Selenium volatilization by rhizospheric bacteria

Dissertation

Submitted in partial fulfillment of the requirement for the award of the degree of Masters of Science in

Biotechnology

By

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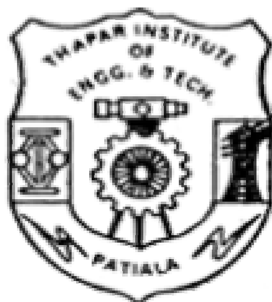

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DEPARTMENT OF BIOTECHNOLOGY & ENVIRONMENTAL

SCIENCES

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Declaration

I hereby declare that the work presented in the dissertation entitled **"Selenium volatilization by rhizospheric bacteria"**, submitted by me in partial fulfillment of the requirement for the award of degree of Masters of Science in Biotechnology to Thapar Institute of Engineering and Technology (Deemed University), Patiala, is an authentic record of my dissertation work during a period of five months from January 2005 to May 2005, under the kind supervision of Dr. N. Tejo Prakash, Assistant Professor, Department of Biotechnology and Environment Sciences. The matter embodied in the dissertation has not formed the basis for award of any degree or diploma.

Nilabh Anand

Place: PATIALA

Date:

This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge.

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This is to certify that the thesis entitled **"Selenium volatilization by rhizospheric bacteria"** submitted by **Nilabh Anand** in partial fulfillment of the requirements for the award of Degree of Masters of Science in Biotechnology to Thapar Institute of Engineering and Technology (Deemed University), Patiala, is a record of the student's own work carried out by him under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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Summary

Selenium in agricultural soils of Punjab has come in public attention lately because of reports from widely divergent backgrounds ranging from research outcome to general awareness campaigns. It was noted that potentially toxic amounts of selenium could be passed on to livestock and human population through fodder and feed resulting in deleterious effects. Although, physico-chemical routes can temporarily provide remediation to seleniferous soils, biological route has been reported to be a potential means to transform selenium to non-bioavailable forms, thus reducing the toxic effects.

Among various biotransformation pathways, volatilization through biomethylation has considerable potential for use as a method of remediation. Most importantly, recent observations that phytoremediation of Se by higher plants is closely associated with the potential of rhizosphere bacteria to biotransform Se to volatile/non-volatile alkylselenides, has increased the need to understand the potential of rhizosphere microbes from tropical soils to reduce/volatilize Se.

Keeping this in view, the present study was carried out to examine the selenium volatilization potential of bacteria isolated from seleniferous and non-seleniferous agricultural soils. The results of the study present the observations with two soil isolates, *Pseudomonas aeruginosa* and *Bacillus subtilis* showing biotransformation Se through reduction vis-à-vis volatilization.

Selenium is a naturally occurring mineral element and is widely distributed in nature found in rocks, soil and water and also gaseous forms in the atmosphere, it was discovered by Berzelius in 1817. Its original source was probably volcanic eruption. It forms compounds with 16 other elements and is found naturally in igneous rocks, volcanic sulphur deposits, sedimentary rocks and some crude oil deposits. It is a group 16 metalloid element as it acquires the characteristic of metals and non-metals. It exist in four different oxidation states i.e., -2(Se^{-2} or selenide), 0 (elemental selenium), +4 (SeO_3^{-2} or selenite) and +6 (SeO_4^{-2} or selenate). Each oxidation state exhibits a different chemical behaviour. Concentration, speciation and association of Se in a given environment depend upon redox conditions, solubility of its salts (selenate are more soluble than selenite) the complexing ability of soluble and solid ligands, biological interactions and reaction kinetics. Selenides and elemental selenium occur in acidic, reducing and organic rich environments. Since, selenides and elemental selenium are not water-soluble, they are not bioavailable. Selenate and selenite are the water soluble and dominant bioavailable forms in soil. Selenium particles move through the soil and are adsorbed on soil particles and organic particles. Selenium occurring in alkaline soils is naturally oxidized to selenate. This highly toxic form easily leaches from the soil and reach the ground or surface waters. Plants can easily absorb and bioconcentrate selenium compounds from water. Bioaccumulation of selenium in fodder and grain crops can result in biomagnifications. Agricultural practices significantly contribute to the bioavailability of selenium to plants trough irrigation.

Selenium in traces is an essential nutrient for humans and animals. But higher concentration of selenium has harmful effects. The borderline between non-toxicity and toxicity of Se is not very much clear as on date due to variable behaviour from region to region and species to species. Exposure to high concentrations of selenium (ADI-400 mg/day) has been observed to cause nausea, vomiting and diarrhea. Chronic oral exposure to high concentration of selenium compounds can produce a disease called Selenosis. Major signs of selenosis are hair loss, keratosis, brittleness of nails and neurological abnormalities. In very small amounts, it is an antioxidant, and is used as anti-cancer agent.

Different microorganisms like bacteria, algae, and fungi play major role in metabolism of selenium in different ways. Use of microbial detoxification of selenium in contaminated environment is an eco-friendly approach. They detoxify selenium by the reduction and/or

biomethylation process either by aerobic or anaerobic mechanisms. A high percentage of the bacteria, actinomycetes and fungi have been found to volatilize selenate and selenite by reduction followed by biomethylation. Volatilization of selenium is preferred form of Se bioremoval as volatile selenium compounds are stable and far less toxic forms than elemental selenium. Elemental selenium can reconvert to toxic anion forms namely selenate and selenite in the presence of favorable conditions.

A number of bacteria have been observed to biomethylate and volatilize selenium in both aerobic and anaerobic conditions such as *Aeromonas sp., Corynibacterium sp., Desulfovibrio vulgaris, Pseudomonas aeroginosa VS*7*, Pseudomonas fluorescens* K27*, Pseudomonas sp.* VW1 etc. Anaerobic bacteria transform toxic forms of selenium as part of their respiratory metabolism to elemental selenium and then to volatile selenium.

In seleniferous soils, concentration of selenium is variable ranging from <0.1 to as high as 8000ug/kg with an average selenium concentration of 0.40μg/kg for 1623 soils through out the world. Selenium occurs in alkaline soils in certain localities of Columbia, Ireland, Israel, South Africa and China. Crop analysis also showed selenium occurring in Argentina, Venezuela, Spain, Bulgaria, Algeria, Morocco, Australia, and New Zealand and in some drier regions of former Soviet Union. The total selenium concentration in Kesterson reservoir, United States of America was found to range from 2.0 to 4.0μg/kg (at 25cm depth) to 40-70μg/kg at topsoil. In seleniferous soils of China, the mean total selenium concentration was found to be ranging from 0.022 to 3.806μg/kg. Pockets of seleniferous soils have been identified in India especially in Northeastern parts of Punjab and Haryana. In the seleniferous areas, the selenium concentration in soils was found to be 4.52μg/kg to 6.79μg/kg, whereas, in non-seleniferous soils areas selenium concentration in soils was found to be 1.08μg/kg.

Due to high rate of selenosis and associated disorders in livestock and human inhabitants in this region, these pockets of the states have in recent past, generated immense interest among researchers and administrators towards mitigation and remediation of soil from selenium. Though, physico-chemical means have been attempted at pilot scale, the soil has been found to be affected after repeated treatments. As an alternative, bioremediation approach has been envisaged. As a preliminary step towards following a biological route to mitigate soil from higher levels of selenium, it is necessary to understand the microbial mechanisms of selenium tolerance, transformation and volatilization, inherently operational in soils, so as to exploit the same towards enhanced removal of the toxic element. Keeping this in view the present study has been carried out to examine above-mentioned mechanisms in a

rhizospheric microorganisms namely *Bacillus cereus* (MTCC 6501) and *Pseudomonas aeruginosa* isolated from soil. *B. Cereus* was selenium tolerant isolate of non-seleniferous soil (Patiala region) where as *P. aeruginosa* was an isolate from seleniferous soils of Nawanshahr region (Simbli).

Selenium is a metalloid *(Wilber, 1983)* as it acquires characteristics of both metals and nonmetals. It forms both organic and inorganic complexes. It may act as an oxidant as well as reductant. Its chemical adaptability accounts for its widespread occurrence in soil, plants, animals and humans *(Bauer, 1997)*. Selenium is chiefly found in igneous rocks, volcanic sulfur deposits, and hydrothermal deposits and in sedimentary rocks *(Bauer, 1997)*. Particles of selenium get adsorbed on the clay particles and are deposited into soil *(Bauer, 1997).* Heterogeneity in its distribution results in its movement among these compartments *(Nriagu, 1989).* Materials known to have the highest selenium concentration are block shale's (around 600mg/kg dry) and phosphate rocks (1-300mg/kg dry) both potentially giving rises to seleniferous soils. Higher oxidation states are predominantly present in alkaline and well-aerated soils. Lower oxidation states are present in poorly aerated soils *(Marian, 1984).*

Main oxidation states of selenium, which are found in environment, are elemental selenium (0), selenite (+4), selenate (+6) and selenides (-2). Selenate and selenite are water-soluble so, they are most abundant forms of bioavailable selenium in the environment. Their oxidation states depend upon the redox potential conditions. Elemental selenium (Se0) exists in crystalline forms and is usually incorporated in soil particles. As selenate and selenite are water-soluble, they are common forms in most of the surface waters. Selenium occurs in water in trace amounts as a result of geochemical processes such as weathering of rocks and soil erosion *(Bauer, 1997).*

Selenium Toxicity in Biological Systems

Selenium is a nutritional component for humans and livestock. Many reviews have appeared describing various biological functions of selenium including its nutritional importance *(Mugesh, et. al., 2001; Birringer, et. al., 2002)*. Global distribution of selenium in nature varies widely from 0.005ppm (Finland) to 8000ppm (Tuva region of Russia) *(Chasteen and Bentley, 2002)*. Selenium in feed and forage plants (at concentrations less than 0.05 to 0.1mg/kg) have been found to cause white muscle disease in livestock *(Muth, et. al., 1958; Sharma and Singh, 1983)*. Accumulation of Se in selenium accumulating plants or ***Beath*** indicators has been historically associated with toxicity to animals that ingested these plants *(Beath, et. al., 1937; Franke, 1934)*. Preliminary reports on chronic Se toxicity as a cause of

hoof and horn disease in livestock in seleniferous region of Punjab (Hoshiarpur and Nawanshahr districts) were given by *Gupta and coworkers (1982).* The malformations appearing in livestock were attributed to feeding of fodders grown in seleniferous soils *(Dhillon, et. al., 1977).* Alkali disease and Blind staggers disease *(O'Toole, et. al., 1996)* have been also observed. Selenosis (selenium toxicity) has been observed in humans as well as animals. High doses of selenium (more than 900μg/day) produces a toxic syndrome of dermatitis, loose hair, diseased nails and peripheral neuropathy *(Beers, et. al., 1999)*

Reduction and Biotransformation of Selenium

Considerable amount of selenium in the agriculture soils and drinking water has become increasingly significant as environmental toxin in agricultural soils and drinking water. Microorganisms can reduce toxic soluble selenium oxyanions species (selenate and selenite) to a much-less toxic insoluble forms, namely elemental selenium. *(Garbisu, et. al., 1995)*. Selenium transformation and mobilization in soils is influenced by microbial (bacteria, fungi and actinomycetes) activity along with the higher plants, *(Stolz, 2002)*. Some rhizosphere bacteria have been also found to reduce selenium and readily transform selenium oxyanion (such as selenate and selenite) into various organoselenium forms like selenoamino acids such as selenomethionine, selenocysteine and selenocystine (*Stolz, 1996*). They are also capable to convert these selenoamino acids into volatile selenides e.g.: - DMSe (Dimethyl selenide) and DMDSe (Dimethyl diselenide) *(Ganther, 1974).* Selenium biotransformation is observed in microbes like bacteria, fungi, plants, rodents, mammals and humans also.

The pathway followed by the soil bacteria for selenium reduction is as follows: -

Selenate → **Selenite** → **Selenide** → **Methylated Selenide**

Selenite ↓ **Elemental Selenium**

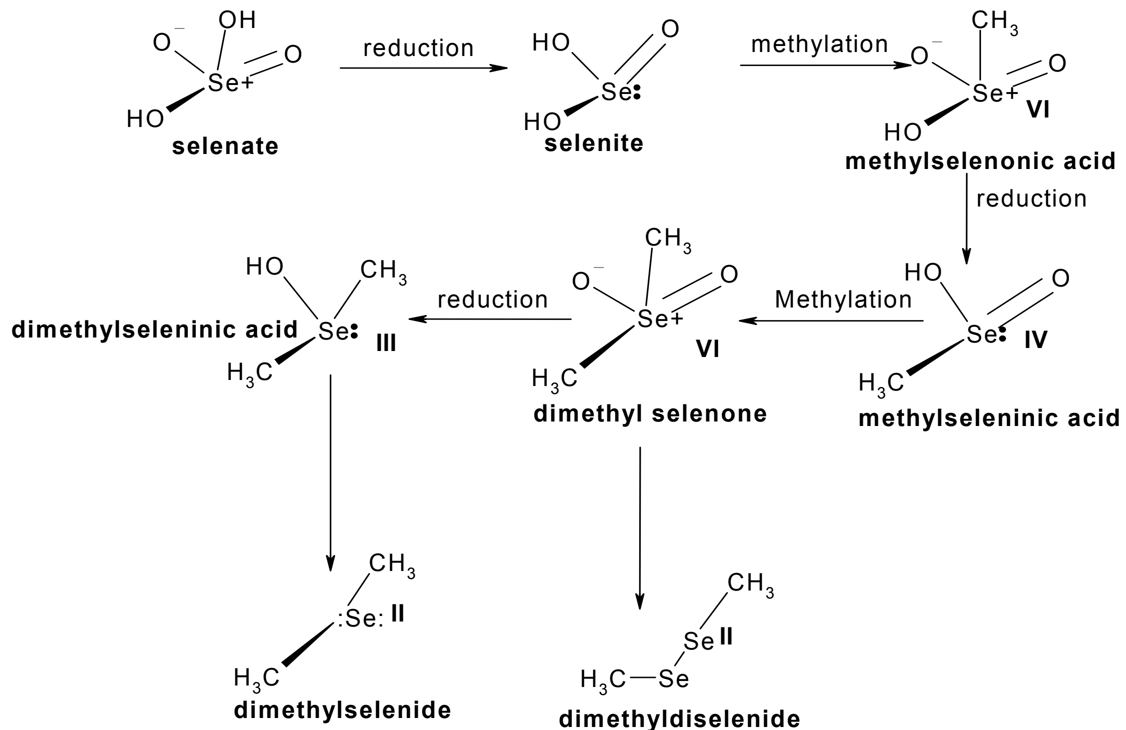
Mechanism of Selenium Volatilization

Selenium volatilization in microbes and plants takes place by reduction and biomethylation process (*Challenger, 1945*). Challenger first proposed microbial methylation of metals in 1945 therefore known as ***Challenger mechanism.*** His hypothesis elucidated the

enzymatic activity responsible for alteration of reductive and methylation reactions (*Challenger, 1951*). Further experiments by his group established that the methyl group of methionine was utilized for selenium methylation *by Scopulariopsis brevicaulis* and *Aspergillus niger* (*Challenger, et. al., 1954*). The enzymes involved are selenocysteine methyl-transferase and molybdenum-dependent methyltransferase (*De Souza, et. al., 1999; Neuhierl, et. al., 1996*). A selenocysteine methyltransferase has been detected in some species of *Brassica juncea* (Indian mustard), *Astragalus sp.* and facultative anaerobes *Pseudomonas fluorescens* (*Thayer, 2002*). Rhizosphere is a potential site of increased microbial activity that may enhance biomethylation of selenium and other trace elements (*Allen, 1991; Anderson, et. al., 1993).*

The Challenger mechanism

(Thayer, 2002; Chasteen and Bentley, 2003)



Bacteria, fungi and algae can volatilize selenium at high rates independently of plants (Table 1). Pure cultures of bacteria, fungi and algae tested under laboratory conditions volatilized selenium at higher rate than plants when expressed per gram dry weight of tissue. *(Fleming, et. al., 1972; Thompson, et. al., 1989; Brady, et. al., 1996; De Souza, et. al., 1997; Fan, et. al., 1997; Real and Frankenberger, 1996).* It was indicated by Zayed and co-workers (1994) that bacteria are involved in plant Se volatilization when detopped roots of Broccoli

treated with Penicillin-G inhibited 95% of the Se volatilization activity from selenate supplied roots, supporting the view that rhizosphere bacteria may be essential for plant Se volatilization. Rhizosphere bacteria enhance selenium accumulation and volatilization by Indian mustard *(De Souza, et. al., 1999).* Rhizospheric bacteria can volatilize selenium *(De Souza, 1997).* In addition, numbers of higher plants have been identified to volatilize selenium either independently or in association with the rhizospheric microorganisms (predominantly bacteria) (Table 1).

Table 1 – Variety of organisms known to volatilize selenium presented along with the products of Se volatilization

BACTERIA

Bacterial species	Se form	DMSe	DMDSe	DMDSeS
*Aeromonas sp.*VS6 *(Chasteen, et. al., 199)*	VI	+	+	-
Corynebacterium sp. (Doran, et. al., 1977)	VI	+	+	-
Aeromonas veronii (Real and Frankenberger, 1996)	IV, VI	Not	Known	-
Rhodobacter spaeroides (Fleet, et. al., 2000)	IV, VI	Not	Known	-
Enterobacter cloacae (Losi, et. al., 1997)	IV, VI	Not	Known	-
Citrobacter freundii KS8 *(Chasteen, 1990)*	IV, VI	+	+	-

FUNGI

Fungal species	Se form	DMSe	DMDSe	DMDSeS
Fusarium sp. (Barkes, et. al., 1974)	IV, VI	+	+	+
Penicillium cittrium (Chasteen, et. al., 1990)	IV, VI	+	+	-
Phragmites australis (Azaizeh, et. al., 2003)	IV, VI	+	Not	Known
Acremonium falciforme (Chasteen, et. al., 1990)	IV	+	+	-
Alternaria alternata (Thompson, et. al., 1989)	VI	+	-	-

ALGAE

Algal species	Se form	DMSe	DMDSe	DMDSeS
Chlorella sp. (Fan, et. al., 1997)	IV	+	+	+
Cyanophyte dominated mat *(Budisa, et. al., 1998)*	IV, VI	+	+	-

HIGHER PLANTS

Plant species	Se form	DMSe	DMDSe	DMDSeS
Arabidopsis thaliana (Ellis and Salt, 2003)	IV, VI	+	+	+
Brassica juncea (de Souza, et. al., 1998)	IV, VI, se-amino acids	+	+	+
Phaseolus vulgaris (Arvy, et. al., 1993)	IV, VI	+	-	-
Neptunia amplexicaulis (Burnell, et. al., 1981)	Se^0, se-amino acids	+	-	-
Astragalus pectinatus (Trelease and Trelease, 1939)	Se^0	+	+	+
Astragalus bisulcatus (Neuhierl, et. al., 1996)	Se^0, IV, VI	+	-	-
Morinda reticulata (Peterson, et. al., 1972)	Se^0, IV, VI	+	+	-
Catheranthus roseus (Eichel, et. al., 1995)	Not known	+	+	+
Coleus blumei (Peter and Straeten, 1995)	Not known	+	+	+
Spartina alterniflora (Anseda and Yoch, 1997)	Se^0, amino acids	+	+	+

IV= selenite; VI= selenate; Se^0= elemental selenium; + = detected; - = unknown; DMSe= Dimethyl Selenide; DMDSe= Dimethyl diselenide; DMDSeS= Dimethyl diselenide Sulfide.

The studies related to volatilization of selenium by bacteria and their association with higher plants in Se biomethylation is very scarce as noted in only few recent reports. In addition, there is no data on the Se biotransformations and especially the aspect of volatilization by bacteria from tropical seleniferous soils. Keeping this in view this study was carried out with focus on examining the extent of volatilization by soil bacteria.

Objective

To examine the selenium volatilization potential of bacteria isolated from agricultural soils.

Work Elements

1) Enrichment and isolation of the microbes from the selenium impacted soil.
2) Observing the selenium tolerance in bacteria.
3) Observing their Selenium volatilization capacity.

Nutrient Medium Preparation

31.0 gm of Tryptic Soya Broth (Hi-media) was dissolved in 200ml of double distilled water and final volume was prepared to 1liter. 100ml of medium was distributed in 250ml of Erlenmeyer flask caped and autoclaved at 121°C at 15 lb for 15min. Similarly solid medium was prepared by dissolving tryptic soya agar into distilled water and autoclaved.

Preparation of Volatile Se Trapping Solution

For preparation of Se trapping solution (6%H_2O_2 at 0.05M NaOH), 100ml. of 30% H_2O_2 was dissolved in 400ml-distilled water, 1.0 gm of crystalline NaOH was also added into 100ml-distilled water and mixed to make final volume 500ml. Thus the alkaline hydrogen peroxide solution is prepared for the experiments.

Isolation and Identification of the Bacterial Stains

Soil samples (rhizospheric soil) were collected from agricultural soils of Nawanshahr and Hoshiarpur (Barwa, Mehindpur and Jounpur villages) districts of Punjab in poly bags. These soil samples were suspended in 100ml of tryptone soya broth (TSB)(Himedia) in 250ml. Erlenmeyer flask containing 100ppm of selenium as sodium selenite (CDH) and sodium selenate (SD Fine). These flasks were kept at 37°C at 120rpm until red colour appeared. For enrichment of selenium reducing microorganisms 1ml of above suspension was re-inoculated in fresh medium containing selenate and selenite for 2-3 times. In final step, Red coloured broth was used to streak on tryptone soya agar (TSA) (Hi-media) plates containing 100ppm of sodium selenate and selenite. Plates were incubated aerobically at 37°C for 24hrs after which red coloured colonies were picked and re-streaked on TSA plates with and without selenate and selenite to ensure that red colour was not produced by the pigments secreted by the isolates. Identity of these cultures was confirmed by gram staining. For further molecular/biochemical characterization the sequence/culture were sent to University of Delhi, South Campus and IMTECH, Chandigarh respectively.

Growth Experiments

A set of growth experiments was conducted to study the tolerance capacity of the microorganism challenged with different concentration of selenate and selenite (25ppm, 50ppm, 75ppm, 100ppm) in liquid medium. For the growth experiment, 100 ml Tryptic Soya Broth was inoculated with 2ml of overnight grown culture (OD_{600}-2.0) and kept at 37^0C under shaking conditions (120rpm). Two different controls were simultaneously maintained i.e., one with inoculum but without selenium and the other as positive control without inoculum and with selenium, to check if there is any chemical induced transformation of selenium in the medium devoid of the organism. The growth experiments were carried out for 12hr with observations taken at 2hr interval. Study beyond 12 hours was avoided due to interference of red colour of elemental selenium with optical density (except in the case of *P. aeruginosa* exposed to selenite).

Selenium Volatilization Experiment

To study selenium volatilization ability of microorganisms, 2ml of 2% inoculum was inoculated in 100ml of TSB media in a round bottom flask containing 50ppm and 100ppm selenite and selenate were separately inoculated with 2ml. 2% of inoculum and kept under aseptic condition at 28°C in incubator. Fresh air was introduced through air pump into culture flasks through a sterilized 0.2μm membrane filter to maintain sterile aerobic conditions. Spent air containing the volatile component was collected in trapping solution (containing alkaline peroxide solution) through outlet. In this experiments two controls were maintained - one with 50ppm selenium without inoculation, the other with 2% inoculum but without selenium. These conditions were maintained for 72 hours to ensure sufficient volatilization of selenium by test culture.

Mass Balance studies

After 72 hours, the spent medium was collected in oak ridge centrifuge tubes and was centrifuged at 8000rpm for 10minutes to separate biomass and supernatant. Biomass pellet, supernatant and trapping solution containing volatile selenium component are acid (1:1 HNO_3: Distilled water) and acid digested. These digested samples were diluted with distilled water and subjected to Se analysis by Graphite furnace-AAS (NOVAA-400, Analytikajena, Deutschland).

Isolation and characterization of bacteria

The studies were carried out on two bacterial strains tolerant to selenium. The two strains were isolated from seleniferous Nawanshahr-Hoshiarpur belt and non-seleniferous soils of Patiala dist. The strains were identified and are reported to be *Pseudomonas aeruginosa* (through gene sequence analysis by University of Delhi); and *Bacillus cereus* (MTCC 6501-through IMTECH, Chandigarh). Following table (Table 2) gives the morphological and biochemical characteristics of the mentioned strains based on which the identifications were confirmed. Figure 1 and 2 show the micrographs of the morphological characteristics of the test strains.

Table 2 – Morphological, physiological and biochemical characteristics of the test strains. (Partially carried out at laboratory and partially obtained from MTCC report on strains)

	Pseudomonas aeruginosa	***Bacillus cereus***
Morphology		
Colony morphology	SNT-3	SNT-1
Configuration	Irregular	Round
Margin	Rhizoid	Wavy
Elevations	Raised	Convex
Surface	Smooth, moist	Rough
Pigment	Light yellow	-
Opacity	Opaque	Opaque
Gram's reaction	-Ve	+Ve
Cell shape	Irregular rod	Rods
Size (µm)	Non-uniform	1.0-1.5
Arrangement	-	Single
Physiological tests		
Growth at temp		
4ºC	-	-
10ºC	-	W
15ºC	+	+
25ºC	+	+
30ºC	+	+
37ºC	+	+
42ºC	+	+
45ºC	-	-
55ºC	-	-
Growth at pH		ND
PH 4.0	-	-
PH 5.0	+	+
PH 6.8	+	+
PH 8.0	+	+

Physiological tests		
PH 9.0	+	+
PH 11.0	+	+
PH 12.0	+	-
Growth on NaCl(%)		
2.0	+	+
4.0	+	+
5.0	+	+
7.0	+	+
10.0	+	+
Growth under anaerobic condition	+	+
Biochemical tests		
Growth on MacConkey agar	NLF (non-lactose fermenters)	-
Indole test	-	-
Methyl red test	+	+
Voges Proskauer test	-	+
Citrate utilization	+	+
Gas production from glucose	-	-
Esculin hydrolysis	-	-
Gelatin hydrolysis	+	+
Starch hydrolysis	-	+
Urea hydrolysis	-	-
Nitrate reduction	+(w)	+
H_2S production (TSI agar)	-	-
Catalase test	+	+
Oxidase test	+	-
Acid production from carbohydrates		
Cellobiose	-	-
Dextrose	-	+
i-Inositol	-	-
Lactose	-	-
Raffinose	-	-
Sucrose	-	+
Xylose	-	-
Sugar utilization as Carbon source		
Glycerol	+	ND
Lactate	-	ND
Fumarate	+	ND

ND = Not Done, +W= weak positive, NLF=Non lactose fermenters.

Figure 1 – Micrograph of *Pseudomonas aeruginosa* showing rod shaped characteristics

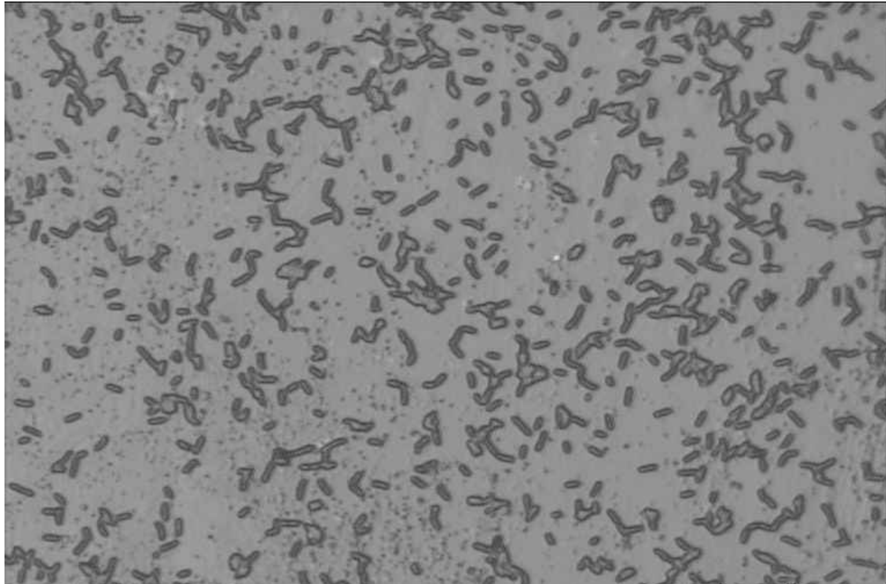
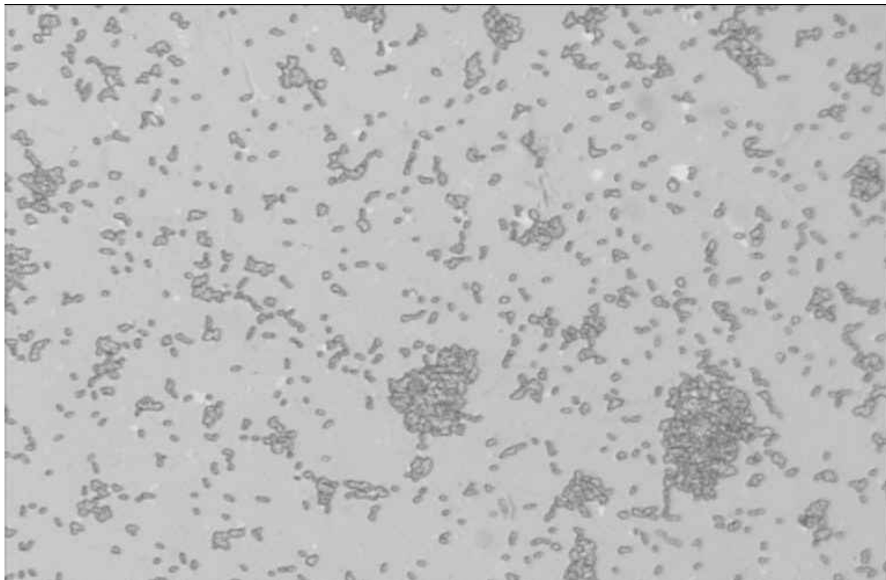


Figure 2 – Micrograph of *B.cereus* showing rod shaped cells, which are slimy and agglomerative in nature



Based on the physiological and biochemical characteristics, it is observed that both the strains are facultative anaerobes. Both the isolates are tolerant to wide range of pH, with *P. aeruginosa* observed to grow effectively even at pH 12. Both the strains are mesophilic in nature and are not thermo tolerant.

Selenium tolerance and growth profile of microbial isolates

A set of growth experiments was conducted to study the growth profile and tolerance capacity of the isolates. The two isolates were challenged with different concentration of selenate and selenite (25ppm, 50ppm, 75ppm, 100ppm) in liquid medium. Growth kinetics was studied on the basis of optical densities of both the strains from the freshly growing cultures in presence of selenite and selenate separately to medium. In the figure-3, there was no lag phase observed when *P. aeruginosa* was supplemented with selenite. The growth studies were extended upto 36 hours if any further variations would occur in growth of the organisms. Figure-4 depicts the growth curve of *P. aeruginosa* in the presence of selenate. Lag phase of two hours has been observed when *P. aeruginosa* was amended with selenate. In the case of selenite, the growth profile study was stopped after 12 hours due to the formation of red coloured precipitation caused by reduction of selenate that interfered with optical density. In either case of selenate and selenite, it has been observed that after 12 hours of incubation, optical density was comparative and similar to control.

Figure 3 - Growth kinetics of *P.aeruginosa* in presence of selenite

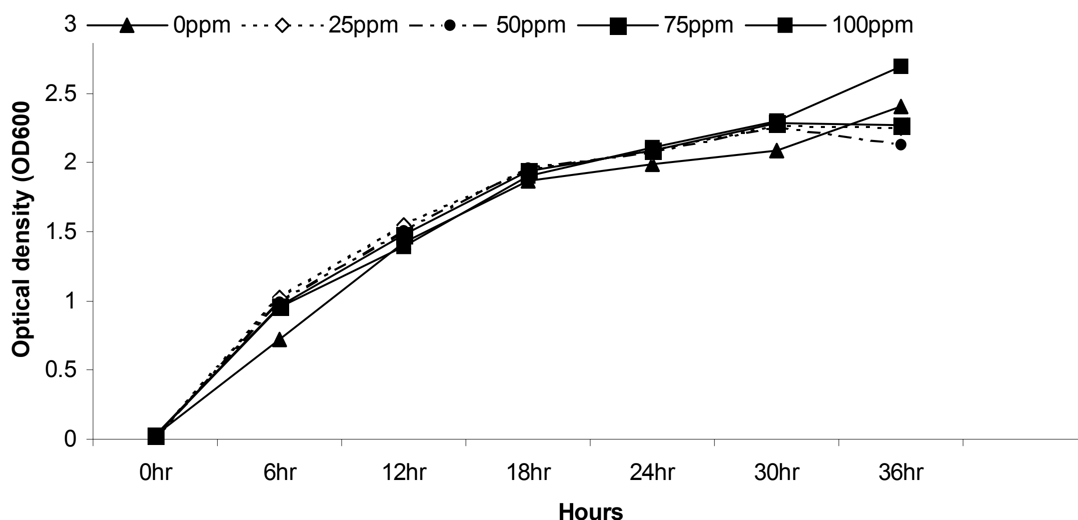
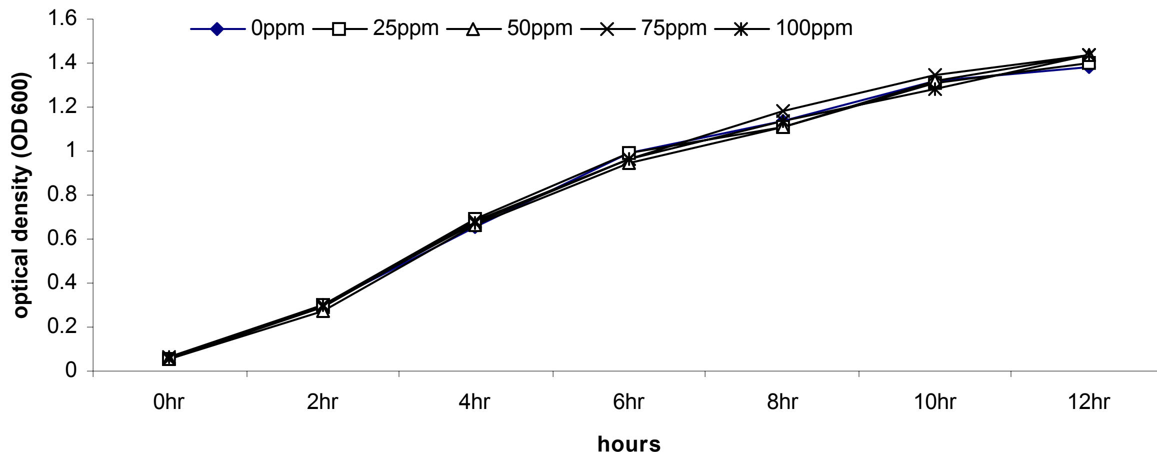


Figure 4 - Growth kinetics of *P. aeruginosa* in presence of selenate**.**



Similar observations have been noted in case of *B. cereus* when amended with different concentration of selenite and selenate. In culture medium amended with selenate a comparatively extended lag phase has been observed longer that the one observed with selenite may be due to delayed induction/initiation of tolerance mechanisms in the presence of selenate. Growth of *B.cereus* was found to follow the similar pattern in case of culture amended with selenite and without selenite and a stationary phase has been observed after 10 hours of incubation. While in case of selenate-amended culture, variability has been observed i.e at higher concentration of selenate, optical density was comparatively lower than that of optical density at 25ppm and without selenate amended culture.

Figure 5 - Growth kinetics of *B. cereus* in presence of selenite

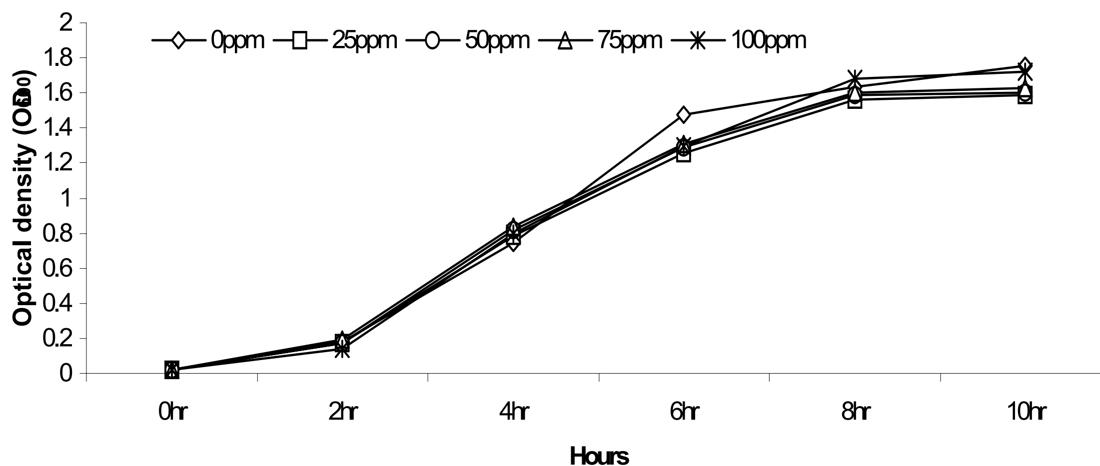
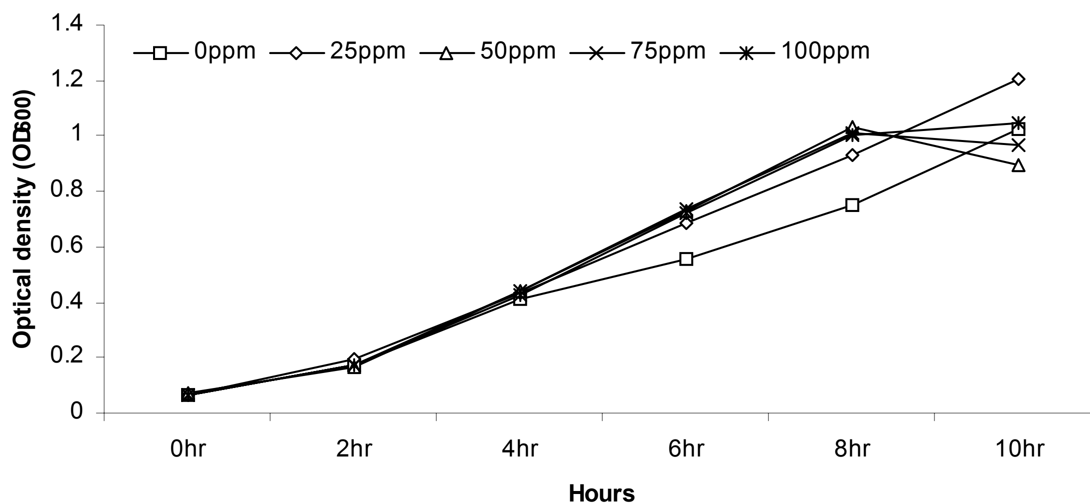


Figure 6 - Growth kinetics of *Bacillus cereus* in presence of selenate



The purpose of the growth profile studies was to understand the response of the test organisms to the presence of two different bioavailable forms of Se oxyanions. Apparently, Se does not present a toxicity problem to either of the organisms at the mentioned Se concentrations, which are about 4 orders of magnitude higher than in the environment from which the organisms have been isolated. The slight increase in the optical density during late exponential and stationary phase in some cases, compared to control, is due to the formation of Se^0. Optical interference of due to formation of Se^0 was visible in the presence of both forms of Se oxyanions. *Dungan and co-workers (2003)* reported such interferences in observation during studies with *Stenotrophomonas maltophilia* and indicated that in the presence of selenite the formation of Se^0 was visible only after 28hr. Se^0 precipitation was also reported in *Enterobacter taylorae* when exposed to 2000 –5000 μg/L *(Zahir, et. al., 2003).* This precipitation, similar to the present study, was noted both in growth medium as well as on the surface of the *E. taylorae* cells. In a study on selenium biotransformations in the culture medium by *E. cloacae* cells, *Losi and Frankenberger (1997),* reported Se^0 was of <0.1μm in diameter either free in the solution or protruding from the outer surface of the cells. *Kessi and coworkers (1999)* suggested that the presence of selenium particles on surface and in solution is an indication of vesicular mechanism to expel the biotransformed selenium. The transformation efficiency of Selenium oxyanions to Se^0 and further is influenced by the oxyanion concentration added to the medium. In a two day experiment, *Losi and Frankenberger (1997)* reported that about 90% of added selenite was reduced to elemental

selenium by *E. cloacae* when the selenite concentration was ranging from 5-100 mg/L, whereas only 62% reduction was observed when the selenite concentration was 1 mg/L which is indicative that the transformation mechanisms are further activated towards protection of the organism as the concentrations increase. *Zahir and co-workers (2003)* further indicated that *E. taylorae* was also capable of reducing newly formed Se^0 to Se^{-2} (selenides) in the high salt conditions. Similar observations were also recorded in the present study where Se was observed to volatilized by both the test organisms the report of which is presented in following discussion.

Selenium Volatilization.

Volatilization through biomethylation has considerable potential for use as a method of remediation. To date, the most investigated application has been the removal of selenium from soils. Removal of selenium by biomethylation and subsequent volatilization has been extensively reviewed *(Thayer, 2002).* The biomethylation of inorganic selenium by microbes, plants and animals is an important pathway by which toxic Se compounds are transformed into relatively non-toxic volatile forms such as dimethyl diselenide and dimethyl selenide *(Frankenberger and Karlson, 1994; Terry, et. al., 2000)*. Methylation/volatilization of Se is a very important process for removing Se form Se-contaminated environments. In a series of studies on Se biovolatilization from Se impacted drainage waters, *Thompson-Eagle, et. al., (1989) and Thompson-Eagle and Frankenberger (1990)* reported that microbial systems transform large amounts of selenite to volatile dimethyl diselenide. Selenium volatilization through the methylation process is thought to be the protective mechanism used by microorganisms to avoid toxicity in seleniferous environments *(Chasteen and Bentley, 2002).* This process permanently removes Se from contaminated soils and water under aerobic conditions, and minimizes its entry and food chain *(Azaizeh, et. al., 2003)*. Rhizospheric zone of plants contain large microbial population have been observed be potential sites for volatilization of selenium. Bacteria in rhizospheric sediments were more important than bulk sediment bacteria in removing Se by volatilization from a constructed wetland receiving selenite-contaminated effluent *(Azaizeh, et. al., 1997)*. Rhizospheric bacteria enhanced Se accumulation and volatilization by *Brassica jun*cea *(deSouza, et. al., 1997)* and *Azospirillum brasilense (Lin et al., 1983).*

Keeping the above aspect in view, the present study focused on Se volatilization potential of the test organisms in the presence of 75 ppm, concentration many times higher

than the environmental concentrations from which the organisms were isolated. In the present study, as elucidated in the methodology, the volatile fraction of Se was trapped in alkaline peroxide solution. On completion of the 72-hour study, Se was estimated using graphite furnace-AAS. The results are presented in following tables.

Table 3 – Se fractions in biomass of *P. aeroginosa*, cell free supernatant and alkaline peroxide trapping solution (volatile fraction), when organisms were exposed to selenium oxyanions

Sample	% Of Se in fraction	
	Selenate supplemented medium	Selenite supplemented medium
Supernatant	38.71	70.34
Biomass	49.12	13.89
Trapping Solution	1.238	1.380

Table 4 – Se fractions in biomass of *B. cereus*, cell free supernatant and alkaline peroxide trapping solution (volatile fraction), when organisms were exposed to selenium oxyanions

Sample	% Of Se in fraction	
	Selenate supplemented medium	Selenite supplemented medium
Supernatant	9.4	10.2
Biomass	87.8	85.9
Trapping Solution	2.3	3.8

In the present study, a definite indication of the volatilization potential was observed in the case of both the test strains. In the 72 hr study, it was noted that in the case of *P. aeruginosa,* the accumulation vis-à-vis transformation of selenium was dependent on the form of selenium oxyanion supplementation. Though the Se fractions indicate variations in the concentrations

in biomass and supernatant, the higher levels in the supernatant may be considered due to Se exudates (Se^0) after reduction by the test organisms. This is supported by the formation and gradual increase of red precipitation in the medium during 72 hr. In the case of *B. cereus*, Se fraction was more in biomass when compared to supernatant or trapping solution which indicative of preference towards accumulation/assimilation and reduction over volatilization in these organisms. Both the organisms have shown the potential to volatilize both selenium oxyanions, as noted in the fraction obtained alkaline peroxide trapping solution. The data is represented in percentages, as the GF-AAS analysis did not exhibit consistency in replicate samples due to inherent interferences in the instrumental technique.

Similar observations of selenium oxyanions to elemental selenium as well as volatile fraction were also noted by *Zahir and co-workers (2003)* during studies with *Enterobacter taylorae.* After 120 hr study, selenate and selenite were observed to be stabilized at lower concentrations, where as concentrations of Se^0 (1800 to 1500 μg/L) reduced in water and simultaneously Se^{2-} increased from 28.5 to 376 μg/L. The researchers concluded that selenate and selenite could be removed from Se-rich agricultural drainage by *E. taylorae* through two pathways: Selenium oxyanion reduction to Se^0, followed by precipitation in the water and Se volatilization to the atmosphere. In the studies examining the potential of rhizosphere microbes isolated from Se-impacted wetlands to biomethylate selenium, *Azaizeh and co-workers (2003)* observed that the total Se volatilized during 15 day of cultivation and the percentage of biomethylated Se from selenate or selenite in the presence of rhizosphere microbes was significantly higher. The authors reported that no Se was detected in the trap solution connected to uninoculated controls supplemented with selenate and selenite. However, the observations during the present study indicated marginal concentration (>0.05%) of Se in the alkaline peroxide solution after 72hr indicating some chemical transformation. Selenium biotransformations by *Stenotrophomonas maltophilia* indicated that Se-tolerant colonies growing on the plates turned red, demonstrating the ability of the organisms to reduce Se oxyanions to elemental Se which coincided with presence of volatile Se compounds *(Dungan, et. al., 2003).* The present studies though confirm the formation of volatile Se thus confirming microbial methylation the forms of Se could not be confirmed. The studies on *S. maltophilia* indicated that Se biomethylates into three alkylselenides namely Dimethyl diselenide (DMDSe), Dimethyl selenide (DMSe) and Dimethyl selenyl sulphide (DMSeS).

As on date, there are know studies indicating the biovolatilization of Se by tropical soil bacteria. The present study thus gives preliminary outlook on the biotransformations of Se,

which seems to be ubiquitous in bacteria from seleniferous and non-seleniferous soils in terms of reduction and volatilization, with either or both the mechanisms existing in the organisms based the ambient environment and growth conditions.

In conclusion, the present study primarily elucidates (i) the observations on Se tolerance by *Pseudomonas aeruginosa* isolated from seleniferous soils and *Bacillus cereus,* an isolate from non-seleniferous soils; and (ii) demonstrates the potential of soil bacteria in biotransforming Se through reduction or volatilization routes. Defining the possible mechanisms of biotransformations need to be further carried out to confirm the potential of these organisms for application in bioremediation protocols.

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