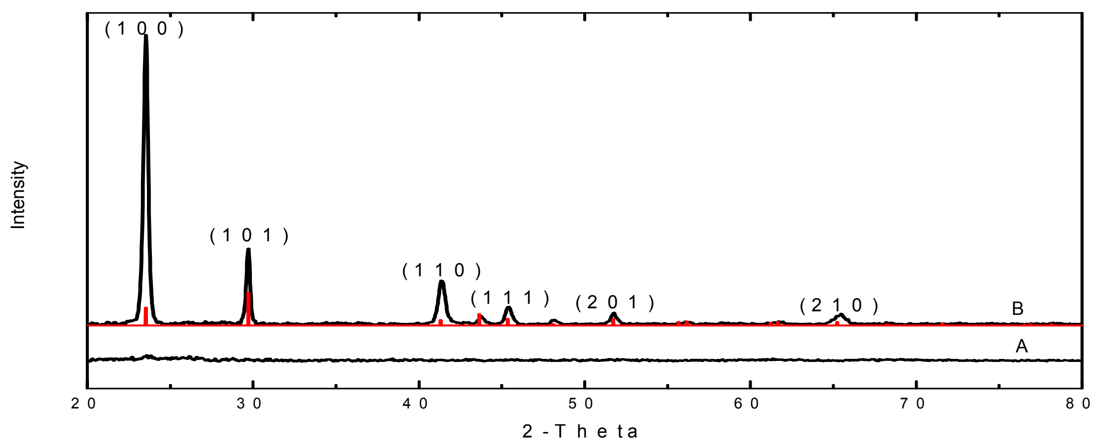


Figure 4.17 XRD of amorphous nano-spheres (A) and crystalline nano-rosettes (B). The peak positions and relative intensities of crystalline t-Se are shown in grey grey)



4.13 Survival of consortium and Se mobilization in Se enriched natural soil

The conditions of the microcosm experiment such as soil moisture content and inoculum size were standardized, and the efficacy of the consortia for transformation of Se oxyanions in soil was then tested. Soil samples (pH 7.3 ± 0.2; organic carbon, 0.273 ± 0.0008%; available phosphorus, 42.40 ± 2.43 mg L^{-1}; total nitrogen, 1568.0 ± 550.7 mg L^{-1}) were treated with selenate and selenite (2.5, 5.0 and 7.5 mg kg^{-1} soil). The consortium containing inocula of the four *Bacilli* strains was grown in Luria broth (100 ml), harvested at log phase, and re-suspended in basal medium (10 ml).

Microcosm was prepared with un-autoclaved soils supplemented with 2.5, 5.0 and 7.5 mg kg^{-1} of selenate and selenite. These were inoculated with standardized cell concentration of 3.9 X 10^9 cells g^{-1} soil. Moisture content of soils was maintained at 30% throughout the experiments. Soil samples (5 g) were withdrawn at regular time intervals in triplicates and analyzed for total residual selenium using neutron activation analysis with Lake Sediment Material (IAEA-SL-1) as reference material. Carbenicillin resistance of the isolates was 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 residual concentration at different intervals in percentage.

In soil microcosm studies, observations on population survival based on carbenicillin resistance, showed an increase in colony forming units (CFU) in all treatments from 40 to 120 days studies (Figs.4.18-4.20). The microbial population in microcosm exposed to 7.5 mg kg^{-1} selenite did not vary (log (5.7; 6.1 and 5.6) cfu gm^{-1}) significantly across the duration of 40, 80 and 120 days. Similar was the trend observed on exposure to selenate. Across all treatments, maximum survival rate was observed in soil supplemented with 2.5 mg kg^{-1} during 120 day period. The corresponding trends in mobilization of selenium over time, on exposure to 7.5 mg kg^{-1} of selenium oxyanions, are also represented in Fig. 4.20. Transformation of selenium oxyanions by microbiota in control soils to that of the soils supplemented with 2.5 mg kg^{-1} selenite revealed 32%, 52% and almost complete removal of Se across 40, 80 after 120 days as compared to control in which the removal was 40% after 120 days as shown in Fig. 4.18. The mobilization of Se from soils was observed to be complete in inoculated soils supplemented with 5 mg kg^{-1} of selenite where as in control soils with same level of Se supplementation; it was 26% after 120 days (Fig. 4.19). At 7.5 mg kg^{-1} of selenite, the

level of removal was relatively less with only 81% when compared to initial concentration. In inoculated soils treated with 2.5 mg kg^{-1} of selenate, the mobilization and removal was 24% at 40 days increasing to 60% at 120 days as compare to control which was 28% after 120 days. The rate of transformation followed a similar trend even in soils supplemented with 5.0 mg kg^{-1}. Transformation rate in soils augmented with 7.5 mg kg^{-1} selenate and consortia was noted to be 68 % after 120 days where as in control it was 33%.

Figure 4.18. Rate of selenium transformation [a – selenite and b – selenate] and corresponding viability of the consortia in soils supplemented with 2.5 mg kg^{-1}. The gray tone and white tone in graphs represent log CFU count and rate of transformation (%) respectively. I represents soils augumented with inoculum and NI represents soils without inoculum of consortia

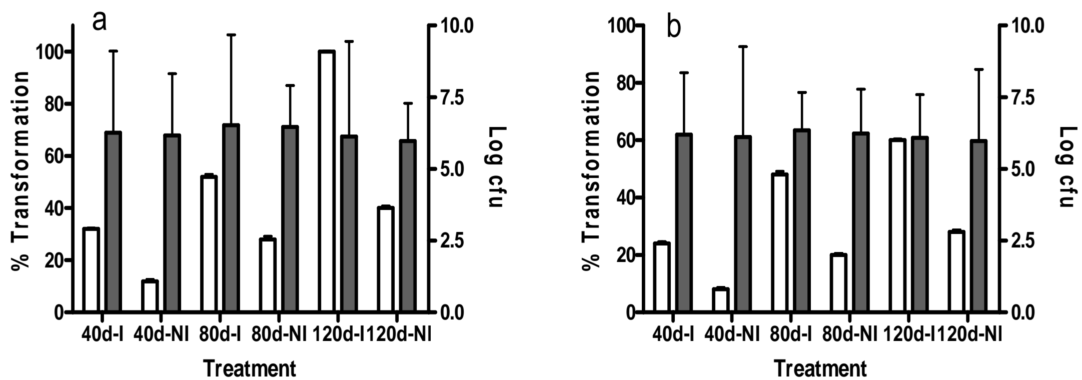


Figure. 4.19. Rate of selenium transformation [a – selenite and b – selenate] and corresponding viability of the consortia in soils supplemented with 5 mg kg^{-1} selenium

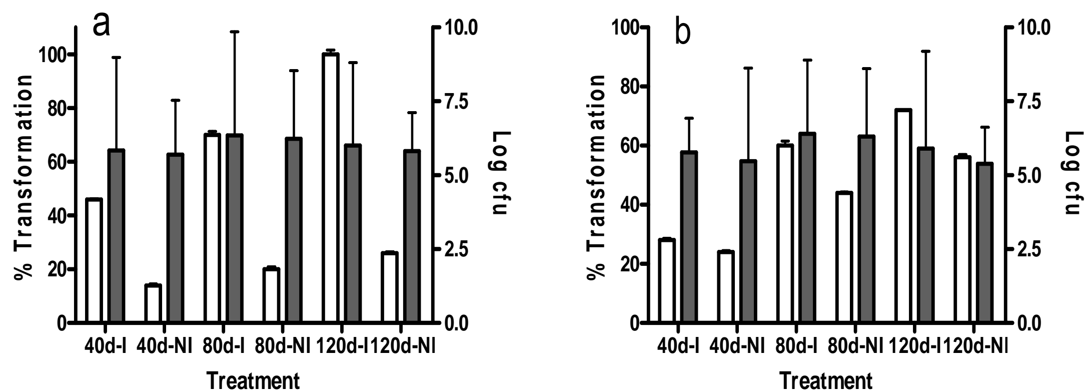
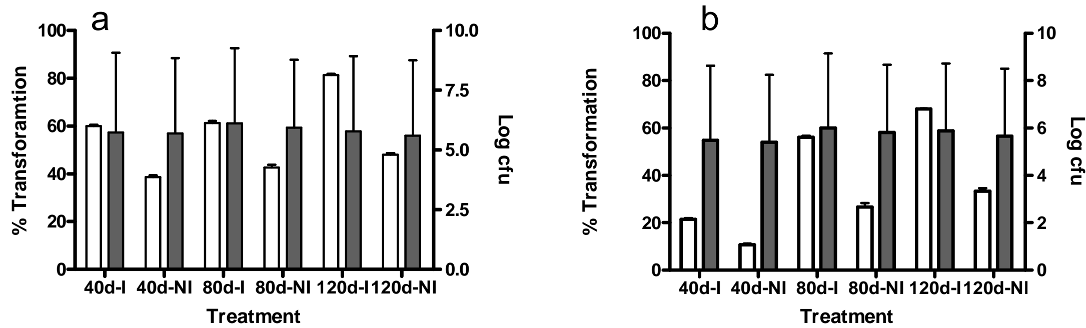


Figure. 4.20. Rate of selenium transformation [a – selenite and b – selenate] and corresponding viability of the consortia in soils supplemented with 7.5 mg kg^{-1} selenium



4.14 Fingerprinting using molecular markers i.e. Rep-PCR

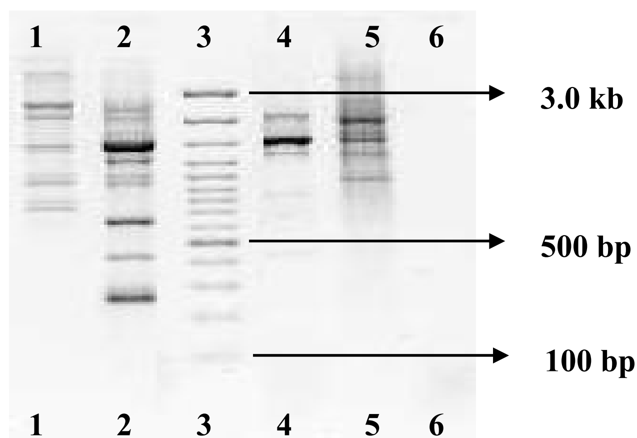
Success of any bio-augmentation study depends on the enumeration of the added microbial population and disappearance of the target compounds. In order to track the added bacteria, it is essential to have enumeration methods that are quick and accurate. DNA fingerprinting of *Bacilli* strains using microbial repeat sequences (REP-PCR) was standardized to develop molecular marker that could become a useful tool in enumerating these isolates in the environment .The profiles of the DNA fingerprints of all the four strains are shown in Fig. 4.21.

Soil samples obtained from soil microcosm inoculated with different isolates (section 3.10) were suspended in saline water (0.85% NaCl) and dilution plated onto LA +carbenicillin plates (40 ug ml^{-1}) to obtain the bacterial count (section 3.10.1). Fifty colonies from these plates were randomly picked per observation from each of the bacterial isolates, genomic DNA isolated (section 3.10.1.2) and subjected to REP-PCR to study the survival of introduced microorganisms.

A combination of PCR conditions was used in the present study to generate strain specific DNA fingerprints to select the population released for bioremediation of selenium contaminated soils.

Figure 4.21 Profile of DNA fingerprints of the strains traced after bioaugmentation

(Lane 1: SNTP-1; Lane 2: NS-2; Lane 3: 100bp marker; Lane 4: NS-3; Lane 5: NS-4)



4.15 Microbial population count in microcosm by REP-PCR

Soil samples obtained from soil microcosm inoculated with different isolates (section 3.10) were suspended in saline water (0.85% NaCl) and dilution plated onto LA +carbenicillin plates (40ug ml^{-1}) to obtain the bacterial count (section 3.10.1). Fifty colonies from these plates were randomly picked per observation from each of the bacterial isolates, genomic DNA isolated (section 3.10.1.2) and subjected to REP-PCR to study the survival of introduced microorganisms.

Population of all the isolates showed a gradual decrease during the incubation time. Fig. 4.22 showed bands of native bacteria present in soil. After 120 days of experiment, with REP-PCR based enumeration, 76, 74 and 70% survival for 2.5 (Fig 4.22), 5 and 7.5 mg kg^{-1} selenite supplemented soils and 60, 36 and 42% survival for 2.5, 5 and 7.5 mg kg^{-1} selenate supplemented soils was obtained for consortia after 120 days. (Table.4.5). The survival data obtained from plate counting was higher as compared to the data obtained from DNA fingerprinting.

Figure 4.22 REP-PCR of three isolates along with amplified strains obtained from inoculated soil samples collected from soil supplemented with 2.5 mg kg^{-1}

(Line 1-4: amplified native strains obtained from inoculated experimental soils which did not show any match with inoculated consortia; Lane 5: NS-2; Lane 6: 100 bp ladder; Lane 7: NS-3; Lane 8: NS-4)

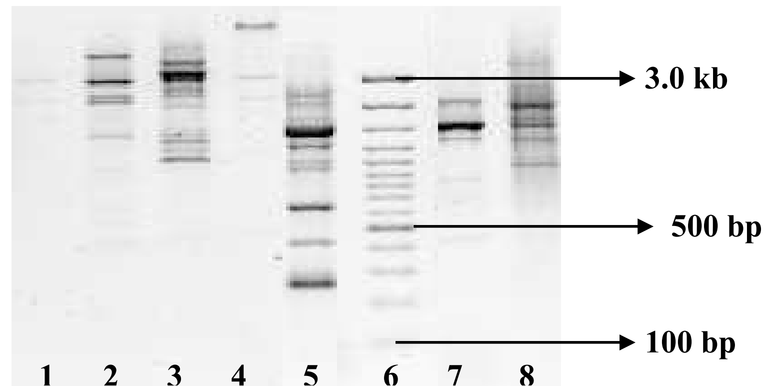


Figure 4.23 REP-PCR of four isolates along with amplified strains obtained from inoculated soil samples

(Lane 1: SNTP-1; Lane 2: NS-2; Lane 3 100 bp marker; Lane 4: NS-3; Lane 5: NS-4; Lane 6-13: amplified strains obtained from inoculated experimental soils; Lane 8, 12 and 13 show prominent bands which showed similarity with original bands of consortia

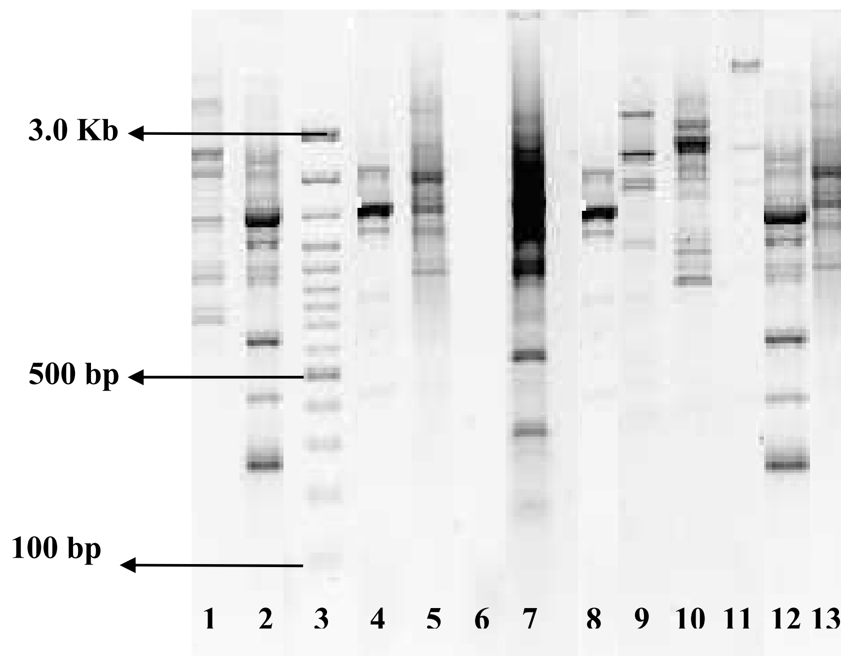


Table 4.5. Survival of bacterial isolates comprising consortia in soil microcosm based on REP-PCR. Values given in parenthesis represent the number of isolates comprising consortia that gave a complete match with standard isolates of consortia versus total number of isolates fingerprinted

Concentration (mg kg^{-1})	40 Days	80 Days	120 Days
2.5 selenite	68 (34/50)	72 (36/50)	76 (38/50)
5 selenite	60 (30/50)	70 (35/50)	74 (37/50)
7.5 selenite	64 (32/50)	66 (35/50)	70 (35/50)
2.5 selenate	50 (25/50)	64 (32/50)	60 (30/50)
5 selenate	44 (22/50)	50 (25/50)	36 (18/50)
7.5 selenate	30 (15/50)	36 (18/50)	42 (21/50)

Thus, in terms of survival rate, based on molecular fingerprinting, survival was observed to be high in 2.5 mg kg^{-1} selenite supplemented soils and low in soils supplemented with 5 mg kg^{-1} selenate after 120 days.

Section B: Determination of the uptake of soil selenium by *Allium* sp. *with and without rhizosphere bacteria*

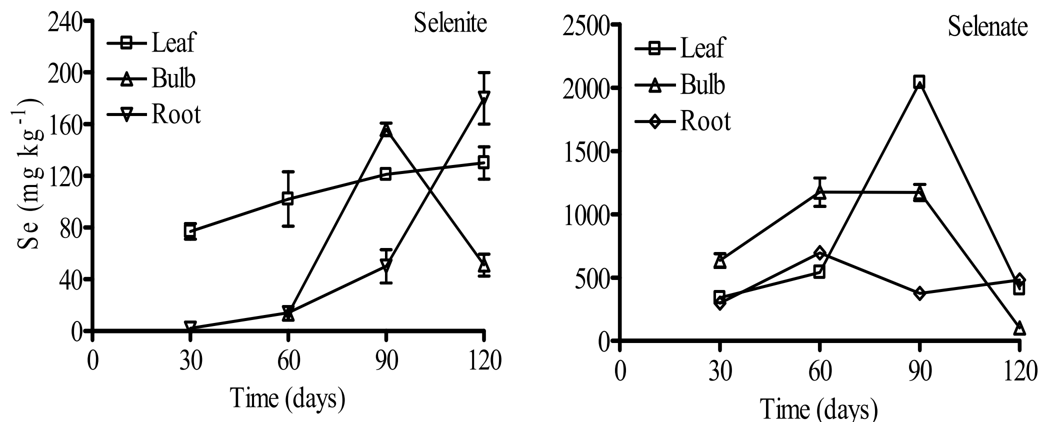
The second objective of the study was to determine the uptake of selenium, supplemented in soil, by the *Allium cepa* plants. These investigations were carried out with and without inoculation of rhizospheric bacteria, isolation of which is detailed in earlier section. The focus was to (a) investigate the influence of rhizosphere bacteria in mobilization of selenium from soil to plants; and (b) examine the uptake potential of *Allium cepa* as a Se hyperaccumulator. The studies were carried out at two levels viz., pots and experimental microcosms and in soils devoid of Se. Experiments were set with soil augmented with known concentration of selenium (as selenate and selenite). Investigations on the influence of rhizosphere bacteria were carried out only in microcosm and not in pots. The estimation of total selenium in plants was carried out using graphite furnace-atomic absorption spectrometer (GF-AAS) in pot experiments and INAA in field experiments.

4.16 Experiments in horticultural pots

Initial studies carried out in pots were to examine the growth profile of *A. cepa* plantlets cultivated in Se supplemented soils in near green-house conditions. The concentration of Se (as selenate and selenite) was selected based on the average levels observed in Se rich soils i.e., 5 mg kg^{-1}. The concentration was also restricted to maximum of 5 mg kg^{-1} to obtain the maximum bulb growth at par with the control samples and that of harvest value.

The overall concentration of selenium in the onion plantlets increased over control during the duration of the growth in response to increasing selenium concentration in the form of selenate and selenite. The distribution of the total selenium was unevenly distributed among the onion tissues analyzed during the exposure to either of the selenium oxyanions. In control plants, selenium concentrations for all tissues ranged from below detection limits to maximum of 7 μ g^{-1} (not indicated in Fig. 4.24). In the present study, the average uptake of selenium was higher in bulb (maximum of 1173 μ g^{-1}), followed by leaf (maximum of 1040 μ g^{-1}) and root (694 μ g^{-1}).

Figure 4.24. Uptake levels of selenium as observed in plant parts of plants exposed to 5 mg kg^{-1} of Se (as selenite and selenate)



The variations in the growth of plant as indicated by the plant dry weight (gm) is presented in Table 4.6. The observations clearly show that although the plant growth relatively gets promoted at the initial stages of the tissue formation i.e., 30 days, further growth is significantly affected by the exposure to either of the selenium oxyanions. The retardation was more prominent in the roots than the above ground tissues viz., bulb and leaves.

In pot experiments, bulbing was visible in most of the plants at the eighth week of plant sampling; however, the bulb formation was observed to be relatively retarded in the case of selenate exposure. In the case of selenite, such retardation was not evident. The plants grew and matured when exposed to selenate. The total selenium concentration in leaf and bulb significantly decreased after 90 days of exposure. The reduction in the total selenium content in the case of bulb and leaves after 90 day duration and prominent formation of bulbs may be attributed to the volatilization which is exhibited by the plantlets after initial accumulation of selenium. The growth profile of the plantlets grown in mentioned conditions are shown in Fig. 4.25 for the duration of the study.

Table 4.6. Dry weight of the parts of plants cultivated in soil augumented with Se (5 mg kg^{-1}). The values after ± represent the standard deviation between three replicates. L, B & R represent leaf, bulb and root of plant.

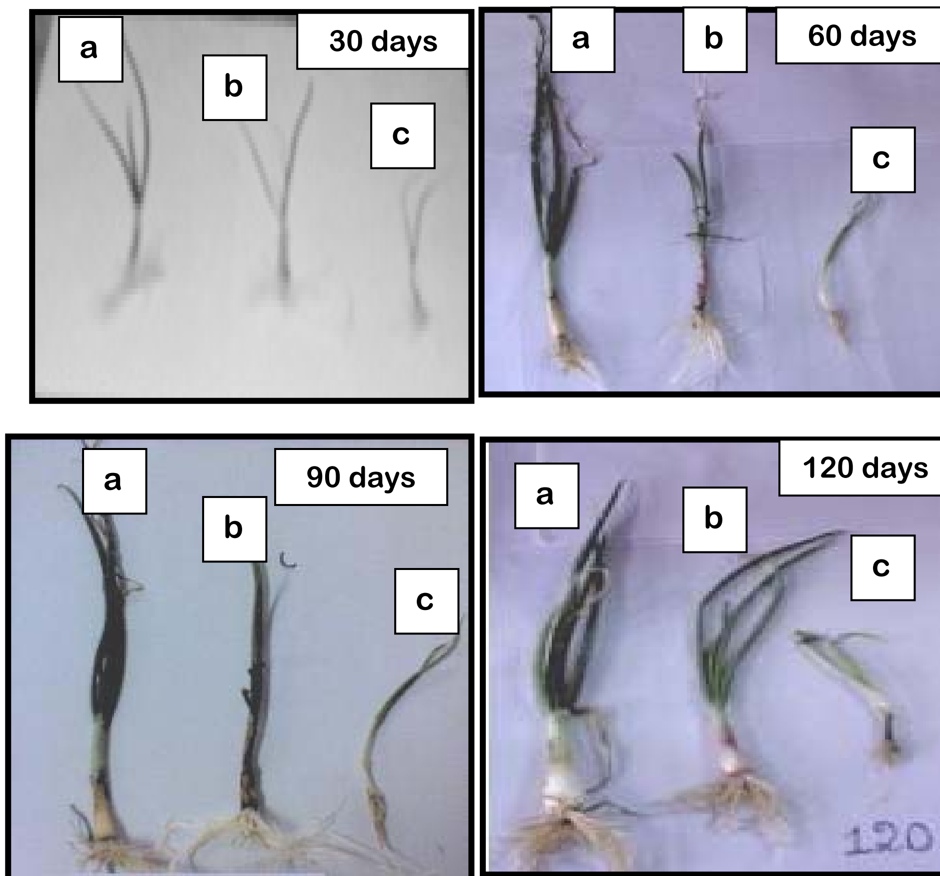
	30 days	60 Days	90 Days	120 Days
Control (L)	102.5±44.3	166.5±18.2	210.7±22.6	101.34±21.6
Selenite (L)	140.6±14.7	132.9±30.7	100.0±5.1	91.62±12.7
Selenate (L)	57.1±16.4	41.1±7.3	22.2±1.4	23.98±3.2
Control (B)		136.0±28.9	88.3±4.4	53.2±2.6
Selenite (B)	No Bulb Formation	92.4±44.6	28.9±7.8	47.37±5.4
Selenate (B)		9.2±1.9	2.30±0.1	6.2±2.7
Control (R)	39.5±26.2	66.8±13.7	113.5±55.0	51.9±6.5
Selenite (R)	29.9±7.9	66.75±24.5	69.6±4.1	42.7±8.8
Selenate (R)	14.6±4.7	16.7±0.6	9.1±2.9	14.6±5.7

The regression and correlation between the uptake of selenium and duration of exposure is given in Table 4.7.

Table 4.7. Statistical verification of the selenium uptake and duration of exposure to different selenium oxyanions. NS – Not significant

Parameter	Selenate	Selenite
Correlation Coefficient	0.92	0.64
Coefficient of Determination	0.85	0.40
Significance ($P<0.05$)	Yes (0.0263)	NS (0.1588)

Figure 4.25. Growth profile of the plantlets grown in control and Se-supplemented soil. Labels a, b and c indicated the plantlets grown in control; selenite and selenate induced conditions respectively.



4.17 Field Investigations

4.17.1 Observations on Se uptake in plants cultivated in soil with inoculation of rhizosphere bacteria

The influence of microbial consortium in the rhizosphere of contaminated soil was evaluated by comparing the main effect on element accumulation in plant tissues. The studies carried out in the field, initially investigated the selenium uptake in onion plantlets when exposed to soil supplemented with different concentrations of selenium oxyanion in presence of consortium of four bacterial isolates (Table 4.8). The range of 2.5 to 7.5 mg kg^{-1} of Se (IV and VI) was chosen to relate Se levels in experimental conditions to the total Se concentration in seleniferous soils which ranged from 2.7 to 6.79 mg kg^{-1} (Sharma et al., 2009). Soil and plants were sampled after regular time

intervals and analyzed for residual selenium along with estimation of population survival by dilution plating on carbenicillin plates and REP-PCR. Preliminary screening of growth investigations indicated that exposure beyond 7.5 mg kg^{-1} resulted in complete mortality of the plants within few days (~7days) after sowing.

Table 4.8. Selenium levels in plant parts of *Allium cepa* grown in soil supplemented with 2.5, 5.0 and 7.5 mg kg^{-1} of selenate and selenite solution. BDL – below detection limit; NA – not analyzed. Concentrations obtained in reference materials for accuracy evaluation also given. * Whole plant

Sample	Control	2.5 mg kg^{-1}		5.0 mg kg^{-1}		7.5 mg kg^{-1}	
		selenite	selenate	Selenite	selenate	Selenite	selenate
40 days							
Plant*	BDL	17.4±0.9	169.0±2.2	37.9±0.9	379.0±3.0	28.0±1.5	524.0±5.3
Soil	2.8±0.5	3.0±0.3	3.5±0.6	3.3±0.5	1.9±0.6	3.8±0.5	2.8±0.5
80 days							
Root	BDL	54.7± 5.6	99.6± 2.9	27.3±1.7	115.2±3.1	59.1 ± 5.2	461.9±11.5
Bulb	BDL	39.2± 3.9	37.6± 1.5	52.4±3.6	507.8±10.1	35.4± 3.3	57.1 ± 1.7
Leaf	BDL	44.9± 2.3	160.2±4.6	79.7±3.2	508.5 ± 7.1	31.6± 4.1	840 ± 10.9
Soil	BDL	7.1± 1.2	BDL	2.1± 0.7	BDL	4.7± 1.6	BDL
120 days							
Root	BDL	33.5± 13.3	65.6±10.1	200.3±23.8	421.3±35.4	NA	125.5±28.9
Bulb	BDL	10.5± 4.1	71.0± 4.4	122.9±17.9	81.5± 5.6	25.2 ± 4.3	254.8 ± 9.9
Leaf	BDL	190.9±13.2	62.7± 3.6	189.4±11.9	655.8 ± 32	297.5±30.6	1462± 74.5
Soil	BDL	BDL	BDL	BDL	BDL	BDL	BDL

Se concentration in reference materials	Present study	Certified value
NRCC CRM DOLT-1	7.43 ± 0.18	7.34±0.42
IAEA RM Sl-1	2.82 ± 0.08	2.9±0.42

Exposure to selenite at 2.5 mg kg^{-1} stimulated the growth of the plant across the duration of the growth period. At 5.0 mg kg^{-1} and 7.5 mg kg^{-1}, although the growth was inhibited at initial phase of the growth (40 days), the plants recovered from the inhibitory effect of selenium (selenite) gaining equal or better biomass to that of the plants in

control conditions. Exposure to selenate at 2.5 mg kg^{-1} and 5.0 mg kg^{-1} inhibited the growth of the plant followed by recovery at later stages. However, at 7.5 mg kg^{-1}, the effect of selenate was significant on plants indicated by the retarded growth when compared to control.

The concentration of total selenium in plants increased compared to control during the duration of growth. The comparison between the total Se levels on exposure to either of the Se oxyanions indicated higher uptake of Se in plants exposed to selenate when compared to selenite. Inhibition of growth in the presence of selenate, correlated with significantly high levels of total Se in the plants reaching >600 μ g^{-1} (at 7.5 mg kg^{-1} exposure) over 120 days.

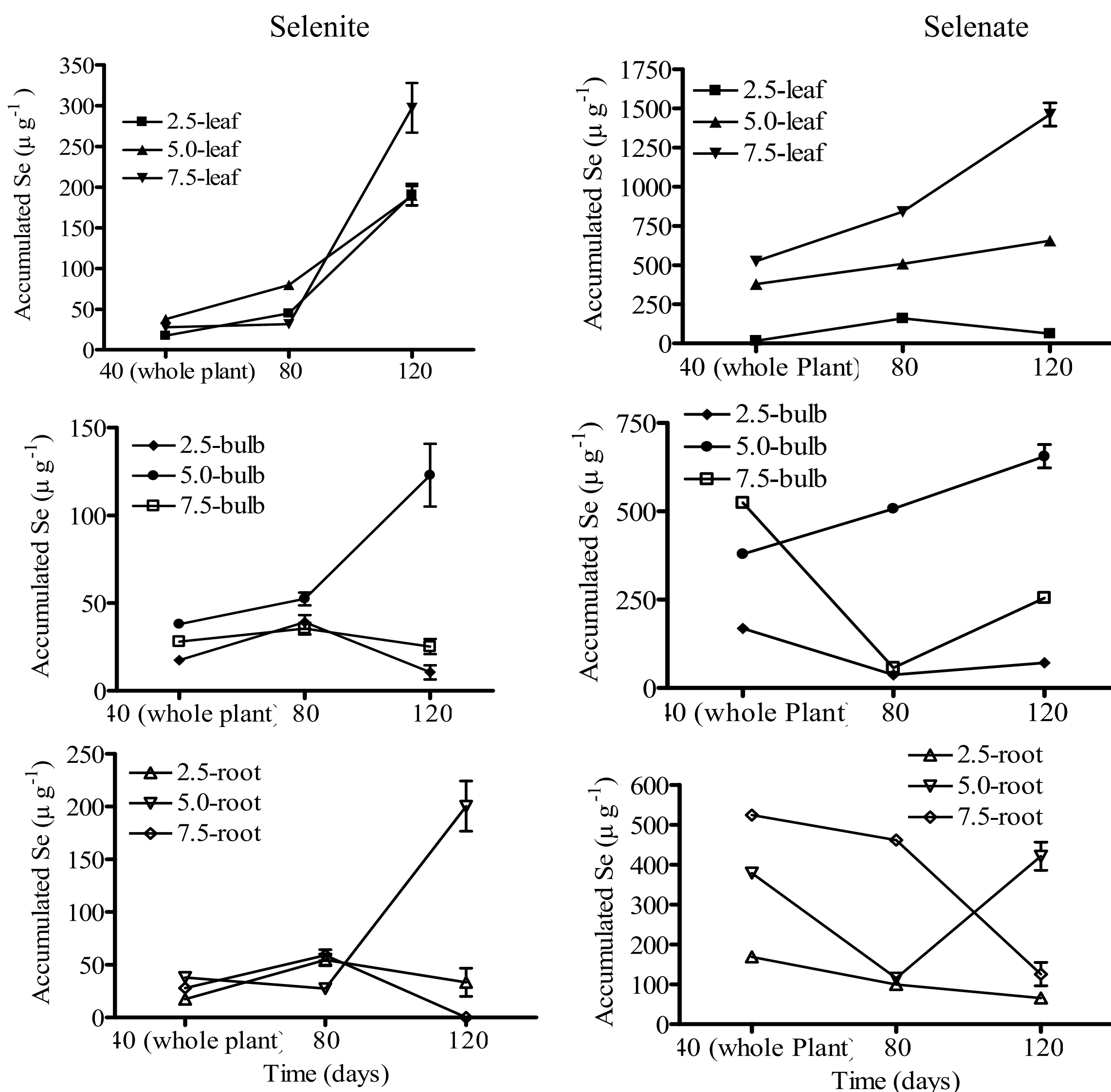
The profile of the accumulation of selenium in different plant parts of the plants exposed to either of the selenium oxyanions is presented in Fig. 4.27. One way analysis of variance (ANOVA) followed by multiple-comparison with Tukey test, carried out to estimate the interactions of selenium levels at various concentrations over time, indicated that there was no significant difference ($p > 0.05$) between the increasing concentrations in soil to the levels obtained in whole plants.

Between the selenium oxyanions, the accumulation of selenite was observably gradual and consistent with limited influence on the growth of the plants, whereas the accumulation of selenate was rapid and high across the growth period, inhibiting the growth, especially at higher concentrations of exposure. Comparison between the fresh weights of the plant to the total selenium levels in the whole plant indicated positive correlations between both the parameters over the duration of growth on exposure to selenite. A comparative profile of the plants grown in soils supplemented with the mentioned concentrations is shown in Fig.4.26. As indicated earlier, the Se levels in 40 day old plants were estimated in whole plants. The increasing uptake of Se by the plants corresponded with the rapid decrease in total Se in rhizospheric zone of soil to below the detection limits.

Fig 4.26. A comparison of the growth of *A.cepa* plantlets in different concentrations of Se oxyanions as selenite (I) and selenate (A) along with control grown in presence of consortium

The uptake of selenium was observably significant in case of exposure to selenate than selenite. In case of exposure to selenite, the overall uptake increased with increasing concentration upto 5.0 mg kg^{-1} (512 µ g^{-1}) followed by decrease towards 7.5 mg kg^{-1} (322 µ g^{-1}) which presumably is due to higher rate of volatilization reported in plants when exposed to selenite. This trend also corresponds to variations in the soil selenium levels. However, the uptake of selenium, on exposure to selenate, was noted to increase in plants (199>1158>1842 µ g^{-1}) with increasing concentration of selenium exposure. The soil Se levels were below detection limits (BDL) exhibiting active accumulation of selenium in plants on exposure to selenate.

Figure 4.27. Profile of selenium accumulation in plant parts exposed to selenate and selenite augmented in soil.



Comparative analysis of the Se accumulation in plant parts indicated no definite trend in distribution of Se among the tissues of the plantlets corresponding to the exposed concentrations (Table. 4.9).

Table 4.9: Selenate and Selenite Contents trend in different plant parts of onion

Concentration	Duration			
Selenate				
2.5^{mgkg-1}	- 80 days	- Se^{leaf}(160.2)	> Se^{root}(99.6)	> Se^{bulb} (37.6)
	- 120 days	- Se^{bulb}(71)	> Se^{root}(65.6)	> Se^{leaf} (62.7)
5.0^{mgkg-1}	- 80 days	- Se^{leaf}(508.5)	> Se^{bulb}(507.8)	> Se^{root} (115.2)
	- 120 days	- Se^{leaf}(655.8)	> Se^{root}(421.3)	> Se^{bulb}(81.5)
7.5^{mgkg-1}	- 80 days	- Se^{leaf}(840)	> Se^{root}(461.9)	> Se^{bulb}(57.1)
	- 120 days	- Se^{leaf}(146.2)	> Se^{bulb}(254.8)	> Se^{root}(125.5)
Selenite				
2.5^{mgkg-1}	- 80 days	- Se^{root}(54.7)	>Se^{leaf}(44.9)	> Se^{bulb}(39.2)
	- 120 days	- Se^{leaf}(190.9)	> Se^{root}(33.5)	> Se^{bulb}(10.5)
5.0^{mgkg-1}	- 80 days	- Se^{leaf}(19.7)	> Se^{bulb}(52.4)	> Se^{root}(27.3)
	- 120 days	- Se^{root}(200.3)	>Se^{leaf}(189.4)	> Se^{bulb}(122.9)
7.5^{mgkg-1}	- 80 days	- Se^{root}(59.1)	> Se^{bulb}(35.4)	> Se^{leaf}(31.6)
	- 120 days	- Se^{leaf}(297.5)	> Se^{bulb}(25.2)	> Se^{root}(N.A)

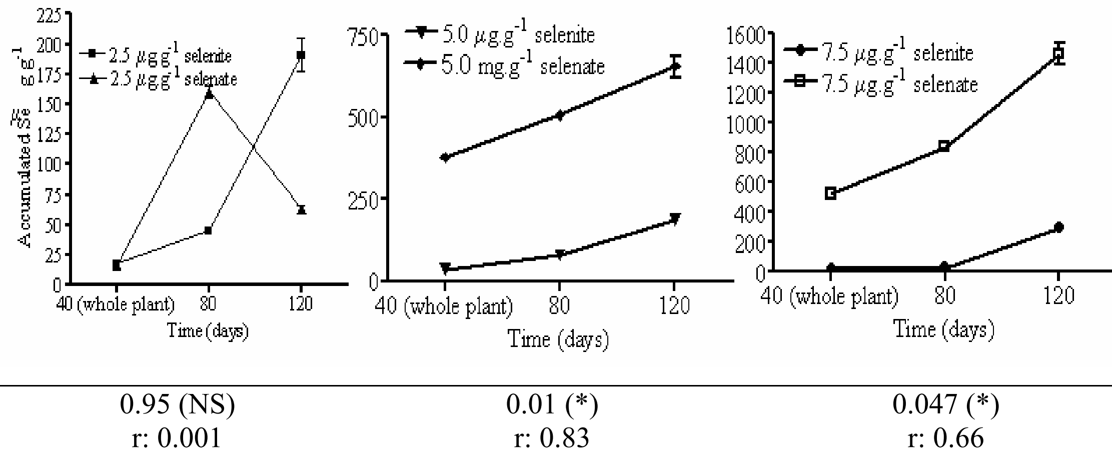
Figure 4.28 (a-c) shows the variations in the accumulation profile of selenium in individual plant parts at 80 and 120 days of growth as compared to whole plant at 40 days. Tests for independent associations (t-test) and ANOVA, on the Se levels obtained over duration of 120 days indicated:

(a) no significant difference (t-test: $p>0.05$) and correlation in the mean levels of accumulation in the various plant parts over concentration of selenium oxyanions, between the oxyanions and duration of exposure (Figure 4.15); and

(b) no significant difference (F test: $p>0.05$) in the mean levels of accumulation between tissues at a particular given concentration over the duration of exposure.

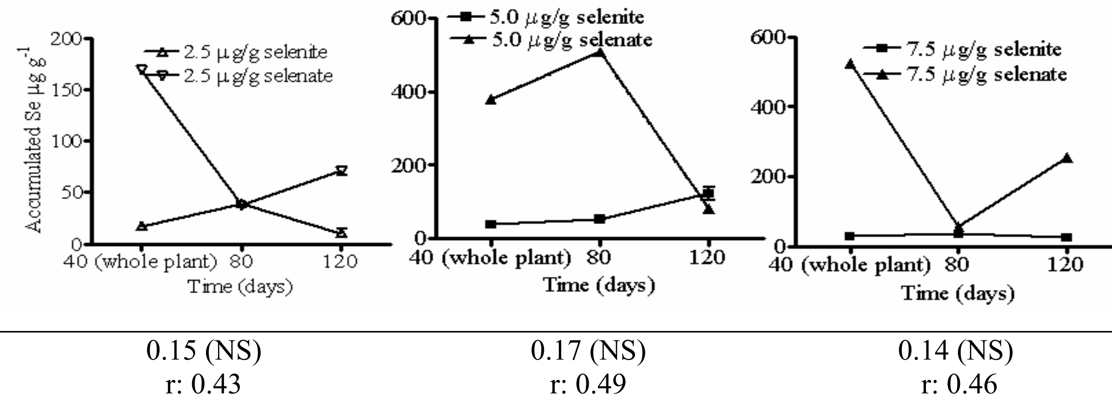
Figure 4.28. Se concentration in leaf (a), bulb (b) and root (c) of plants grown in different concentrations of selenite and selenate. Statistical significance (p<0.05) and correlation between levels of selenite and selenate is given below the graph (NS: not significant: * : level of significance)

(a) Leaf



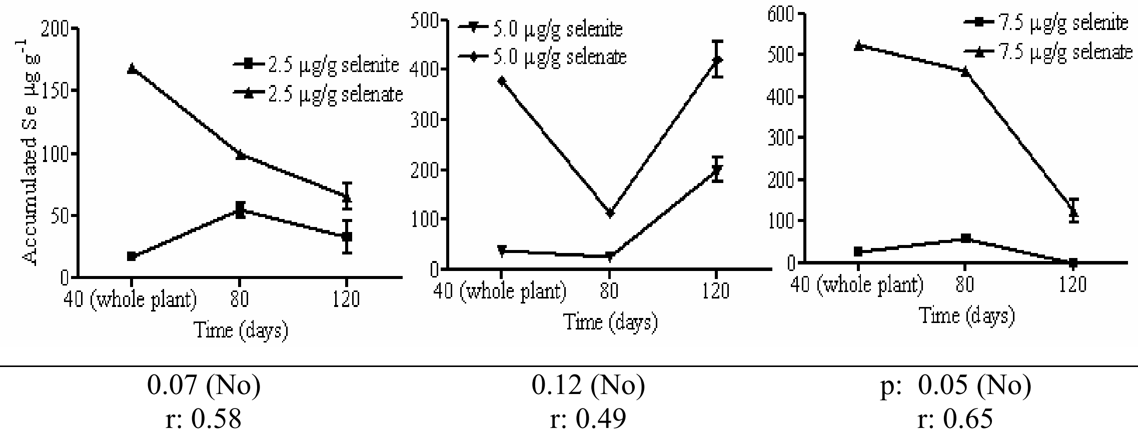
0.95 (NS)	0.01 (*)	0.047 (*)
r: 0.001	r: 0.83	r: 0.66

(b) Bulb



0.15 (NS)	0.17 (NS)	0.14 (NS)
r: 0.43	r: 0.49	r: 0.46

(c) Root



0.07 (No)	0.12 (No)	p: 0.05 (No)
r: 0.58	r: 0.49	r: 0.65

Exceptions were only observed in the relationship of selenium levels between oxyanions over time in leaves of plants exposed to 5.0 $\mu g\ g^{-1}$(r: 0.83) and 7.5 $\mu g\ g^{-1}$(r: 0.66) wherein the trend in the accumulation was observably similar.

4.17.2 Observations on Se uptake in plants cultivated in soil without inoculation of rhizosphere bacteria

The second set of experiment simultaneously carried out in the field involved observations on the selenium uptake in onion plantlets when exposed to soil supplemented with different selenium oxyanion concentrations and devoid of any bioaugmented bacterial consortium. Soil and plants were sampled in triplicates after regular time intervals and analyzed for selenium content as mentioned above in section 4.17.1.

In this study, the toxic effect of selenate was more prominent with retardation in terms of growth and fresh weight observed in case of plants exposed to all concentrations of selenate. The toxic effect of selenate was more significant in absence of microbial consortium across all concentrations accompanied by mortality in plants exposed to selenate. Whereas, in case of selenite treatment, exposure to selenite at 2.5 mg kg^{-1} stimulated the growth of the plant across the duration of the growth period, even in the absence of inoculants. Whereas at 5.0 mg kg^{-1} and 7.5 mg kg^{-1}, although the growth was inhibited at initial phase of the growth (40 days), the plants recovered from the inhibitory effect of selenium (selenite) gaining higher or equal biomass to that of the plants in control conditions. Exposure to selenate at 2.5 mg kg^{-1} and 5.0 mg kg^{-1} inhibited the growth of the plant followed by recovery at later stages. However, at 7.5 mg kg^{-1}, the effect of selenate was significant on plants indicated by the retarded growth when compared to control. Supplementation of 7.5 mg kg^{-1} of Se(VI) to soil showed maximum Se uptake in plants (476 $\mu\ g^{-1}$; 556 $\mu\ g^{-1}$; 665 $\mu\ g^{-1}$) at 40, 80 and 120 days (Table 4.10). In case of Se (IV), higher uptake (218.21 $\mu\ g^{-1}$) was observed at 5 mg kg^{-1} and the lowest (20.74 $\mu\ g^{-1}$) uptake was shown by 2.5 mg kg^{-1} across the duration of exposure.

The concentration of total selenium levels in plants increased compared to control, and plants exposed to selenate accumulated higher levels of selenium when compared to selenite (Table 4.10) as is observed in plants treated in presence of consortium (Table 4.8). Retardation in growth in the presence of selenate correlated with failure in bulb formation and high selenium levels in the plants reaching ~665 μ g^{-1} (at 7.5 mg kg^{-1} exposure) over 120 days.

The distribution of total selenium in different parts of plant viz., root, bulb and shoot examined after 80 and 120 days of growth did not show any definite trend (Table 4.11), as shown below, in the concentrations during the growth period in presence of either of the selenium oxyanions.

Table 4.10. Selenium levels in whole plants of *Allium cepa* grown in soil supplemented with 2.5, 5.0 and 7.5 mg kg^{-1} of selenate and selenite solution. BDL – below detection limit; NA – not analyzed.

Sample	Control	2.5 mg kg^{-1}		5.0 mg kg^{-1}		7.5 mg kg^{-1}	
		Selenite	Selenate	selenite	Selenate	Selenite	selenate
				40 days			
Plant	BDL	20.74±1.2	237.0±0.8	40.48±2.4	297.0±1.0	128.36±3.5	476.0±9.3
Soil	BDL	2.23±0.6	2.05±0.8	2.88±0.9	3.2±0.3	4.9±0.6	5.75±0.5
				80 days			
Plant	BDL	56.45±2.56	113.0±9.04	100.56±6.08	128±2.06	163.66±2.56	556.0±3.68
Soil	BDL	1.49± 0.3	1.04±0.2	1.67±0.7	2.05±0.9	3.00± 0.9	3.24±1.33
				120 days			
Plant	BDL	91.48±4.43	290±9.83	218.21±15.1	NA	137.32±8.44	665±10.62
Soil	BDL	BDL	BDL	1.08±0.5	BDL	2.12±0.8	1.50±0.8

In the absence of microbial inoculum, the accumulation of selenite was observably consistent and having limited effect on the growth of the plants as compare to selenate which had inverse effect across the growth period.

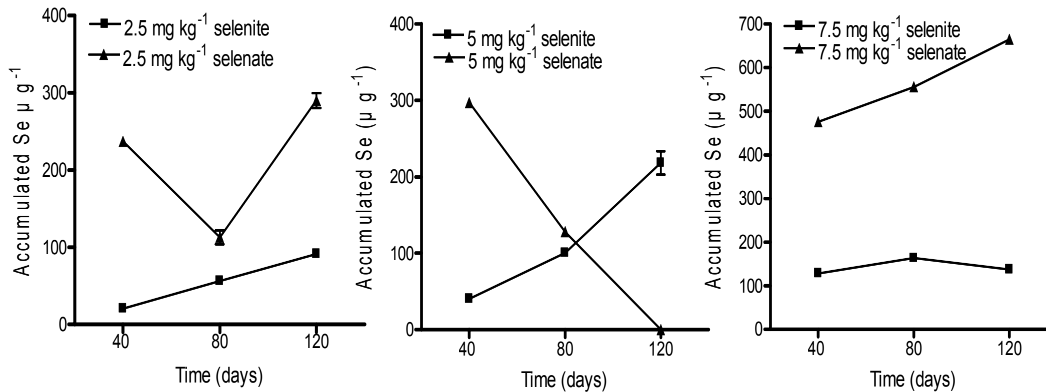
Table 4.11: Selenate and Selenite Contents trend in different plant parts of onion

Selenate (Only whole plant)			
40 days	- 7.5 $^{mgkg-1}$ (476)	>5.0 $^{mgkg-1}$(297)	>2.5 $^{mgkg-1}$(237)
80 days	- 7.5 $^{mgkg-1}$(556)	>5.0 $^{mgkg-1}$(128)	>2.5 $^{mgkg-1}$(113)
120 days	-7.5 $^{mgkg-1}$(665)	>2.5 $^{mgkg-1}$(290.9)	

Selenite				
2.5 $^{mgkg-1}$	- 80 days	- Se^{bulb}(84.92)	> Se^{leaf}(51.47)	> Se^{root}(32.98)
	- 120 days	- Se^{bulb}(122.56)	> Se^{root}(113.15)	> Se^{leaf}(38.73)
5.0 $^{mgkg-1}$	- 80 days	- Se^{root}(123)	> Se^{bulb}(104)	> Se^{leaf}(74.7)
	- 120 days	- Se^{leaf}(260)	> Se^{root}(204)	>Se^{bulb}(190.65)
7.5 $^{mgkg-1}$	- 80 days	- Se^{bulb}(335)	> Se^{leaf}(99.6)	>Se^{root}(56.4)
	- 120 days	- Se^{bulb}(215.6)	> Se^{leaf}(126.78)	> Se^{root}(69.6)

The concentration of total selenium accumulated in plants across growth period was significant in case of exposure to selenate than selenite (Fig.4.29).

Figure 4.29. Profile of selenium accumulation in *Allium* plants exposed to selenate and selenite augmented in soil



In case of exposure to selenite, the overall uptake increased with increasing concentration upto 5.0 mg kg^{-1} (218.2 μ g^{-1}) followed by decrease towards 7.5 mg kg^{-1}

(137.3 μ g^{-1}) which presumably is due to higher rate of volatilization reported in plants when exposed to selenite. This trend also corresponds to variations in the soil selenium levels. As per the present study, at all concentrations of selenate, onion foliage was visibly shorter, bulb size smaller and root mass much reduced when compared to control and selenite treatment accompanied by complete mortality of plants exposed to 5.0 mg kg^{-1} concentration of selenate at 120 days. The soil Se levels did not deplete at the rate, they were observed in case of plants exposed to Se treatments in presence of consortium thereby indicating the role of plant microbe interaction in mobilization of Se in soil.

4.17.3 Influence of bioaugmentation of Se tolerant rhizosphere bacteria on Se uptake in plants

A comparative representation of Se level in the plant vis-à-vis the fresh weight in the presence and absence of microbial inoculum is given in Fig 4.30 along with growth profile in Fig 4.31. The results indicate higher uptake of selenium in plants without inoculum (WoI) when compared to the same in the presence of rhizosphere bacteria (WI). However, varying observations were seen in growth profile of plants, as indicated by fresh weights of plants grown in the presence and absence of microbial inoculum. In the plants cultivated in the soil devoid of microbial inoculum, corresponding decrease in the fresh weight showed inhibition of the growth by Se. In contrast, inoculation of rhizosphere bacteria in soil was observed to facilitate plant growth by reducing the uptake of Se by the plants vis-à-vis its influence on the growth profile of the plant.

The bioaccessible levels of elements like selenium in edible crops like *Alliums* are affected both by availability of element in soil and its fractionation in various plant parts. The results of the present study, therefore, hints at importance of understanding (a) the levels of accumulation at various edible parts of the plant; and (b) the influence of Se tolerant rhizobial bacteria on the Se accumulation profile of the plants.

Figure 4.30. Comparison of the fresh weight of the plant with reference to the selenium uptake in plants cultivated in soil with (WI) and without (WoI) augmentation of microbial inoculum

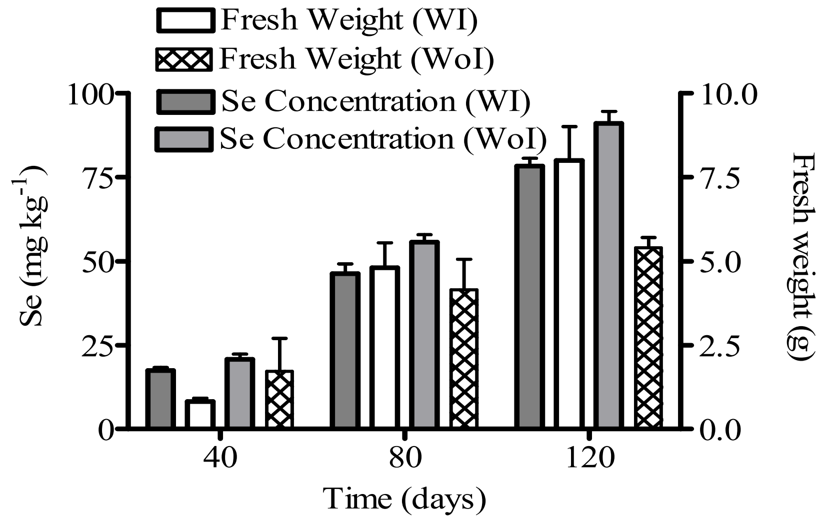
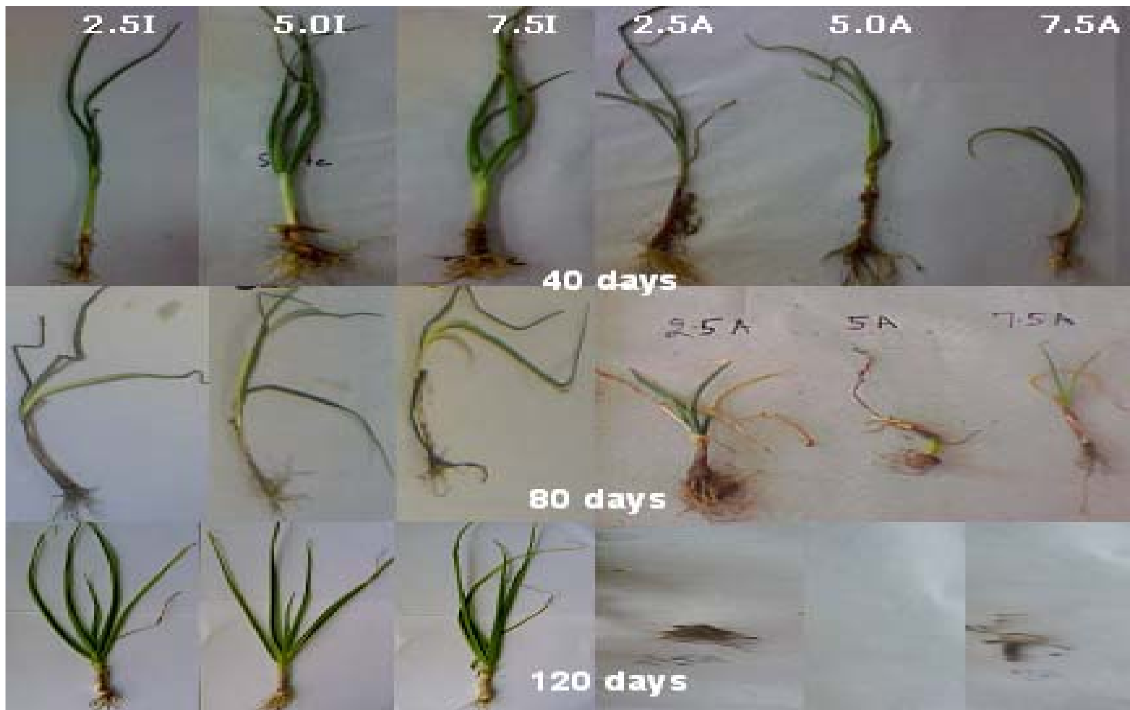


Fig 4.31. A comparison of the growth of *A.cepa* plantlets in different concentrations of Se oxyanions as selenite (I) and selenate (A) without consortium



4.17.4 Survival studies of bacteria grown in Se supplemented soils with plants

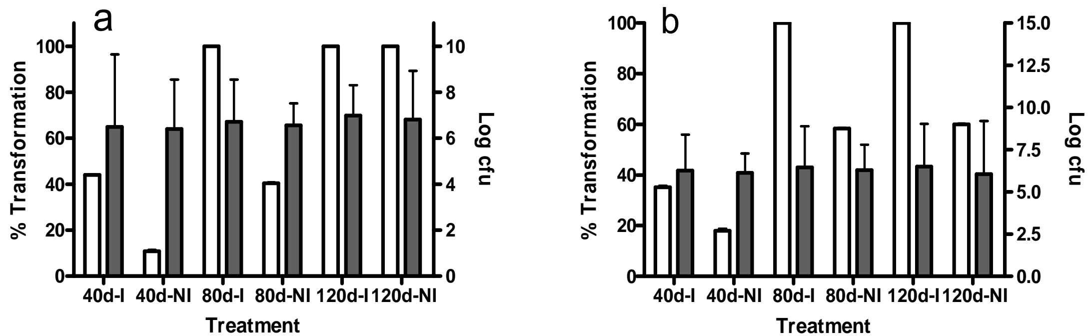
Similar microcosm conditions were maintained in current study as was mentioned in section 4.13, but with cultivation of *Allium* plants, to examine the survival of bacteria in the presence of a rhizosphere zone of plants. Population dynamics based on the antibiotic resistance, of the consortia showed an increase in the population after 120 days of incubation in selenite treated soils and there was increase in colony forming units after 80 days followed by declining trend in number after 120 days in selenate treated soils across all treatments. Population of consortia showed an increase from log (6.4) cfu gm^{-1} after 40 days to log (6.56) cfu gm^{-1} after 80 days that increased gradually to log (6.98) cfu gm^{-1} after 120 days in 2.5 mg kg^{-1} selenite treated soils (Fig. 4.32A) while population increased from log (6.46) cfu gm^{-1} at 40 days to log (6.49) cfu gm^{-1} after 80 days that came down to log (6.26) cfu gm^{-1} after 120 days in 2.5 mg kg^{-1} selenate treated soils treated soils. Similar trend was observed in population survival of 7.5 mg kg^{-1} selenite [(log) 6.30, 6.37 and 6.42 cfu gm^{-1}] and selenate [(log) 6.30, 6.37 and 6.42 cfu gm^{-1}] treated soils respectively during growth period (Fig. 4.32C). It was observed that the microbial population based on LA + carbenicillin plates in control microcosm of all treatments showed a declining trend after 120 days. Maximum survival rate was observed in soil supplemented with 5 mg kg^{-1} treatment during 120 day period. The corresponding trends in mobilization of selenium over time, on exposure to selenium oxyanions, are also represented in Fig. 4.32 B.

Transformation of selenium oxyanions by microbial consortia in soils supplemented with selenite treatments revealed complete removal of Se across 120 days as compared to control in which the removal was 78 and 80% in 5.0 and 7.5 mg kg^{-1} selenite supplemented soils without consortia after 120 days as shown in Fig.4.32B and 4.32C. Similar trend was observed in selenate treated soils supplemented with consortia attaining complete transformation after 120 days. At 7.5 mg kg^{-1} of selenite, the level of removal was 80% after 120 days in uninoculated soils, which was attained in inoculated soils augmented with same concentration after 80 days. In uninoculated soils treated with 2.5 mg kg^{-1} of selenate, the mobilization and removal was 18% at 40 days increasing to 60% at 120 days as compare to 5 mg kg^{-1} which was higher with value of 76% after 120 days. Transformation rate in soils augmented with 7.5 mg kg^{-1} selenate without consortia

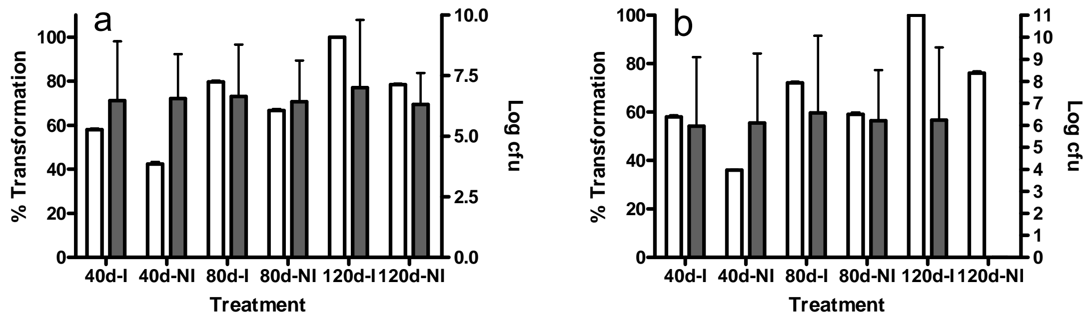
was noted to be 23 % after 40 days which increased to 71% after 120 days with highest transformation rate among selenate supplemented soils.

Figure. 4.32. Rate of selenium transformation [a – selenite and b – selenate] and corresponding viability of the consortia in soils supplemented with different concentrations of Se.

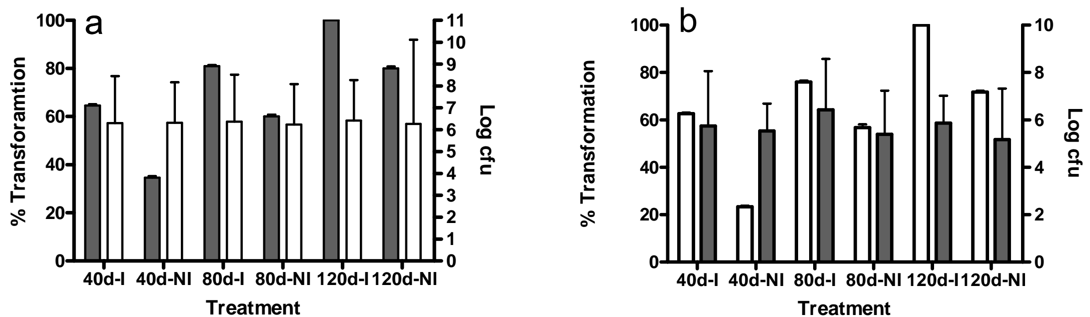
A. 2.5 mg kg^{-1}



B. 5.0 mg kg^{-1}



C. 7.5 mg kg^{-1}



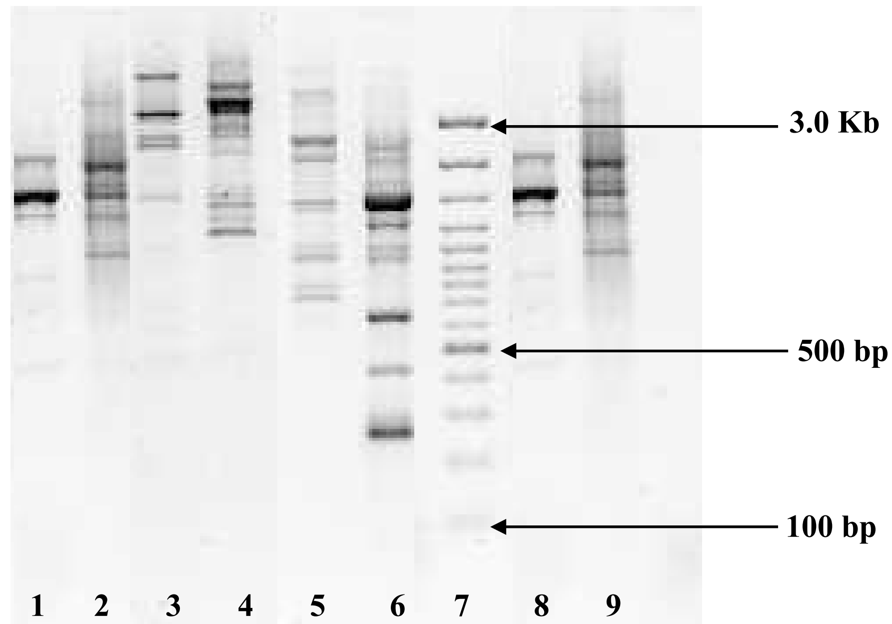
The gray tone and white tone in graphs represent log CFU count and rate of transformation (%) respectively. I represents soils augumented with inoculum and NI represents soils without inoculum of consortia

4.17.5 Microbial population count in microcosm by REP-PCR

Population of all the isolates showed a gradual increase after 80 days followed by decline after 120 days across all treatments except 5.0 mg kg^{-1} selenite as determined by molecular fingerprinting. After 40 days enumeration, maximum survival rate was noted in 2.5 mg kg^{-1} selenite supplemented soil. After 120 days of experiment, with REP-PCR based enumeration, maximum survival rate of 60% was observed for 2.5 mg kg^{-1} selenate supplemented soils followed by 5.0, 2.5 (Fig. 4.30) and 7.5 mg kg^{-1} selenite supplemented soils with 56, 50 and 46% survival rate respectively after 120 days. (Table 4.12).

Fig. 4.33. REP-PCR of bacterial strains isolated from Se supplemented (2.5 mg kg^{-1} selenite) soil collected after 120 days

Lane 1: NS-3; Lane 2: NS-4; Lane 3, 4: mismatch strains; Lane 5: SNTP-1; Lane 6: NS-2; Lane 7: 100 bp ladder; Lane 8 and 9 represents amplified strains obtained from inoculated samples showing similarity with first two lanes.



A combination of PCR conditions was used in the present study to generate strain specific DNA fingerprints to select the population released for bioremediation of selenium contaminated soils.

Table 4.12. Survival of bacterial isolates comprising consortia in soil microcosm based on REP-PCR. Values given in parenthesis represent the number of isolates comprising consortia that gave a complete match with standard isolates of consortia versus total number of isolates fingerprinted

Concentration (mg kg^{-1})	40 Days	80 Days	120 Days
2.5 selenite	80 (40/50)	84 (42/50)	50 (25/50)
5 selenite	78 (39/50)	64 (32/50)	56 (28/50)
7.5 selenite	76 (38/50)	80 (40/50)	46 (23/50)
2.5 selenate	70 (35/50)	84 (42/50)	60 (30/50)
5 selenate	64 (32/50)	78 (39/50)	40 (20/50)
7.5 selenate	40 (20/50)	54 (27/50)	38 (19/50)

The lowest survival rate obtained from plate counting as well as DNA fingerprinting was for 7.5 mg kg^{-1} selenate augmented soils thereby supporting the toxic nature of selenate oxyanion, despite the protection of rhizosphere zone of plants.

Section C: Evaluation of anti-oxidant and related properties in plants grown in seleniferous soils

The modulations in pro and anti-oxidant properties as TBARS levels and GSH-Px activity respectively were monitored with respect to the corresponding levels of accumulated Se in plants. These observations were taken both at pot and field experiments.

The anti-oxidant activity of the plant extracts was attempted following thiobarbituric acid (TBA) assay (Miller and Aust, 1989) and GSH-Px activity was determined by a modification of the method of Flohe and Gunzler (1984).

4.18 Pot Experiments

There are limited observations reported on Se-induced anti-oxidant activity in plants. As discussed in earlier section, in pot experiments the plants were exposed to concentration of 5 mg kg^{-1} of selenium supplemented in form of selenate and selenite and studies were carried out for period of 120 days. TBARS assay were carried out in samples of 120 day harvest.

The first contact of the plant with selenium occurs through the roots that act as a transport systems for movement of nutrients to plants, followed by the roots are bulbs as underground tissues of the plant. Therefore, the concentration of malondialdehyde (MDA), an oxidation product of polyunsaturated fatty acids, in the roots and bulb was determined to examine the level of oxidative damage caused by selenium oxyanions amended in soil. Table 4.13 shows that after the exposition of the plants to selenate, MDA in the underground tissues (root and bulb) increased about ~ 40% with respect to control and ~15% with respect to selenite exposed plants. Interestingly, the plants showed a different reaction pattern depending on the treatment. In the root-bulb part of the selenate supplied plants, the MDA concentration was the highest, whereas lowest MDA concentration was noted in leaves of control plants. Selenate was observed to induce higher MDA generation when compared to selenite, resulting in higher GSH-Px response in leaves of the exposed plants. Enhanced pro-oxidant activity in the plants was observed to significantly retard the growth of the plants prominently in the presence of selenate followed by selenite.

Table 4.13: Concentration of MDA in over and underground tissues of *Allium cepa* plantlets harvested after 120 days

Leaves	MDA (μmoles/mg)
Control	0.07×10^5
Selenite	0.16×10^5
Selenate	1.2×10^5
Bulb+Root	**MDA (μmoles/mg)**
Control	0.041×10^5
Selenite	0.26×10^5
Selenate	1.5×10^5

4.19 Field Investigations

Further to the observations on the Se induced levels of TBARS, as a function of corresponding levels of selenium oxyanions in whole plants cultivated in pots, experiments were carried out in soil microcosms supplemented with selenium at various concentrations (Tables 4.14 A-C). Without supplementation of selenium, the levels of TBARS and GSH-Px indicated decrease up to 80 days sample followed by increase at 120 days sample. Supplementation of 2.5 mg kg^{-1} of selenite to soil and corresponding increase in selenium uptake by plants induced TBARS generation upto 80 days, during which the GSH-Px levels were observed to be lower. The levels of TBARS showed a downward trend over time on exposure to 2.5 mg kg^{-1} of selenite, suggesting expression of anti-oxidant properties by plants exposed to lower concentrations of selenium, as selenite, during growth. This was also indicative over 120 days, where in TBARS levels reduced with corresponding increase in GSH-Px levels indicating initiation of anti-oxidant activity. The trends significantly correlated with the levels of Se accumulation during this period. Exposure of plants to soil supplemented with 2.5 mg kg^{-1} of selenate, also indicated similar trend. In general, it was observed that selenite application induced higher values in case of TBARS when compared to selenate, while GSH-Px activity was relatively higher to TBARS during the exposure to selenate.

In contrast to the observations at low concentration (2.5 mg kg^{-1}) of Se exposure, TBARS levels were observably higher at induced soil concentration of 5.0 and 7.5 mg kg^{-1} of selenium. Amongst the Se oxyanions, selenate induced higher concentration of TBARS then selenite. In terms of GSH-Px, at 7.5 mg kg^{-1} of selenite, was found to

induce higher activity of GSH-Px enzyme. At 5 mg kg^{-1}, there was no definite trend observed in TBARS levels on exposure to selenate where as reducing trend in the same was noted with selenite. The modulations in GSH-Px levels correlated with Se levels in whole plants exposed to 5.0 and 7.5 mg kg^{-1} of selenate, which is in contrast to marginal or no correlation in the presence of selenite. Thus, the data related to GSH-Px coincided with the accumulated levels of selenium, agreeing with the induction of antioxidant properties of selenium only at higher concentrations of exposure.

Amongst the plants exposed to Se oxyanions in soil, analysis of variance (ANOVA) between levels of Se, TBARS and GSH-Px indicated variation in mean levels of these parameters at all concentrations of Se supplementation. The high Se dosage, in general, was observed to boost the GSH-Px activity to neutralize the Se induced oxidative stress recorded as a distinct increase in TBARS values.

Across the study, in the control plants cultivated without selenium, lipid peroxidation increased significantly with senescence, which was observed to reduce in the presence of selenium. During senescence, the accumulation of TBARS mostly remained lower in selenium treated plants. In case of 2.5 mg kg^{-1} concentration, the TBARS levels were recorded to be lowest in case of both oxyanions as compare to control across the growth period, except selenite treatment at 120 days which was observed to be higher then control plants. At 5.0 mg kg^{-1}, the TBARS values followed the same trend in case of both oxyanions with lower values during growth as compare to control, but marginal high concentration was examined in case of selenite at 40 days and 120 days in case of selenate treated plants.

At higher selenium supplementation and end of growth period of plants, the decrease in lipid peroxidation was smaller indicating that antioxidative effect of selenium was diminished in senescence plants.

Table 4.14 Modulations in Selenium accumulation and pro (TBARS)/anti-oxidant (GSH-Px) properties in whole plants of *Allium cepa* cultivated in soils supplemented with different concentrations of Se (ND – not detected; (NS – non significant)

A. 2.5 mg kg^{-1}

Days	Se Conc. (mg kg^{-1})			TBARS (MDA moles mg^{-1})			GSH-Px (nmol 100 mg^{-1})		
	Control	Selenite	Selenate	Control	Selenite	Selenate	Control	Selenite	Selenate
40	ND	17.4±0.9	169.0±2.2	347±3	266±3	299±1.5	3.1±0.2	2.8±0.5	3.5±0.7
80	ND	46.3±3.9	99±3	1485±4.6	454±3	296±5	3.3±0.5	2.3±0.2	2.5±0.3
120	ND	78.3±10.2	66.4±6.0	243±0.5	479±1	205±1	3.0±0.2	2.8±0.3	3.0±0.2

Correlation	**Selenite**	**Selenate**
TBARS (Con.) / TBARS (Exp.)	0.00	0.54
GSH-Px (Con.) / GSH-Px (Exp.)	-0.99	-0.68
Se Accumulation. / TBARS	0.90	0.76
Se Accumulation / GSH-Px	0.25	0.66
TBARS / GSH-Px	-0.20	0.01

t-Test Between oxyanions

TBARS	NS	($p>0.05$)
GSH-Px	NS	($p>0.05$)
Se accumulation	NS	($p>0.05$)

B. 5 mg kg^{-1}

Days	Se Conc. (mg kg^{-1})			TBARS (MDA moles mg^{-1})			GSH-Px (nmol 100 mg^{-1})		
	Control	Selenite	Selenate	Control	Selenite	Selenate	Control	Selenite	Selenate
40	ND	37.9±0.9	379±3	347±3	387±0.7	272±6	3.1±0.2	2.9±0.4	3.8±0.7
80	ND	53.1±2.8	377±7	1485±4.6	273±1.4	289±3	3.3±0.5	2.6±0.4	2.6±0.4
120	ND	170±17	386±25	243±0.5	186±0.3	253±1.3	3.0±0.2	3.0±0.4	3.0±0.2

Correlation	**Selenite**	**Selenate**
TBARS (Con.) / TBARS (Exp.)	0	0.89
GSH-Px (Con.) / GSH-Px(Exp.)	-0.98	-0.51
Se Accumulation. / TBARS	-0.88	-0.95
Se Accumulation / GSH-Px	0.76	-0.01
TBARS / GSH-Px	-0.36	-0.29

t-Test Between oxyanions

TBARS	NS	($p>0.05$)
GSH-Px	NS	($p>0.05$)
Se accumulation	**	($p<0.01$)

C. 7.5 mg kg^{-1}

Days	Se Conc. (mg kg^{-1})			TBARS (MDA moles mg^{-1})			GSH-Px (nmol 100 mg^{-1})		
	Control	Selenite	Selenate	Control	Selenite	Selenate	Control	Selenite	Selenate
40	ND	28±1.5	524±5.3	347±3	326±0.5	245±0.4	3.1±0.2	4.1±0.2	2.2±0.4
80	ND	42.03±4.2	453±8.0	1485±4.6	238±1.3	1000±5.7	3.3±0.5	3.5±0.4	2.4±0.3
120	ND	161±11.6	614±37	243±0.5	256±2.3	416±2.5	3.0±0.2	4.2±0.2	3.3±0.4

Correlation	**Selenite**	**Selenate**
TBARS (Con.) / TBARS (Exp.)	-0.60	0.96
GSH-Px (Con.) / GSH-Px (Exp.)	-0.99	-0.63
Se Accumulation. / TBARS	-0.40	-0.68
Se Accumulation / GSH-Px	0.58	0.82
TBARS / GSH-Px	0.50	-0.16

t-Test Between oxyanions		
TBARS	NS	($p>0.05$)
GSH-Px	*	($p<0.05$)
Se accumulation	**	($p<0.01$)

With reference to the trend in anti-oxidant enzyme, GSH-Px, in plants cultivated without selenium, it was observed to increase during plant senescence to counteract oxidative reactions. In the plants supplied with lower concentrations i.e 2.5 and 5.0 mg kg^{-1} of selenium oxyanions, GSH-Px activity did not increase significantly as compare to control plants. In selenite treated plants, GSH-Px levels were found to be lower in 2.5 and 5.0 mg kg^{-1} treated plants across the growth period, with exception in 5.0 mg kg^{-1} treated plants, which was observed to have enzyme activity equal to that of control at 120 days. In case of selenate amended plants, GSH-Px activity was higher at initial stage followed by decline at 80 days period and attaining values equal to that of control plants at 120 days. At the high Se level i.e 7.5 mg kg^{-1}, GSH-Px increased in case of selenite treated plants to counteract the Se-evoked oxidative stress.

Inverse relationship was observed between the TBARS and GSH-Px levels of control and experimental plants indicating the influence of Se exposure on the modulation of both pro and anti-oxidant activity. The positive correlation between the Se concentrations and antioxidant activities reported here for onion plantlets also agrees with the earlier reports. The observations in the present study, thus apparently indicate that Se

lowers the oxidative stress and promotes anti-oxidant activity at short exposures and/or not to higher levels. However, at higher levels of exposure, Se acts as a pro-oxidant prompting immediate response of anti-oxidant activity. In addition, senate is more efficiently phyto-available and therefore more hyperaccumulated inside the plant when compared to selenite, thus affecting the biological response vis-à-vis growth of the plant.

5.0 Discussion

5.1 Characterization of seleniferous soils and Se tolerant bacteria

5.1.1 Characterization of soils

Rhizospheric soil samples collected from agricultural soils from seleniferous site of Nawanshahr-Hoshiarpur districts of Punjab, India and non-seleniferous soil from Thapar Technology Campus, Patiala (Punjab) was processed and subjected to physicochemical characterization.

The binding of selenium onto soils and sediments depends on many factors e.g pH, EC, Se species, competing anions, iron oxides and type of clay minerals (Dhillon and Dhillon, 2003; Seby et al., 1997). Changes in the pH of soil solutions alter the relative proportion of available and unavailable form of minerals. Nutrients in soil are strongly affected by soil pH which in turn influences their availability. Even where supply of all minerals is adequate, deficiency can occur due to other factors also. The pH of soil samples collected from seleniferous sites ranged from acidic to alkaline. Whereas in case of soil samples collected from Patiala region, pH was found to be towards alkaline range (7.7). The pH was observed to be in the neutral range in untreated soils of Barwa region while in Gypsum treated soils of Barwa region, pH value was marginally acidic (6.6). The soil sample collected from Jainpur indicated neutral (7.1) to alkaline (8.8) range.

The chemical form 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). In poorly aerated acidic soils, inorganic Se predominates as the relatively insoluble selenide and elemental forms. Se as selenate is 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). The present study also resulted in similar observations noted in terms of relationship between Se levels in plants to that of pH, wherein higher concentration of Se was noted in plants

cultivated in alkaline soils of Jainpur when compared to those cultivated in soils of near neutral pH as that in Barwa.

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 Jainpur region, EC value were towards range of 221-393 $\mu S\ cm^{-1}$ where as in Patiala region value was 190 $\mu S\ cm^{-1}$. In un-treated soil of Barwa, EC values were found to be higher (209 $\mu S\ cm^{-1}$) while in gypsum treated soils, value was found to be lower with value of 115 $\mu S\ cm^{-1}$. Clay minerals and organic matter play an important role in chemical processes controlling the mobility of Se in soils (Dhillon and Dhillon, 2003). 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 to be 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 with sand content ($r = -0.69$), 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, K uptake by plants is limited by high levels of Ca in some soils. High levels of K can in turn, limit Mg uptake even if Mg

levels in soil are high. Gypsum addition reduced pH hence reduced the cation exchange capacity accompanied by low potassium content in soils.

In Jainpur soils, the CEC values observed to increase (510 meq/100g) with increase in pH of the respective soil samples of the region. Similar changes were noted in gypsum treated soils of Barwa in terms of CEC values which were found to be lower (199 meq/100g) as compared to untreated soils of same region. Similar observations were found with value of 250 meq/100g in soil samples collected from Patiala. The potassium levels in samples collected from Jainpur region was observed to be in range of 38.1 - 44.6 mg kg^{-1}. In untreated soils of Barwa, the concentration was 42.6 and in Gypsum treated soils of the Barwa region potassium level was found to be lower (32.3 mg kg^{-1}) as compare to untreated soils followed by Patiala soils with lowest potassium content of 14.20 mg $kg.^{-1}$ 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). Balisterieri and Chao (1987) investigated the effects of various anions on selenite adsorption by goethite and observed the following selectivity sequence: phosphate > silicate > citrate> molybdate > bicarbonate/carbonate > oxalate > fluoride > sulfate.

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 Jainpur region, soil samples phosphorus content was found to be in range of 1.6-4.65 and lowest of 1.6 was observed

in soil samples having pH of 8.1 showing that phosphorus levels decrease with increase in pH. Similar decline in levels of phosphorus was observed in alkaline soils of Patiala with value of 2.60 mg $kg.^{-1}$ In untreated soils of Barwa, phosphorus content was more then 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).

5.1.2 Selenium content in crops cultivated in seleniferous soils

Plants can accumulate significant levels of selenium in their tissues. However, the role of this element in growth and metabolism of the plant is still being debated upon. Plants and their produce are the major sources of Se intake into animal and human system. The level of intake plays a critical role in the health of the animal and human system.

The intake of selenium through diet has been found to play an important role in expression of anti-oxidant properties in the organism. However, there is limited information available regarding status of nutritive constituents of the food/vegetable plants grown in Se rich soils and modulations in cellular properties due to Se rich diets (Saggoo et al., 2004). Selenium content of soil samples analyzed by INAA, collected from two seleniferous sites of selenium impacted region was found to contain 2.7 and 6.5 mg kg^{-1} of selenium respectively (Sharma et al., 2009). Crop produce from this region was found to contain high concentration of selenium when compared with global data on Se in food crops. The higher end of the range observed in the present study was above the estimated threshold level of 4 mg kg^{-1} (Engberg et al., 1998). The Se levels were observed to vary within the region with Se rich and Se adequate regions dispersed in small tracts/pockets (Sharma et al., 2007).

The selenium content observed in the wheat grain and husks was found to be quite high with value of ~115.2 mg $kg.^{-1}$ Amongst all the crop produce examined, Se levels in mustard seeds was found to be highest viz., 670 mg kg^{-1}. Selenium uptake in rice crops indicated a lower uptake in grains with concentration of 16.21 mg $kg.^{-1}$ Similar findings

were observed in terms of selenium uptake from moderately affected region with highest levels in grains/seeds of mustard averaging at 594 mg $kg,^{-1}$ and in wheat grains, selenium content was lowest as compare to mustard with value of 41.1 mg kg^{-1}.The reduced uptake by the wheat was attributed to changes in agronomic practices and depth of water source (Sharma et al., 2009). In reference to selenium levels in foods observed in other parts of the world, wheat grain produced in North and South Dakota in the USA was reported to contain more than 2000 μg kg^{-1}. Grain Se concentrations were proposed to be in the ranges of <25, 25-40, 40-1000 and >1000 μg kg^{-1} represent deficient, marginal, moderate to high and excessive concentrations in China respectively (Combs, 2001; Tan, 1989). In addition, based on the data reviewed of various crop produce from European countries (Ventura et al., 2007), the observations obtained in the present study clearly indicated a substantially high uptake of selenium in the crop produce from the seleniferous soil of the region under study. Mustard, mushrooms, alliums (onion and garlic), broccoli and Brazilian nuts have the ability to accumulate selenium from soil to significantly high levels (Sharma et al., 2007; Reilly, 1998). Selenium levels ranging from 0.3 to 0.5 mg kg^{-1} was reported in different varieties of onions grown in Japan (Noda et al., 1983). The typical levels of selenium in vegetables and fruits in Portugal, New Zealand, Finland and Mainland China ranged from 1-25 μg kg^{-1} (Ventura et al., 2007; Combs, 2001; Diaz-Alarcon et al., 1996). The recommended dietary allowances (RDA) of selenium for adults, both male and female, are 55 μg d^{-1} with tolerable upper intake levels (TUL) of 400 μg d^{-1} (Goldhaber, 2003). If the RDA values are to be considered, the concentrations obtained in grains are exceeding the recommended values (FAO, 2001).

Plant foods are the major dietary sources of selenium in most of the countries throughout the world. The bioavailable selenium content of the soil where plants are grown or animals are raised does influence their selenium content and thus that of human food, as demonstrated by this study.

5.1.3 Isolation and characterization of bacteria

Four isolates prominent in Se oxyanions reduction were isolated from rhizospheric soils of wheat from selenium contaminated regions of Nawanshahar and Hoshiarpur districts. Their selection was based on their potential to tolerate up to 100 mg

L^{-1} of selenium as selenate (Na_2SeO_4) and selenite (Na_2SeO_3) and was designated as SNTP-1, NS-2, NS-3 and NS-4. These bacterial isolates were tested for growth in the presence of different concentrations (5 mg L^{-1}, 15 mg L^{-1} and 25 mg L^{-1}) of Na_2SeO_4 and Na_2SeO_3 respectively. Similar growth rate was observed in all the isolates in the presence of either of the selenium oxyanions viz., selenate and selenite as determined by optical density and total viable counts (colony forming units). In case of control culture (0 mg L^{-1}), growth rate was faster as compare to oxyanion treated cultures. The cultures grown in the presence of different concentrations of selenium oxyanions, showed red color formation in the media as growth progressed signifying the reduction of selenium oxyanions to elemental red selenium. 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). Preliminary studies on the reducing potential of these strains indicated they dominantly reduce selenium to Se (0) in *in vitro* conditions (Yadav et al., 2008). Similarly, experiments were carried out with consortia of four isolates that were characterized earlier, to examine if the potential to the reduction of selenium oxyanions gets enhanced with concurrent activity of consortium. The results indicated that the Se reduction potential was more prominent as a consortium than as individual groups of organisms.

5.1.4 Biochemical Characterization

Biochemical and morphological tests that were carried out indicated differences in the strains and exhibited varying sets of biochemical responses. NS-2 showed negative results in catalase and nitrate test and therefore was notably not a facultative anaerobe in nature, while other three isolates showed characteristics consistent with that of typical facultative anaerobic bacteria. 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 their isolate belonging to genus *Bacillus.*

5.1.5 Antibiotic profiling

The antibiotic profiling studies were carried out for all isolates using different concentrations of 28 antibiotics which gave a resistance profile of the isolates in presence of different 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. Some of the antibiotics which inhibited the growth of isolates at 1 mcg were erythromycin, kanamycin, tetracycline, oxacillin, cloxacillin and certain other antibiotics. All isolates under study showed resiatance towards carbenicillin, even at higher concentration of upto 300 mcg except NS-3 which showed resistance upto 40 mcg.

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. So 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 (Lawrence, 2000; Verma et al., 2000; Bhattacherjee et al., 1988). Even within a single species, strains can exhibit different degrees of antibiotic resistance. Bacteria 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 current study of antibiotic profiling as depicted by resistance of isolates against carbenicillin, a relation between heavy metal and antibiotic resistance in

anticipated due to significant tolerance of the test strains towards diverse variety of antibiotics tested.

5.1.6 Effect of pH on growth of organism and selenium reduction

In formulation of bioremediation approaches, it is important to understand the behavior of soil bacteria towards the presence of Se in prevailing soil and water conditions. Behavior 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 have indicated the potential of bacteria to reduce both oxyanions in neutral to alkaline range of pH 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 strains except SNTP-1 showed retardation in growth at early exponential phase of the growth profile studies at higher pH conditions (pH 10) followed by recovery of the growth profiles at delayed exponential phase but rate of recovery varied in case of each type of strain and oxyanion treatment. In case of NS-2 and NS-3, retardation in terms of growth was observed on exposure to selenite at early exponential phase, followed by recovery at late exponential phase only in case of NS-3. While in case of NS-4, growth inhibition was prominent on exposure to both oxyanions. On exposure to acidic conditions (pH 4), no reduction was observed in 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 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 ranges values are expected to prevent the growth of *B. cereus*. In case of *Enterobacter taylorae,* which can reduce selenium as selenate, the activity was observed

to be significant in high salt and high pH selenate contaminated agricultural drainage water (Zahir et al., 2003).

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 pH at higher alkaline levels (pH 10) than the reported species and also tolerate impact of Se in ambient environments.

5.1.7 Effect of temperature on growth of organism and selenium reduction

In order to understand the mechanism of the bacterial biotransformations 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 behavior in terms of reduction potential. All strains showed slow growth and delayed reduction at 20°C. While at 50°C, decline in growth was observed along with absence of Se reduction. Similar observations were also reported by Fermanian et al. (1994), where in 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 behaviors in similar soil conditions. With tropical soil temperatures, reaching higher than 35°C in certain areas of this seleniferous belt, the persistence and selenium mobilizing activity by these organisms would invariably be influenced by the ambient temperature. As-on-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).

5.1.8 FAME based identification

The evidence that the profile of cellular fatty acids could be used for identifying bacteria was given by Abel et al. (1963). In the present study, through clustering based on the FAME data, a defnite separation was found between isolates identified as *B. pumilus* and *B. licheniformis*, which is in agreement with the results of Kampher (1994).

Identification tools will hardly be able to distinguish between the *B. cereus*-related species. This can be deduced from the highly similar fatty acid profiles of *B. cereus* and *B. thuringiensis*; from genetic rRNA, gene and plasmid sequence analysis; from population genetic studies and comparative genomic analysis; and because these species can only be differentiated based on their morphology, phenotype and pathogenicity (Tourasse et al., 2006; Bavykin et al., 2004; Kampher, 1994; Drobniewski, 1993).

Gas chromatography of bacterial cellular fatty acid methyl esters is primarily used as a means of identifying many important gram-negative bacteria such as *Pseudomonas* (Mukwaya and Welch, 1989) and *Campylobacter* (Lambert et al., 1987). It has also been applied to *Lactobacillus* (Gilarova et al., 1994; Rizzo et al., 1987). The first genus-wide fatty acid methyl ester (FAME) analysis of the genus *Bacillus* was done by Kampher (1994) who concluded that fatty acid analysis allows the assignment of *Bacillus* strains to species group but is not as useful for exact species identification. Species of *Bacilli* are divided into six groups (A-F) based on the FAME studies carried out by Kaneda (1977). Strains NS-2, NS-3 and NS-4 placed under Kaneda group E on the basis of presence of abundant cellular fatty acids. The fatty acid methyl ester profiles based on Microbial Identification System (MIS) software and database (TSBA 50 library) revealed that all isolates belong to *Bacilli* group as mentioned in Table 4.4.

5.1.9 DNA fingerprinting and 16S rDNA analysis

PCR provides a sensitive and specific method of identifying a known DNA sequence. Multiplex PCR, a modification of the basic PCR process, has the added advantage of having multiple primer pairs specific for varied targets in the amplification reaction (Shangkuan et al., 2001). Approach for PCR-mediated strain fingerprinting is based on the targeting of repeated DNA sequences with outwardly-directed oligonucleotide primers. The general methodology is referred to as enterobacterial repetitive intergenic consensus-PCR, or ERIC-PCR was used to type various *B. cereus* group bacteria (Shangkuan et al., 2000). This method also allows differentiation of *B. anthracis* from other *B. cereus* group bacteria (Shangkuan et al., 2001).

In the present study, unique ERIC-PCR based genotypic fingerprints of different isolates from selenifereous soil samples were found. Four representative isolates were found to be genetically dissimilar. The genomic fingerprints showed certain intense

amplimers. To further confirm, the genetic variability in the representative isolates, 16S rDNA was amplified and sequenced. All isolates showed 96- 99% similar with closely related matches as shown in figure 4.11.The biochemical, ERIC-PCR and 16S rDNA results were in concordance and showed variability at each level. An earlier report demonstrated that a betaproteobacterium *Thauera selenatis* could respire selenium anaerobically (Rech and Macy, 1992) however; three strains isolated in this study were facultative anaerobes. Members of the genus *Bacillus* are in general well known for conferring resistance to various other metals and metalloids (Nies 1999; Stolz et al., 2006) as was shown by isolates under study towards Se oxyanions upto concentration of 100 mg $L.^{-1}$ Phylogenetic analysis revealed that the selenium-tolerant microorganisms in present study belong to *Bacilli* group. Similar studies were reported by Ghosh et al. (2008) for selenium tolerant microorganims which stated that these were common soil bacteria belonging to the classes of *b-Proteobacteria* and *Bacilli* and thus may offer an insight into the tolerant bacterial population present in tropical selenium contaminated sites.

5.1.10 Selenium reduction of Se tolerant *Bacillus* strain and biogenesis of nanoparticles

The test strain NS-3 examined for Se reduction potential in aerobic and anaerobic conditions showed that this transformation takes place in the both the mentioned conditions confirming the facultative nature of the strain. As on date, majority of the observations of Se reduction were studied in anaerobic bacteria with limited observations in aerobic strains (Ghosh et al., 2008; Losi and Frankenberger, 1997; Garibasu et al., 1996). Amorphous Se nanospheres, such as those produced by the *Bacillus* sp., comprise both disordered [-Se-]*n* chains and Se_8-rings and are relatively unstable at ambient temperatures (Peled and Hadziioannou 1991; Kasap and Yannacopoulos, 1989). However, the biogenic Se nanospheres produced in this study remained stable until the organic component was removed in the post-preparative washing step, suggesting that the biomass was acting as a stabilizing agent. The amorphous red Se nanospheres were then transformed to crystalline trigonal trapezohedral (hexagonal) selenium (*t*-Se) (Fig 4.15 a) in the presence of organic solvents, via hexagonal, platy nano-structures (Fig 4.14 a). The development of Se nanorods in the presence of simple organic solvents has been observed

before (Takahashi et al., 2006; Zhou and Zhu 2006; Gates 2002b), although the size of the crystal clusters and the enhanced transformation rates (60 min.) are exceptional in this case (Prakash et al., 2009). In previous investigations, the nucleation of the *t*-Se nanorods appeared to occur on hexagonal platelets, which here may have be either neo-formed *t*-Se or an intermediate monoclinic phase (Gates 2002b; Mayers et al., 2001; Peled and Hadziioannou 1991; Miyata et al., 1978). Figs 4.13A, 4.14A, 4.15A in this study show a similar transformation. *t*-Se is very stable and comprises ordered helical [$-Se-_n$] chains linked together by inter-chain Van der Waals forces (Ren et al., 2004; Corb et al., 1982). The nature of the chain structure parallel to the 001 *c*-axis direction in *t*-Se results in one dimensional growth of Se and favors the acicular crystal development seen here. Preferential growth is expected from crystals orientated along the c-axis and the formation of the relatively large radiating rosettes suggests restricted development of *t*-Se nuclei (Zhou and Zhu, 2006; Zhang et al., 2003).

5.1.11 Survival of Consortium and Se mobilization in Se enriched natural soil

The consortia of the four bacterial isolates obtained in this study were examined for their viability and Se mobilizing potential in Se enriched soils in competition with the native microbiota. Microcosm studies were carried out i*n situ* in plots supplemented with 2.5, 5.0 and 7.5 mg kg^{-1} 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 microbiota in soils supplemented with selenite oxyanion without plants revealed total removal of Se across 120 days in case of 2.5 and 5 mg kg^{-1} followed by approximately 80 % in soils supplemented with 7.5 mg kg^{-1} of Se as selenite. Mobilization of selenate was found in the range of 60% for 2.5 and 5 mg kg^{-1} and 68% for the 7.5 mg kg^{-1} supplemented soils after 120 days. Further, the population of all the four cultures was in the range of 5.39 – 6.13 (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 harbors more microorganisms capable of enhanced xenobiotic degradation abilities.

The survival of these inoculated organisms has been studied using different methods viz., antibiotic profiling and REP-PCR. Antibiotic plating is the most common method used (Yang et al., 2005; Mallick et al., 1999). Observations on population survival based on carbenicillin resistance, maximum survival rate was observed in soil supplemented with 2.5 mg kg^{-1} during 120 day period. Dilution plating with selective antibiotics was utilized to monitor the survivability of strains of *A. baumannii* and it was seen that the population of $6.5\pm0.13\times10^{8}$ cfu g^{-1} soil at day zero (just after bioaugmentation) decreased to $2.09\pm0.08\times10^{8}$ cfu g^{-1} soil after 90 days of incubation, confirming the stability of modified organism in soil (Mishra et al., 2004). The survival of an antibiotic-resistant *Pseudomonas putida* enumerated in field and microcosm soils at 7- to 14-day intervals over 49 d showed survival upto 7 weeks in microcosm soils at a density of 10^{4} cfu g^{-1} soil, whereas in field soils the population declined to 10^{3} cfu g^{-1} soil by the fourth week while higher cell densities in the rhizosphere (10^{6}–10^{5} cfu g^{-1} fresh root mass) was found when inoculated with seed coating, with no subsequent decrease in numbers (Hirkala and Germida, 2004). Multiple antibiotic resistances were also observed for *Pseudomonas aeruginosa* (Okoh, 2003) and a number of other bacterial isolates from soil.

Antibiotic plating for estimation of population survival has its known advantages and disadvantages as explained earlier in section 5.1.5. Hence, the combination of soil microcosms with molecular fingerprinting techniques can be very useful as this allows many replicates to be performed simultaneously with relatively high reproducibility and a useful way of investigating effects of various abiotic and biotic parameters on bacterial community structure. By combining different techniques more detailed information and higher taxonomic resolution can be obtained for the inoculated strains and thus simplifying the estimation of their actual survival in the contaminated soil.

Keeping the above aspect in view, DNA fingerprints based on REP-PCR of isolates were developed to observe the population dynamics in conjugation with carbenicillin resistance. DNA of the native isolates and the isolates from soil were subjected to REP-PCR and the original DNA fingerprints of the isolates were compared with the fingerprints of microorganisms isolated from the selenium contaminated soil microcosms to score the survival of the population. Molecular fingerprinting profiles

indicated that the survival based on antibiotic plates showed higher results than that by REP-PCR.

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, with the exception of some studies regarding selenium, 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).

5.2 Determination of uptake of soil selenium by *Allium* sp. with and without rhizosphere bacteria

Plants can accumulate good amount of selenium in their tissues though it is not required for their metabolism. Food plants are important source of its entry into animals and human beings but its levels in crops is critical for animal as well as human health. Since there is limited information available regarding status of nutritive constituents and genotoxic hazards of the food/vegetable plants grown in Se rich soils (Yadav et al., 2007; Saggoo et al., 2004), the present investigation examined (a) the selenium uptake by

Allium cepa, one of the important ingredient in global culinary, in pot and field experiments; in presence and absence of bacteria; and (b) the anti-oxidant property of the *Allium* plant extracts.

5.2.1 Selenium modulated plant growth in pot experiments

In the present study, the growth profile of plantlets did not get affected in initial stages of growth (on 30 days) on exposure to either of oxyanions as observed in terms of dry weight of plants. But after thirty days of growth period, there was decrease in the biomass in response to selenium concentrations and the effect was more prominent in case of roots followed by bulb and leaves. Plants exposed to selenate showed retardation in bulb formation but no such observation was noted on exposure to selenite.

The observations of Kopsell and Randle (1997) reported that selenium concentrations in nutrient medium (tested from 0.5 to 2.0 mg L^{-1}) have a visible effect on plant growth of *A.cepa*. According to the observations of the above authors, at the 2.0 mg L^{-1} sodium selenate concentration, onion foliage was visibly shorter, bulb size smaller and root mass much reduced when compared to control and lower concentrations. Decrease in plant growth and yield with increased Se application were also reported for alfalfa (*Medicago sativa*) and subterranean clover (*Trifolium subterraneum*) (Broyer et al., 1966).

The observations of Lefsrud et al. (2006) also indicate that the growth of the leaf tissues of kale (*Brassica oleracea* var. *acephala*) responded to increase in concentrations of selenium and that the influence of selenate was more prominent than selenite. Kopsell and Randle (1999) reported decrease in dry mass of shoot to the extent of 23% in the case of exposure of *Brassica juncea* upto the concentration of 4.0 mg Se l^{-1} supplemented as sodium selenate in the growth medium under hydroponic conditions. Similarly, Banuelos et al. (2002) showed decrease in shoot dry weight in *B. napus* (canola) grown in high-Se soil (40 mg of Se kg^{-1}) when comparing plants grown in low-Se soil (0.1 mg Se kg^{-1}). Similar to observations obtained on *A.cepa* in the present study, radishes (*Raphanus sativus*) grown in selenium-enriched culture media did not apparently exhibit intense symptoms of toxicity. However, a reduction of ~ 25% in the growth of roots of the plants exposed to selenite was observed. Ximenez-Embun et al. (2004) also observed similar trends in studies with lupine, sunflower and Indian mustard plants. Recent observations

by Dhillon and Dhillon (2009b) on the selenium upake and growth profile of various vegetable crops viz., radish, turnip, potato, cauliflower, brinjal, tomato and pea indicated that these crops vary significantly in their sensitivity to the presence of selenate-Se in soil.

The accumulation of total selenium in different parts of onion plantlets did not follow a definite trend in presence of either of oxyanions. In general, it was observed that selenate application induced higher accumulation of Se then selenite. Selenate exposed plants showed marked reduction in bulb formation suggesting that plants were sensitive to selenate treatment. However, no such decline in bulb formation was noted in case of selenite exposure. The difference in growth profile of plants exposed to different oxyanion treatments under same conditions might be linked to toxicity of selenate. Accumulation of selenium was highest in selenate treated plants having maximum accumulation in bulb followed by leaf and root. The findings are consistent with observations of Hurd-Karrer (1937) who reported high selenium distribution in wheat exposed to soils supplemented with selenate. The tissue Se concentration in *Brassica juncea* (Hanson et al*.,* 2003), *Astragalus bisulcatus* (Rosenfeld and Beath 1964) and *Stanley pinnata* (Feist and Parker, 2001; Pickering et al*.,* 2000; De Souza et al*.,* 1998) was also reported to be more prominent in the case of selenate. Xu and Hu (2004) and Smrkolj et al. (2006) reported that such variable accumulation can also be observed if the selenium is foliarly sprayed instead of growing the plantlets in Se-spiked soils. Their observations showed that proportions of Se translocated to leaves and stems of plants are quite significant. Observations of Lefsrud et al. (2006) showed higher Se accumulation in leaf tissues of Kale plantlets in the case of selenate exposure in contrast to lesser accumulation on selenite exposure.

The concentration of total accumulated selenium in bulb and leaf part showed a declining trend after ninety days of growth which may be linked to volatilization process in plants. *Alliums* (garlic and onion) are known to accumulate selenium and form organo-selenium compounds which to significant extent volatilize as dimethyldiselenides (DMDSe) and dimethylselenides (DMSe). Other selenium compounds which are known to form in *Alliums* include selenocystine, selenomethionine, selenocytathionine, selenomethionine selenoxide, etc. (Whanger 1989). With reference to the speciation of selenium in the various plant parts of *Allium cepa*, Wrobel et al. (2004) reported that

incorporation of selenium to the high molecular fraction, which was more pronounced in the leaves relative to the bulbs. This incorporation was better with selenate enrichment (33% in leaves and 26% in bulbs) than with selenite (3% and 5% respectively). The chemical forms of selenium in plants or plant products can vary markedly. From 1% to 50% of the selenium was selenite in 20 different vegetables grown on Se-rich medium (Whanger 2002; Cappon, 1981).

5.2.2 Field studies

5.2.2.1 Se uptake in plants cultivated in soil with inoculation of rhizosphere bacteria

This phase of the study was carried out to understand the influence of selenium oxyanions on the growth and associated anti and pro-oxidant activities induced during the growth of plants in Se supplemented soils. The focus was to examine the role of rhizophere bacteria and their influence on Se accumulation levels in plants.

Over 120 day growth period, the effect of Se on plant growth was noted to be dependent on dosage and the chemical form in which selenium was being supplemented. Similar to the observations in the pot experiments, among the selenium oxyanions, the impact of selenite on the growth of the plants was observably lesser than selenate. In case of selenate treatment, the accumulation of Se was rapid, inhibiting the growth, especially at higher concentrations of exposure with significantly high accumulation (~600 μ g^{-1}) in the plants cultivated in soil supplemented with 7.5 mg kg^{-1} of selenate. These results are consistent with other reports referring to different plants, grown in microcosms in which selenate provoked a greater selenium accumulation in plants then did selenite (Pedrero et al., 2006; Cartes et al., 2005; Chen et al., 2002; Lee et al., 2001; Lee and Park, 1998). In terms of growth, selenite had stimulating effect on growth of plants at lower concentration of 2.5 mg kg^{-1}. At higher levels of soil Se supplementation i.e 5 and 7.5 mg kg^{-1} as selenite, growth was retarded during initial stage followed by normal growth at later stages. Similar observations were obtained, with plants showing positive growth pattern over control at 2.5 mg kg $^{-1}$ selenate supplementation during early stage of growth. In case of selenite treatment, the accumulation of selenium increased with increasing concentration up to 5.0 mg kg^{-1} followed by decline towards 7.5 mg kg^{-1}.

However, the accumulation of selenium followed an increasing trend in plants with increasing concentration of selenate exposure.

The inhibition of the plant growth was also significant in the presence of selenate when compared to selenite as indicated by gradual decrease in plant fresh weight. This observation was in contrast to that of Rios et al. (2008) in lettuce plants, wherein selenate was observably less toxic than selenite across various levels of exposure. The incorporation of selenium as selenate was more prominent in leaves followed by bulb of the plantlets as also reported by Wrobel et al. (2004). The accumulation of Se when exposed to selenate was reported to be higher in plants such as *Lactua sativa, Allium fistulosum, Allium schoenoprasum* and *Brassica juncea* grown in presence of selenite (Rios et al., 2008; Kapolna and Fodor, 2007; De Souza et al., 1998).

There was no definite trend observed in terms of accumulation of total selenium in different plant parts on exposure to either of oxyanions except leaf part which showed an increasing trend at all concentrations of both oxyanions with an exception of 2.5 mg kg^{-1} selenate which decreased after 80 days. The absence of a definite trend in the accumulation pattern of Se in plant parts was also reported by Kopsell and Randall (1999); Saggoo et al. (2004) and Di Gregario et al. (2006) in the case of *Brassica* sp. and *Allium* sp.

The augumentation of rhizosphere bacteria to soils have observably reduced the uptake of Se in plants during exposure to either of the Se oxyanions. The levels and the extent of mortality in plants were observably reduced in soils inoculated with these isolates, thus indicating a synergistic and protective effect of these strains on the growth of plants exposed to Se.

5.2.2.2 Se uptake in plants cultivated in soil without inoculation of rhizosphere bacteria

The effect of different oxyanions treatment at different concentrations in absence of bacterial inoculum was studied in this part of experiment. In the absence of consortium the accumulation of oxyanions in plants was found to be higher then in plants grown in presence of consortia. Similar trend of accumulation was followed with highest concentration in selenate exposed plants during the growth period then selenite treatment as is observed in earlier section 5.2.2.1. The effect of selenate on growth of plants at all

concentrations was more distinct showing retardation in growth of plantlets accompanied by loss of bulb and root formation and/or complete mortality in case of 5.0 mg kg^{-1} augmented plants after 120 days. In case of selenite treated plants, no definite trend was noted in accumulation profile in different plant parts which was similar to observations of inoculum treated plants. Selenate was observed to show more toxic effects in uninoculated soils across all concentrations, accompanied by failure in proper bulb, root and leave formation in *Allium* plants.

5.2.2.3 Influence of bioaugmentation of Se tolerant rhizosphere bacteria on Se uptake in plants

Remediation of metal compounds presents a different set of problems when compared to organics. Organic compounds can be degraded while metals normally need to be physically removed or be immobilised (Kroopnick, 1994). Furthermore, the physio-chemical technologies used for soil remediation render the land useless as a medium for plant growth as they also remove all biological activities, including useful microbes, such as nitrogen fixing bacteria and mycorrhizal fungi, as well as fauna. There is a need to develop suitable onsite techniques for the removal of non-volatile and non-mobile soil contaminants (Wheeler, 1994). Plants that uptake heavy metals from soil offer alternative and less expensive method to strip heavy metals directly from the soil. Plants have constitutive and adaptive mechanisms for accumulation or tolerating high contaminant concentrations in their rhizospheres. Microorganisms can mobilize and remove metals/metalloids through diverse variety of mechanisms (Gadd, 2004).

The main objective of in situ bioremediation trial in plots with plants was to study the effect of plant-microbe interaction on enhanced transformation of selenium oxyanions. *Allium* plants were chosen because it is an established selenium hyperaccumulator and exhibit definite anti-oxidant properties. Yadav et al. (2007) reported that *Allium cepa* can accumulate selenium in proportion to available selenate concentration in soil. The authors envisaged that allium species could be introduced as commercially viable crops in seleniferous region for the selenium mobilization vis-à-vis fortification. Comparison of mobilization rates of both selenium oxyanions in plots with plants indicated complete transformation after 120 days and the population of all the four cultures was in the range of 5.87 – 6.71 (log value), thus indicating enhanced plant-

microbe interaction, i.e. plots showing higher transformation of Se oxyanions also showed more microbial presence. Population dynamics of inoculated organisms in plots showed that survival of bacterial organism was always better in plots with plants as compared to the plots without plants, leading to enhanced plant-microbe interaction in the rhizospheric soil and so, enhanced transformation of Se oxyanions in soils with *Allium* plants. Factors leading to certain decline in the microbial population in the field studies over a period of time could be due to nutrition status of the soil, moisture levels, microbial antagonism etc. However, actively growing roots of the plant release organic compounds, which support growth of the microbial community in the rhizosphere and alter the microbial community creating a distinct community structure in bulk soil with enhanced population density of inoculated strains (Kent and Triplett, 2002; Bowen and Rovira, 1991; Bolton et al., 1991; Lynch and Whipps, 1990).

The accumulation of selenium in plants during the growth period was higher in plants grown in absence of consortia and fresh weight also showed a declining trend in absence of consortia. The plant fresh weight of plants grown in selenate supplemented soils was significantly lesser when compared to the ones grown in bioaugumented soils at the same concentration. Further, exposure of plants to higher concentrations of Se as selenate resulted in mortality when cultivated in absence of rhizosphere inoculum where as the plants grew in the presence of inoculum, which confirms the protective and stimulating role of rhizosphere inoculation of Se tolerant microorganisms.

Similar findings were reported in terms of inoculation effects of plant growth promoting rhizobactera *Methylobacterium oryzae* strain CMBM20 and *Burkholderia sp*. strain CMBM40, isolated from rice (*Oryza sativa*) tissues, on tomato (*Solanum tuberosum*), grown in nickel and cadmium-treated soil (Madhaiyan et al., 2007). These bacterial strains significantly reduced the toxicity of both metals in tomato and promoted the plant growth under gnotobiotic and pot culture conditions. Phytovolatilisation of selenium is carried out by both plants and microorganisms and involves reduction of selenate to selenite (and/or selenide) (Terry and Zayed 1998; Frankenberger and Karlson 1994).

Microbially mediated reduction and oxidation processes can also modify the solubility of metals and metalloids. Microbial reduction results in mobilisation of iron and manganese, but can immobilise elements such as uranium, chromium (Gadd 2004),

and selenium (Di Gregorio et al., 2006). The concentration of accumulated selenium was lower in selenite treated plants. Se is metabolized in plants by the S assimilation pathway and its distribution and assimilation depend on the form and concentration of selenium form present in environment (Elles and Salt, 2003; Terry et al., 2000). Thus, most of Se taken up as selenate remains unchanged while plants supplied with selenite accumulate organic forms of selenium (Kahakachchi et al., 2004; de Souza et al., 1998; Zayed et al., 1998). The reduced inorganic selenium is assimilated into organic forms such as selenomethionine and selenocysteine which are then methylated to nonvolatile dimethylselenium compounds and finally converted to volatile dimethylselenide (DMSe) and dimethyldiselenide (DMDSe) (Zhang and Frankenberger 2000). Rhizosphere bacteria seem to be also responsible for enhancing plant-mediated selenium volatilisation (De Souza et al., 1999a).

The choice of examining *Allium* was based on the confirmatory reports showing therapeutic applications of organoselenium compounds in *Allicaceous* species. Ip et al. (2000) demonstrated that organoselenium compounds in Se-enriched garlic and onion are more active and effective in suppression of mammary tumors in cancer treated mice. Cai et al. (1995) also reported that enhanced levels of seleno-amino acids (selenocysteine) were responsible for the reduction of mammary tumor growth in carcinogen treated mice. Furthermore, there are potentially scores of Se analogs of specific organosulphur compounds in *Allium cepa* that prompt investigation for phytopharmaceutical activity.

5.3 Evaluation of anti-oxidant and related properties in plants grown in seleniferous soils

5.3.1 Pot Experiments

The present study attempts to explore the possibilities of growing the Se fortified crops as commercially viable bio-fortified crops. This work presents the study carried out to examine the anti-oxidant activity of over ground (leaves) and underground (bulb and root) tissues in Se enriched soils. Selenate treatment induced increased concentration of MDA in the plants. The higher values of MDA were reported in the underground tissues

than in leaves of control plants. Across all tissues of plants exposed to different concentrations of selenium oxyanions, similar observations were found with highest MDA concentrations in plants supplied with selenate followed by selenite and control leaves, thus supporting the fact that selenate treated plants showed more anti-oxidant activity followed by selenite exposed plants.

The anti-oxidative and growth promoting effect of selenium on lettuce was extensively studied by Xue et al. (2001). The authors hypothesized that senescence, an integral part of plant development, may coincide with the production of free oxygen radicals and be regulated by a variety of environmental and autonomous factors. In young and senescing plants, the antioxidative effect of Se was associated with the increased activity of glutathione peroxidase (GSH-Px). In the senescing plants, the added Se strengthened the antioxidative capacity also by preventing the reduction of tocopherol concentration and by enhancing superoxide dismutase (SOD) activity. Similarly, the pro- and anti-oxidant activity of selenium was examined by Hartikeinen et al. (2000) in ryegrass (*Lolium perenne*) by cultivating at very low to very high Se additions, and the changes in the activity of GSH-Px were monitored along with other associated parameters, as a function of Se quantity added. In order to study the biological role in higher plants, ryegrass (*Lolium perenne*) was cultivated in a soil without Se or amended with increasing dosages of H_2SeO_4 (0.1, 1.0, 10.0 and 30.0 mg Se kg^{-1}). Selenium was observed to exerted dual effects - At low concentrations it acted as an antioxidant, inhibiting lipid peroxidation, whereas at higher concentrations, it was a pro-oxidant, enhancing the accumulation of lipid peroxidation products. The antioxidative effect was associated with an increase in glutathione peroxidase (GSH-Px) activity. In the second yield, the diminished lipid peroxidation due to a proper Se addition coincided with promoted plant growth. The oxidative stress found at the Se addition level $\leq$ 10 mg kg^{-1} resulted in drastic yield losses. This result indicates that the toxicity of Se can be attributed, in addition to metabolic disturbances, to its pro-oxidative effects at higher concentrations of selenium.

5.3.2 Field Investigations

The GSH-Px and TBARS levels in control plants increased up to 80 days followed by decrease by 120 days similar to the observations reported by Hartikeinen et

al. (2000). In the present study too, amongst the plants exposed to Se oxyanions in soil, analysis of variance between levels of Se, TBARS and GSH-Px indicated variation in mean levels of these parameters at all levels of Se supplementation. Rotruck et al. (1973) identified Se to be an essential component of the enzyme glutathione peroxidase (GSH-Px). The high Se dosage boosted the GSH-Px activity to neutralize the Se induced oxidative stress recorded as a distinct increase in TBARS values. Such variations during the growth were also reported by Hartikeinen et al. (2000) in rye-grass grown in soils supplemented with 10 mg kg^{-1} as selenite. Se acts as an antioxidant at lower levels as manifested by decrease in TBARS and increase in GSH-Px levels in ryegrass upto addition of 1.0 mg kg^{-1} and pro-oxidant at higher concentrations. (Se-10 mg kg^{-1}). Similar observations were reported for TBARS levels in *Lettuce* plants supplemented with 1 mg kg^{-1} of selenite (Xue et al., 2001).

In the present study, GSH-Px increased in plants exposed at the 7.5 mg kg^{-1} selenite which is presumed to counteract the Se-evoked oxidative stress during growth period. This notion was also supported by the positive response shown by GSH-Px enzyme to selenite levels up to 10 mg L^{-1} in case of bitter gourd seeds (Chen and Sung, 2001). In general, the enhanced antioxidant capacity of Se-treated plants is associated with improved activity of glutathione peroxidase (GSH-Px) to inhibit oxidative damage to cells (Seppanen et al., 2003; Pennanen et al., 2002; Xue et al., 2001). The increase in MDA concentrations in case of selenate augmented plants across all concentrations was similar to previous findings who reported increase in this parameter on increasing the external application of selenium (Rios et al., 2008; Xue et al., 2001; Hartikeinen et al., 2000).

The concentration of malondialdehyde (MDA), an oxidation product of polyunsaturated fatty acids, in the plants can be taken to indicate the level of oxidative damage caused by selenium in growth media. Inverse relationship was observed between the TBARS and GSH-Px levels of control and experimental plants indicating the influence of Se exposure on the modulation of both pro and anti-oxidant activity. Studies carried out on protective effects of Se to other toxic elements in *Brassica oleracea* showed that 10 days after the exposition of the plants to Se, MDA in the roots increased to about 50%. After 30 days, in the plants supplied only with selenium, the level of MDA was the lowest (Pedrero et al., 2008). Luo reported that the GSH-Px activity in the leaves

of cucumber and the yield were significantly increased, whereas the content of malondialdehyde (MDA) decreased, after the soil was fertilized with selenium at 0.026 mg kg^{-1} (Luo et al., 2000).

The positive correlation between the Se concentrations and antioxidant activities reported here for onion plantelets also agrees with the earlier reports on broccoli (Pedrero et al., 2008), lettuce and ryegrass (*Lolium perenne*) (Cartes et al., 2006; Hartikeinen et al., 2000) and germinating barley seeds (Huang et al., 1994). Smrkolj et al. (2006) observed that electron transport activity was highest in the leaves of young plants of *Pisum sativum* with the highest concentration of Se (591 ng g^{-1}) and attributed it to an increased GSH-Px activity in plant cell organelles such as mitochondria. Other reports (Xue and Hartikainen, 2000; Hartikainen et al., 2000; Xue et al., 2001) have shown similar observations that Se exposure increased GSH-Px activity. Se accumulation can improve the biochemical activity in *S. platensis* by enhancing its production of biomass, photosynthetic pigments, anti-oxidant activity and protein concentrations (Chen et al. 2006 b; Li et al. 2003; Zheng et al. 2003 a, b; Huang et al. 2002, 2001) but is also found to have toxic effects on the growth of this blue green algae depending on Se levels (Chen et al. 2005, 2006a, b). Chen observed that exposure of *Spirulina platensis* to higher Se concentrations (≥175 mg L^{-1}) led to higher Se accumulation induced lipid peroxidation (LPO) with simultaneous increase in activities of GSH-Px and SOD, along with other anti-oxidant enzymes (Chen et al., 2008). However, the authors indicated that increase in activities of the antioxidant enzymes were not sufficient to protect cell membranes against Se stress. Hence, TBARS levels and corresponding GSH-Px were observed to be reliable and stable indicators for monitoring the modulations in pro and anti-oxidant activity induced by selenium accumulation and assimilation by the plants.

Conclusions

In summary, the work aimed at examining the following objectives

- Characterize seleniferous soils and isolation of selenium tolerant bacteria
- Determine the uptake of soil selenium by *Allium* species with rhizospheric bacteria
- Examine the anti-oxidant and related properties in plants grown in seleniferous soils.

The salient findings in the study are as follows:

A. Characterize seleniferous soils and isolation of Se tolerant bacteria

1. The soil samples 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): 110-220 µS, cation exchange capacity: 499.65- 833.25, available P: 3.59-8.16 mg L^{-1} and potassium levels were found to be 33.3-44.6 ppm respectively.

2. The soil samples obtained from two sites from selenifereous region contain 6.5 and 2.7 mg kg^{-1} of selenium respectively. The selenium levels observed in the crop products (15-670 mg kg^{-1}) in this region are significantly higher than the global data on Se in food crops.

3. The bacterial isolates (SNTP-1, NS-2, NS-3 and NS-4) were tested for growth in the presence of different concentrations (5 mg L^{-1}, 15 mg L^{-1} and 25 mg L^{-1}) of selenate (Na_2SeO_4) and selenite (Na_2SeO_3). Similar growth trend was observed in all the test strains in the presence of either of the selenium oxyanions viz., selenate and selenite over a period of 12 h as determined by total viable counts (colony forming units).

4. Morphological and gram characteristics of isolates revealed that isolates were gram positive rods. All isolates showed characteristics of *Bacillus* species based on results obtained from biochemical tests.

5. Antibiotic profile of isolates revealed that all isolates were resistant to carbenilcillin (300 µg) except NS-3 which showed resistance upto 40 mcg.

6. 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

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

8. The molecular identification based on 16S rDNA and FAME analysis lead to characterization of isolates. All isolates belong to *Bacillus* species based on mentioned identification systems.

9. ERIC-PCR based identification revealed that four isolates were found to have different genotypic fingerprints.

10. *Bacilli* isolate NS-3, a facultative anaerobe had the potential to reduce and transform toxic Se (IV) anions to elemental selenium. The red-amorphous α-Se was observed to be spherical in nature which when subjected to abiotic experimental conditions was observed to yield *t*-Se which was pure crystalline Se.

11. Complete transformation of selenium oxyanions by microbiota was observed in soils supplemented with 2.5 and 5 mg kg^{-1} selenite oxyanion across 120 days followed by 80 % in soils supplemented with 7.5 mg kg^{-1} of Se as selenite which was higher then selenate supplemented soils. Antibiotic based survival rate revealed highest survival in 2.5 mg kg^{-1} selenite and lowest in 7.0 mg kg^{-1} selenite augmented soils after 120 days. In terms of survival rate, based on molecular fingerprinting, maximum survival rate was observed in 2.5 mg kg^{-1} selenite supplemented soils and lowest survival in soils supplemented with 5 mg kg^{-1} selenate after 120 days.

B. Determine the uptake of soil selenium by *Allium* species with rhizospheric bacteria

1. In study conducted in pots, the observations indicated that accumulation of the total selenium did not follow any trend among the onion tissues and total selenium concentration in leaf and bulb significantly decreased after 90 days of exposure. The average uptake of selenium was higher in bulb, followed by leaf and root.
2. In field studies, carried out in presence of bacteria, the accumulation of selenate was maximum accompanied by growth inhibition in plants. Maximum accumulation

was noted in the plants cultivated in soil supplemented with 7.5 mg $kg^{-1}$of selenate. Selenite had positive impact on growth of plants at lower concentration of 2.5 mg kg^{-1}. Similar trend was observed on exposure to higher levels of soil Se supplementation where initial retardation during early stage followed by normal growth at later stages. The consortium was having positive impact on growth of plants accompanied by lesser accumulation of Selenium in plants.

3. In field studies carried out without bacteria accumulation of selenium was prominent across all treatments but did not follow any specific trend and accumulation was higher in selenate treated plants. Complete mortality was observed in 5.0 mg kg^{-1} selenate exposed plants.
4. Survival rate which was recorded, based on the antibiotic resistance of the consortium, showed an increase in the population after 120 days of incubation in selenite augmented soils while in selenate treated soils that had plants, there was initial increase in colony forming units after 80 days followed by declining trend in number after 120 days. Highest survival was observed in soil supplemented with 5.0 mg kg^{-1} Se.
5. On the basis of molecular fingerprinting better survival of the isolates was noted in 2.5 mg kg^{-1} when compared with observations in soil with 7.5 mg kg^{-1} selenate after 120 days. REP-PCR based enumeration revealed maximum survival rate of 60% in 2.5 mg kg^{-1} selenate supplemented soils followed by 5.0, 2.5 and 7.5 mg kg^{-1} selenite supplemented soils with survival rate of 56, 50 and 46% respectively. The lowest survival rate obtained from plate counting as well as DNA fingerprinting was for 7.5 mg kg^{-1} selenate. Complete mobilization of Se oxyanions was noted in plants augmented with consortium across all treatments at the end of experiment (120 days).

C. Determination of anti-oxidant and related properties in plants grown in seleniferous soils

1. In pot experiments, the anti-oxidant activity (TBARS assay) was more prominent in the case of exposure to selenate followed by selenite despite the retarded plant growth in the presence of selenium.

2. In field studies, at lower concentration of 2.5 mg kg^{-1}, selenite application induced higher values in case of TBARS when compared to selenate while GSH-Px activity was observed to be higher in plants on exposure to selenate. At higher concentrations (5.0 and 7.5 mg kg^{-1}), TBARS levels were observably higher and selenate induced higher concentration of TBARS then selenite. In terms of GSH-Px, maximum activity of GSH-Px enzyme was noted in 7.5 mg kg^{-1} of selenite supplemented plants. The changes observed in antioxidant activities determined in terms of TBARS and GSH-Px across all treatments was dependent on accumulation of selenium in plants.

In conclusion, the work carried out in the study could primarily:

(a) identify and characterize the selenium tolerant bacteria isolated from seleniferous soils and determine their role in mobilizing selenium from soils;

(b) examine the Se-uptake potential of *Allium cepa* cultivated in Se-enriched soils and the influence of Se tolerant rhizosphere bacteria on uptake; and

(c) examine the pro/anti oxidant properties induced by the uptake of Se in the plants

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Media Composition

Minimal Salt Medium

Ingredients	Quantity (g l^{-1})
K_2HPO_4	2.5
KH_2PO_4	2.5
$(NH_4)2HPO_4$	1.0
$MgSO_4.7H_2O$	0.2
$FeSO_4.7H_2O$	0.01
$MnSO_4.7H_2O$	.007
Sucrose	10.0

Final pH at 25º C (7)
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min

Tryptic Soy agar

Ingredients	Quantity (g l^{-1})
Tryptone	17.0
Soytone-Enzymic digest of soyabean meal	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5
Agar	15

Final pH at 25º C (7.3±0.2)
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Tryptic Soy Broth

Ingredients	Quantity (g l^{-1})
Tryptone	17.0
Soytone-Enzymic digest of soyabean meal	3.0
Sodium chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5

Final pH at 25º C (7.3±0.2)
Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Luria-Bertani (LB) Medium

Ingredients	Quantity (g/L)
Nacl	10
Beef extract	5
Tryptone	10
Agar	10

pH 7 adjusted with 5N NaOH. Sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min. (Added filter sterilized ampicillin 50 µg/ml to prepare LB - Ampicillin plates)

Buffers and solutions

Modified Universal buffer (5X)

Tris (hydroxyl methyl) amino methane	3.025 g
Maleic acid	2.90 g
Citric acid	3.50 g
Boric acid	1.57 g
NaOH (1N)	122 ml
Water	up to 250 ml
pH	5.5

Phosphate buffer

Stock solution A

2 M monobasic sodium phosphate, monohydrate (276 g/L)

Stock solution B

2 M dibasic sodium phosphate (284 g/L).

Mixing an appropriate volume (ml) of A and B as shown in the table below and diluting to a total volume of 200 ml, a 1 M phosphate buffer of the required pH at room temperature.

A	B	pH
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5

TBE Buffer (10x)

Tris-HCl 0.09 M (pH 8)
Boric acid 0.9 M
EDTA 0.02 M (pH 8)

Plasmid extraction solution I (10X)

Tris-HCl 25 mM (pH 8.0)
Glucose 50 mM
Na2EDTA 10mM

Plasmid extraction solution II

NaOH 5M
SDS 10%

Plasmid extraction solution III

5.0 M K-acetate (pH 4.5)

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

Ligation reaction of amplicon in pGEM-Teasy

Plasmid pGEM-Teasy (50ng/µl) 1µl
Insert (75ng/µl) 1µl
Buffer (2X) 5µl
T4 Ligase 1µl
H_2O 2µl

Primers

M13 forward primer
5'-GTAAAACGACGGCCAGT-3'

M13 reverse primer
5'-CAGGAAACAGCTATGAC-3'

T7 primer
5'-TAATACGACTCACTATAGGG-3'

SP6 primer
5'-ATTTAGGTGACACTATAG-3'

Rep forward primer
5’-IIIICGICGICATCIGGC -3’

Rep reverse primer
5’-ICGICTTATCIGGCCTAC - 3’

16S rDNA Forward primer
5’-AGAGTTTGATCCTGGCTCAG-3’

16S rDNA reverse primer
5’-ACGGGCGGTGTGTTC-3’

16S rDNA forward primer (V3 region)
5’-GACTCCTACGGGAGGCAGCAG -3’

16S rDNA reverse primer (V3 region)
5’-ATTACCGCGGCTGCTGG -3’

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