Characterization of two metal resistant *Bacillus* strains isolated from slag disposal site at Burnpur, India

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Abstract

Two strains of *Bacillus* sp. resistant to arsenate and lead designated as AsSP9 and PbSP6, respectively were isolated from the slag disposal site. They were identified to be related to *Bacillus cereus* cluster on the basis of 16S rDNA based sequence analysis and phenotypic characteristics. Both were rod-shaped (AsSP9, 2-5 µm and PbSP6, 2-4 µm), aerobic, salt tolerant (2-8% NaCl), endospore forming bacteria with minor differences like the AsSP9 showed sporangial bulging and PbSP6 had positive lipase activity. The temperature range for their growth was 20-40°C and pH range 6.0-9.0 with an optimum temperature of 37°C and pH of 7 for both strains. The principal nitrogen sources for AsSP9 and PbSP6 were DL-Tryptophan and L-Phenylalanine, respectively. The suitable carbon source for AsSP9 was lactose and for PbSP6 sucrose. The heavy metal accumulation efficiency was found to be 0.0047 mg g^{-1} of dry mass for AsSP9 and 0.686 mg g^{-1} of dry mass for PbSP6.

Key words

Slag disposal, *Bacillus* strains As and Pb resistant, Phylogram

Introduction

Contamination of heavy metals in the environment is one of the major concerns because of their toxicity and threat to human and other forms of life. Among the most potent heavy metals arsenic and lead are considered to be extremely toxic to all forms of life. Arsenic is a metalloid which ranks twentieth in abundance of elements in the earth crust (Mandal *et al.*, 1996). Arsenic can be present in soils, air and water as chemical compounds of both inorganic and organic forms. Environmental arsenic pollution is increasing due to its mobilization from geological sources and anthropological and industrial activities (Nordstrom, 2002; Bhattacharjee and Rosen, 2007). In soil, arsenate and arsenite are the two most often encountered forms of arsenic by the living system (Cullen and Reimer, 1989; Balasoiu *et al.*, 2001). One of the major concerns is also the potential mobilization of arsenic in ground water. In India, West Bengal state is most affected from arsenic contamination in ground water. It has been estimated that six million people in West Bengal and 57 million people in Bangladesh have been exposed to arsenic through contaminated wells (British Geological Survey, 2001; Smedley and Kinniburgh, 2002).

Both forms of inorganic arsenic (arsenite and arsenate) are toxic to organisms; arsenite disrupts sulfhydryl groups of proteins and interferes with enzyme function, where as arsenate acts as phosphate analog and can interfere with phosphate uptake and transport. While arsenite shows greater toxicity and is more mobile under most environmental conditions, arsenate is the thermodynamically favourable form in aerobic waters (Jackson and Harrison, 2005).

Despite its toxicity, a number of microorganisms are capable of using either the oxidized form of inorganic arsenic (V) or the reduced form of arsenic (III) in their metabolism (Silver and Phung, 2005). Microbial arsenic detoxification may involve reduction of arsenate to arsenite by arsenate reductase followed by arsenite efflux by arsenite transporters (Chauhan *et al.*, 2009).

Since medieval times, lead has been used in piping, building materials, solders, paint, type metal, ammunition and castings. Recently, lead has been introduced in the environment from a variety of sources such as, storage battery, lead smelting, tetraethyllead manufacturing and mining, plating, ammunition, ceramic and glass industries (Kadirvelu *et al.,* 2001).

In mammalian systems, lead compounds have been shown to impair a number of physiological parameters including central nervous system (CNS) development and synthesis and functioning of neurotransmitters (Meredith *et al.,* 1988), reproduction (Goyer, 1991) and metabolic processes (Ma, 1991). Severe exposure to lead has been associated with sterility, abortion, still birth and neonatal deaths (Goyer *et al.,* 1972). Elevated levels of lead in soils may decrease soil productivity and a very low level of lead may inhibit some vital plant processes, *viz.*, photosynthesis, mitosis and water absorption with toxic symptoms of dark green leaves, wilting of older leaves, stunted foliage and brown short roots (Kabata-Pendias, 2001; Yang *et al.,* 2004). Lead resistant bacteria are known which can remove toxic lead from the environment. For example, *Pseudomonas marginalis* shows extracellular lead exclusion and *Bacillus megaterium* has intracellular lead accumulation efficiency (Roane *et al.,* 1999). Pb(II)-resistant strains of *Staphylococcus aureus* and *Citrobacter freundii* that accumulate metal as an intracellular lead-phosphate have also been isolated and studied (Levinson *et al.,* 1998).

Bacterial arsenate reduction and lead accumulation represents a potential environmental implication to bioremediation. So, in the present study, we have attempted to isolate and characterize two bacterial strains belonging to *Bacillus* sp. that are capable of accumulating considerable amount of lead and arsenate and can thus be employed in bioremediation.

Materials and Methods

Isolation of metal resistant bacterial strains: Slag samples (100 g) were collected in sterile plastic bags from IISCO (Indian Iron and Steel Company) slag disposal site which is located in West Bengal, India at 23° 40' 0 N latitude and 86° 55' 60 E longitude (Pandey and Maiti, 2008). The samples were mixed properly and enriched for arsenate and lead resistant clones by incubating 10 g of slag in 90 ml of sterile water amended with 10 ml Luria Bertani medium and 20 μg ml^{-1} sodium arsenate (NaH$_2$AsO$_4$) and lead acetate [Pb (CH$_3$COO)$_2$] at 37°C for 2 hr (Higham and Sadler *et al.,* 1984). Supernatants were plated at 10^{-2} dilution by spread-plate method on LB agar medium containing 20 μg ml^{-1} of NaH$_2$AsO$_4$ and Pb (CH$_3$COO)$_2$. The plates were then incubated at 37°C. The colonies which appeared after 3 days of incubation were further screened at higher concentrations (20-800 μg ml^{-1}) of each heavy metals [NaH$_2$AsO$_4$ and Pb (CH$_3$COO)$_2$]. Finally, two strains named AsSP9 and PbSP6 were selected for further studies.

Optimization of culture condition: Optimum temperature, pH and salt concentrations preferred by these bacteria were determined by growing the cells in LB broth at variable pH,
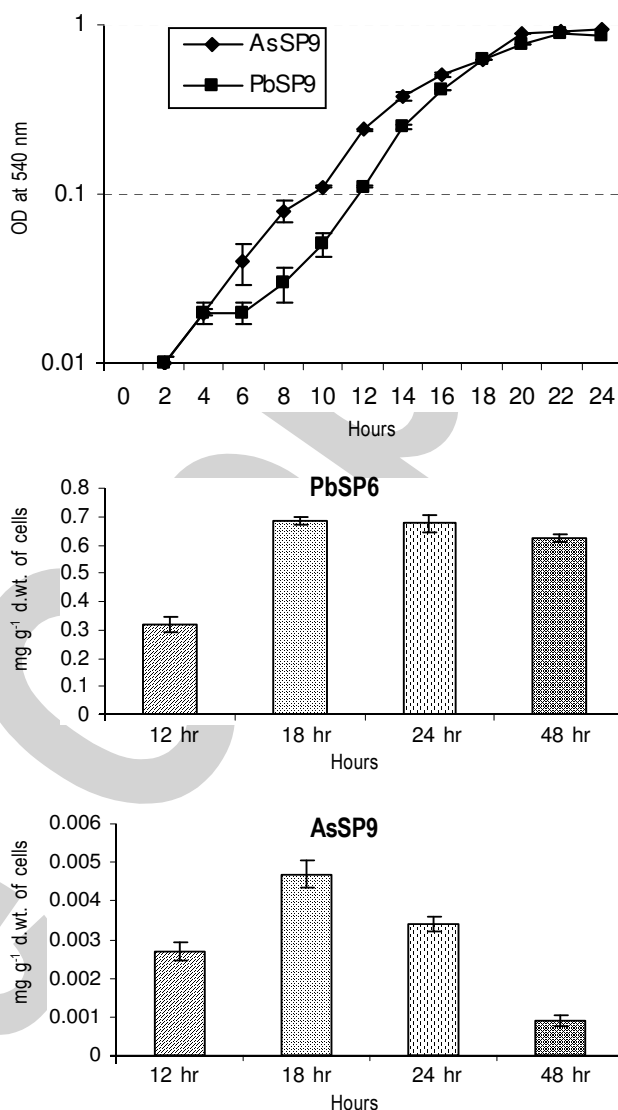

Fig. 1: A. Standard growth curve of the bacterial isolates; B and C. Accumulation efficiency of PbSP6 and AsSP9 at different growth stages. Values are mean of 5 replicates ± SE

temperature and salt concentrations. Growth patterns were determined for both under predetermined optimum conditions. The most utilizable nitrogen and carbon sources were determined by growth in Davis-Mingioli's medium (Davis and Mingioli, 1950) supplemented with respective nitrogen or carbon sources at concentrations of 0.1 and 1%, respectively. The growth was measured at 540 nm.

Determination of maximum tolerance limit and accumulation efficiencies: The maximum tolerance limit (MTL) was determined for the selected strains AsSP9 and PbSP6 in comparison to the sensitive strain of *E. coli* as control. For this the bacteria were grown in LB broth containing gradually increasing concentrations of sodium arsenate and lead acetate, respectively ranging from 100 -1200 μg ml^{-1} for resistant clone and 5-50 μg ml^{-1} for *E. coli* .
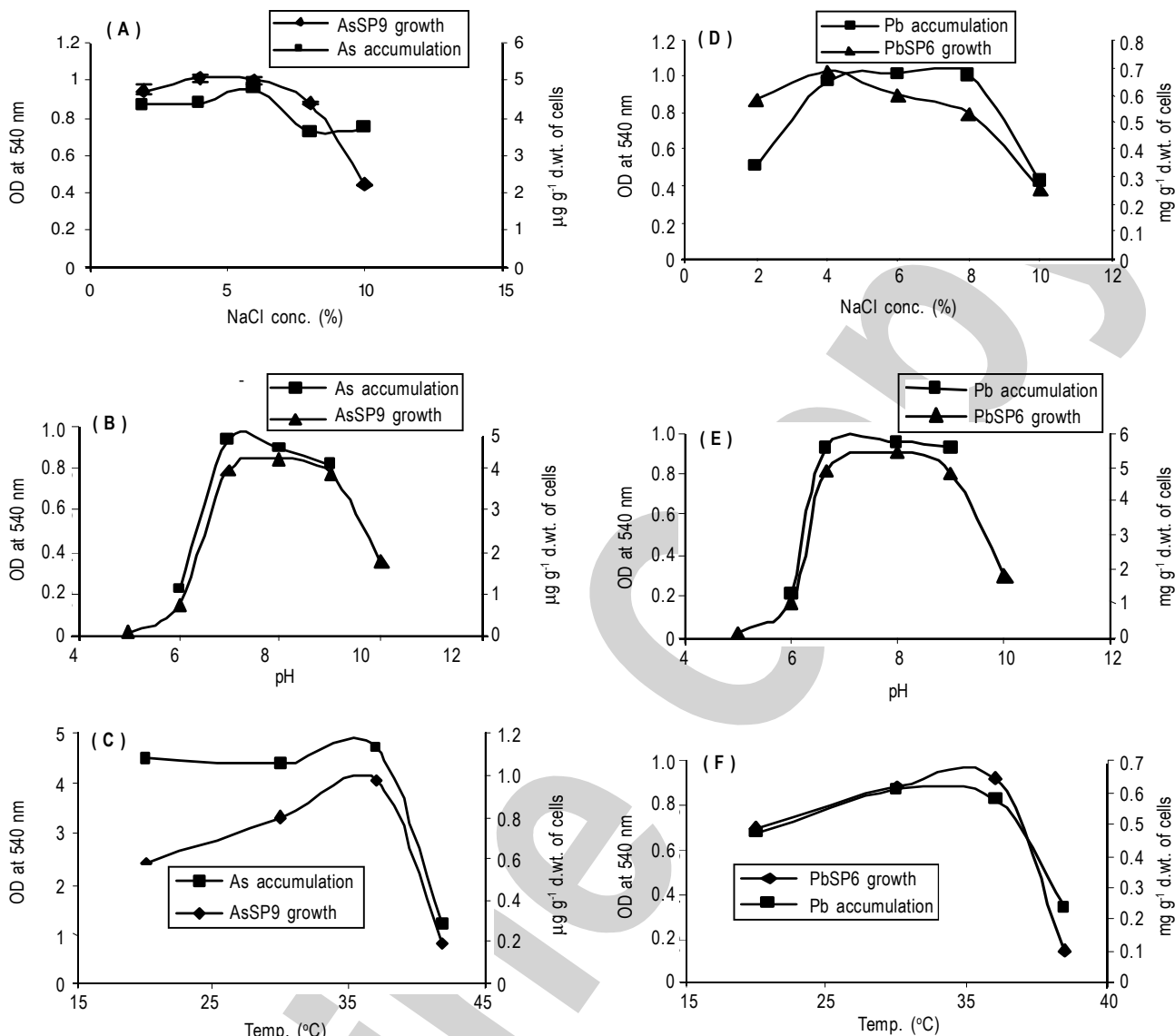
Fig. 2: Optimum conditions for growth and accumlation. A = Effect of NaCl, B = Effect of pH, C = Effect of temperature on AsSP9; D = Effect of NaCl, E = Effect of pH and F = Effect of temperature on PbSP6

The accumulation efficiencies of AsSP9 and PbSP6 were measured by Atomic Absorption Spectroscopy (AAS). For this 500 mg cell pellets obtained at different time interval were suspended in 20 ml miliQ water added with 5% concentrated HNO_3 and 0.5% concentrated HCl. The cell suspensions were then digested in Anton Paar MDS (Rotor Model No. 12HF100) according to User 001H of Perkin Elmer Application Note (Instruction Manual, Perkin Elmer AAnalyst 700).

Phenotypic studies: Phenotypic studies such as Gram staining, endospore staining, motility behaviour, biochemical tests, acid production tests etc were done following standard protocols (Benson, 1990).

Statistical analysis: Values are mean of 5 replicatesof ± SE . All data were subjected to student's *t*-test analysis with significance level of $p < 0.05$ using SPSS software package.

Scanning electron microscopy (SEM): For SEM overnight grown cultures were harvested by centrifugation at 4000 rpm and washed three times with 0.1 M PBS. Then the cells were fixed in 2.5% glutaraldehyde prepared in phosphate buffer for 1 hr. Cells were then washed with 0.1 M PBS three times to remove all traces of gluteraldehyde. After this - the cells were dehydrated, by treatment, in the following graded solutions of alcohol 30, 50, 70, 90 and 100%. Each treatment was given 10 min and the last one for 30 min. The cells were then mounted on cover slips with proper dilution and subjected to scanning electron microscopy (Watson *et al*., 1984).

DNA extraction and phylogenetic study: PCR amplification of the 16S rDNA was carried out using primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). Amplified PCR products were
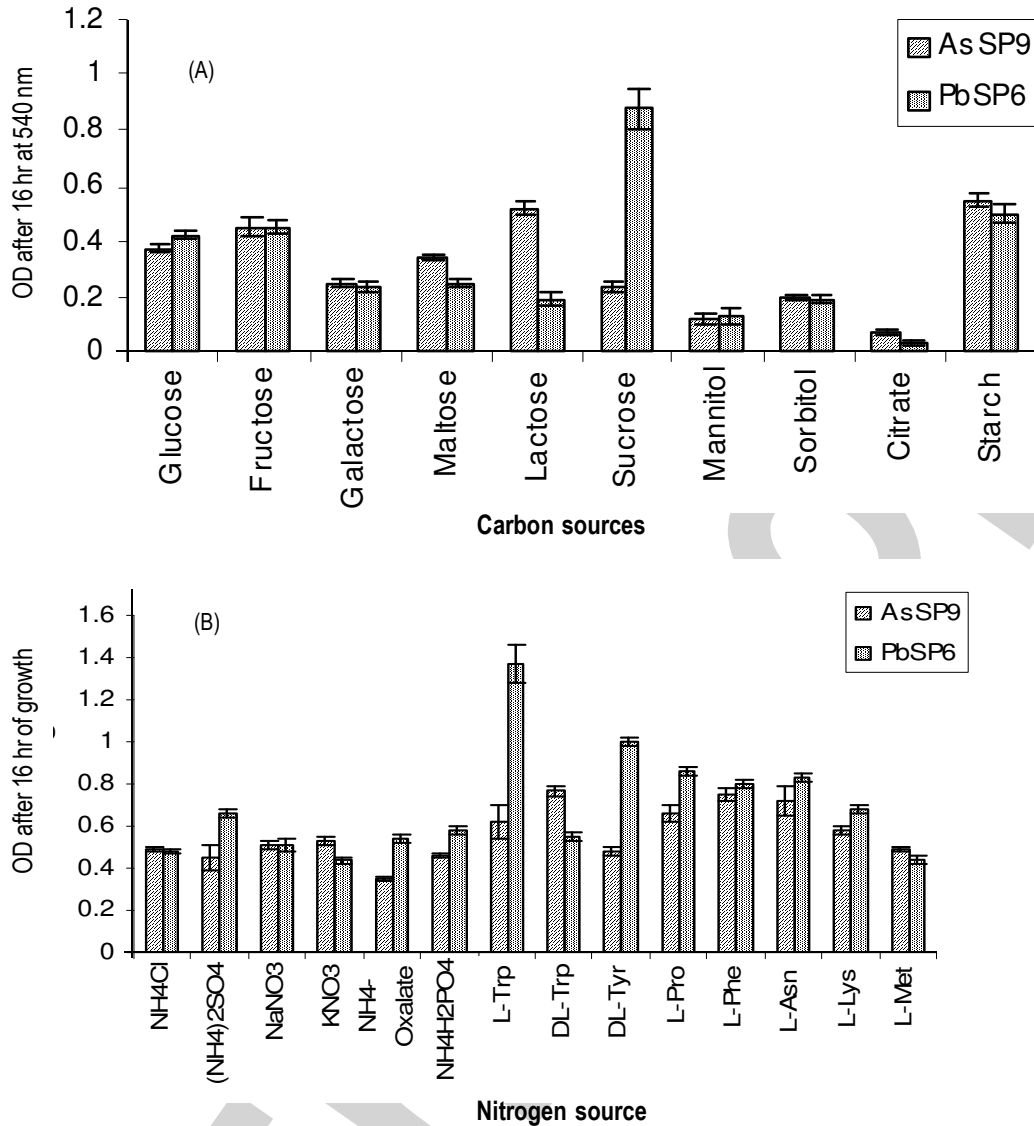
Fig. 3: Carbon and nitrogen source utilization. (A) shows different carbon source utilization and (B) shows different nitrogen source utilization

purified and sequenced and continuous sequences of 791 and 862 nucleotides of 16S rRNA gene for AsSP9 and PbSP6, respectively were used to search for homology through GenBank and RDP database (Saha and Chakraborty, 2006). Neighbour-Joining phylogenetic tree was constructed by TREECON software package with correction of Jukes and Cantor (Jukes and Cantor, 1969) using *Bacillus subtilis* strain DSM10T as an out group. Overall stability of the tree topology was determined by bootstrap analysis of 100 replications.

Results and Discussion

The growth pattern of AsSP9 and PbSP6 has been shown in the Fig. 1A. The result indicated a doubling time of about 100 min for both, however, the PbSP6 picked up slower than AsSP9. The stationary phase reached at about 22 hr in both. The optimum temperature, pH and NaCl concentrations required for growth and

accumulation were found to be 37°C, pH 7 and 4-6%, respectively (Fig. 2). The isolates, however, showed normal growth up to pH 9. The alkaliphilic nature and salt tolerant abilities of the strains were significant with respect to their application in metal contaminated, salinated alkaline soil for bioremediation. Moreover, the slag, the natural habitat from which these bacteria were isolated also had a pH of 9.8 (Pandey *et al.*, 2008). The increased metal tolerance in relation to pH has been reported in *Aspergillus* sp. by Faryal *et al.* (2007) . The arsenate and lead accumulation efficiencies as determined by Atomic Absorption Spectroscopy were found to be 4.7 μg g^{-1} of dry cells for AsSP9 and 0.686 mg g^{-1} of dry cells in PbSP6 (Fig. 1B,C). The accumulation efficiency of AsSP9 was low but its maximum tolerance level was about 800 μg ml^{-1} suggesting that the strain might have an efflux mechanism (Cervantes *et al.*, 1994) by which it is releasing the arsenate in its reduced form *i.e.* arsenite. Thus, AsSP9 survives in a medium that contains both
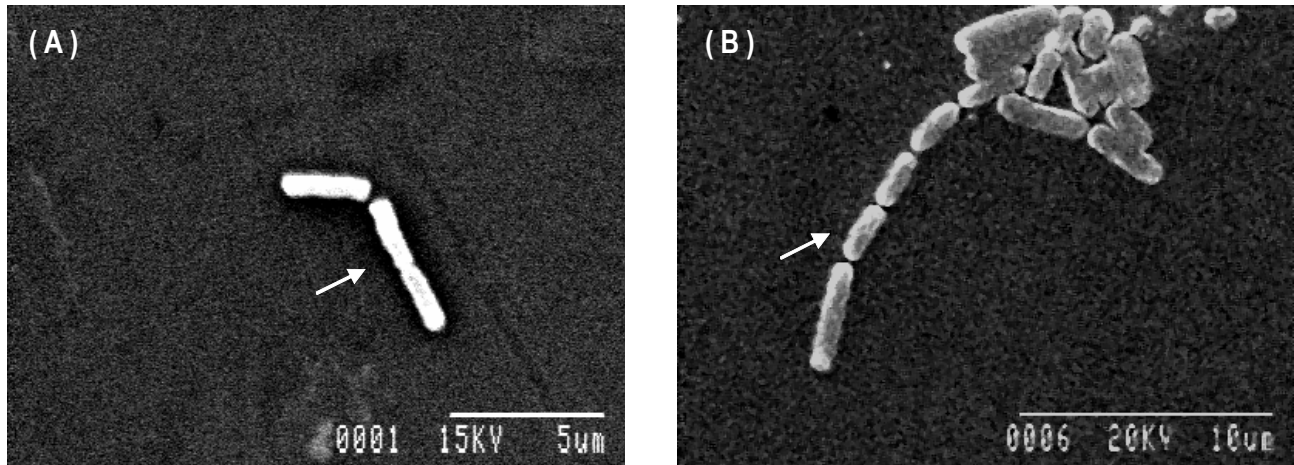
Fig. 4: Scanning Electron Micrograph at magnification x 5000 indicating phenotypic characteristics of two bacterial isolates. (A) PbSP6 showing rod shaped cells (size, 2-5 μm) in short chain; (B) AsSP9 showing rod shaped cells (size, 2-4 μm) in long chain. Chain length has been indicated by arrows
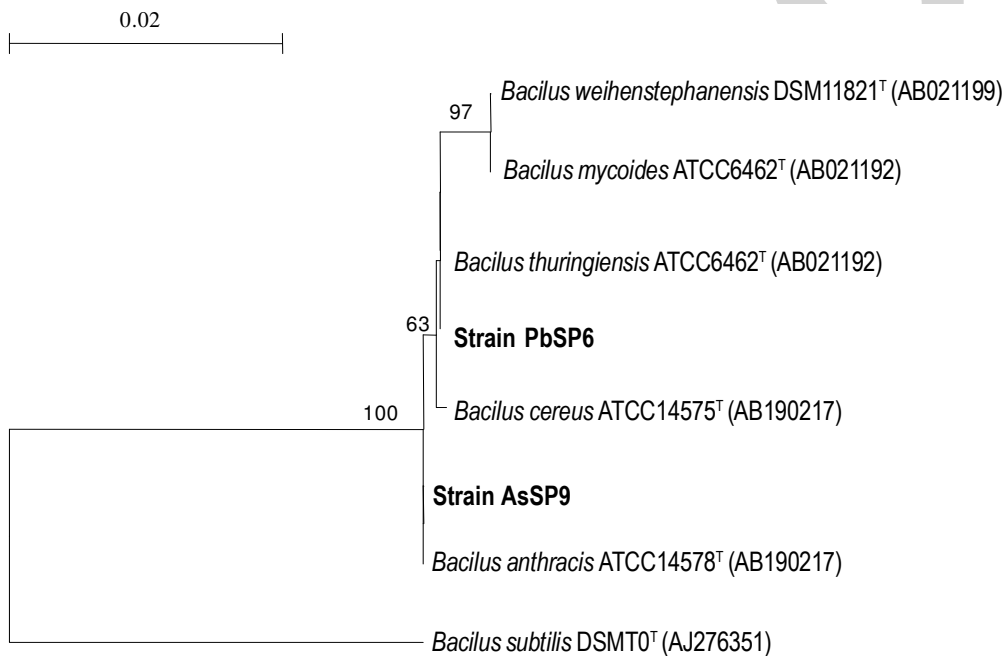

Fig. 5: Phylogenetic tree based on neighbour-Joining method showing the relationship between strains PbSP6, AsSP9 with various members of *Bacillus cereus* group. Bootstrap values of 100 replications (only values above 60) are shown at the nodes. The tree was generated using TREECON software. Sequence from *Bacillus subtilis* strain DSM10^T was taken as out group. Bar, 0.02 base substitutions per site

arsenate, which was added and arsenite, which was released from the cell. It is also purely resistant to arsenite (200 μg ml^{-1}). Reduction of arsenate to arsenite has been reported in several Gram - positive and Gram - negative bacteria such as *Bacillus arsenicoselenatis* (Switzer Blum *et al.*, 1998), *Bacillus selenitireducens* (Switzer Blum *et al.*, 1998) , *Chrysiogenes arsenatis* (Yamamura *et al.*, 2003), *Desulfosporosinus auripigmentum* (Newman *et al.*, 1997), *Shewanella trabarsenatis* (Saltikov *et al.,* 2003) *etc*. In PbSP6, the accumulation occured in the stationary phase, as well as the death phase. So a passive means of adsorption might be the mechanism involved in this case (Puranik *et al.*, 1999).

Since it was found that the accumulation efficiencies in both the cases were directly related to growth rate or biomass, we determined the most preferred nitrogen and carbon source for growth. The most suitable nitrogen source for the isolate AsSP9 was DL-tryptophan and for PbSP6 was L-tryptophan when these amino acids were used as sole source of nitrogen. The most suitable carbon source also varied. While AsSP9 preferred lactose as the best carbon source for growth, the PbSP6 preferred sucrose as the most suitable carbon source when used as the sole source of carbon in DM's medium (Fig. 3). A study of this kind may help us in designing cost effective nutrient media for these isolates.

Table - 1: Phenotypic characteristics of the isolates AsSP9 and PbSP6

Characteristics	AsSP9	PbSP6
Gram's reaction	+	+
Cell shape	Rods	Rods
Endospore	+	+
Motility	(+)	(+)
Biochemical tests		
Indole test	-	-
Methyl red test	+	+
Voges Proskauer test	-	-
Citrate utilization	+	+
H_2S production	-	-
Gas production	(+)	(+)
Casein hydrolysis	+	+
Esculin hydrolysis	+	+
Gelatin hydrolysis	+	+
Starch hydrolysis	+	+
Nitrate reduction	+	+
Catalase test	+	+
Oxidase test	+	+
Arginine dihydrolase	+	+
Tween 20 hydrolysis	-	+
Tween 40 hydrolysis	-	+
Tween 80 hydrolysis (Lipase test)	-	+
Acid production from		
Glucose	+	+
Maltose	+	+
Xylose	-	-
Salicin	+	+
Mannose	-	-

+ = Positive; - = Negative; (+) = Weak positive

The results of the phenotypic studies have been depicted in the Table 1. Although, both the strains showed almost similar phenotypic characteristics, they differed with respect to sporangial bulging and lipase activity (Tween 20, Tween 40 and Tween 80 utilization). PbSP6 was lipase positive whereas AsSP9 was not. They also differed slightly with respect to cell size and arrangement as evident from the scanning electron micrograph (SEM) data which indicates a chain-like arrangement of the bacilli with AsSP9 forming longer chains (Fig. 4B) than PbSP6 (Fig. 4A). This chain-like arrangement is in accordance to their relationship with *Bacillus anthracis* as evident from the phylogram also, however, unlike anthracis the isolates showed motility.

Analyses based on partial 16S rRNA gene sequences indicated that both the strains have a degree of similarity in sequence (99.9%) between them. The strain PbSP6 showed an absolute 100% sequence similarity with closest *Bacillus thuringiensis* strain ZJOU-010 and *Bacillus thuringiensis* IAM 12077^T. While the strain AsSP9 showed 100% sequence similarity with *Bacillus cereus* strain ZQP3 and *Bacillus anthracis* ATCC 14578^T. Phylogenetic analysis was carried out using 16S rRNA gene sequences from type strains of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus thuringiensis* and *Bacillus weihenstephanensis*. The two heavy metal resistant strains showed greater than 99.0% sequence identity with type strains of *Bacillus cereus* group.

The neighbour joining phylogenetic tree (Fig. 5) revealed that the strain AsSP9 along with *Bacillus anthracis* formed a cluster while the strain PbSP6 formed a clade in between *Bacillus cereus* and the cluster formed by *Bacillus thuringiensis - Bacillus mycoides - Bacillus weihenstephanensis*. The strains, although showed high extent of 16S rRNA sequence similarity with members of *Bacillus cereus* group, in the absence of overall genome comparison data and detailed chemotaxonomic data, they could not be given a species status. These strains were concluded as *Bacillus* sp.

It has been shown that microbes have the capability to reduce arsenate, but whether they reduce it for detoxification or for growth is a matter of concern. In a sample of agricultural soil, arsenate reduction was not coupled to growth (Jones *et al.*, 2000), rather it could be linked to detoxification. However, in the JMM-4 strain of *Bacillus* sp., a close relative of *Bacillus arsenicoselenatis* isolated from arsenic contaminated mud in Australia had been capable of oxidizing lactate to acetate while reducing arsenate to arsenite (Santini *et al.*, 2002). The strain JMM-4 is also a haloalkaliphilic organism similar to the AsSP9. Regarding lead resistance there are reports that level of resistance for lead is also directly related with the biomass which is in accordance with our study (Konopka *et al.*, 1999).

Conclusively, two bacterial isolates belonging to the genus *Bacillus* sp. have been isolated from a slag disposal site of an iron and steel factory and characterized in the present study. The strains designated as AsSP9 is arsenate resistant and PbSP6 is lead resistant. They have been characterized with respect to their growth requirements and heavy metal accumulation efficiencies and a direct relation of biomass and accumulation could be established. Identification was based on phenotypic characteristics and partial 16S rRNA genes and thus the specific epithet could not be assigned. The 16S rRNA gene sequences obtained have been submitted to GenBank with an accession numbers of FJ798592 and FJ798593 respectively for PbSP6 and AsSP9.

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