ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING BACTERIA FROM SELENIFEROUS SOIL

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submitted in fulfilment of the requirement
for the award of degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

Under the guidance of

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CERTIFICATE

Certified that the thesis "**ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING BACTERIA FROM SELENIFEROUS SOIL**" which is submitted by Miss Akansha Chadha, in the fulfilment of the requirement for the award of the degree of mater of science in biotechnology in the department of biotechnology (DBT), Thapar Institute of Engineering and Technology, Patiala is a record of the candidate's own independent and original research work carried out by her under our guidance and supervision. The matter embodied in this thesis has not been submitted in part or full to any other Institute or University for the award of any degree.

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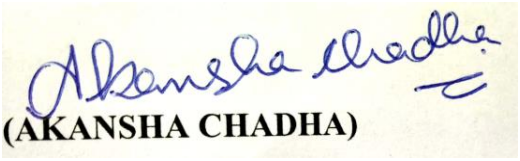
DECLARATION

I hereby declare that the work which is being presented in the thesis "**Isolation and characterization of plant growth promoting bacteria from seleniferous soil"** submitted by me for the award of the degree of Master of Science in the department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is true and original record of my own independent and original research work carried out under the supervision of **Prof. Dr. M. Sudhakara Reddy**, Professor, Department of Biotechnology. The matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any degree in India or Abroad.



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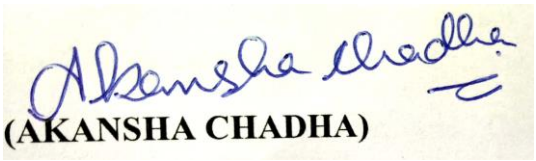

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ABSTRACT

Due to the increased human activities and a number of natural conditions, the environment is getting contaminated. One such factor is the formation of the seleniferous soil which plays a vital role in the contamination of an ecosystem. Patches of seleniferous soil have been found in north-eastern parts of Punjab. The underground water of this region is highly rich in seleniferous materials as it gets transported from Siwalik hills through small rivulets. Not only humans, the livestock and plants are also affected by the increased concentration of selenium. There are a number of microorganisms which are not only able to tolerate its high concentration but also promote the growth of the plants in that soil by releasing various metabolites. The objective of the present work was to isolate the selenium-tolerant bacteria and to determine its role in plant growth promotion by using selenium-contaminated soil. The present work also demonstrates the amount of selenium accumulated by the isolated bacteria as well as in plant tissues which were treated with it.

In the present study, two Se-tolerant bacteria were isolated and were further screened for plant growth promotion. The two strains, B_{49} and B_{71} identified as *Leclercia adecarboxylata* and *Cedecea neteri* respectively have been able to tolerate upto 300 mM of sodium selenite and show IAA production, phosphate solubilization and ammonia production. They were able to produce 46.89 and 69.91µg /ml of IAA respectively. The phosphate solubilization index shown by them is 1.26 and 1.39 respectively.

These isolates have also been found to transform one form of selenium into another. Two months nursery trials were also conducted to check selenium tolerance and plant growth promotion *in vivo.* A remarkable increase in the plant height, root length, shoot length, shoot biomass and root biomass as compared to control plants with no inoculum were observed.

Therefore, these bacterial strains can be potentially used in the soils contaminated with selenium for plant growth promotion and bioaccumulation of selenium by plants for increased tolerance and further practices such as biofortification of food grains.

TABLE OF CONTENTS

ABBREVIATIONS

Abbreviation	Word (s)
CFU	Colony Forming Unit
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic acid
IPTG	Isopropyl-thiogalactoside
MQ	Mili Q
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
X-Gal	5-Bromo-4-chloro3-indolyl-D-galactoside
mg	Milligram
µg	Microgram
µl	Microlitre

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Chapter-1

Introduction

There are a number of natural as well as anthropogenic activities such as disposal of fly ash, mining activities, burning of fossil fuels, weathering, volcanic activities which have been contaminating the environment as well as affecting the redistribution of various elements (Gailer 2002). As a result of which there are a number of areas which are rich in these elements while many are deficient of these. One such example of it is the occurrence of seleniferous soil which is not uniformly distributed across the globe. Selenium is a metalloid which belongs to the chalcogen family i.e. it is a member of Group 16 (VIA). It is an essential trace element which is required for the proper functioning of our body. It may be present in an inorganic form: (selenite, selenate or selenide) or in organic form such as selenomethionine, selenocysteine.

A naturally occurring organic form of Se i.e., Selenocysteine is thought to be the most important chemopreventive compound. It also helps in increasing the activity of various enzymes like glutathione peroxidase, glutathione S transferase and thioredoxin reductase (Zheng et al. 2008). In humans, Se plays a vital role in various metabolic pathways like thyroid hormone metabolism, immune function, and antioxidant defense systems.

The soils containing 5 mg Se kg^{-1} are considered as seleniferous soils. Patches of seleniferous soils have been found in northeastern parts of Punjab (Barwa, Simbly, Jainpur, Panam) which almost covers about 100 ha of area. Some parts of Haryana, Assam, Meghalaya and West Bengal are also rich in selenium (Dey et al. 1999). Interestingly, the amount of selenium in this region ranges from 2.12 ± 1.13 mg kg^{-1} on the surface and 1.16-0.51 mg kg^{-1} on subsurface which is almost 5 times higher than that in the case of non-seleniferous soil (Dhillon and Dhillon 1999). The reason for this high level of Se in this region is said to be because of underground water which is highly rich in seleniferous materials and is transported from the Siwalik hills through small rivulets (Dhillon and Dhillon 2003).

If the consumption of Se is less than 0.05-0.1 mg Se kg^{-1}, it may lead to its deficiency while the consumption of more than 2-5 mg Se kg^{-1} may lead to its toxicity (Gissel-Nielson et al 1984). The intoxication symptoms includes wandering of animals in circles, tongue paralysis, weakening of legs, abdominal pain, impairment of vision or a varying degree of blindness, excessive salivation and ultimately death. Deficiency of selenium may cause Keshan disease

which is characterized by cardiomyopathy along with multiple foci of necrosis. It mainly affects young children and women. Although selenium is essential in small amounts but its higher amounts lead to toxicity (Dumont et al. 1994). Therefore, a balance has to be established to avoid such harmful effects.

Selenium is required in very small amounts for plant growth, its high concentration in crops and fodder may affect humans and animals. For animals, the minimum requirement of Se is about 0.05-0.10µg Se g^{-1} and if the consumption is below this value then it may lead to deficiency. Some symptoms of Se toxicity involve snow-white chlorosis with pink coloration on the lower sides of leaves and sheath. It has been observed that wheat plants which shows the varied amount of toxicity contains Se ranging from 107.5-262.4 mg kg^{-1} and 29.2-66.5 mg kg^{-1} in shoots and roots respectively (Dhillon and Dhillon 2003).

Se toxicity is very common in the case of animals who consume fodder grown on seleniferous soil. However, Se intoxication can be classified as acute or chronic poisoning. According to a research, if Selenium is consumed <40 µg per day then it may cause deficiency while if it is consumed >400 µg per day then it proves to be toxic (Zheng et al. 2014). Because of the different chemical composition of these types of soil, it is evident to find out the different microbes with naturally adapted mechanisms (Ghosh et al. 2007).

Microorganisms are present everywhere and therefore they are also present in these selenium contaminated areas. Environmentally, these are very important as they are metal tolerant in nature and can be helpful in various aspects (Ghosh et al. 2007). It has been observed that there are various bacteria which are capable of transforming one oxidation state of metal into another (from toxic to less toxic form) (Zheng et al. 2014). Bacterial strains effectively reduce Se(IV) to Se(0) which is evident from the change in color of the reduction medium to orange–red/ red due to the generation of an allotropic form of elemental selenium during the exponential growth phase (Mishra et al. 2011).

Microbes also play a very important role in the mobility and bioavailability of selenium. They have an impact on the bioavailability by changing the oxidation state of an element which directly influences the solubility of the compounds (Fernández-Martínez and Charlet 2009). Other mechanisms involved in the biotransformation are reduction, oxidation, alkylation,

dealkylation, methylation, and demethylation. Different pathways for the reduction of selenium oxyanion into Se(0) or Se(-II) have been reported. In energy metabolism, bacteria can use selenate and selenite as terminal electron acceptors (dissimilatory reduction). They can also reduce and assimilate Se in the organic compounds (assimilatory reduction) (Fernández-Martínez and Charlet 2009).

Few bacteria can also replace selenium with sulfur present in the proteins or could also form volatile methylated compound (Darcheville et al 2008). Microbes can alter selenium behavior by changing geochemical characteristics like $E_{H,}$ pH and metal speciation. It has been reported that microbes may cause retention of Se in the soil which may result in the decrease of Se mobility in the soil (Darcheville et al 2008).

Many bacteria like *Klebsiella, Arthrobacteria, Pseudomonas, Enterobacter* etc., showed plant growth promoting activities (Labandera and Gonzalez 2000), which are referred as PGPR (Plant Growth Promoting Rhizobacteria). PGPR can have direct as well as indirect effects on the plant growth promotion (Glick 2003). Direct effects include the production of plant hormones like gibberellins, auxin etc. while the indirect one involves the prevention of bacterial, fungal and nematode growth by the production of HCN, ammonia, siderophore, antibiotics, etc.

Microbes have the ability to reduce or oxidize a number of selenium compounds (Levine 1924). They include *Arthrobacter, Aeromonas, Bacillus* (Burton et al. 1987) and many more. In 1981, Sarathchandra et.al reported that microbes can reduce elemental selenium to selenide and could also oxidize elemental selenium to selenate and selenite. In 1982, Doran described that microbes are capable of transforming in soil, sewage, sediment and in water.

OBJECTIVES:

To study the role of bacterial isolates in alleviating the adverse effects of selenium in plants

Work Elements:

- Isolation of selenium tolerant bacteria from seleniferous soil
- Effect of bacterial isolates on plant growth promotion

Chapter- 2
Review of Literature

There are many human activities which have been contaminating the environment for ages as a result of which the ecological balance of an ecosystem is getting disturbed (Ghosh et al. 2007). One such activity is agriculture, which by the use of various chemical fertilizers and pesticides is causing a huge damage to the environment (Vejan et al .2016). Also, the agricultural discharge is one of the primary sources of different toxic forms of metals and non-metals (Ghosh et al. 2007). These metals include lead, nickel, arsenic, lead, selenium, and iron. This leads to the uneven distribution of these metals. Let's take an example of selenium which is present unevenly across the globe. Moreover, its distribution is site-specific (Wang and Gao 2001). It is because of the unequal distribution only that a stretch of merely 20 km (Fordyce 2007) between the seleniferous soils and selenium-deficient soils in countries like China and Brazil results in selenium poisoning and selenium deficiency respectively (Lenz and Lens 2009).

2.1 Selenium: an "essential poison"

Discovered in 1818, Se is an essential trace element of biological importance. It resembles sulfur in many of the chemical properties (Trofast 2011).

Selenium is a crucial constituent of vital metabolic pathways which includes thyroid hormone metabolism, immune function, and antioxidant defense system. Selenium is also integrated at the active site of many selenoproteins in the form of selenocysteine. Some examples of these selenoproteins are glutathione peroxidase, thioredoxin reductase and iodothyronine deiodinase (Brown and Arthur 2001).

According to the recommendations given by WHO, 50-55µg/day of Se is essential for the humans (Gupta and Gupta 2017). According to SCF (Scientific Committee of Food) of European Commission, the daily intake of Se by adults should also be 55 µg/day. In humans, selenium deficiency may cause damage to the bone metabolism, growth abnormalities, and malfunctioning in the functioning of thyroid (Reeves and Hoffman 2009).

In 1930's, the rural district of North-West China (known as Keshan) was affected with cardiomyopathy along with multiple foci of necrosis. Later, this disease was called as "Keshan disease" which occurs due to the deficiency of selenium i.e. when the consumption

of Se is less than 12 µg/day (Zhao et al. 2013). It mostly affects women of childbearing age and young children. There are several other diseases as well which are associated with selenium deficiency. Few of them are Kashin-Beck disease (chronic and endemic form of osteochondropathy) and myxedematous endemic cretinism which occurs due to selenium and iodine deficiency (Dumont et al. 1994).

According to UK department of health, the maximum unassailable selenium intake should not be more than 450 µg per day. But in seleniferous regions, the total intake of selenium ranges from 300-724 µg/day (Foster and Sumar 1997). Moreover, the selenium content in the crops of seleniferous regions of Punjab ranges from 14-670 mg kg^{-1}. The corresponding selenium concentration in this region by both men and women is 4.4 mg kg^{-1} in nails and 2.5 mg kg^{-1} in hairs which are higher than the normal values and hence results in selenosis (Hira et al. 2004).

Selenium toxicity has been reported in humans, plants, and livestock. Few symptoms of selenium toxicity (selenosis) in humans include changes in nails, hair loss, garlic-like odor (Foster and Sumar 1997), nausea, vomiting and diarrhea (Lenz and Lens 2009).

Although the exact mechanism for the selenium toxicity is not reported yet it is suspected that it triggers the formation of free radicals which in turn damages DNA (Letavayova et al 2006).

Thus, Selenium, like the moon, has two faces as it is both toxic and an essential micronutrient.

2.2 Selenium cycle

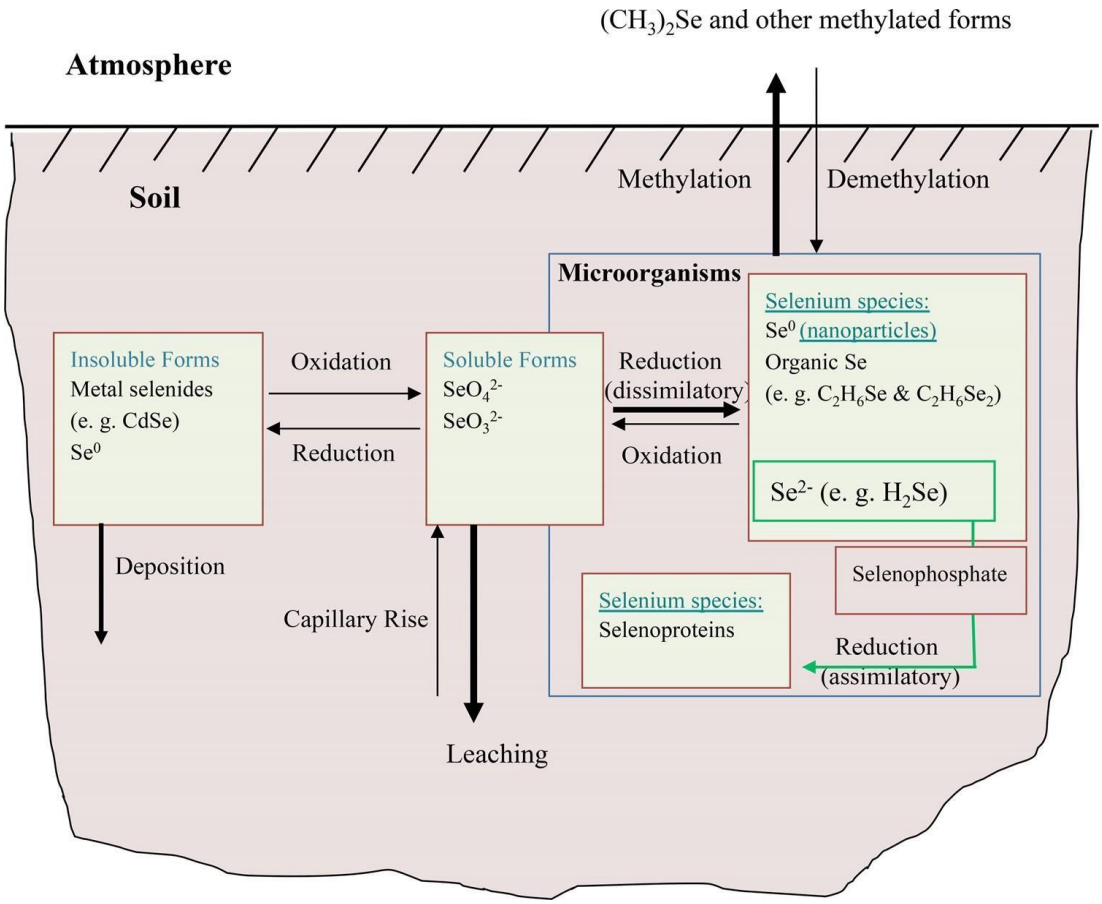
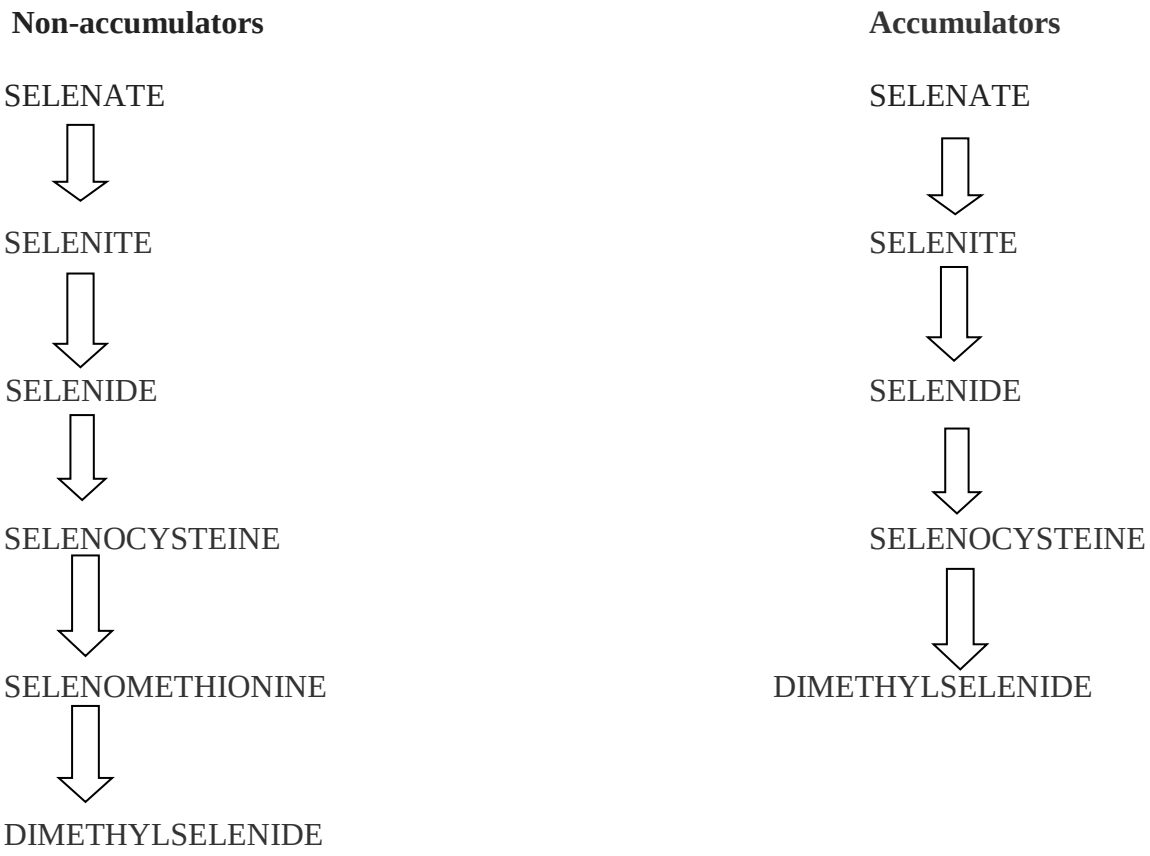


Fig 2.1 Se cycle in soil and the effect of biotransformation carried out by microbes (Eswayah et al. 2016)

2.3 Role of selenium in plants

Plants can be classified as hyperaccumulators, accumulators or non-accumulators depending upon the concentration of selenium present in their tissues. Non-accumulators are those which contain less than 25 µg Se g^{-1} and these include forage, crop plants and grasses (Brown and Shrift 1982). Accumulators can further be divided into primary and secondary accumulators. Primary Se accumulators generally contain 1000 mg kg^{-1} of selenium while secondary accumulators like Grindelia, Atriplex, Castilleja etc. contain about 50-100 mg kg^{-1} of Se. Selenium hyperaccumulators like Stanleya pinnata and Astragalus bisculatus could accumulate 1000-15000 mg kg^{-1} of Se in them (Saha et al. 2017). In the maximum number of cases, 70% of selenite and 85% of selenate is found in the aerial tissues of plants. Some plants like Aster and Atriplex which contains an average amount of selenium are toxic to animals.

Plants take selenate ten times more as compared to selenite (Mehdi et al. 2013). Further, these compounds are metabolized in chloroplast via same metabolic pathway as that of sulfur. If selenate is present then it is first converted into selenite. Selenate is firstly activated by ATP sulfurylase-adenosine 5'-phosphoselenate (APSE) which is further reduced to selenite by adenosine 5'-phosphosulfate reductase which by reduction with glutathione gets converted into selenide. Different plants have different end products of Se metabolism depending upon the accumulating capacity of the plant. For example, in case of non-accumulating plants, five steps are involved in the formation of dimethylselenide (DMSE) while in Se accumulating plants, dimethylselenide is formed as an end product (Saha et al. 2017).



2.3.1 Selenosis in plants

Although there are many forms of Se present in the soil but only inorganic anions like selenate (SeO_4^-) and selenite (SeO_3^-) are proven to be toxic in nature. This toxicity could be due to the following reasons:

- Roots can absorb both selenate as well as selenite as a result of which it may get translocated to other parts of the plant
- The metabolism of both the anions takes place within the cell
- The organic selenium metabolites may act as analogs to sulfur and therefore may interfere with the cellular biochemical reactions (Brown and Shrift 1982).

Some symptoms of Se toxicity involve snow-white chlorosis with pink coloration on the lower sides of leaves and sheath. It has been observed that wheat plants which shows the varied amount of toxicity contains Se ranging from 107.5-262.4 mg kg^{-1} and 29.2-66.5 mg kg^{-1} in shoots and roots respectively.

2.4 Selenium in soil

Table 2.1 Different forms of selenium in soil **(**Chabroullet (2007); Haygarth (1994); Losi and Frankenberger (1997)

SPECIES	CHEMICAL FORMULA	CHARACTERISTICS
Inorganic species		
Selenate [Se(VI)]	$H_2SeO_4^0$, $HSeO^{4-}$, SeO_4^{2-}	Most soluble form and exists in alkaline and oxidized environment
Selenite, Selenium dioxide [Se(IV)]	$H_2SeO_3^0$, $HSeO_3$ SeO_3^{2-}, SeO_2	It can be reduced to elemental selenium and mostly exists in an environment with a neutral pH
Elemental selenium [Se(0)]	Se^0	Insoluble, non-toxic and impervious to oxidation and reduction
Selenide [Se(-II)]	H_2Se	Occurs in acidic, reducing and organic-rich environment
Organic species		
Selenocysteine (SeC)	$HSeCH_2CHNH_2COOH$	Main selenium species in organic tissues
Selenomethionine (SeM)	$CH_2Se(CH_2)_2CHNH_2COOH$	Predominant species in plants

2.5 Selenium problem in soil

2.5.1 Seleniferous soils

Selenium is present in rocks, soil, water, and air. But rocks form the chief source of selenium among them (Wang and Gao 2001). It mainly exists in four oxidation states: selenide (Se^{2-}), elemental selenium (Se^{0}), selenite (SeO_4^{2-}) and selenate (SeO_4^{2-}) (McNeal and Balistrieri 1989).

In soils, selenium concentration is inconsistent and ranges from >0.1 – 8000 mg/kg throughout the world (Ure and Berrow 1982). Soils accommodating 5 mg Se kg^{-1} or above are known as seleniferous soils while the soils containing less than 5 mg Se kg^{-1} are termed as normal or non-seleniferous soils (Dhillon and Dhillon 1991). They are also formed just like the normal soils i.e. due to the active and passive factors of soil formation (Dhillon and Dhillon 2003).

Surprisingly, soils containing even 0.10 mg Se kg^{-1} could bear vegetation with toxic levels of selenium. The large variation of the occurrence of selenium could be due to the different soil properties which are responsible for selenium absorption. According to the three level classification of soil given by National Research Council, the seleniferous soils are mostly alkaline in nature and contain free calcium carbonate. However, topography and leaching plays an important role in the formation of seleniferous soils. Areas from which leaching takes place forms the selenium-deficient regions while the areas in which leachate gets deposited becomes selenium-enriched regions (Dhillon and Dhillon 2003).

2.5.2 Distribution and behavior of selenium in the environment

Selenium belongs to the chalcogen family and thereby exhibits homogeneous behavior as that of sulfur. Due to which it is related to natural sulfides like pyrite and chalcopyrite (Wiberg et al. 2001). Economically, there is no selenium ore present on Earth (Butterman and Brown 2004). It is present in the high-sulfur coals which contain around 43 mg Se kg^{-1} (Yudovich and Ketris 2006). Volcanic tuff and black slates also contain 22-32 mg kg^{-1} of selenium in them (Kunli et al. 2004).

In earth's crust, selenium is present with the minimum concentration of 0.05-0.09 mg kg^{-1} (Neal and Sposito1989). However, rocks are the chief source of selenium. Moreover, rocks contain about 40% of the total selenium found in the earth's crust.

Among all forms of selenium, selenate is the most mobile form. When present in the form of elemental selenium, it becomes unavailable to plants. Thus, selenium biogeochemistry, as well as environmental parameters has a strong impact on the mobility, concentration, and distribution of selenium (McNeal et al. 1989). Also, there are many factors which influence the distribution of selenium and these factors include burning of fossil fuels, weathering, and precipitation of minerals, volcanic eruptions, mining operations and disposal of fly ash (Dhillon and Dhillon 2003).

Selenium is present unevenly across the globe. Moreover, its distribution is site-specific. Usually, the regions with high selenium are present in the form of spots and the regions with low selenium exist in the form of a belt.

2.5.3 Geographic distribution of selenium toxic soils

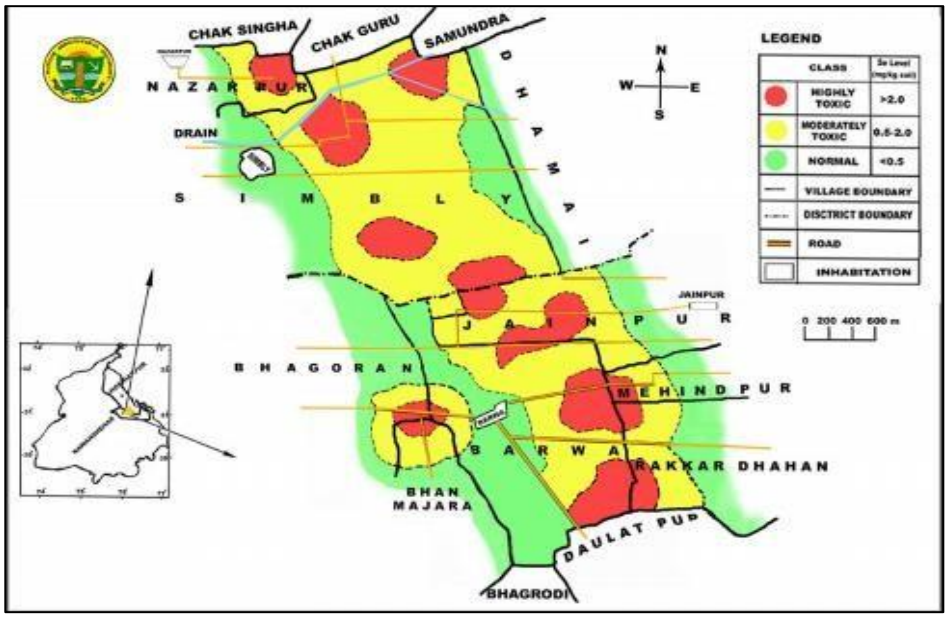


Fig 2.2 Map of Punjab showing seleniferous regions in red (Dhillon and Dhillon 2003)

Some areas of seleniferous soils have been found in Hoshiarpur and Nawanshahr regions of Punjab. In these areas, the total and water-soluble Se ranged from 0.23-4.55 and 0.02-0.16 mg

kg^{-1} respectively. Soils of these regions are alkaline in nature and contain calcium carbonate (Dhillon and Dhillon 2004). In Haryana, soils with 10 mg kg^{-1} of Se have also been reported (Singh and Kumar 1976) but no case of selenium toxicity in humans and animals have been reported so far. The toxic pastures of West Bengal contain 1.45-2.25 mg kg^{-1} of selenium.

The distribution of Se is variable even on the surface and sub-surface of the soil. The areas with high selenium contain 1.5-20 mg kg^{-1} and 0.7-16 mg $kg^{-1}$of Se on the surface and sub-surface of the soil respectively (Dhillon and Dhillon 2003).

The Kesterson Reservoir and Lahontan Valley in California accommodates 4-25 and 0.7-1.5 mg kg^{-1} of Se respectively in the upper 20 cm of soil. In many parts of the world like Mexico, Canada, Ireland, and Columbia, selenium poisoning has been reported and the Se content in these regions varies from 0.3-324 mg kg^{-1}.

The maximum amount of Se i.e. 98 kg^{-1} has been recorded in Western United States (Rosenfeld 1964).

It has been observed that patches of seleniferous soils are situated near seasonal rivulets which could be responsible for carrying the finer material from the Siwalik hills into these low-lying areas. Because these selenium-rich areas are low-lying, this could also be a reason for their enrichment with the finer deposits brought down by the run-off water (Dhillon and Dhillon. 1991). Moreover, the only irrigation source in these areas is underground water which contains 2.54-69.53 µg l^{-1} of Se which is about nine times more than that found in the non-seleniferous soils. Hence, high level of selenium in the irrigation water also plays an important role in the selenium toxicity occurring in these areas. In central parts of California, high concentrations of Se in irrigation and drainage water caused toxicity in aquatic birds (Presser and Barnes 1985).

2.6 Abiotic transformations of selenium

Selenium mainly exists in four oxidation states: selenide (Se^{2-}), elemental selenium (Se^{0}), selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}). Each oxidation state has its own physical and chemical properties. Out of these, selenate is the most mobile and soluble form in a liquid state and is thermodynamically stable in alkaline and oxidizing environment. It gets easily leached in the

soil and becomes readily available to plants due to which it is considered to be the most dangerous forms of selenium in the terms of environmental pollution (McNeal et al. 1989).

Elemental selenium is insoluble in nature and impervious to both oxidation and reduction and is therefore non-toxic in nature (Sarquis and Mickey 1980). Selenite is mostly found under oxidizing environments and is less soluble than selenate (McNeal et al, 1989). It mostly exists in the form of selenous acid (H_2SeO_3), which is one of the weak acids. It can also exist as $HSeO^{3-}$ or SeO_3^{2-} depending upon the pH of the solution (Seby et al. 2001). In an acidic environment, it can also be reduced to elemental selenium by various microorganisms and by different reducing agents like ascorbic acid or sulfur dioxide (Sarquis and Mickey1980).

In reducing environments, selenide occurs in the form of hydrogen selenide gas (McNeal et al. 1989) which is fifteen times more dangerous as compared to hydrogen sulfide gas. At the same time, it can get easily oxidized to elemental selenium (non-toxic) in the alveoli of lungs and on the mucous membrane of a nose (Sarquis and Mickey 1980).

2.7 Biotic transformations of selenium

2.7.1 Selenium tolerance in bacteria

Microbes are involved in a number of transformations and it has been reported that they are accountable for about one-third of the elemental transformations occurring in the environment. They may do so by either changing the valency of the element (oxidation or reduction) or by changing its chemical form (solid, liquid or gas). These transformations include various processes like assimilatory, dissimilatory, methylation or detoxification processes (Stolz et al. 2002).

2.7.2 Mechanism of selenium tolerance in bacteria

During assimilation, various permeases help in the transportation of selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) inside the cells which are further reduced to selenide (Se^{2-}) (Karle and Shrift 1986). Furthermore, bacteria with the help of selenophosphate synthase, synthesizes selenophosphate. Eventually, serine reacts with selenophosphate and selenocysteine is synthesized. In cellular components, sulfur is usually replaced with selenium when the latter is present in excess (Dungan and Frankenberger 1999). In soil, water and sediments, reduction

of selenate and selenite are of great importance as it helps in removing toxic oxyanions. Since it involves little selenium fluxes, the assimilatory reduction does not contribute much (Eswayah et al. 2016).

Under anoxic conditions, microbes may follow a different approach in which they might make use of unusual electron acceptors (Lenz and Lens 2009) like sugars, alcohol, organic acids and hydrogen (Zhang et al. 2008). This anaerobic process is known as dissimilatory reduction. These reducers have certain enzymes which are not hampered by the existence of compounds like nitrate, nitrite, and sulfate. It has been elucidated that the energy produced in selenite and selenate reduction is higher than that produced in the case of sulfate. Nevertheless, nitrate reduction yields the highest amount of energy (Lenz and Lens 2009). Therefore, in order to reduce selenium to elemental selenium, a great electron donor dosage is required. This process of dissimilation is very important in the context of bioremediation (Zhang et al. 2008).

2.7.2.1 Reduction of selenate

In various cases, brick-red coloration is observed which is due to the reduction of selenate to elemental selenium and there are various bacteria which can utilize selenate as an electron acceptor and these includes *Sulfurospirillum barnesii, Bacillus arseniciselenatis and Thauera selenatis* (Stolz et al. 1999). The reduction of selenate (Debieux et al. 2011) could be explained as follows:

$$SeO_4^{2-} + 2e^- + 2H^+ \longrightarrow SeO_3^{2-} + H_2O$$

$$SeO_3^{2-} + 4e^- + 6H^+ \longrightarrow Se^0 + 3H_2O$$

If the selenate respiring bacterial cells are observed under TEM (Transmission Electron Microscope), then the selenium nanospheres could easily be seen in the extracellular media as well as inside the cell (Oremland et al. 2004). When the selenium enters the cell for assimilation, it gets reduced in the cytoplasm which results in the formation of selenium precipitates which are then transported outside the cell (Nancharaiah and Lens 2015).

In the bacterial species, various enzymes like selenate reductase (Ser), periplasmic nitrate reductase (Nap), membrane-bound nitrate reductase (Nar) catalyzes the selenium reduction

(SeO_4^{2-} to SeO_3^{2-}). Selenate reductase (Ser) is a heterotrimer which has been isolated from *Thauera selenatis*. It is present in the periplasm and contains various subunits like Ser A (96 kDa), Ser B (40 kDa) and Ser (23 kDa). It also contains several prosthetic groups such as iron, molybdenum and acid-labile sulfur (Schroder et al. 1997). It has been illustrated that selenate reductase does not utilize nitrate, nitrite, sulfate as electron acceptors and is certain for the reduction of SeO_4^{2-} to SeO_3^{2-}. On the other hand, molybdenum is also found in the active site of selenate reductase found in the membrane of *Sulfurospirillum barnesii* (Schroder et al. 1997).

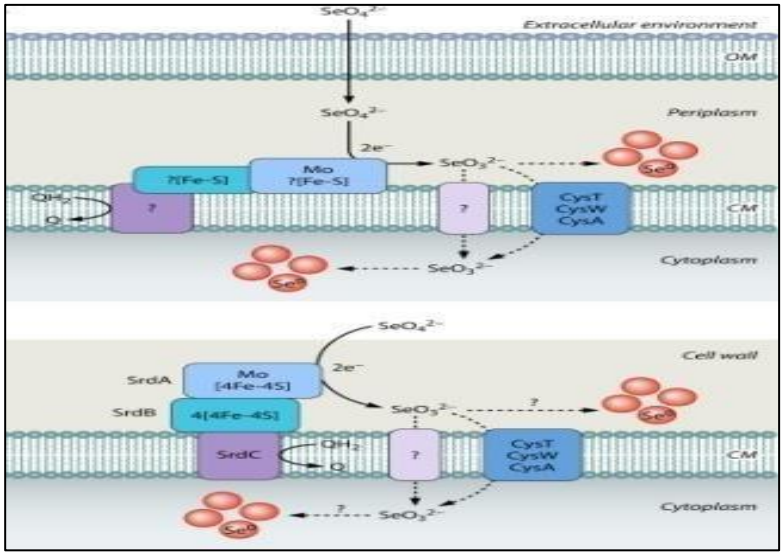


Fig 2.3 Mechanism of selenate reduction in *Enterobacter cloacae* (Nancharaiah et al. 2015)

In *Enterobacter cloacae* (facultative anaerobe), Ser is situated in the membrane fraction. It is nearly 600 kDa and is heterotrimeric in nature. It contains various prosthetic groups like heme, nonheme iron, and molybdenum. It also exhibits activity on chlorate and bromate (Ridley et al. 2006). It was also demonstrated that it's selenate reductase activity could be inhibited by tungstate but molybdate could help in its activation. (Yee et al. 2007) demonstrated that Ser present in the bacteria is regulated by FNR gene (fumarate nitrate reduction) which is usually induced during suboxic conditions (Yee et al. 2007).

2.7.2.2 Reduction of selenite

There are different mechanisms which can be used by microorganisms for the conversion of selenite into elemental selenium (Kessi 2006, Kessi and Hanselmann 2004). A group of enzymes called reductases are involved in it and these are named sulfite reductase, periplasmic nitric reductase, and dimethyl sulfoxide reductase (Hunter and Manter 2009). The reduction is also mediated by thiols which are present in the cytoplasm (Turner et al. 1998). Selenite reacts with glutathione (GSH) to form selenodiglutathione (GS-Se-SG). The latter is reduced by NADPH glutathione reductase and forms selenopersulfide of glutathione (GS-Se) which on hydrolysis forms elemental selenium and reduced GSH.

Microorganisms reduce selenite into elemental selenium by using various mechanisms and some of them are: 1. Thioredoxin reductase system 2. sulfide-mediated reduction 3. Painter-type reactions 4. Siderophore-mediated reduction and 5. Dissimilatory reduction (Zannoni et al. 2008)

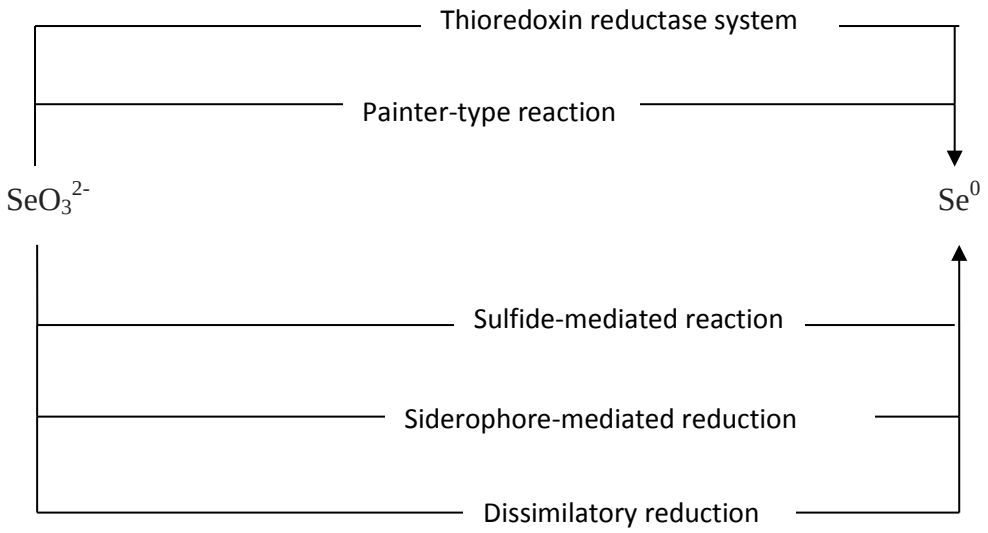


Fig 2.4 Types of mechanisms by which bacteria reduce selenite (Nancharaiah et al. 2015)

Painter-type reaction: This reaction was named after the name of the scientist "Painter" who discovered that SeO_3^{2-} and thiols are highly reactive towards each other. He also illustrated the formation of selenotrisulfide (RS-Se-SR) in the cells of *Escherichia coli* which were brought in contact with SeO_3^{2-}. It could be represented as follows (Painter 1941):

$$4RSH + SeO_3^{2-} + 2H^+ \longrightarrow RS\text{-}Se\text{-}SR + RSSR + 3H_2O$$

Thioredoxin reductase system

The *E.coli* cells were grown in the SeO_3^{2-} containing medium in which the concentrations of thioredoxin reductase and reduced thioredoxin [$Trx(SH)_2$] were elevated (Yamada et al . 1997). The reaction between reduced thioredoxin and selenodiglutathione leads to the formation of oxidized thioredoxin ($Trx\text{-}S_2$), selenopersulfide anion and reduced glutathione. Hence, it was hypothesized (Bjornstedt et al. 1992) that reduced thioredoxin is involved in the selenite reduction.

$$Trx(SH)_2 + GS\text{-}Se\text{-}SG \longrightarrow Trx\text{-}S_2 + GSH + GS\text{-}Se^- + H^+$$

$$Trx\text{-}S_2 + NADPH + H^+ \longrightarrow Trx(SH)_2 + NADP^+$$

Siderophore mediated reduction

Pseudomonas stutzeri produces an iron siderophore called pyridine -2,6-bis (thiocarboxylic acid) $[C_7H_3O_2S_2]^{2-}$ through the process of reduction detoxifies the selenite and forms the insoluble precipitates of elemental selenium (Zawadzka et al. 2006). Pyridine-2,6-bis (thiocarboxylic acid) on reduction gives dipicolinic acid and hydrogen sulfide (H_2S) which acts as a reducing agent (Zannoni et al. 2008) and leads to the formation of Se^0.

$$[C_7H_3O_2S_2]^{2-} + 2H_2O \longrightarrow [C_7H_3O_4]^{2-} + 2HS^- + 2H^+$$

It has been reported that when *T.selenatis* is provided with only a single electron acceptor (selenate) then only a part of the produced selenite is reduced whereas when both NO_3^- and SeO_4^{2-} are provided as electron acceptors then the total reduction of selenite takes place.

Increased reduction of NO_3^- and SeO_3^{2-} takes place when the levels of nitrate reductase are increased while the absence of periplasmic NO_3^- activity leads to the reduction of either NO_3^- or SeO_3^{2-}. Hence, these observations lead to the conclusion that nitrate reductase plays an important role in the reduction of selenite (Decker and Macy 1993).

2.7.2.3 Reduction of selenium to selenide by bacteria

Few microorganisms have been reported which can reduce many of the selenium species into selenide (Se^{2-}) i.e. they undergo dissimilatory reduction. For example, *Thiobacillus ferrooxidans*, an obligate acidophile can reduce elemental selenium into hydrogen selenide

under anaerobic conditions (Bacon and Ingledew 1989). The remarkable amount of selenide is produced by *Bacillus selenitireducens* (selenium–respiring bacteria) when it is supplied with elemental selenium (Se^0). It can also reduce selenite and finally lead to the formation of selenide (Pearce et al. 2009 and Herbel et al. 2003).

2.7.2.4 Oxidation of selenium by bacteria

Diverse microbes have been examined which can oxidize elemental selenium and selenite present in the soil. Moreover, various bacteria have also been found which can utilize the selenic acid (H_2SeO_4) (product of Se^0 oxidation) as an energy source. *Bacillus megaterium* has been found to oxidize elemental selenium into selenite and selenate (Sarathchandra and Watkinson 1981). The pathways which lead to the oxidation of selenium are still unknown.

Various studies have also concluded that the rate of oxidation is very slow and leads to the formation of selenate and selenite (Losi and Frankenberger 1998). In oxic soil slurries, oxidation leads to the production of both selenate and selenite but selenite forms the main product. Reports suggest that heterophilic bacteria and sulfur-oxidizing bacteria are the chief sources of Se^0 oxidation (Dowdle and Oremland 1998).

2.7.2.5 Methylation of selenium by bacteria

This type of mechanism is adopted by the microbes to eliminate selenate and selenite in the form of volatile compounds like dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe). This process is also important for the detoxification and natural Se cycling. Microbes produce DMDSe and DMSe from various selenium sources like water, sewage sludge, and soil.

There are a number of methylated species which are formed by different bacteria and they include dimethyl selenone [$(CH_3)_2SeO_2$], dimethyl triselenide ($CH_3SeSeSeCH_3$), dimethyl selenyl sulfide (CH_3SeSCH_3) and dimethyl diselenyl sulfide ($CH_3SeSeSCH_3$).

Various methylation pathways have been put forward by different scientists but till date, the pathway given by Challengers is the most promising one.

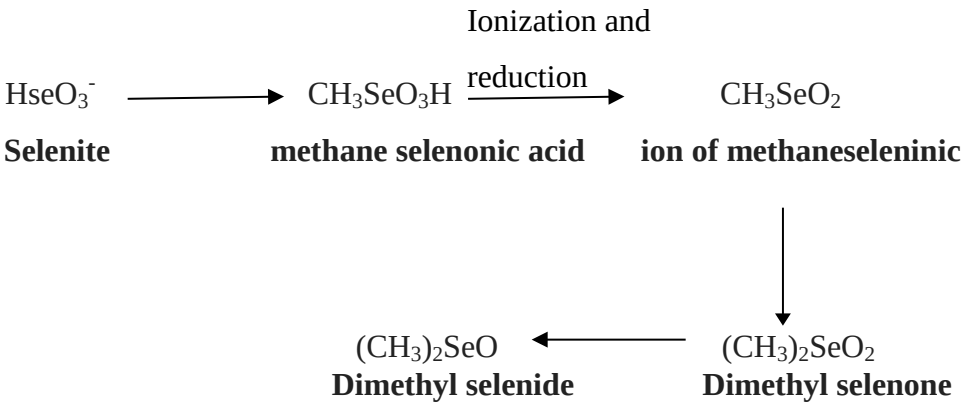


Fig 2.5 Methylation pathway in bacteria (Eswayah et al. 2016)

2.7.2.6 Demethylation of selenium compounds by bacteria

There are many microbes which can use dimethyl diselenide (DMDSe) and dimethyl selenide (DMSe) as the carbon and energy sources. In 1977, Doran and Alexander isolated *Xanthomonas* and *Corynebacterium* which can grow by utilizing these compounds. Various scientists have investigated the reason behind it but the precise pathway is still missing. It has been reported that under anoxic conditions, demethylation of DMSe takes place by microbes and methane (CH_4), carbon dioxide (CO_2), hydrogen selenide (H_2Se) are formed. Generally, methanogens and sulfate-reducing bacteria are involved in this process (Oremland and Zehr 1986).

2.8 Effects of various selenium tolerant bacteria on plant growth promotion

2.8.1 Indole acetic acid (IAA) production

IAA production is one of the significant characteristic shown by various rhizospheric bacteria. It is the product of tryptophan and is one of the most important auxins. Generally, auxins are produced by bacteria for their own defense (Shih-Yung 2010). By synthesizing IAA, bacteria disturbs the physiological activities of host and helps in the increased uptake of nutrients by producing longer roots and by increasing the number of root hairs. It also helps in the development of embial cavity and promotes fruiting and flowering in plants (Zhao, 2010).

The physiological confirmation of different Trp-dependent pathways was found in *Azospirillium brasilense* (Carreno-Lopez et al. 2000). The pathway can be explained (Matsukawa et al. 2007) as follows:

Tryptophan ⟶ indole-3-acetamide (IAM) ⟶ IAA

In 1985, Horemans and Vlassak reported that when *Azospirillium brasilense* is made to grow aerobically in the absence of tryptophan then the highest amounts of auxins are produced. In 2008, Ahmad et al isolated *Azobacter, Pseudomonas, Mesorhizobium and Bacillus* from different soils and studied their plant growth promoting effects. They also reported that more than 80% of isolates of *Azobacter, Pseudomonas and mesorhizobium* produced high amounts of IAA while only 20% of *Bacillus* species were able to produce auxin.

2.8.2. Phosphate solubilization

Phosphorus is one of the crucial mineral required for the growth of a plant. But only a small amount of the applied phosphorus is available to plants (Yu et al. 2011) due to which it becomes one of the limiting nutrient. Hence the need for phosphorus fertilizer arises which creates a lot of pollution and is also not cost-effective (Reddy et al. 2002). Fortunately, various phosphate solubilizing bacteria were isolated which were capable of phosphate solubilization. These bacteria isolates not only makes insoluble phosphorus available to plants but also helps in its growth and development. The various processes involved in the solubilization are chelation, production of organic acids and acidification (Chung et al. 2005). They not only reduce the cost but also helps in the mobilization of various fertilizers supplied to the soil. Furthermore, they also release various secondary metabolites which help in the plant promotion (Kaur and Reddy 2014).

Masanori et.al isolated and characterized a gram positive and facultative anaerobe: *Bacillus sp*. from selenium-contaminated deposits. It was able to grow by taking selenate as an electron acceptor. It was able to reduce upto 1mM of selenate into elemental selenium. Due to its reduction properties, this strain could be used in the process of bioremediation.

Sharma et al. (2009) isolated a facultative anaerobe; *Pseudomonas aeruginosa* was isolated from the rhizospheric soil of Jainpur region of Hoshiarpur. This strain was able to reduce 53%

of sodium selenite and 21% of sodium selenate into elemental selenium. Furthermore, this microbe was also capable of volatilizing the 4.7% of sodium selenite and 5.1% of sodium selenate.

Avenden et al. (2016) reported the ability of *Pseudomonas putida* to form selenium nanoparticles. This strain aerobically reduced selenite but was unable to reduce selenate into elemental selenium.

Since, the high levels of selenium are posing severe effects on plants, humans and livestock which led to the interest of various scientists. Although many scientists have worked on it till date but more is yet to come.

Chapter-3
Materials and Methods

3.1 Collection of soil sample

To isolate selenium tolerant bacterial strains, agricultural land of village Jainpur (31.13539N, 76.18536E) in Nawashahr region of Punjab affected by elevated level of selenium was selected. The rhizospheric soil sample of wheat (*Triticum aestivum*) grown in that village was collected. Soil sample was drawn carefully upto 5-10 cm in depth and was collected in plastic bags, brought to the laboratory and stored at 4℃ until used for isolation of selenium tolerant bacterial strains. Rest of the soil was used for determining various physiochemical characteristics and selenium concentration in soil sample.

3.2 Estimation of selenium (Se) in soil sample

Reagents

- Soil sample – 1 g
- HNO_3 + $HClO_4$ (3:1)
- 6N HCl
- Double distilled water
- MQ water (pH- 1.8 adjusted with HCl)
- MQ water
- 2,3-Diaminonaphthalene (DAN) dye solution – 0.1% in 0.1N HCl

Procedure

- 100 ml of 0.1 N HCl was taken in an amber colored bottle
- 100 mg of DAN was added to 500 mg of hydroxylaminehydrochloride and both were mixed thoroughly by shaking
- The bottle was kept in water bath at 50^{o}C for 30 minutes
- The crude mixture was poured in a separating funnel
- 20-25 ml cyclohexane was added at the top of the crude dye solution
- Separating funnel was shaken vigorously, two layers were allowed to separate and settle properly
- The DAN dye was extracted in the aqueous layer and it was collected in a bottle
- The upper layer of cyclohexane was discarded

- Again fresh cyclohexane was added to collect DAN. This step was repeated 4-5 times till the layer of cyclohexane became clear
- Extracted DAN solution was collected in a bottle covered with an aluminium foil or amber colored bottle and stored at $4^{o}C$

STABILIZING SOLUTION

- Added 10 g of hydroxylamine hydrochloride powder to 40 ml double distilled water and mixed properly.
- Added 4 ml of 1 M EDTA solution and volume was made upto 100 ml by double distilled water.

Procedure

- 1 g of soil sample was taken in a teflon tube and HNO_3- $HClO_4$ mixture (3:1) was added to the tube.
- The tubes were digested in microprocessor digestor and the mixture was digested at $200^{o}C$ for 30 minutes.
- After digestion, the tubes were allowed to cool.
- Added 5 ml of 6N HCl and again the tubes were tightened and placed in the digestor.
- The digestion was done at $150^{o}C$ for 15 minutes.
- Then the volume was made upto 50 ml with distilled water.
- The solution was gently mixed by inversion to allow the debris to settle.
- The experiment was performed in four replicas.
- In each glass tube, 5-10 ml of HCl (pH-1.8), 0.5-1 ml of digested samples and 0.1 ml of stabilizing solution was added.
- Vortex the tubes for 1 minute and placed them in water bath for 10 minutes. Standard stock solution of sodium selenite (100 ppb = 100 ng/ml) was made. It was used to make standard sodium selenite solutions ranging from 10-50 ng as follows:

Standard	Stock solution (µl)	MQ water (pH-1.8) in µl
10	300	2700
20	600	2400
30	900	2100
40	1200	1800
50	1500	1500

3.3 Isolation of selenium tolerant bacterial strains

- Isolation of selenium tolerant bacterial strains was done by serial dilution method on tryptone soya agar plates.

COMPOSITION OF TRYPTONE SOYA AGAR

Ingredients	g/L
Casein enzymic hydrolysates	15
Papaic digest of soyabean meal	5
Sodium chloride	5
Agar	15
Final pH (at 25℃)	7.3±0.2

Procedure

- All the ingredients were dissolved in 1000 ml distilled water and autoclaved at 15 Psi for 15 minutes.
- After autoclaving sodium selenite was added.
- Pouring of medium was done in the sterilized petriplates.
- After solidification of medium, plates were used.

- Plates were formed under aseptically conditions i.e., laminar air flow to avoid any type of contamination.

3.3.1 Enrichment of the media

100 ml of TSB was enriched with 10 g of the soil sample and sodium selenite and was incubated at 37^{o}C till the media turns red in color.

3.3.2 Serial dilution method

- For isolation of bacterial strains, dilutions were made upto 10^{-6}in test tubes containing 9 ml sterile physiological normal saline (0.85% NaCl in distilled water) in each test tube.
- 1 ml starter culture (enriched media) was taken and serial diluted upto 10^{-6}
- From each dilution 100 µl cultures was spread on tryptone soya agar plates amended with sodium selenite.
- Plates were incubated for 24 hours at 37^{0}C.

3.3.3 Subculturing of isolated bacterial strains

- Streaking was done for the isolation of pure strains from the obtained mixed culture.
- Colonies grown on tryptone soya agar plate amended with sodium selenite were maintained by making patches on the other TSA plate amended with sodium selenite.

3.3.4 Determination of selenium tolerance level of pure bacterial strain

Tolerance level of bacterial isolates was checked at different concentrations of sodium selenite in tryptone soya broth.

COMPOSITION OF TRYPTONE SOYA BROTH

Ingredients	**g/L**
Pancreatic digest of casein	17
Papaic digest of soyabean meal	3
Sodium chloride	5
Dextrose	2.5

Dibasic potassium phosphate	2.5
Final pH (at 25℃)	7.3±0.2

Procedure

- Purified prominent isolates were screened for their selenium tolerance.
- Bacterial colony was inoculated in a tube containing peptone water and incubated it overnight.
- After incubation, 1 ml of culture was transferred to fresh peptone water.
- Incubated it at 37^{o}C until the turbidity of the solution matches with the turbidity of Mcfarland's standard.
- Within 50 minutes, transferred 1 ml of the culture to the tryptone soya broth containing sodium selenite at different concentrations (0 mM to 400 mM).
- Incubated it at 37^{o}C for 24 hours.
- Serial dilution of each concentration was done till 10^{-7} and the same (100 µl) was spread on tryptone soya agar plates and incubated at 37^{o}C.
- Next day, the colony forming units (Cfu) at each concentration was calculated.

3.4 Plant growth promotion by highly tolerant bacterial isolates

3.4.1 Quantification of Indole Acetic Acid (IAA)

Quantitative analysis of Indole acetic acid was done according to Brick et al.1991

Reagents

COMPOSITION OF YEAST MALT DEXTROSE BROTH

Ingredients	**g/L**
Peptic digest of animal tissue	5
Yeast extract	3
Malt extract	3
Dextrose	10
Final pH (at 25^{o}C)	6.2±0.2
L-Tryptophan (100µg/ml)	

Salkowski's reagent (2% 0.5M $FeCl_3$ in 35% perchloric acid)

Procedure

- Yeast malt dextrose broth with 100µg/l was prepared and autoclaved.
- Inoculated the broth with the bacteria under sterile conditions.
- Kept it for incubation at 37^oC for 5 days.
- After 5 days, culture was centrifuged at 8000 rpm for 10 minutes.
- 1 ml of supernatant was taken in a test tube to which 2 ml of salkowski's reagent was added and kept in dark room for 30 minutes.
- Absorbance was taken at 540 nm using a spectrophotometer.
- To prepare standard curve, standards of 5, 10, 20, 50, 100 µg/l were made as follows:

CONCENTRATION (µg/l)	AMOUNT OF STOCK ADDED (ml)	AMOUNT OF MEDIA ADDED (ml)
0	0	10
5	0.05	9.5
10	0.1	9.9
20	0.2	9.8
50	0.5	9.5
100	1	9

- Added 2 ml of salkowski's reagent and stored in dark for 30 minutes. Absorbance was measured at 540 nm after pink color was formed.
- Concentration of unknown sample was drawn from the standard curve by making regression equation.
- Experiment was carried out in three replicates.

3.4.2 Estimation of phosphate solublization by bacteria

Method for qualitative estimation of phosphate solublization was done on Pikovskaya medium containing insoluble tricalcium phosphate suggested by Pikovskaya, 1948.

COMPOSITION OF PIKOVSKAYA AGAR

Ingredients	**g/L**
Glucose	2.5
Ammonium sulphate	13
Sodium chloride	0.1
Potassium Chloride	0.2
Yeast extract	0.5
Manganese sulphate (trace)	0.0001
Ferrous sulphate	0.0001
Agar	7.2

Procedure

- Pikovskaya agar plates were made and the inoculation was done by stabbing the bacteria in it.
- Plates were incubated at 37˚C for 3-6 days.
- Colonies form halo zone around them, indicated the phosphate solublization.
- Then phosphate solublization index (SI) was calculated using following formulae (Premono et al, 1996):

$$\text{Solublization index (SI)} = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony diameter}}$$

3.4.3 HCN production by bacteria

Determination of HCN production was done according to Bakker and Schippers (1987).

Reagents

- 2% (w/v) of sodium carbonate
- 0.5% (w/v) of picric acid
- 4.4 g/l glycine

Procedure

- Inoculation of bacteria was done on tryptone soya agar plates amended with 4.4 g/l glycine.
- Whatmann filter paper was dipped in 2 % w/v sodium carbonate in 0.5 % (w/v) picric acid solution and was placed inside the lid of the petriplates.

- Plates were sealed with parafilm and incubated at 37^{o}C for 4 days.
- Color change of the filter paper from yellow to brownish indicates the HCN production.

3.4.4 Production of ammonia by bacteria

COMPOSITION OF PEPTONE WATER

Ingredients	**g/L**
Peptic digest of animal tissue	10
Sodium chloride	5
Nessler's reagent	

Procedure

- Inoculated the bacteria in peptone water with the help of an inoculating loop.
- Incubated it at 37^{o}C for 48-72 hours.
- Added nessler's reagent to it and observed the color change.

3.5 To check plant growth promoting activities of high selenium- tolerant bacterial isolates in nursery conditions

3.5.1 Nursery experiment

To check the effect of isolated selenium tolerant bacterial strains on crop production, field experiment of maize crop was carried at TIFAC Core, Thapar Institute of Engineering and Technology Patiala, Punjab. This site is situated at 30.30 N˚ latitude and 76.38 E˚ longitude. The region possessed tropical hot and dry climatic conditions followed by dry and hot summer and very cold in winter. The used soil in experiment was bought from seleniferous region of Nawanshahr region of Punjab having sandy loamy texture.

3.5.2 Seed inoculation

Seed inoculation of selenium tolerant bacterial isolates were done by mixing the bacterial in 10 percent sugar and 40 percent Gum arabic to form a slurry in which seeds were dipped to form uniform coating of inoculum around the seeds. The inoculated seeds were dried overnight in shade and used for sowing.

Methodology of seed inoculation

- Selenium tolerant bacterial isolates were grown in tryptone soya broth.
- The culture was centrifuged at 8000 rpm for 5 minutes and supernatant was discarded.
- The obtained pellet was suspended in the autoclaved distilled water.
- Then, 50 g sugar was taken in 500 ml beaker and solution was heated for 15 minutes.
- 200g of gum arabic was added in hot sugar solution, mixture was allowed to cool to room temperature.
- Surface sterilization of seed inoculation was done by treating seeds in 95% ethanol for 3 minutes followed by 3 % sodium hypochlorite for 5 minutes, then washing was done with sterile distilled water for 4-5 times.
- Seeds were added to the slurry and mixed properly to form uniform coating around the seeds.
- Surface sterilized seeds coated with 40 % gum arabic and 10 % sugar solution but without inoculum served as a control.
- Seeds were dried in shade for overnight.

3.5.3 Experimental preparation and cultivation of plants

Nursery trials were conducted at Core, Thapar Institute of Engineering and Technology. In nursery trails, plastic cups were used and 3 treatment each with ten replicates.

Treatment consisted of

- Soil
- Soil + B_{49} (Se tolerant isolate no. 1)
- Soil + B_{71} (Se tolerant isolate no. 2)

Maize variety was cultivated in seleniferous soil in the summer season. All cups were irrigated regularly.

3.6 Plant analysis after harvesting

After 2 months of harvesting, various parameters were measured: root and shoot length, number of leaves, dry weight of shoot and root, height of plant was measured and recorded.

3.6.1Estimation of selenium in plant tissue

Selenium bioaccumulation of B_{49} and B_{71} was checked by using fluorescence spectrometry.

Procedure

- Bacterial isolates were allowed to grow in tryptone soya broth with different concentrations of sodium selenite.
- The bacterial culture was centrifuged at 8000 rpm for 5 minutes.
- The supernatant was stored separately.
- The obtained pellet was dried and weighed.
- Then, selenium accumulation was estimated by fluorescence spectrometry by taking 100 mg bacterial pellet grown at different sodium selenite concentrations.
- Selenium accumulation was also estimated in the crushed roots and shoots of maize plant tissue.
- Free selenium which was not accumulated by bacterial isolate was also estimated in the supernatant by fluorescence spectrometry.

3.7 Morphological and biochemical characterization of highly selenium-Tolerant bacterial isolates

3.7.1 Gram staining

- A thin bacterial smear was made with the help of a loop or a needle. Heat fixed the smear with the help of a flame.
- Flooded each smear with crystal violet for 30 seconds and then washed the slide by using distilled water.
- Added Gram's iodine solution for 60 seconds and then washed it off with the help of ethyl alcohol. Ethyl alcohol was added drop by drop until no more color flows from the smear. Rinse it with distilled water.

- Rinsed secondary stain, safranin at the smear and left it for 1 minute and then again rinsed it with water.
- The slides were air-dried and observed under microscope by using oil- immersion objective.
- If the bacteria appears purple in color (retains primary stain), then they are called Gram positive bacteria while the bacteria which appears reddish-pink color (retains secondary stain) are known as Gram-negative bacteria.

3.7.2 Motility test

- A semi-solid media of nutrient broth with 0.4% agar was made and autoclaved.
- The media was poured in the test tubes and left undisturbed for some time.
- The bacterial colonies were inoculated in the media with the help of an inoculating loop.
- Incubated the tubes at 37^{o}C for 24 hours.
- Analysed for the depth of turbidity appeared.

3.7.3 Catalase test

- A small bacterial colony was placed on the clean slide with the help of an inoculation loop.
- Added few drops of hydrogen peroxide (H_2O_2).
- Bubbling indicated the evolution of O_2 i.e., Positive results.
- No bubbling or a few scattered bubbles i.e., Negative results
- Later appearance of bubbles was ignored.

3.7.4 Oxidase test

- This test was performed by using oxidase discs manufactured by HiMedia, India.
- Took a fully isolated colony from the plate on clean glass slide.
- Placed oxidase test disc on to the slide above bacterial colony.
- Reaction was observed within 5-10 seconds at 25-30°C.

- Positive reaction showed deep purplish blue coloration.
- A change later than 10 seconds or change at all is considered negative reaction.

3.7.5 Nitrate reduction test

- Nitrate agar is used to test the ability of organisms to reduce nitrate to nitrite using enzyme nitrate reductase.
- For this test, nitrate discs were used manufactured by HiMedia, India.
- The bacterial isolates were grown on nutrient agar plates.
- Placed Part A (disc) on suspected colony and added a drop or two of part B (rehydrating fluid) on the disc.
- The nitrate reduction was observed after incubation at 37°C for 24 hours.
- Red or pink color formation on addition of discs indicates positive reaction while negative reaction indicates no color change.

3.7.6 Starch hydrolysis test

COMPOSITION OF STARCH AGAR

Components	**g/L**
Starch (soluble)	20.0
Peptone	5
Beef extract	3
Agar	15
pH	7.0±0.2

COMPOSITION OF IODINE SOLUTION

Components	**g/L**
Iodine	1
Potassium iodide	2.9

A homogeneous solution of iodine and potassium iodide was made and stored in amber – colored bottle.

Procedure

- By using an inoculation loop, a double streak inoculation of each Se-tolerant bacteria was done on the starch agar plate.
- Incubated the inoculated plates at 37^0C for 48 hours.
- After incubation, flooded the surface of the plates with iodine solution for 30 seconds.
- Poured off excess iodine solution from the petri-plate.
- The plates were examined on the basis of the color change of the media around the bacteria.
- Starch media turns dark-blue in the presence of iodine and a yellow zone around the colony indicated the amylolytic activity.

3.7.7 Fermentation of Carbon substrate by bacterial isolates

The fermentation broth contains the same ingredients as that of nutrient broth. Additionally, a specific carbohydrate and a pH indicator (phenol red) are also added. Phenol red is red at neutral pH but it turns yellow at or below pH 6.8 because of the production of organic acids.

PREPARATION OF FERMENTATION BROTH

Components	**g/L**
Peptone	10.00
Carbohydrates	5.00
Sodium chloride	15.00
Phenol red	0.018
pH	7.3

(Carbohydrates used were glucose, lactose, maltose and mannitol)

Procedure

- Fermentation broth was taken in test tubes and durham tubes were added in an inverted position.
- Sterilized the media by autoclaving at 121^oC for 3 minutes.
- Inoculated the media with the test organism and kept one tube uninoculated as a comparative control.

- Inoculated all the tubes (including control) at 37^{o}C for 24 – 48 hours.
- Observed the changes that took place in inoculated tubes and compared them to the uninoculated one (control).
- Change in color of inoculated tubes indicated the production of acid alone. But appearance of bubbles along with color change confirms the production of acid along with gas i.e., fermentation.

3.7.8 Urease test

UREA AGAR COMPOSITION

Ingredients	**g/L**
Peptic digest of animal tissue	1.00
Dextrose	1.00
Sodium chloride	5.00
Disodium phosphate	1.200
Monopotassium phosphate	0.800
Phenol red	0.012
Agar	15.00
Final pH	6.8±0.2

Procedure

- Medium was sterilized by autoclaving at 121^{o}C for 15 minutes.
- Allowed to cool at 50^{o}C and added 50 ml of 40% urea solution (filter-sterilized) in it and mix well.
- Made the slants by dispensing the media in the tubes and allowing them to stand in a slanting position.
- Inoculated the test organism in the urea agar slants.
- Incubated the slants at 37^{o}C for 24 – 48 hours.
- Presence of urease is indicated by red or cerise color while yellow color indicates the absence of urease activity.

3.7.9 IMViC tests

IMViC tests comprises of four different tests

- Indole production
- Methyl-red test
- Voges – Proskauer test
- Citrate utilization test

3.7.9.1Indole production test

- Prepared 1% tryptone broth by dissolving 10 g of tryptophan in one litre of distilled water.
- Sterilized the media by autoclaving at 121^{o}C for 15 minutes.
- Inoculated the broth with the test organism and incubated at 37^{o}C for 48 hours.

  After incubation, added 1 ml of Kovac's reagent to all the tubes including control.
- Tubes were shaken after 10-15 minutes.
- A cherry (deep) red color on the top layer of the tube indicated the positive result while the absence of the red color confirmed that the test organism was indole negative.

3.7.9.2 Methyl - red and Voges-Proskauer tests

MR-VP BROTH COMPOSITION

- Methyl red pH indicator
- VP reagent I (napthol solution)
- VP reagent II (40% potassium hydroxide)

PREPARATION OF MR-VP BROTH

Components	**g/L**
Peptone	7.00
Dextrose	5.00
Potassium phosphate	5.00
pH	6.9

Procedure

- Dispensed 5 ml broth in two sets of test tubes and autoclaved it for 15 min.
- Inoculated the one set of tubes with the test organism and kept at 37^{o}C for 48 hours.

- After incubation, added 5 drops of methyl red indicator to all the tubes (including indicator).
- Appearance of red color indicated the positive result while the yellow color indicated the negative result.
- To another set of test tubes, added 12 drops of VP reagent I (Napthol solution) and 2-3 drops of VP reagent II (40% potassium hydroxide)
- Shaken the tubes for 30 seconds by removing the caps so that the media is exposed to oxygen.
- Observed the tubes after 15-30 minutes.
- Crimson to ruby pink color indicated positive test while no change in color indicated negative test.

3.8 Molecular methods for identification of highly tolerant selenium isolates

3.8.1. Isolation of genomic DNA from selenium tolerant bacterial isolates (Chen WP, 1993)

- Prepared 5 ml TSB in test tube and autoclaved it at 121^{o}C.
- Inoculated it with the bacteria and kept it at 37^{o}C for 24 hours.
- Centrifuged 5 ml culture at 12,000 rpm for 10 minutes.
- Discarded the supernatant and allowed the pellet to dry so that no media is left behind.
- Suspended the pellet in 300 µl SE buffer (0.15 M NaCl, 0.1 M EDTA, 2% SDS) and incubated at 65^{o}C for 1 hour (invert mix after every 10 minutes).
- Added equal volume of phenol and chloroform and gently invert mix.
- Centrifuged at 13500 g for 20 minutes.
- Transferred the aqueous layer in a fresh eppendorf.
- Added equal volume of chloroform and repeated till no white layer is visible.
- Added 3µl RNase and incubated at 37^{o}C for 30 minutes.
- Added equal volume of 24:1 chloroform :isoamyl alcohol and centrifuged at 13500 g for 10 minutes.
- To the supernatant, NaCl was added such that it becomes 1.6 M when added.

- Added equal volume of water saturated diethyl ether, mix and centrifuged at 10,000 g for 10 minutes.
- To the supernatant, 95% of ethanol was added (double volume) and incubated at room temperature for 15 minutes.
- Washing of the pellet was done with 70% ethanol.
- Suspended the pellet in 30 µl of MQ water.

3.8.2 Qualitative Analysis of isolated DNA (Agarose Gel Electrophoresis)

Materials

- 0.5X TBE buffer, agarose gel (1%), DNA (100 ng) sample, loading dye, DNA ladder, ethidium bromide staining solution, gel doc, gel tray, cast tray, electrophoresis unit, glass flasks, measuring cylinder, micropipettes, sterilized tips.

Procedure

- 100 ng of DNA sample was loaded on 1% agarose gel prepared in 40ml TBE (0.5X) buffer, ethidium bromide was added in the gel.
- After gel solidification, DNA sample was loaded in the wells with 6X loading dye.
- Nucleic acids were electrophoresed at 50 volts (3volts/cm) for 40-60 minutes.
- Gel was visualized on U.V. trans-illuminator.

3.8.3 Quantitative Analysis of Isolated DNA

Materials

- Nano drop 1000 spectrophotometer (Thermo scientific, Wilmington, DE),

Procedure

- Quantitative analysis was done by using a Nano drop 1000 spectrophotometer.
- Quantity of DNA sample was evaluated by measurement of A260/280 ratio.
- Ideally, A260/A280 ratio should be in range of 1.8-2.0. Ratio less than 1.8 indicates protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

3.8.4 Amplification of 16S rDNA And Purification of PCR Products

Materials

Eppendorfs , DNA samples (template), Deoxynucleotide triphosphates (dNTPs), 10X Buffer, DNA polymerase (Taq polymerase), Magnesium chloride (Fermentas), Forward and Reverse primer, sterile MQ water, sterile PCR tubes, micropipettes, sterilized tips.

For a gene of 1.5 kb, primers used were

Forward primer: 5'-AGA GTT TGA TCC TGG CTC AG-3'

Reverse primer 5'- ACG GGC GGT GTG TTC-3'

Composition of reaction mixture

Component	**Concentration**	**Volume for one reaction (in µl)**
MQ Water		12.2
PCR Buffer	10X	2
dNTPs		1.5
DNA template	10 ng	1
$MgCl_2$	1.5 mM	1
Forward primer	0.2 mM	1
Reverse primer	0.2 mM	1
Taq Polymerase (Fermentas, USA)	2.5 U/µl	0.3

Reaction conditions for PCR

Cycle	**Temperature**	**Duration**
Initial Denaturation	92°C	2 min
Final Denaturation	92°C	1 min
Annealing	50°C	30 sec
Initial extension	72°C	2 min
Final Extension	72°C	7 min

Protocol

- Amplification of the above 20 µl of reaction mixture was performed in GenAmp 2700 thermocycler (Applied bio-system USA) with 35 cycles of amplification.
- A Control reaction containing no DNA template was included in each amplification process so as to check for the presence of contamination of reagents and buffer.
- Aliquots (6µl) of amplification products were electrophoresed in 1% agarose gel and visualized on a UV transilluminator.

3.8.5 Purification of PCR products

Materials

- QIAquick gel extraction kit

  (QiageInc., USA)

- PCR samples to be purified.

Procedure

- PCR products were purified by gel excision purification.
- 0.8% agarose gel was made and run.
- The band was cut with the help of a scalpel and the purification was done.
- Protocol is as per manufacturer's instructions.
- Purified PCR products were then suspended in 30 µl MQ water and used for TA cloning.

3.8.6 Ligation of 16S DNA in TA cloning vector pMD20T

The purified PCR products were cloned using Takara cloning kit as per manufacturer's instructions. The reaction was made as follows:

Plasmid (pMD20T)	1µl
Insert	2 µl
Mighty mix	5 µl
MQ water	2 µl

3.8.7 Genetic transformation of bacteria

All genetic manipulations were carried out as per standard protocol by Sambrook**.**

Materials

- Luria broth (HiMedia, India)

Components	**g/L**
Casein enzymic hydrolysates	10.00
Yeast extract	5.00
Sodium chloride	5.00
pH	7.0±0.2

- Luria Agar + Ampicillin plates

Components	**g/L**
Caesin enzymic hydrolysates	10.00
Yeast extract	5.00
Sodium chloride	5.00
Agar	15.00
Ampicillin	100 µg/mL

Procedure

- Inoculated 100 µl of DH10 β cells in 20 ml Luria broth in 250 ml borosil flasks and incubated it for 16-20 hours at 37^{o}C under shaking condition (120 rpm).
- Aseptically transferred 1% of the above saturated culture into a fresh 20 ml Luria broth and incubated at 37^{o}C for 2-3 hours with vigorous shaking at 120 rpm.
- O.D. was taken at 600 nm which should be 0.5 so as to confirm growth of bacteria.
- Transferred the culture to sterile ice-cold falcon (50 ml).
- Placed the falcons on ice for 10 more minutes.
- Recovered the cells by centrifugation at 5000 rpm for 10 minutes at 4^{o}C.
- Decanted the media i.e., supernatant and allowed it to stand for 1 minute.
- Now, Re-suspended the cells in 1 ml of ice cold 0.1 M $CaCl_2$ and washed 2-3 times by centrifuge conditions of 4000 rpm for 10 minutes at 4^{o}C.
- The cells were then stored on ice for 12-24 hours.

- Aliquots of 100 µl suspension of competent cells were transferred to sterile and prechilled eppendorfs and added 80% glycerol to the cells.

3.8.8 Blue-White Screening of Competent Cells

- Added previously cloned product in 100µl of E. coli competent cells including 1 control with no plasmid and another control with insert.
- Mixed the contents of the eppendorfs gently.
- Stored the eppendorfs on ice for 30 minutes for the binding of the plasmids.
- Incubated the eppendorfs in water bath pre-heated at 42^{o}C for 2 minutes.
- Rapidly transferred the eppendorfs on ice for 1-2 minutes for heat shock.
- Added 1 ml of Luria broth and incubated at 37^{o}C for 45-60 minutes to allow bacteria to recover and express antibiotic resistance marker encoded by plasmid.
- 100 µl of transformed cells were spread on L.A + ampicillin (100µg/ml) plates by spread plate method.
- The plates were pre-spread with 40 µl of each isopropyl beta-D-thiogalactopyranoside (IPTG) and X-gal (5-bromo-4-chloro-3-indoyl β-D galactosidase).
- X-gal and IPTG were used for the screening of colonies containing a recombinant plasmid.
- The plates spread with transformed cells were then incubated overnight at 37^{o}C in upright position.
- The plates were then checked for the appearance of either recombinant or nonrecombinant colonies after 16-20 hours.
- Basically white colonies indicated transformed and recombinant colonies with gene of interest while blue colonies indicated the untransformed or the non-recombinant ones.
- Random white colonies were picked i.e., 3 or 4 for each isolate and the colony PCR was performed by using M_{13} primers.

  M_{13} primers used were:

  - Forward primer: 5'-GGT TTT CCC AGT CAC GAC-3'
  - Reverse primer: 5'-GGA AAC AGC TAT GAC CATG-3'

Composition of reaction mixture

Components	volume for 1 reaction
M.Q.water	6µl
Buffer	2µl
dNTPs	1.5 µl
$MgCl_2$	1 µl
Forward primer	1 µl
Reverse primer	1 µl
Template	3 µl
Taq polymerase	0.3 µl

The reaction mixture was prepared and heated at 98^{o}C to allow the lysis of cells.

Reaction conditions for PCR

Cycles	Temperature	Duration
Initial denaturation	92^{o}C	2 minutes
Denaturation	92^{o}C	1 minutes
Annealing	50^{o}C	30 seconds
Elongation	72^{o}C	2 minutes
Final elongation	72^{o}C	7 minutes
Dwelling temperature	4^{o}C	

The obtained PCR products were stored at -4°C.

3.8.9 Plasmid DNA isolation (alkali lysis method) and insert amplification

Reagents

Solution I: 50mM glucose + 10 mM EDTA + 25mM tris-HCl (pH 8.0)

Solution II: 0.2 M NaOH and 1% SDS

Solution III**:** 60 ml of 5M potassium acetate + 11.5 ml of glacial acetic acid + 28.5 ml of distilled water.

Procedure

- Prepared 10 ml Luria Broth in a test tube and sterilized using autoclave.
- Once the media got cooled, added ampicillin (100µg/l) to the media.
- Inoculated single bacterial colony in the test tube and incubated at 37^{o}C for 16-24 hours.

- After that, took 5 ml of culture and centrifuged at 8000 rpm for 5 minutes to pellet down the cell culture.
- Added 200 µl of ice-cold solution I and mixed using vortex (to ensure that bacterial pellet was completely dispersed in the solution).
- Allowed it to stand at room temperature for 3 minutes.
- Added 400µl of freshly prepared solution II and mixed by inversion.
- Kept the tubes on ice for 10 minutes.
- Added 300 µl of ice-cold solution III and invert mixed gently.
- Placed the tubes on ice for 10 more minutes.
- Centrifuged the contents at 12000 rpm for 10 minutes at 4^{o}C.
- Transferred the supernatant to a fresh tube and added equal volume of phenol: chloroform: isoamyl alcohol (25:24:1).
- The suspension was then again centrifuged at 10,000 rpm for 20 minutes.
- Upper aqueous layer was then transferred to a fresh tube and equal volume of isopropanol was added to it.
- Gently inverted the tubes few times and allowed them to stand for 10 minutes at room temperature.
- Centrifuged the contents at 8000 rpm for 10 minutes and discarded the supernatant.
- Added 300µl of 70% ethanol to the cell pellet for washing and centrifuged at 8000 rpm for 5 minutes.
- Decanted the supernatant and allowed the pellet to air-dry.
- Suspended the obtained pellet in 30 µl of MQ water.
- After plasmid isolation, again colony PCR was performed and the samples were checked on agarose gel (1.0% w/v) electrophoresis.

3.9 16S rDNA Sequencing

The 16S rDNA inserts were sequenced for both strands using M13 forward and reverse primers, used for pMD20T vectors. The sequence was generated by using an applied biosystems automatic sequencer (DNA sequencing facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

3.9.1 Analysis of Sequenced Data

The 16s DNA gene sequences of isolates were compared with those available in EZ taxonomy. The strains with closely related sequences were aligned by using Multalin. Phylogenetic tree was constructed by maximum parsimony method by using MEGA-7 program (Tamura et al. 2011).

3.10 Physico-chemical characteristics of soil

3.10.1 Determination of soil pH

- 2g soil sample was weighed and taken in 100ml beaker.
- 50ml of distilled water was added and mixed thoroughly using glass rod.
- Sample was mixed for 2-5 minutes manually for proper mixing.
- Then, suspension was allowed to settle down for 5 minutes.
- pH was determined by immersing the electrode in supernatant solution and reading was recorded when stabilized (usually after 30 sec).
- The experiment was repeated 3 times.

3.10.2 Available phosphorus (P)

Available phosphorus in the seleniferous soil was determined according to Olsen et al. (1954)

Reagents

- 0.5 M $NaHCO_3$ extracting solution: 42 g of sodium bicarbonate was added in distilled water and final volume was made up to 1 litre. The pH was adjusted to 8.5 with the help of 1N NaOH.
- Sulphuric acid (2.5 M): 140 ml of conc. H_2So_4 was diluted with 1 litre of distilled water.
- Reagent A**:** 12 g of ammonium molybdate was added to 250 ml of distilled water and 0.2908 g of antimony potassium was added to 100 ml of distilled water. Both the solutions were added to

1000 ml of 2.5 M of sulphuric acid, mixed thoroughly and the volume was made up to 2 litre with distilled water.

- Reagent B (freshly prepared): 1.058 g of ascorbic acid was added to 200 ml of reagent A and mixed thoroughly.
- Stock standard P solution (50 ppm)**:** 0.2917 g of KH_2PO_4 was dissolved in 1 litre of distilled water.
- Working standard P solution (1 ppm): 20 ml of stock was diluted to 1 litre.

Procedure

- 2.5 g of soil sample was weighed and 50 ml of extracting solution was added to it.
- The suspension was kept on shaker for 30 minutes followed by filtration with the help of whattman filter paper no. 42
- 10 ml of filtrate was taken in a beaker and 1 ml of 2.5 M H_2SO_4, 15.5 ml distilled water, 8 ml of reagent B and again 15.5 ml of distilled water were added.
- After 10 minutes, O.D. of the sample was measured at 882 nm against blank.
- Blank was prepared as above but does not contain soil.
- To prepare standard curve, 0, 2, 5, 10, 15, 20 ml of 1 ppm working standard solution was added. Added 10 ml of extracting solution, 1 ml of 2.5 M H_2SO_4, 8 ml of reagent B and the final volume was made upto 50 ml with the help of distilled water. The P concentrations of these solutions were 0.4 ppm, 0.1 ppm, 0.2 ppm, 0.3 ppm and 0.4 ppm respectively. After 10 minutes, O.D. of these solutions was taken at 882 nm.

3.10.3 Total phosphorus (Kitson and Melon, 1944)

(Vandomolybdophosphoric Yellow color method)

Principle

Ammonium molybdate reacts with acidic soil and forms a heteropoly acid and molybdophosphoric acid. Yellow color vanadomolybdophosphoric acid is formed. Intensity of yellow colour is directly proportional to phosphate concentration.

Reagents

- Vanadomolybdate solution
  - Solution A- 25g ammonium molybdate [$((NH_4)_6Mo_7O_{24}.4H_2O$] was dissolved in 300ml distilled water in 500ml beaker.

- Solution B- 1.25 g ammonium (meta) vandate (NH_4VO_3) was dissolved in 300ml boiling water. Cooled, added 250 ml concentrated HNO_3 and cooled again. Then solution A was added to solution B and final volume was made up to 1000ml in volumetric flask resulting in final vanadomolybdate solution.

➢ Phosphorus stock standard solution (50 mg/I P)

Dried KH_2PO_4 was dissolved in distilled water and mixed thoroughly. Acidifed with 7N H_2SO_4 and volume was made 1 litre to get 50 mg/I P solution. Toulene was added to prevent the microbial activity.

Procedure

- 10 g acid digested soil sample was taken in 50 ml volumetric flask. Added 10 ml of vanadomolybdate solution and diluted to 50 ml.
- Mixed properly and phosphorus concentration was noted on spectrophotometer after 10 minutes at 420 nm.
- Working concentration of 0, 1, 2, 3, 4 and 5 ml from per litre stock solution in 50 ml volumetric flasks and developed the yellow color mentioned above.
- Spectrophotometer was calibrated with known standard phosphorus concentration and calculated for sample.

Calculations

$$P\ (mg/kg) = \frac{\text{Vol make up after digestion}}{\text{vol of digest used to develop color (ml)}} \times \frac{50 \times P\ (mg/l)}{\text{weight of sample (g)}}$$

3.10.4 Organic carbon

Total organic carbon (%) was estimated as per the protocol given by Walkley and Black, 1934.

Reagents

- 1 N potassium dichromate ($K_2Cr_2O_7$): 49.04 g of $K_2Cr_2O_7$ was dissolved in distilled water and final volume was made up to 1 litre.
- 0.5 N ferrous ammonium sulfate (freshly prepared): Dissolved 198 g of ferrous ammonium sulfate in distilled water and final volume was made upto 1 litre.
- Di phenyl amine indicator (DPA): Dissolved 0.5 g of DPA in the mixture of 20 ml water and 100 ml conc. H_2SO_4.
- Concentrated sulphuric acid

Procedure

- Weighed 1 g of soil sample and added 10 ml of 1N $K_2Cr_2O_7$.
- The mixture was swirled to assure the proper mixing of the soil and reagent.
- 20 ml of concentrated H_2SO_4 was added and the flask containing the mixture was left undisturbed for 30 minutes.
- Added 200 ml of distilled water and 1 ml of di phenyl amine indicator to it.
- The contents were titrated with 0.5 N ferrous ammonium sulfate (freshly prepared) till the end point is observed from blue violet to green.
- The blank was also prepared as above but does not contain the soil.

Calculations

$$\text{Organic carbon (\%)} = \frac{10\,(B - T) \times 0.003 \times 100}{B \times \text{weight of soil (g)}}$$

Where,

- B is volume of ferrous ammonium sulfate solution required for blank titration
- T is volume of ferrous ammonium sulfate solution required for soil sample
- Because organic matter contains 58% carbon, so
- Organic matter (%) = Organic carbon (%) × 1.724 (van Bemnelen factor)

3.10.5 Total nitrogen

Total nitrogen in soil sample was estimated by Kjeldahl method by Piper (1960).

Reagents

- Anhydrous sodium sulfate
- 0.5 gm Copper sulfate (catalyst)
- Concentrated sulphuric acid
- 0.02 N Sulphuric acid: 0.272 ml of concentrated sulphuric acid in 500 ml.
- Boric acid solution: 20g of boric acid dissolved in 1000 ml distilled water.
- Mixed indicator solution: 0.066 g of methyl red and 0.099 g of bromo cresol green was dissolved in 100 ml distilled water.

- 0.25 N standard sodium hydroxide solution: 13.201 ml of NaOH solution in 200 ml distilled water and final volume made upto 1 litre.
- Digestion mixture: 10 g sodium sulfate, 0.5 gm copper sulfate, 30 ml concentrated H_2SO_4.

Procedure

- 2g of soil sample was taken in kjeldahl flask, 0.5 gm copper sulfate was added in 30 ml H_2SO_4 and after digestion 50 ml water was added.
- Flask was kept in digestion chamber at 100 C˚ for 2-3 hours.
- Color changed was monitored from dark brown to greenish white, contents were cooled and 50 ml distilled water was added.
- Boric acid was taken in a beaker into which tubes were dipped.
- Glass beads were added in distillation flask containing digested sample.
- The volume of liquid in the distillation flask should not exceed half the capacity of flask to prevent the frothing.
- Sodium hydroxide was added to the distillation flask through the open end of the condenser attachment and stoppered. Water flow was maintained through the condenser.
- Condenser should be arranged to dip the tube in the beaker containing 50 ml boric acid.
- Distillate one third of the total volume of the solution in the flask.
- Assembly was cooled and dismantled.
- Distillate was collected through receiver tube containing 15 ml boric acid and 2-3 drops of mixed indicator was added.
- The distillate containing ammonia was titrated against sulphuric acid till grass green color changes to steel grey.

Calculations

$$\text{Nitrogen (\% by mass)} = \frac{1.4 \times V \times N}{W}$$

Where,

- V is the volume in ml of standard sulphuric acid used in titration.
- N is the normality of standard sulphuric acid.
- W is the mass in g of sample taken for the test.

Results

4.1 Collection of soil sample

Seleniferous soil samples were collected from Jainpur village in Nawanshahr region of Punjab. The collected sample was rhizospheric soil of wheat (*Triticum aestivum).* It was used for the isolation of selenium tolerant and plant growth promoting bacterial isolates. Figure 4.1 shows the site of sample collection

Fig 4.1 Site of sample collection i.e. seleniferous soil of Jainpur (village of Nawanshahr)

4.2 Estimation of selenium

Selenium in the soil sample was estimated with the help of fluorescence spectrometry (Perkin Elmer). It is the simple and fastest method. It is usually used for the determination of concentration of compound in the solution. In this, both emission as well as excitation spectra are used. With the increase in the concentration of compound, intensity of emission also increases.

Selenite reacts with DAN (dye) to form a fluorescent complex called piazselenol whose absorbance can be measured at 520 nm. Selenium concentration is determined through a standard curve.

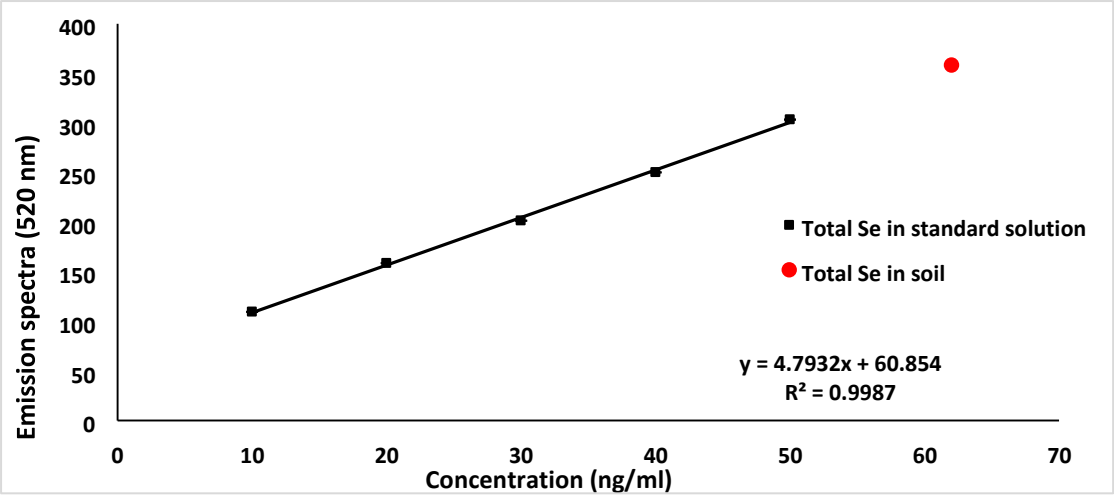
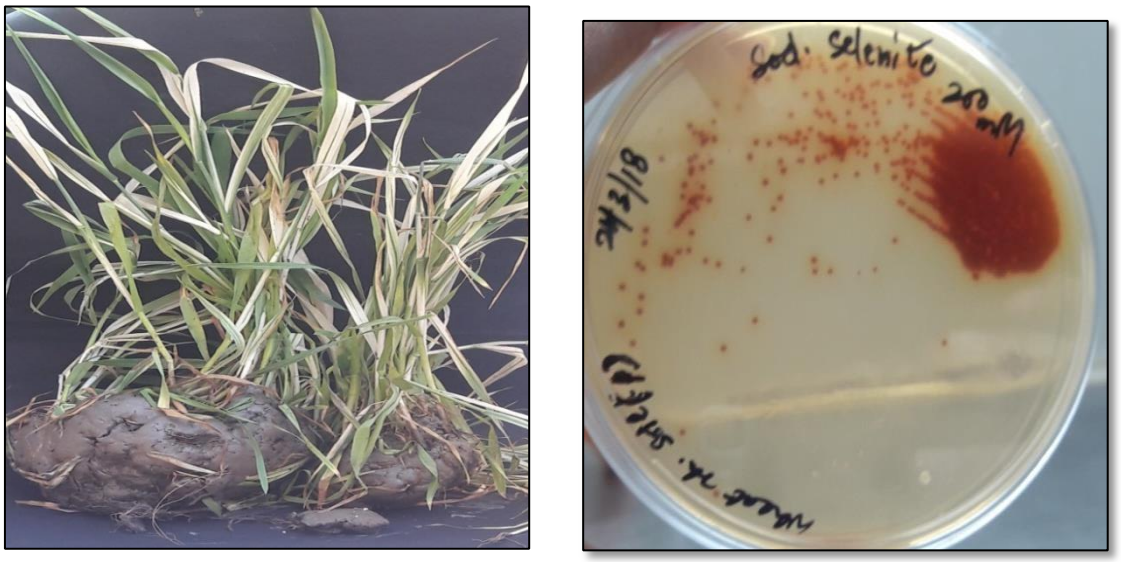


Fig 4.2 Linear graph showing different standards (seleniferous soil sample) at different concentrations

The selenium content in the collected soil sample came out to be 5 mg/kg.

4.3 Isolation of selenium-tolerant bacteria

Tryptone soya broth (TSB) was used for the isolation of selenium-tolerant bacteria. Enrichment followed by serial dilution till 10^{-6} was done and the final dilution was spread on the tryptone soya agar (TSA) plates supplemented with sodium selenite. In order to get pure isolates, streaking of the selected bacterial isolates was done (Figure 4.3(b)).



a) b)

Fig 4.3 Isolation of selenium-tolerant bacteria a) Rhizospheric soil sample of wheat b) Pure isolates obtained by streak plate method

Different bacterial isolates were selected according to their morphology i.e. shape, size and elevation and the patches of the same were made with the help of an inoculation loop. The plate was incubated at 37^{o}C for 24 hours and then was transferred to 4^{o}C for further use.

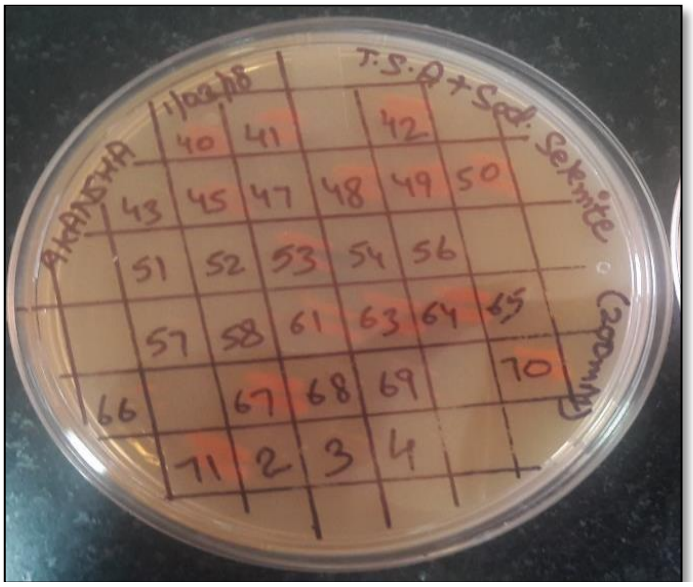


Fig 4.4 Different bacterial isolates growing on selenium amended plate

A total of 71 bacterial isolates were selected based on their morphological characteristics which were further used for determining their selenium tolerance. All the bacteria were designated as SeB

4.4 Selenium tolerance

The tolerance of bacterial isolates was checked by counting the Cfu (colony forming units). The concentrations at which the bacterial isolates were examined are: 50mM, 100mM, 150 mM, 200mM, 250 mM, 300mM, and 400 mM. Randomly 10 isolated bacterial strains were screened for selenium tolerance at different concentrations.

Table 4.1 Tolerance studies of bacterial isolates (log cfu/ml)

S.no	**Bacteria**	**log cfu/ml at different concentrations of sodium selenite**			
		Control	**100 mM**	**200 mM**	**300 mM**
1.	SeB 40	10.89	10.29	9.17	No growth
2.	SeB 42	10.99	9.32	No growth	No growth
3.	SeB 48	10.98	9.71	9.36	No growth
4.	**SeB 49**	**11.14**	**9.77**	**9.68**	**8**
5.	SeB 53	10.81	8.54	9.23	No growth
6.	SeB 56	10.85	8.86	9.68	No growth
7.	SeB 63	10.98	8.94	9.55	No growth
8.	SeB 64	10.87	8.59	9.25	No growth
9.	SeB 70	10.92	8.76	9.22	No growth
10.	**SeB 71**	**11.07**	**9.82**	**9.30**	**8.77**

Since, SeB_{49} and SeB_{71} showed high tolerance (300 mM), they were selected for further analysis and were termed as highly selenium-tolerant bacteria

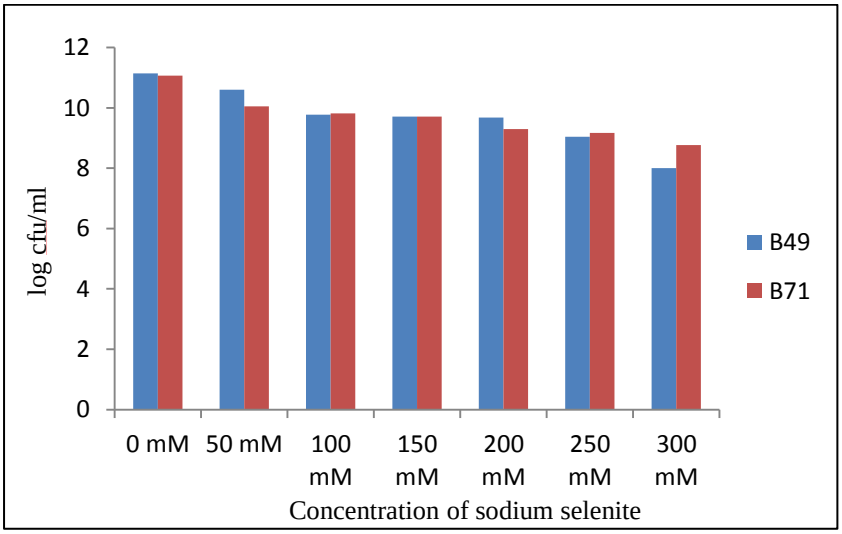


Fig 4.5 Effect of increase in the concentration of selenium on bacterial growth

From the above graphs, it is concluded that with the increase in the concentration, the growth of bacteria decreases. Both the highly-tolerant bacterial isolates (B_{49} and B_{71}) showed selenium tolerance upto 300 mM.

It was observed that with the increase in the concentration of sodium selenite, growth of the bacteria decreases and finally gets arrested at concentration 400 mM. Both the highly tolerant bacteria (B_{49} and B_{71}) were further screened for plant-promoting activities.

4.5 Plant growth promoting activities

4.5.1 IAA production by bacteria

IAA is one of the active auxins which is produced by various microorganisms including bacteria and helps in the plant growth. It is produced by L-tryptophan and helps in plant promotion by producing longer roots and increasing number of root hairs which in turn helps in more nutrient uptake.

IAA production in the bacterial isolates was determined in yeast maltose dextrose broth supplemented with 0.1% tryptophan. After five days of incubation the bacterial culture was centrifuged and 1 ml of the supernatant was taken. 3 ml of salwoski's reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$) was added to 1 ml of the bacterial supernatant and incubated for 30 minutes in dark. The optical density was taken at 530 nm. The pink color of the broth indicated the presence of IAA produced by the bacteria. The pink color is due to the formation of Tris-(indole-3-aceto iron III) complex.

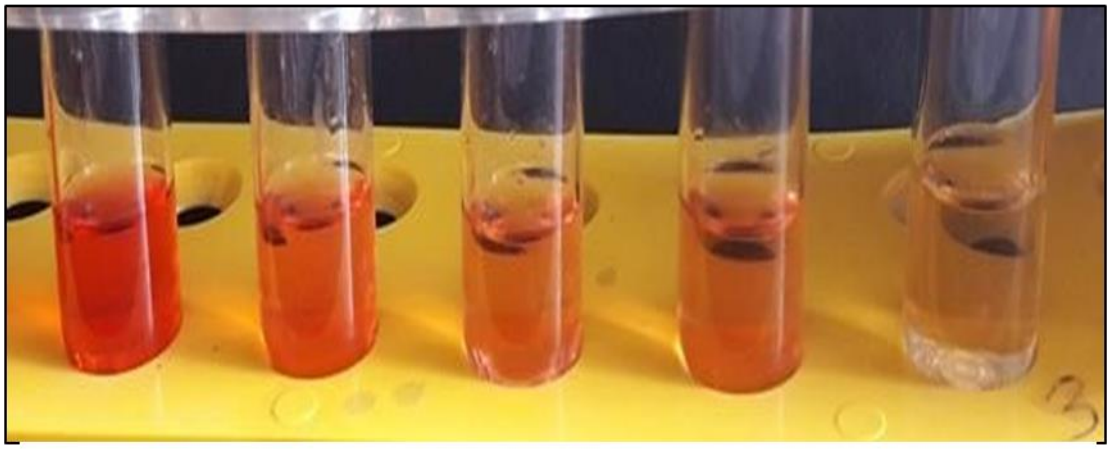


Fig 4.6 Standards of IAA (in µg/ml) a)100 b)50 c)20 d)10 e)0

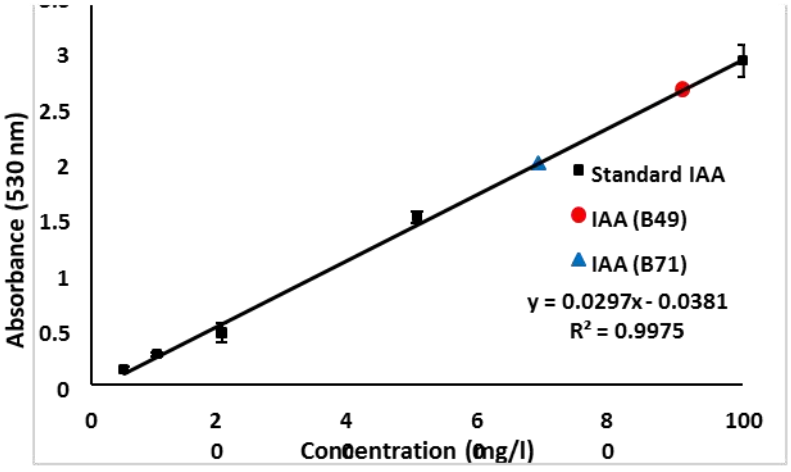


Fig 4.7 Standard curve of IAA production

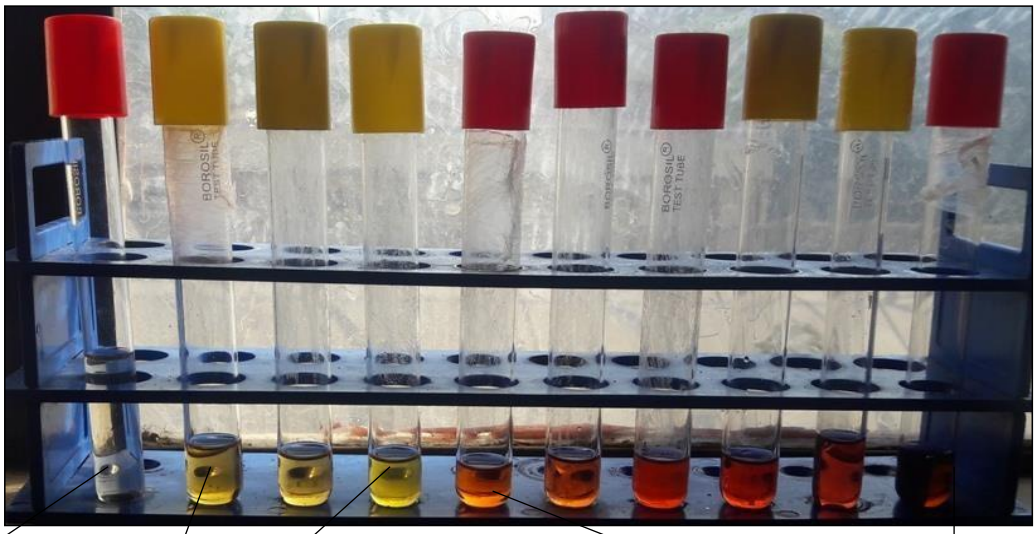


Figure 4.8 Determination of IAA production by highly tolerant bacterial isolates

A total of 10 bacterial isolates were screened for IAA production, out of which only three isolates showed IAA production.

4.3.2. Phosphate solubilization by bacteria

Various microbes have the capability of improving plant's growth by solubilizing the insoluble phosphate into the soluble form. In this process, various processes like production of organic acids, chelation, and acidification are involved which results in the formation of halos.

The phosphate-solubilizing capacity of bacterial isolates was determined by using Pikovskaya agar on which the bacterial isolates were point inoculated and incubated at 37^{o}C for five days.

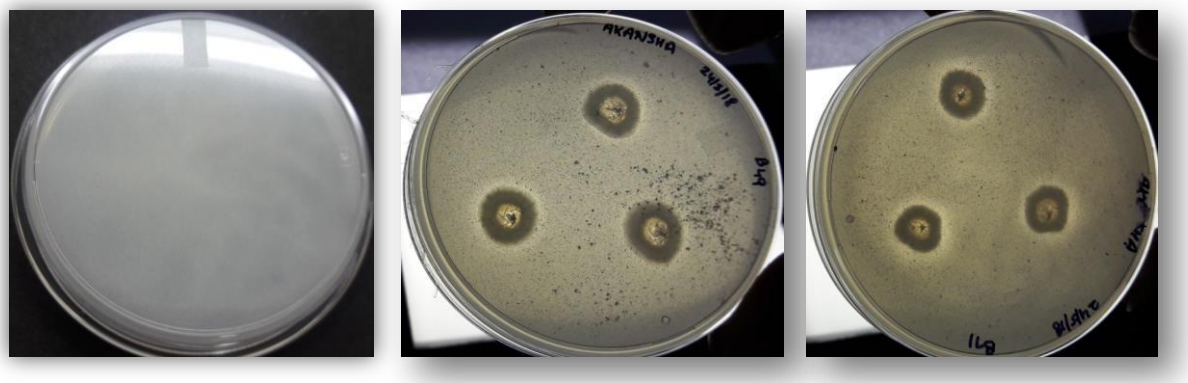
After incubation, the halo zones around the bacteria were measured and the solubilization index (SI) was calculated by using the following formula:

$$\text{Solubilization Index (SI)} = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

Again, ten isolates were selected for the screening of phosphate solubilization, out of which eight bacterial isolates were able to solubilize insoluble phosphate into the soluble form.

Table 4.2 Solubilization index of bacterial isolates

Bacterial isolates	Solubilization index (SI) (mm)
B_{40}	1.11
B_{42}	1.19
B_{48}	1.23
B_{49}	**1.26**
B_{53}	No formation of halo zone
B_{56}	1.21
B_{63}	1.0
B_{64}	1.16
B_{70}	No formation of halo zone
B_{71}	**1.39**



(a) (b) (c)

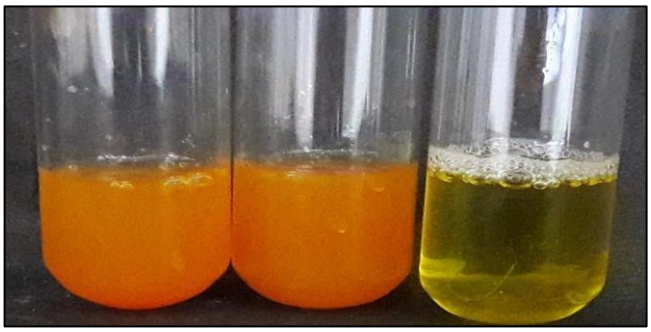
Fig 4.9 Phosphate solubilization by bacterial isolates a) Control b) B_{49} and c) B_{71}

4.3.3 Production of ammonia by bacteria

Ammonia production of bacterial isolates was determined in peptone water. The isolates were inoculated in peptone water and incubated for 48-72 hours. Nessler's reagent was added the bacterial culture and the production of ammonia was examined by the change in color.

Nessler's reagent is usually prepared by adding potassium iodide and mercuric chloride which is alkaline in nature. When Nessler's reagent react with ammonia fumes, the color of the media changes.

Ten different bacterial isolates were screened for ammonia production, out of which only two were able to produce ammonia.



a) b) c)

Fig 4.10 Production of ammonia by bacterial isolates a) and b) Ammonia production by B_{49} and B_{71} and c) Control

4.3.4 HCN production by bacteria

HCN production in bacterial isolates was checked by preparing the nutrient agar plates amended with glycine. The plates were inoculated with bacteria and the whatman paper dipped in 2% w/v sodium carbonate and 0.5% picric acid was placed inside the lid of the petriplate. The plates were observed after 3-4 days after incubation. The change in the color of the strip from yellow to red ensures the production of HCN by bacteria. HCN is highly toxic for plant pathogens and thus acts as a biocontrol agent and helps in plant growth promotion. Many plants have the ability to produce cyanogenic glycosides which release cyanohydric acid (HCN) on hydrolysis. The presence of these cyanogenic glycosides can be determined by using filter paper strips impregnated with picric acid. All the ten bacterial isolates were screened for HCN production but none of them was able to produce cyanogenic glycosides.

(a) (b) (c)

Fig 4.11 HCN production by bacterial isolates a) Control plate, b) & c) Negative reaction by both bacterial isolates B_{49} and B_{71}

4.4 Bioaccumulation of selenium in bacteria

Both the bacterial isolates (B_{49} and B_{71}) were grown overnight in tryptone soya broth amended with sodium selenite. Three concentrations i.e. 100 mM, 200 mM and 300 mM of media amended with sodium selenite were used. The culture was centrifuged at 8,000 rpm for 10 minutes to obtain the bacterial pellet which was allowed to dry till whole of the liquid gets evaporated. The pellet was suspended in distilled water which was further digested in a microdigestor processor. The digested product was further processed for estimating the amount of selenium accumulated by bacterial cells through fluorescence spectrometry.

Table 4.3 Selenium accumulation by bacterial isolates

Concentrations	**B_{49} (µg/mg)**	**B_{71} (µg/mg)**
100 mM	655.8± 0.8	613.0 ± 0.2
200 mM	407.7± 0.3	327.5 ± 0.4
300 mM	195.5 ± 0.4	76.0 ± 0.1

Table 4.4 Two way ANOVA analysis

	Sum of squares	**Degree of freedom**	**Mean square**	**F (DF_n,DF_d)**	**P value**
Interaction	4416	2	2208	F(2,12)=0.42	P=0.6610
Bacterial isolates	747157	2	373579	F(2,12)=72.52	P<0.0001
Concentration	29384	1	29384	F(1,12)=5.704	P=0.0342
Residual	61819	12	5152	-	-

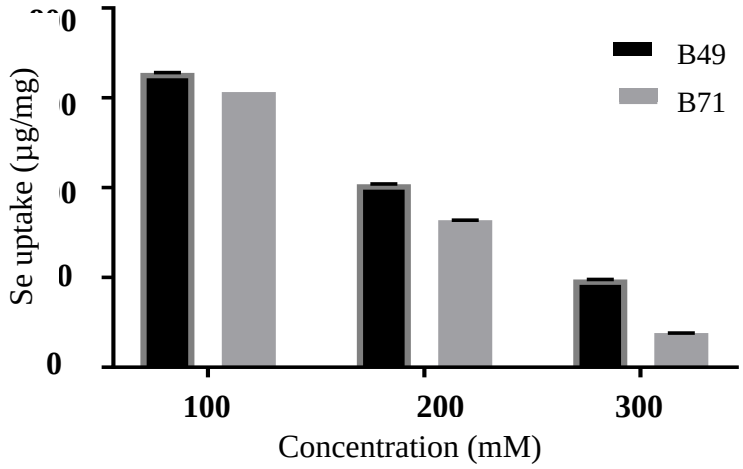


Fig 4.12 Selenium uptake by bacterial isolates at different concentrations

4.6 Bioaccumulation of selenium in plants

Table 4.5 Effect on root biomass inoculated with bacterial isolates (B_{49} and B_{71})

Treatments	Dried root biomass (mg)
Control	6.1±0.1
B_{49}	7.5±0.3
B_{71}	11.6±0.2

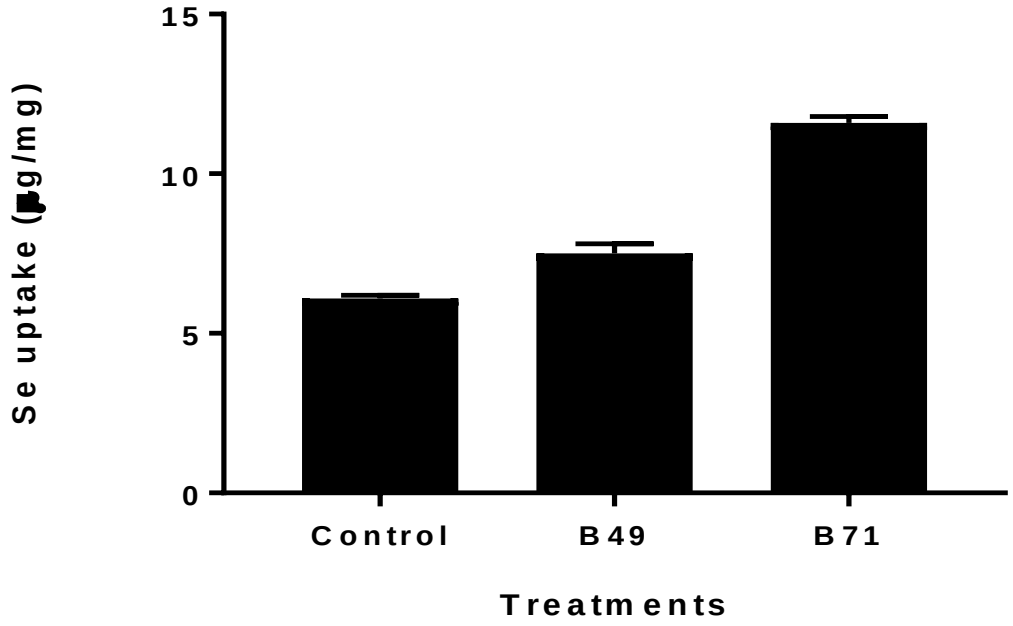


Fig 4.13 Effect of bacterial isolates (B_{49} and B_{71}) on root biomass

Table 4.6 Effect on shoot biomass inoculated with bacterial isolates (B_{49} and B_{71})

Treatments	Shoot biomass (mg)
Control	4.2±0.1
B_{49}	4.6±0.5
B_{71}	8.6±0.5

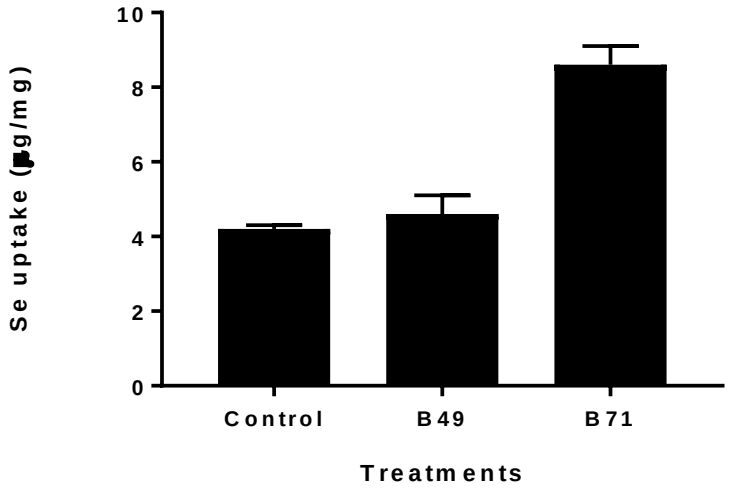


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Fig 4.14 Effect of selenium uptake by shoots when treated with bacterial isolates

4.4 Plant growth promotion by bacterial isolates (nursery experiment)

4.4.1 Nursery experiment

In order to check the in vivo plant-growth promoting activities of bacterial isolates, a nursery experiment was conducted in which the maize plants were grown in the plastic cups containing the seleniferous soil brought from the village of Jainpur (Nawanshahr). The experiment was conducted in TIFAC-CORE, Thapar Institute of Engineering and Technology, Patiala. In the cups, the holes were made for allowing the proper aeration and the removal of excess water.

4.4.2 Inoculation of seeds

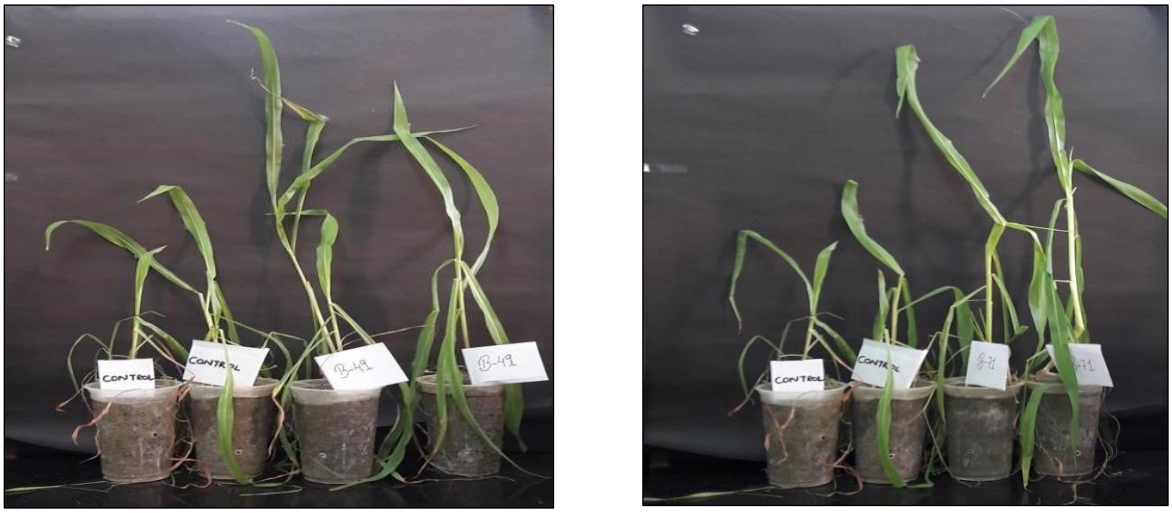
In order to carry out the field experiment, two bacterial isolates (B_{49} and B_{71}) with high tolerance and maximum number of plant-growth promoting activities were selected. The maize seeds were mixed with bacterial culture in 10% sugar and 40% gum arabic which were allowed to dry overnight.

4.4.3 Experimental nursery preparation

The seleniferous soil was filled in the cups and 2-3 seeds with inoculum were sowed in each cup

The experiment was done in ten replicas. The experiment consisted of:

- Soil with seeds but no inoculum (Control)
- Soil with seeds coated with B_{49} inoculum
- Soil with seeds coated with B_{71} inoculum



a) b)

Fig 4.15 Comparison of two months old plants with control plants. a) Control plants compared with the plants containing B_{49} b) Comparison of control plants with B_{71} plants .

4.4.4 Analysis of maize plant after harvesting

The maize plants were harvested after two months and the following parameters were calculated:

- Whole plant length
- Shoot length
- Root length
- Dry weight of shoot
- Dry weight of root

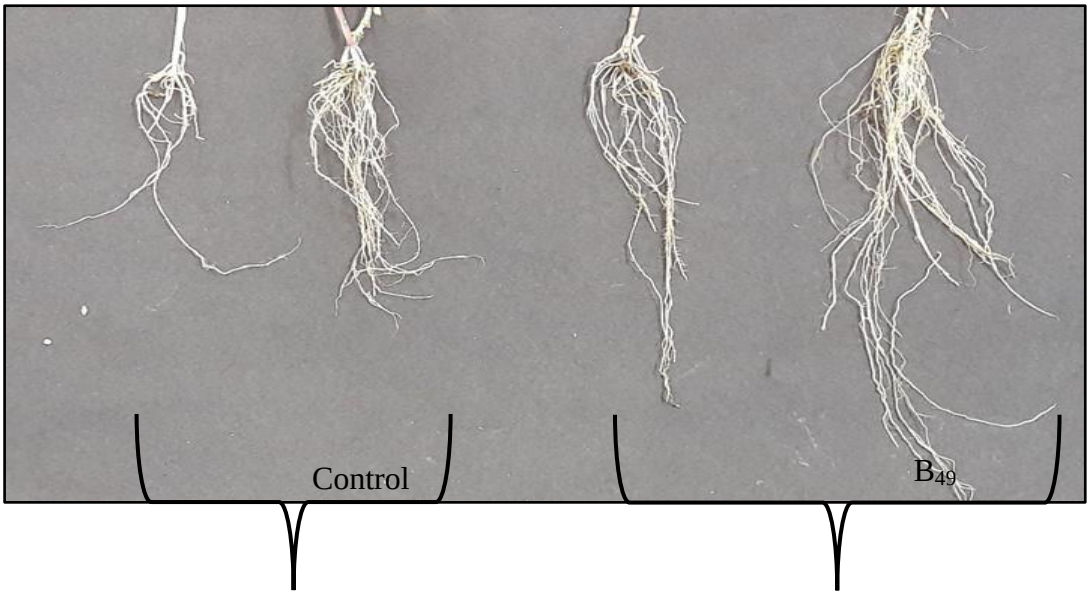


Fig 4.16 Comparison of roots of control plants with the plants containing bacterial isolate (B_{49})

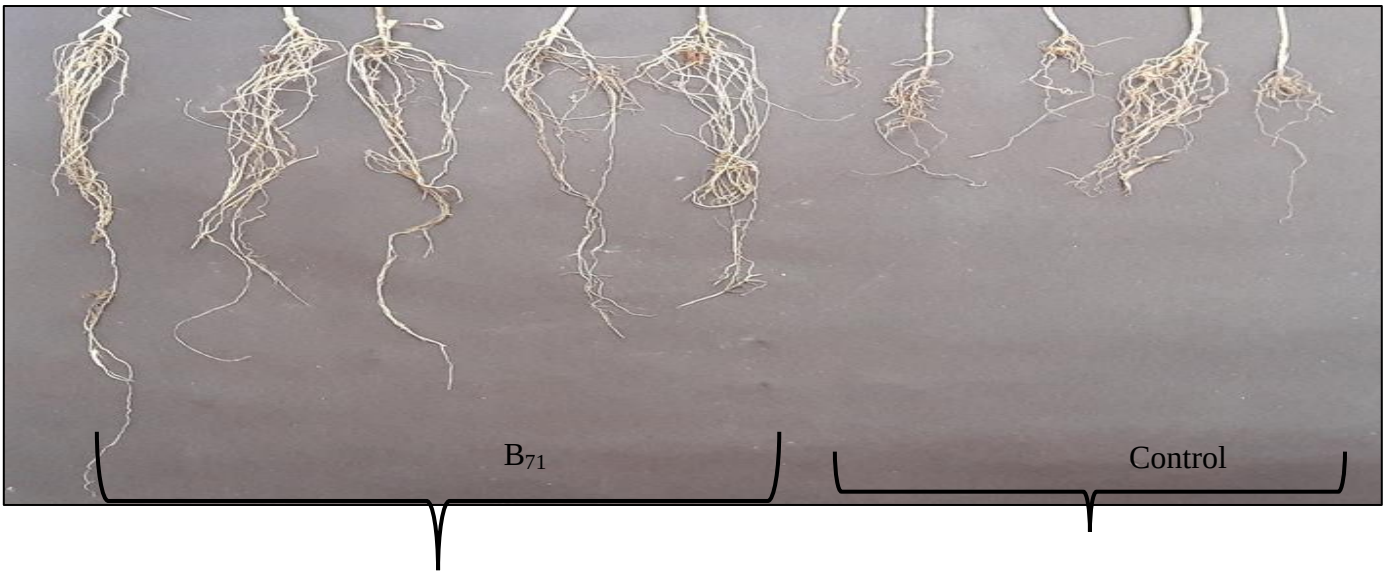


Fig 4.17 Comparison of roots of control plants with the plants containing bacterial isolates (B_{71})

Table 4.7 Effect on various plant tissues inoculated with bacterial isolates (B_{49} and B_{71})

Treatments	Shoot height (cm)	Length of roots (cm)	Dry weight of shoots (mg)	Dry weight of roots (mg)
Control	17.1± 0.03c	13.0±0.05c	505 ± 0.03c	108± 0.07c
B49	21.4± 0.09b	21.42± 0.05b	623 ± 0.05b	202± 0.05b
B71	27.1± 0.04a	31.4± 0.07a	961± 0.08a	230 ± 0.01a

4.5 Molecular methods for the identification of selenium-tolerant bacteria

4.5.1 Isolation of genomic DNA of bacterial isolates

The quality of genomic DNA was determined by agarose gel electrophoresis. This technique is commonly used to separate DNA, RNA and proteins. 0.8% gel was made for visualizing the DNA bands and the gel was allowed to run for 60 minutes. After which it was visualized in Gel doc, under UV light.

For the better and sharp visualization of DNA bands, usually 8% agarose gel is preferred. It is so because with the increase in the concentration of agarose, pore size of the gel decreases which makes the DNA fragments harder to move. During this technique, ethidium bromide (florescent stain) is used which binds with the DNA molecules and fluoresces in the presence of UV light.

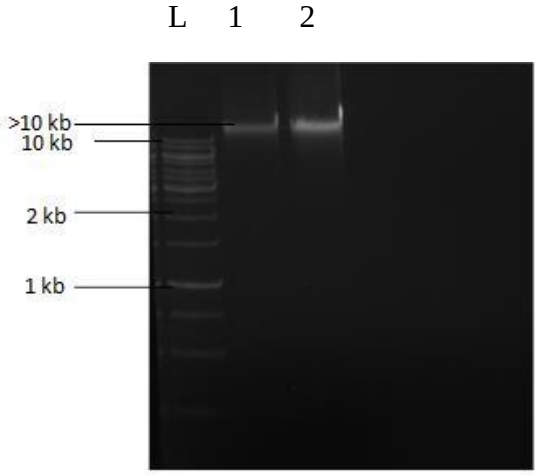


Fig 4.18 Bands showing genomic DNA of bacterial isolates. Lane 1- Ladder, Lane 2- B_{49} and Lane 3- B_{71}

4.5.2 Quantitative analysis of isolated DNA

The concentration of an isolated DNA was determined by using nanodrop. It also gives the purity of the product by giving 260/280 nm ratio. The absorbance is taken at 260/280 because nucleic acids i.e. DNA and RNA absorb at this region while proteins absorb at 280 nm. The results of the nanodrop are as follows:

Table 4.8 Quantitative estimation of isolated DNA

Name of the sample	Concentration (ng/µl)	Absorbance 260/280
B_{49}	41.2	1.85
B_{71}	76.7	1.80

From the above results (260/280) ratio, it is clear that the DNA of B_{71} and B49 is of high quality.

4.5.3 Amplification of 16S rDNA

Since the concentration of an isolated DNA is not enough for the ligation, the 16s rDNA region of DNA was amplified by using PCR (polymerase chain reaction). After the completion of PCR, numbers of DNA copies were obtained and therefore the concentration also increased.

Since 16s rDNA is a highly conserved region in prokaryotes, it is used for the identification and classification of bacteria. Therefore, it is also known as a reliable molecular clock.

After the amplification, the amplified samples were made to run on agarose gel (0.8%) and the size of the bands was determined by comparing it with ladder

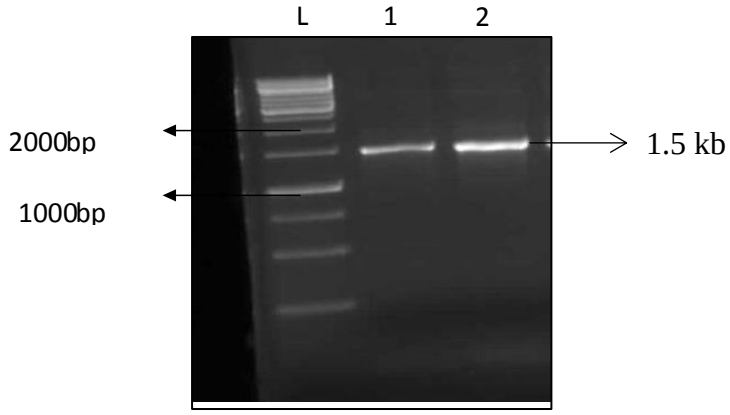


Fig 4.19 Amplified DNA bands of bacterial isolates. Lane 1: Ladder (1 kb) Lane 2: B_{49} and Lane 3: B_{71}

4.5.4 Purification of amplified product

Since the extracted DNA contains many impurities like RNA and proteins, purification of the amplified product was done to get the pure DNA. QIAquick PCR purification kit was used for the easy and efficient purification of the samples and again the reading was taken at nanodrop to get the purification status.

The absorbance at 260/280 came out to be 1.8 for both the samples which ensured that the DNA is free from RNA and protein contamination.

4.5.5 TA Cloning and ligation of the PCR amplified product

The PCR-amplified products contain 3'–A overhangs while the T-vector used in cloning adds dT at the 3' end. As a result of which the insert was allowed to ligate in the vector more efficiently and easily. Ligation is the joining of nucleic acids to form recombinant DNA which is inserted into a plasmid (pMD20-T).

4.5.6 Genetic transformation and blue-white screening

Genetic transformation is the uptake and incorporation of free DNA by bacterial cells.

The ligated product was transformed in the competent cells of Escherichia coli DH 10β and the resultant was plated on the LA + ampicillin plates. The plates were incubated for 16 hours at 37^{o}C. The plates contained blue and white colonies. Blue colonies represented the untransformed cells while the white colonies depicted the transformed cells. The colony PCR of these transformed (white) colonies was done to ensure that the picked colonies contain plasmid with a desired insert.

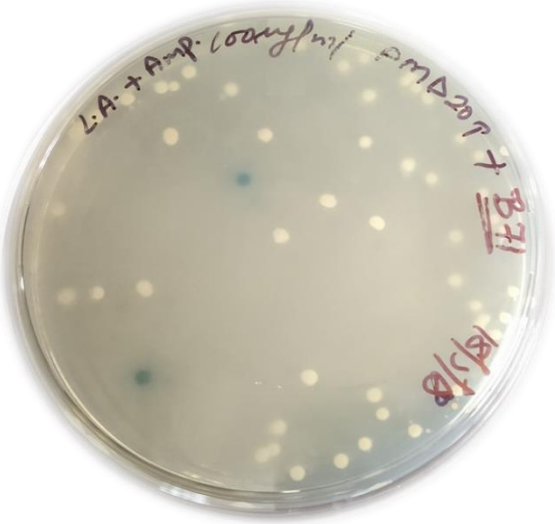


Fig 4.20 Plate showing blue (untransformed) and white (transformed) cells**.**

4.5.7 Plamid isolation and insert amplification

Plasmid DNA was isolated by alkali-lysis method. The bacteria of interest was first grown in LB and then the cells were lysed by using an anionic detergent, sodium dodecyl sulfate (SDS). After the disruption of cells, potassium acetate was used which only the plasmid DNA to reanneal and stay solubilized.

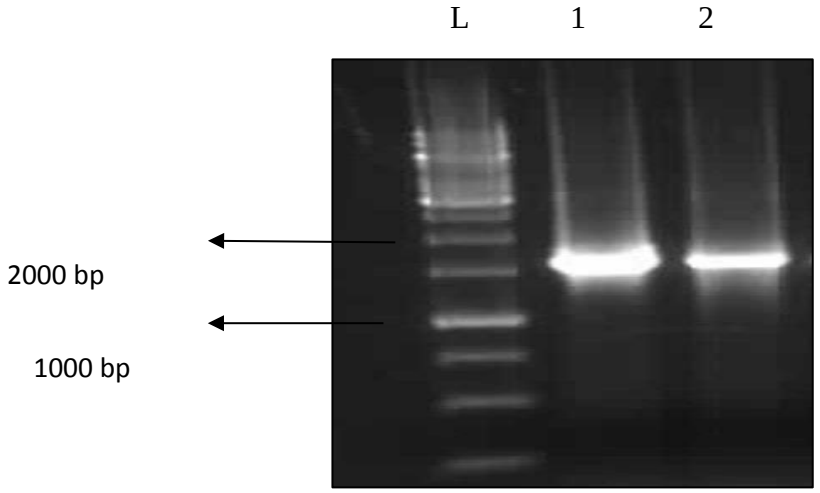


Fig 4.21 Plasmid DNA of bacterial isolates. L-Ladder, Lane 1: B_{49} and Lane 2: B_{71}

4.6 16S rDNA sequencing

After the plasmid isolation, bacterial isolates containing plasmids (16 Se B_{49}, 16 Se B_{71}) were sent for sequencing to DNA sequencing facility, Department of Biochemistry, New Delhi, India.

The results obtained after sequencing are as follows:

B_{49}

CTCGGTACCCGGGGATCCGATTAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGG
CAGGCCTAACACATGCAAGTCGAGCGGTAGCACAGGGAGCTTGCTCCTGGGTGACG
AGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTAC
TGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGG
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TCAGCGGGGAGGAAGATGTTGAGGTTAATAACCTCAGCAATTGACGTTACCCGCAG
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AAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTA
GAGGGGGGTAGAATTCCAGGTGTAGCGGTGAATGCGTAGAGATCTGGAGGAATACC

GGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGA
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AGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAG
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CTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAAC
TGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACAC
ACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTC
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CCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGG
GAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTG

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Leclercia adecarboxylata isolate PSB8 16S ribosomal RNA gene, partial sequence	2719	2719	98%	0.0	99%	HQ242721.1
Leclercia adecarboxylata isolate PSB10 16S ribosomal RNA gene, partial sequence	2713	2713	98%	0.0	99%	HQ242723.1
Uncultured bacterium clone bb2s1 16S ribosomal RNA gene, partial sequence	2700	2700	98%	0.0	99%	DQ068877.1
Enterobacter sp. E3-5 16S ribosomal RNA gene, partial sequence	2699	2699	98%	0.0	99%	KP058389.1
Enterobacter sp. B7(2013) 16S ribosomal RNA gene, partial sequence	2699	2699	98%	0.0	99%	KF010358.1
Enterobacter cloacae complex sp. ECNIH7, complete genome	2697	21145	98%	0.0	99%	CP017990.1
Enterobacter asburiae strain ATCC 35953, complete sequence	2697	20970	98%	0.0	99%	CP011863.1
Enterobacter ludwigii strain FGC63 16S ribosomal RNA gene, partial sequence	2697	2697	98%	0.0	99%	KF358445.1
Bacterium HLB-1 16S ribosomal RNA gene, partial sequence	2697	2697	98%	0.0	99%	GU451180.1
Endophytic bacterium HB02 16S ribosomal RNA gene, partial sequence	2697	2697	98%	0.0	99%	FJ205659.1
Endophytic bacterium HA04 16S ribosomal RNA gene, partial sequence	2697	2697	98%	0.0	99%	FJ205656.1
Enterobacter sp. B13(2013) 16S ribosomal RNA gene, partial sequence	2693	2693	98%	0.0	99%	KF010360.1
Pantoea sp. y2 16S ribosomal RNA gene, partial sequence	2693	2693	98%	0.0	99%	GQ395336.1
Enterobacter cloacae complex 'Hoffmann cluster IV' strain DSM 16690, complete genome	2691	21129	98%	0.0	99%	CP017184.1
Enterobacter asburiae strain ITM L4311 16S ribosomal RNA gene, partial sequence	2691	2691	98%	0.0	99%	KC568144.1

Fig 4.22 BLASTN showing associated taxa of query sequence

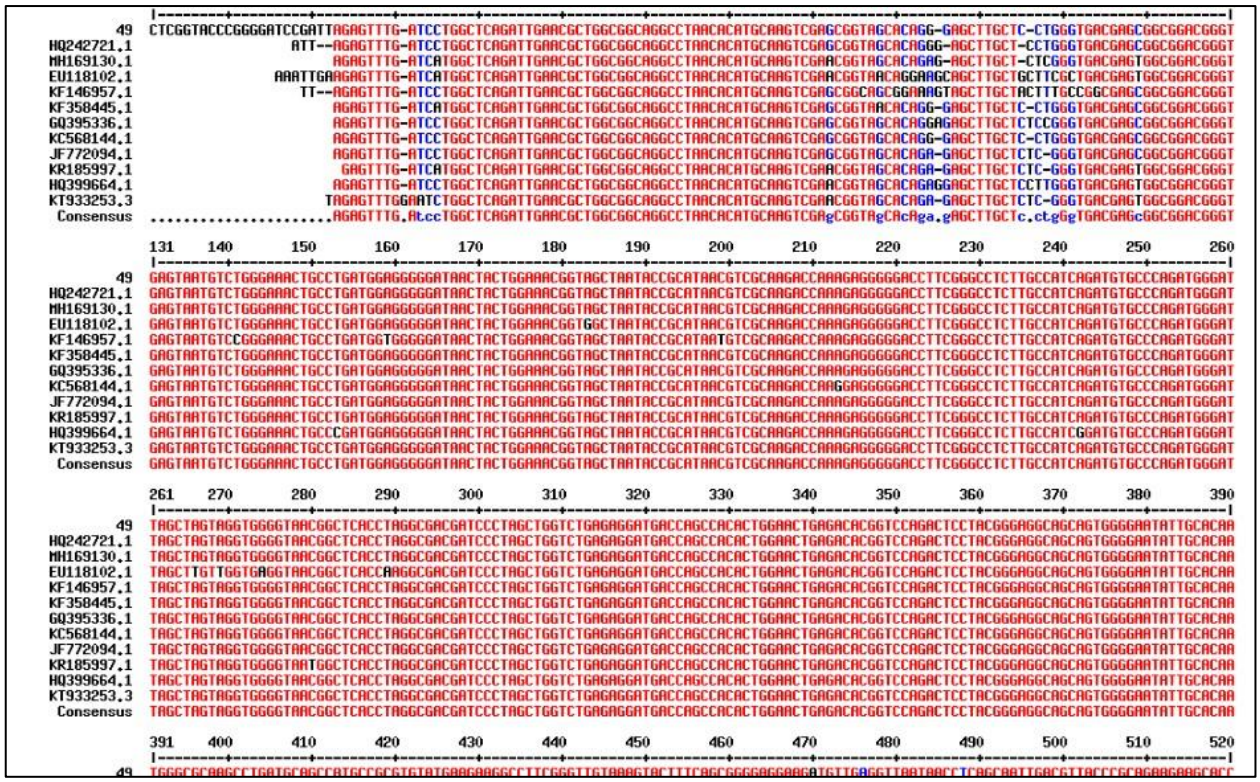

Figure 4.23 MultAlin showing multiple sequence alignment

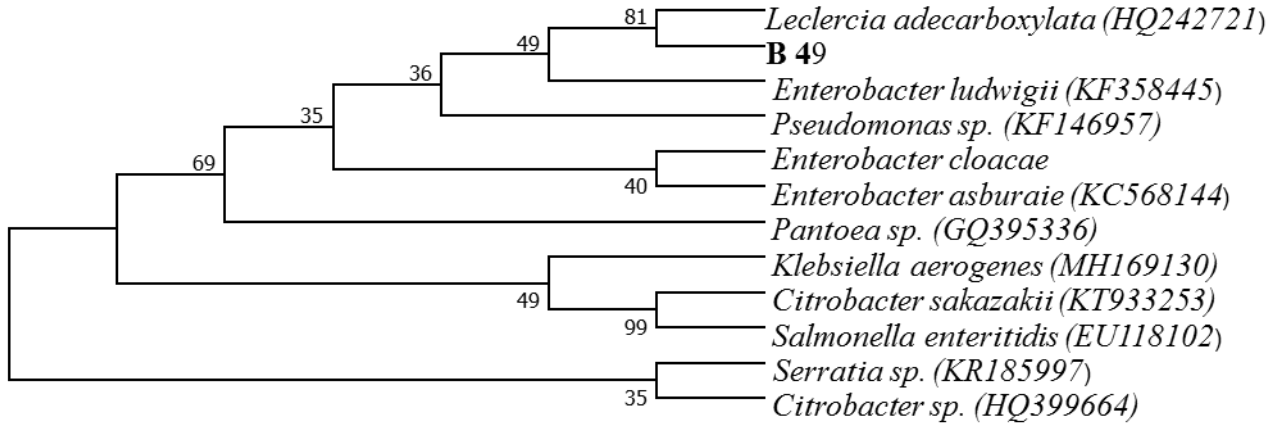

Fig 4.24 Phylogenetic tree showing relatedness with the query sequence

>16s71

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ATGAACCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGG
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GGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGG
TTGTAAAGTACTTTCAGCGAGGAGGAAGGCATTAAGGTTAATAACCTTAGTGATTGACGTTACTCGC
AGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGG
AATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCGGATGTGAAATCCCCGGGCTCAACC
TGGGAACTGCATTCGAAACTGGCAAGCTTGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCG
GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACG
CTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
TCGACTTGGAGGTTGTGCCCTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCACCGCCTGGGGA
GTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATTGGTT
AATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGG
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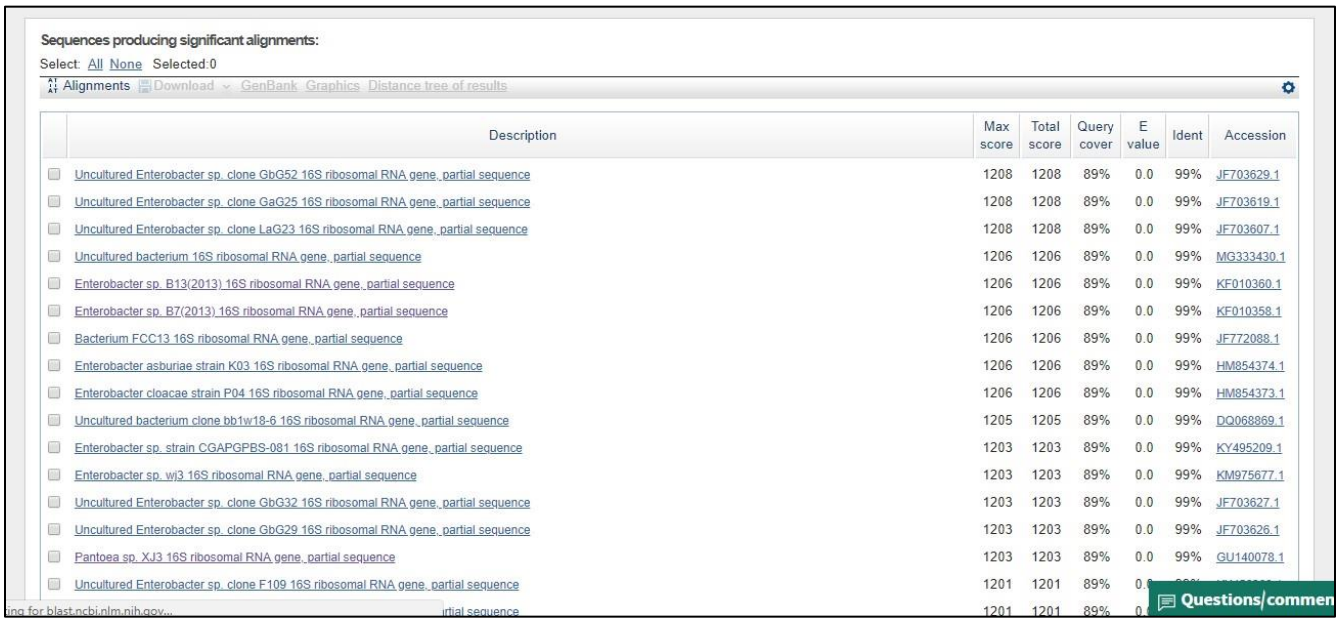

Fig 4.25 BLAST n showing associated taxa of query sequence

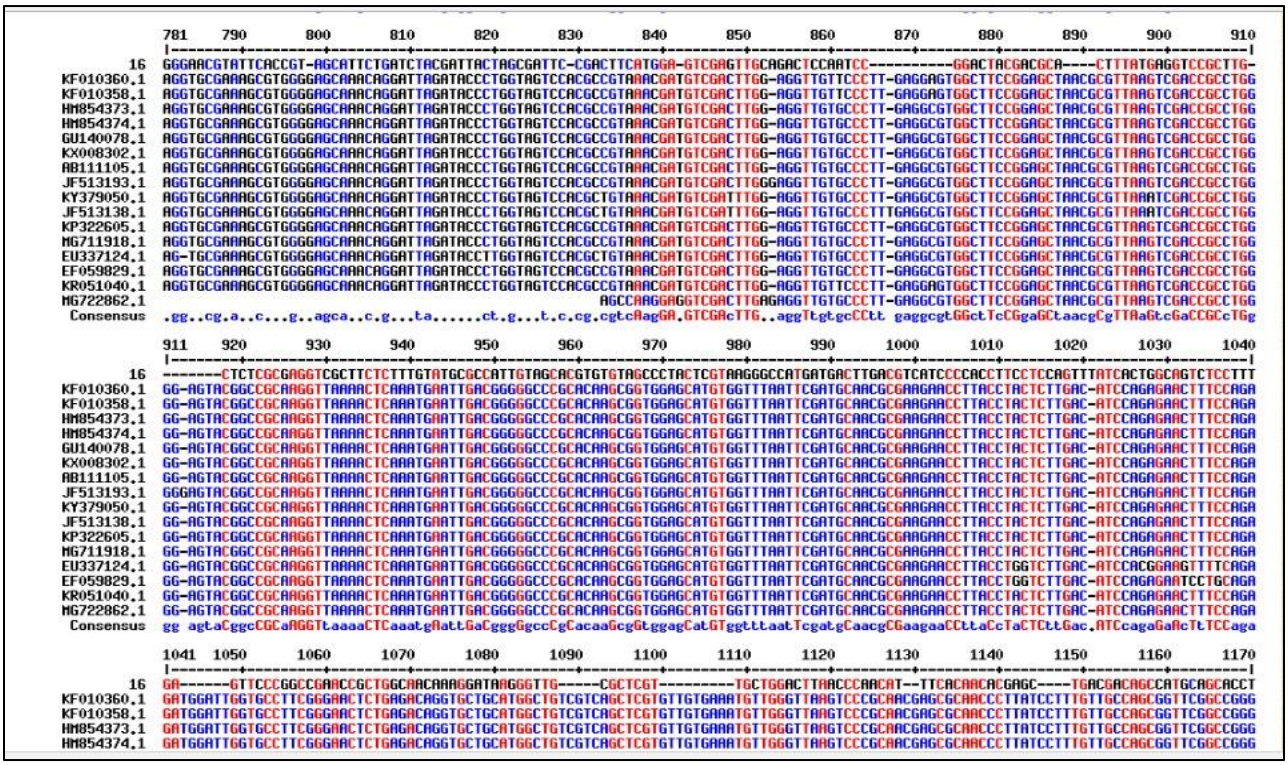

Figure 4.26 MultAlin displaying multiple sequence alignment

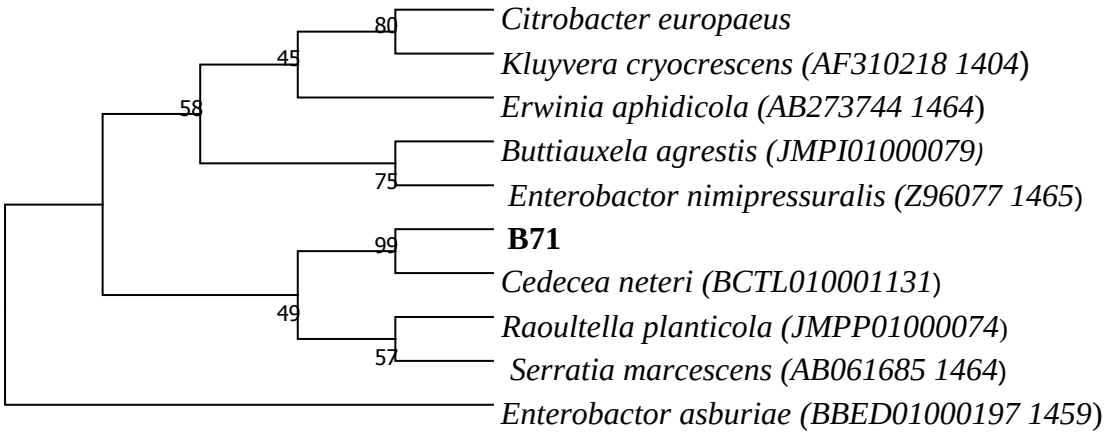

Fig 4.27 Phylogenetic tree of B_{71}

Biochemical characterization of bacterial isolates

Biochemical reactions	B_{49}	B_{71}
Gram staining	-	-
Morphology	Bacilli	Bacilli
Motility	+	+
Oxidase	-	-
Catalase	+	+
IMViC test		
Indole production	+	-
Methyl red	+	+
Voges-Proskauer	-	+
Citrate utilization	-	+
Urease	-	-
Nitrate reduction	+	+
Starch hydrolysis	-	-
Sugar fermentation		
Glucose	+	+
Lactose	+	-
Maltose	+	+
Mannitol	+	+
Gas production during sugar fermentation		
Glucose		
Lactose	+	+
Maltose	-	-
Mannitol	+	+
	+	+

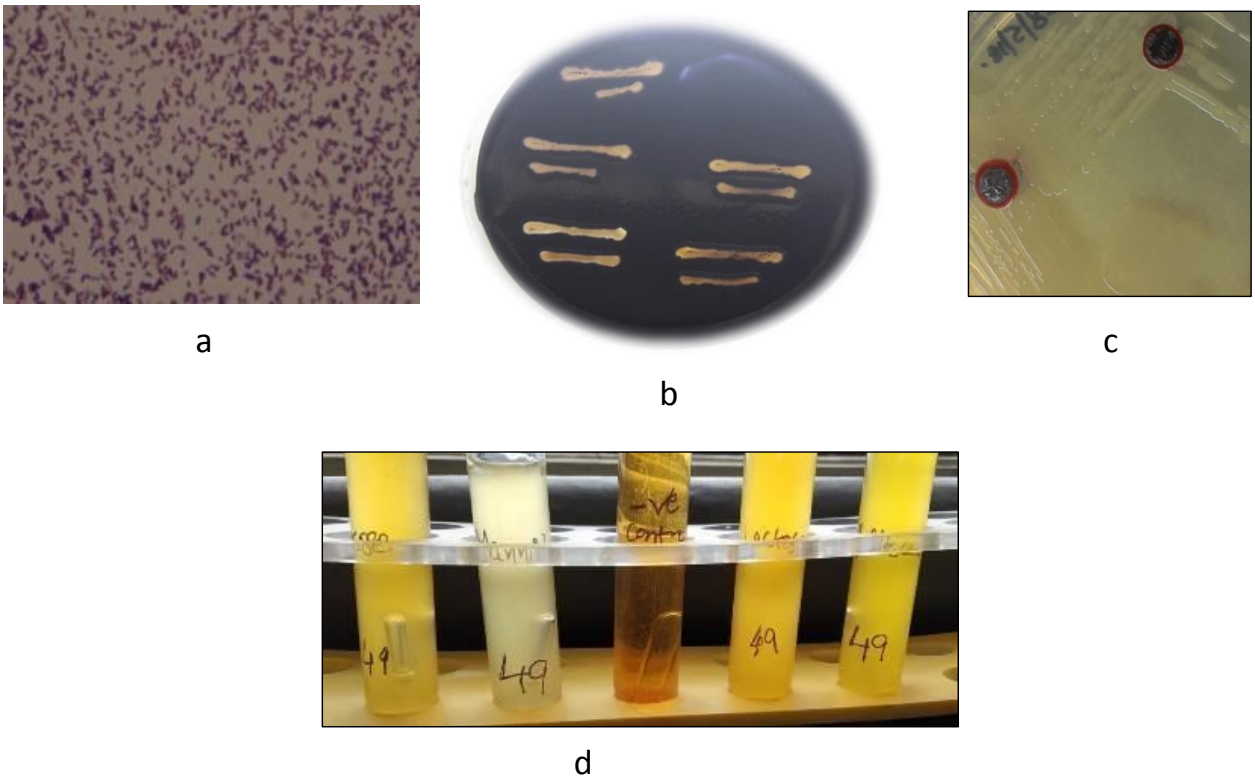

Figure 4.28 Biochemical characterization of bacterial isolates a) gram staining b) Starch hydrolysis test c) nitrate reduction test d) sugar fermentation

4.8 Physiochemical characteristics of soil sample

S.NO.	Physiochemical characteristics	Sample (wheat)
1.	Soil pH	8.38 ± 0.21
2.	Available phosphorus	0.3021 ± 0.491
3.	Total phosphorus	2.148 ± 0.004
4.	Organic carbon	15.50%
5.	Nitrogen	0.48 ± 0.03

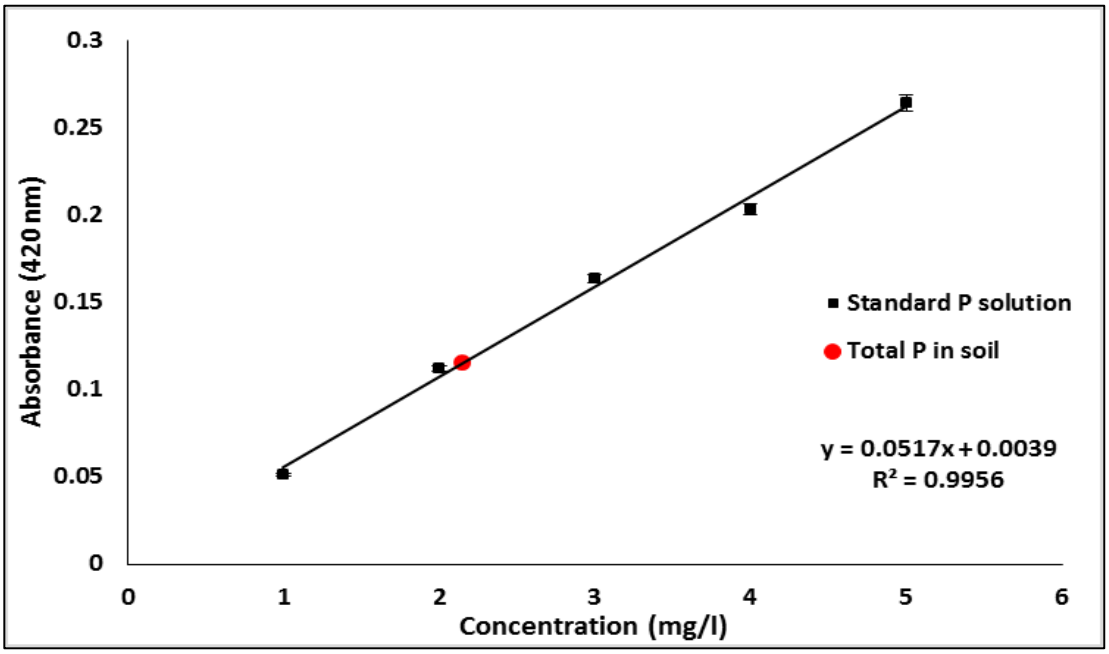

Fig 4.28 Standard curve of total phosphorus

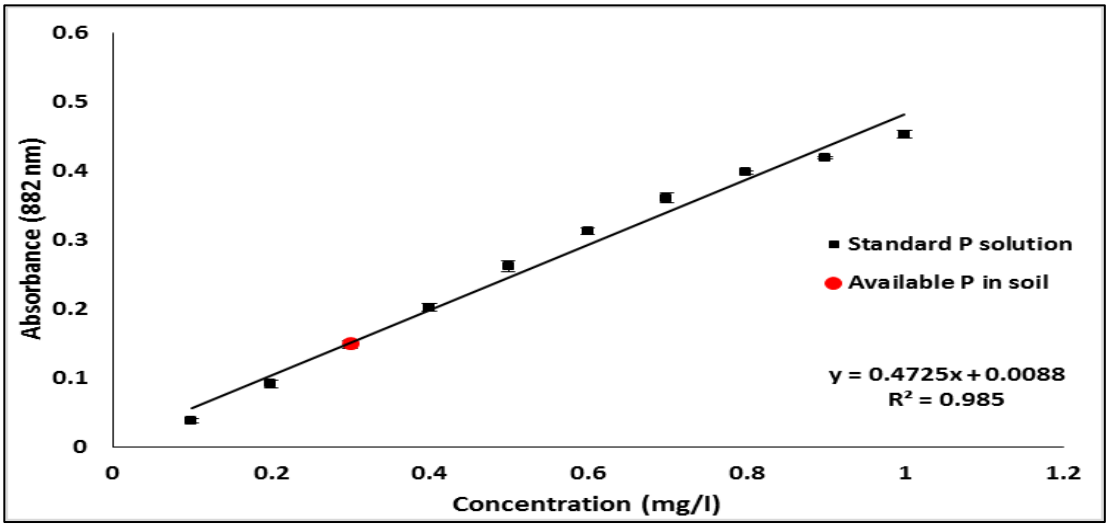

Fig 4.29 Standard curve of available phosphorus

DISCUSSION

Seleniferous soils have been widely distributed across the globe. The Nawashahr and Hoshiarpur regions are rich with selenium. The soil sample was contained from Jainpur, village of Nawashahr which contains 5 mg/kg of selenium. In 2003, Dhillon and Dhillon reported that soils containing >0.5 mg kg^{-1} is known as seleniferous soil.

Soils are highly rich in microorganisms which are able to tolerate selenium at different levels. In the present study, the isolated bacteria are *Leclercia adecarboxylata* and *Cedecea neteri.* These isolates are able to tolerate selenium upto 300 mM. Mishra et al. (2011) reported *Bacillus megaterium* which is able to reduce selenite into elemental selenium. Ghosh (2011) reported eight bacterial strains with minimum inhibitory concentration upto 350-750mM.

The isolates were also screened for various plant-growth promoting activities like IAA production, HCN production, phosphate solubilzation and ammonia production. Both the isolates were positive for IAA production, Ammonia production and phosphate solubilzation. Ahmad and Khan (2008) reported 72 bacterial isolates which included *Azotobacter, Bacillus* and *Pseudomonas*. More than 80% of the bacterial isolates were able to show plant growth promotion.

Masanori et.al isolated and characterized a gram positive and facultative anaerobe: *Bacillus sp*. from selenium-contaminated deposits. It was able to grow by taking selenate as an electron acceptor. It was able to reduce upto 1mM of selenate into elemental selenium. Due to its reduction properties, this strain could be used in the process of bioremediation. Sharma et al. (2009) isolated a facultative anaerobe, *Pseudomonas aeruginosa* from the rhizospheric soil of Jainpur region of Hoshiarpur. This strain was able to reduce 53% of sodium selenite and 21% of sodium selenate into elemental selenium. Furthermore, this microbe was also capable of volatilizing the 4.7% of sodium selenite and 5.1% of sodium selenate.

CONCLUSIONS

Seliniferous soil is widely distributed across the globe which needs the proper management to avoid any kind selenium toxicity in living beings. In the present study, seleniferous soil sample was collected from Jainpur village, Nawashahr, Punjab and was used in isolating seleniumtolerant and plant growth promoting bacteria. The soil sample was also used for determining the amount of selenium present in the soil sample which was found to be 5 mg/kg. The various physiochemical characteristics of soil like pH, total phosphorus, available phosphorus, organic carbon and nitrogen content were also determined. The pH of soil was 8.38 ± 0.21 which means it is alkaline in nature. The available phosphorus, total phosphorus, organic carbon and nitrogen content was 0.3021 ± 0.491, 2.148 ± 0.004, 15.50% and 0.48 ± 0.03 respectively.

The seleniferous soil was enriched and serial diluted in order to isolate selenium-tolerant bacteria. A total of 75 bacteria were obtained of which some were able to tolerate upto 300 mM of sodium selenite. Further, these bacteria were screened for various plant growth-promoting activities like IAA production, phosphate solubilization, ammonia production and HCN production.

Two highly-tolerant bacteria (B_{49} and B_{71}) along with maximum plant-growth promoting activities were selected. These bacteria were tolerant upto 300 mM of sodium selenite and showed maximum IAA production with 69.91µg/ml and 46.20 µg/ml respectively. They were also able to solubilize insoluble phosphate into soluble phosphorus with a solubilization index of 1.16 and 1.23 respectively.

Both the bacterial isolates were rod-shaped and gram-negative in nature. Various biochemical tests were also conducted and both the isolates were positive for citrate utilization, VP, catalase, motility, nitrate reduction and sugar fermentation. On the other hand, the isolates were negative for indole production, methyl-red test, oxidase, urease and starch hydrolysis.

In order to check the plant growth properties of these isolates outside the lab in real time conditions, nursery trials of these bacterial isolates were conducted in which the maize seeds were coated with bacterial culture and seeded in the cups containing seleniferous soil of Jainpur village. The experiment was done in ten replicas. The cups were irrigated every 2^{nd} day and were allowed to grow for two months. After which the plants were harvested and the various parameters like root length, shoot length, whole plant length, dried shoot weight and dried root weight were calculated and compared with

control. The plants inoculated with bacterial isolates showed increase in the root length, number of root hairs, and height of whole plant. The plants accumulated 657.25 µg/ng, 661 µg/ng and 649.2 µg/ng in 100 mM, 200 mM and 300 mM respectively.

Therefore, the study undertaken indicated that these two bacterial isolates *Cedecea neteri* and *leclercia adecarboxylata* are capable of bioaccumulating significant amount of sodium selenite in their biomass and reducing sodium selenite into non-toxic elemental selenium. So they can be used in the process of bioremediation. Moreover they also show a number of plant growth promoting activities in vitro and in vivo i.e. they can be used in the seleniferous soil as potential biofertilizer and bioremediator candidates.

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