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VERTICAL ZONATION AND SEED GERMINATION INDICES OF CHROMIUM RESISTANT CELLULOLYTIC AND NITROGEN FIXING BACTERIA FROM A CHRONICALLY METAL EXPOSED LAND AREA

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Abstract

Twenty eight cellulolytic and 25 nitrogen fixing bacteria were isolated from 20, 40 and 60 cm depths of the chromium contaminated land area. The cellulolytic as well as nitrogen fixing microbial communities in soil profiles were dominated by genus *Bacillus*. More diverse nitrogen fixing bacterial isolates belonging to different genera *Paenibacillus, Corynebacterium* and *Pseudomonas* were observed as compared to cellulolytic bacterial community. Majority of the cellulolytic bacteria were found inhabitants of 20 cm soil layer while 40 cm depth was the preferred zone for the nitrogen fixing bacteria. Screening of the bacterial isolates for chromium resistance showed that isolates designated as ASK15 and ASK16 were able to resist up to 1800 mg/l of chromium while the nitrogen fixing isolates which offered a maximum resistant level up to 1650 mg/l of chromium were ASNt10 and ASNS13. Nitrogen fixing isolates enhanced seed germination by 33% and expressed efficient nitrogenase activity up to 0.80 (C_2H_2 nmol/ml/hr). Growth promoting assay proved ASNt10 a potential isolate which produced 90 µg/ml of indoleacetic acid (IAA). Though cellulolytic isolates did not affect seed germination, a significant influence on root length similar to that of ASNt10 and ASNS13 with nearly 5-fold increase in comparison with uninoculated control was observed. The isolates ASK15, ASK16 were identified as *Bacillus cereus* while ASNt10 and ASNS13 as *Paenibacillus barcinonensis* and *Bacillus megaterium*, respectively.

Key words: Bioremediation, Rehabilitation, Metal pollution, Chromium reduction.

Introduction

Industrial proliferation in parallel to rapid urbanization has posed a serious situation regarding environmental health. Heavy metals' generated pollution is an emerging environmental deterioration factor. Developing countries have drastically been affected in this regard owing to poor management of industrial effluents. Detrimental effects of the contaminants over vast area of agricultural lands of the world make it a high attention seeking matter, particularly in developing countries where major economy is based on agricultural products (Sinha *et al*., 2006; Jamali *et al*., 2007). Disturbances of naturally occurring cleaning processes of pollutant recipient environments due to high accumulation of heavy metals have furthered the substantial threat to agro-ecosystems.

Chromium and its compounds are widely used in industries. The generated wastes contaminate both terrestrial as well as aquatic environments thus ultimately affecting plants, animals and even humans. Chromium loaded wastes in the form of stagnant ponds may result in seepage through soil. Long-term exposure of soil to chromium stress has been reported to affect its various physical, biochemical as well as microbial properties. It imposes a selective pressure that favours proliferation of microbes that are tolerant / resistant to the stress (Diaz-Ravina & Baath, 1996; Hutchinson & Symington, 1997).

Soils may suffer fertility losses due to depletion of soil nutrients under drastic effects of heavy metals on soil microbiota (Wani & Khan, 2010; Krujatz *et al*., 2011). It is commonly accepted that toxic metals, their chemical derivatives, metalloids and organometals can have significant effects on microbial populations and under toxic conditions more or less every index of microbial activity can be affected (Giller *et al*., 1998; Violante *et al*., 2008; Gadd, 2009). Nitrogen fixing bacteria are central to the nitrogen cycle while cellulose degraders recycle organic carbon. Deleterious effects of heavy metals on such microbes reduce soil fertility and hence crop yields (Panda, 2003; Kamnev *et al*., 2005).

Despite metal toxicity, many microbes grow and flourish in apparently metal polluted environments and a variety of mechanisms contribute to their resistance (Avery, 2001; Holden & Adams, 2003). Resistance to varying levels of the metal and ability to reduce the more toxic form of chromium make a variety of microorganisms effective biological tools for controlling the contamination by detoxifying chromate polluted waste waters and soils (Shakoori & Makhdoom, 2000; Megharag *et al*., 2003; Ahmed *et al*., 2005; Rajkumar *et al*., 2005; Sultan & Hasnain, 2006; Qazi *et al*., 2010). Majority of the studies, however, addressing isolation of metals' resistant microorganisms from polluted soils, depend on sampling from surface or certain depths (Bahig *et al*., 2008; Chihching *et al*., 2008; Karelová *et al*., 2011). While it is well known that microbial diversity and structure involved in different biogeochemical cycles vary along different soil depths (Fierera *et al*., 2003). It is pertinent here to note that deposition of a pollutant might have exaggerated the soil vertical physicochemical and thus microbiological variations. Therefore, it is important to study different depths of a given contaminated soil for isolation of particular bacteria of one's interest.

Vast land area around 400 acres adjoining leather tanning units in Kasur city had remained exposed to toxic chromium containing effluents. As for about a half century, the area had remained in the form of shallow ponds which received the industrial discharges. The objectives of the present study were to isolate cellulolytic and nitrogen fixing bacteria from different soil depths that could be employed for detoxifying chromium contaminated soils which may escalate nutrient turnover in the contaminated areas and thus promote plant growth. Agricultural land bioremediation is essentially a new field responding to the emerging and rapidly increasing environmental health concern.

Materials and Methods

Sample collection: Soil samples were collected from a land area called Deen Ghar, Kasur. Adjoining to the study area is a rich cluster of tanneries (leather tanning industries). The study location had been receiving effluents of the tanneries for a few decades and a decade before the whole area was resumed the look of shallow ponds withholding pinkish effluents. Thereafter, the tanneries effluents' drainage was improved and the area was also drained out. The area is situated in southern east of Kasur, a city of Punjab province of Pakistan with an area of 3,995 km^2, elevation 218 m (715 ft), located at 31.12° North latitude, 74.45° East longitude and a population density of 595/km^2.

Duplicate soil samples (A and B) from the subject area were collected from freshly dug out pits. Soil from a given point was sampled from 0−20, 20−40 and 40−60 cm depths in sterile glass bottles. Samples were immediately brought to the laboratory and processed for the isolation of bacteria. Soil from the described depth was sampled from vertical surface of a freshly dug out pit by pressing directly a sterile open mouthed glass bottle against the surface to avoid cross contamination.

Soil analyses: Soil pH was measured by making soil slurry in water in a ratio of 1:2. It was kept for one hour and pH was measured with the help of a pH meter. Moisture contents of the collected soil samples were determined as described by Gupta (2000). Five grams of a soil sample was sieved through 20 mesh sieve, weighed and placed in an oven at 80ºC. Weight of the soil was measured every day until a consistent weight achieved. Soil samples were also processed for determination of different metals concentrations. Total metal concentrations in soil were determined following EPA standard soil digestion method for chromium, lead and copper. For this purpose, soil was digested in a solution of nitric acid and perchloric acid in a ratio of 1:3 to analyse total Cr, Pb and Cu. Soil samples were processed in triplicate through atomic absorption spectrophotometer (AAS-Perkin Elmer AAnalyst 400). Soil organic carbon was estimated following the method of Ben-Dor & Banin (1989).

Isolation of cellulose degrading and nitrogen fixing bacteria: For isolation of cellulolytic bacteria, 1 g of a soil sample was suspended in 10 ml autoclaved distilled water. Then 0.1 ml of the serially diluted suspensions was spread on the selective cellulose-agar medium containing cellulose as a sole carbon source (Ogbonna *et al*., 1994). The composition of slightly modified cellulose-agar medium was (g/l): K_2HPO_4, 1.0; $NH_4H_2PO_4$, 5.0; NaCl, 5.0; $MgSO_4.7H_2O$, 0.2; yeast extract, 5; microcrystalline cellulose (Sigma), 10; and agar, 15. Cellulose degrading bacteria were isolated by incubating the inoculated plates at 37°C for 24 hrs.

The soil samples were processed in a similar way for isolation of nitrogen fixing bacteria on nitrogen free glucose-agar medium after Benson (1994). However, the inoculated plates were incubated for three days at 30°C. Following appearance of growth on selective media, colonies with varying morphological characteristics were selected and processed for routine pure culturing and preservation. Isolates were characterized morphologically (spore, morphology, gram characteristics and motility) and biochemically (catalase, oxidase, VP test, gelatin liquefaction, carbohydrate utilization; D-glucose, D-arabinose, D-lactose, D-galactose and xylose) and then identified in accordance with the methods recommended in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) and Microbiological applications (Benson, 1994).

Chromium resistance and reduction potential of the bacterial isolates: Cellulolytic and nitrogen fixing bacteria isolated from different soil depths were screened for chromium resistivity. For this purpose, chromium (K_2CrO_4) amended nutrient agar plates were prepared. Bacterial isolates were revived from the stocks and streaked on the nutrient agar plates harbouring 100, 250 and 500 µg/ml of Cr(VI). Growth of the isolates as an indicative of chromium resistivity was recorded for 10 days.

Determination of minimum inhibitory concentrations (MICs) of metals: For finding MICs of Cr, Cu, Pb and Hg for the bacterial isolates, microdilutions of double strength nutrient broth according to the required metal concentrations were prepared. The metal salts used were K_2CrO_4, $CuSO_4.5H_2O$, $Pb(NO_3)_2$ and $HgCl_2$. Two percent of fresh bacterial growth was inoculated into metal amended media and incubated at 37ºC for 48 hrs. Optical density of the growth was then recorded at 600 nm on a UV spectrophotometer.

Chromium reduction: For finding Cr(VI) reduction potential of bacterial isolates, 20 ml of nutrient broth spiked with 50 µg/ml of Cr(VI) as K_2CrO_4 was prepared in 100 ml culture bottles and inoculated with 10% of fresh (overnight incubated) bacterial culture. Inoculated bottles were incubated at 37°C in shaker at 120 rpm. Uninoculated broth processed similarly served as control. Following 48 hrs of incubation, 1.5 ml of a bacterial culture was taken and centrifuged at 10000 rpm for 5 min. Supernatant was analysed for residual Cr(VI) in the media by colorimetric method using diphenylcarbazide (DPC) reagent (Rehman *et al*., 2005). Percent Cr(VI) reduction was determined by the following formula:

% Cr(VI) Reduction = Final Cr(VI) Conc. after 48 hrs – Initial Cr(VI) Conc. at hr 0 × 100 / Initial Cr(VI) Conc. at h 0

Molecular characterization of the bacterial isolates: Isolates selected on the basis of high resistance against metals and efficient chromium reduction potential, were identified through 16S rRNA gene sequencing. For this purpose, genomic DNA was extracted from overnight grown cultures of bacterial isolates following Li *et al*., (2005). Primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) were used to amplify 16S rRNA. 50 µl total reaction volume was used containing 5 µl DNA extract, 5 µl each of 25 mM $MgCl_2$, 1 mM dNTPs, 5 pmol forward and reverse primers, 2 U/ml DNA *Taq* polymerase and 1X *Taq* buffer. Remaining volume was adjusted with DNA free water. The PCR cycle with denaturation for 3 min at 94°C following 35 cycles of denaturation for 30 sec at 95°C, annealing step of 2 min at 60°C and 1 min extension at 72°C with a final extension step of 30 min at 72°C was run in a thermal cycler (USA). PCR product was separated by electrophoresis on 1% agarose gel stained with ethedium bromide in TAE buffer. 1.5 kb amplified bands were visualized under UV (Gel Doc, Bio-Rad Laboratories, USA) and excised for purification using Gene Purification Kit (Fermentas) following the manufacturer's instructions. They were then got sequenced using Big Dye Terminator v3.1 cycle sequencing ready reactions (Macrogen, Korea) at the DNA Sequencing Facility, Korea. 16S rRNA gene sequences were assembled with phrap (version 0.990319). Homology searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). 16S rDNA sequences determined in this study were submitted to GenBank for obtaining accession numbers.

Characterization of selected nitrogen fixing isolates for nitrogen input and plant growth enhancement

Acetylene reduction assay: Nitrogen fixing ability of the bacterial isolates was determined through assessment of nitrogenase activity using acetylene reduction assay (ARA). Bacteria were grown in 20 ml of N-free medium in a 50 ml serum vial at 30°C for 10 days. Then 10% acetylene-rich environment was provided to the cultures through injection. Acetylene was prepared by dissolving calcium carbide in water while an equal volume of air was removed from cultures before injecting acetylene to generate space. For the analysis of acetylene reduction, 2 ml of gas from the head space was removed from incubated cultures and processed biochemically for nmol/ml/h estimation of ethylene following the method of Larue & Kurz (1973).

Indoleacetic acid production: Nitrogen fixing bacterial isolates were incubated in N-free broth supplemented with 0.1 g/l ammonium chloride and 100 ug/ml L-tryptophan at 30°C for 3 days with aeration at 120 rpm. Culture supernatant was then collected by centrifugation at 6000 rpm for 20 min. Indolic compounds were measured by mixing supernatant with Salkowski reagent in 1:1. The mixture was kept for 30 min in dark and O.D. was then measured at 535 nm (Vanieraki *et al*., 2011).

Ammonia production: Ability of the bacterial isolates to fix atmospheric N_2 to NH_3 was assessed qualitatively following the method of (Dye, 1962). Nitrogen fixing isolates were grown in 10 ml of peptone water (g/l: peptone, 10; NaCl, 5; Disodium hydrogen phosphate, 9; Potassium dihydrogen phosphate dodecahydrate, 1.5) and incubated for 48 hrs at 37°C. Following the incubation 0.5 ml of Nessler's reagent was added to the cultures. A yellow-brown colour after addition of Nessler's reagent indicated the formation/ presence of ammonia. Uninoculated peptone water served as control.

Characterization of cellulolytic bacteria

Cellulose hydrolysis zones: The cellulose degrading bacterial isolates were preliminary screened for their cellulolytic activity on the cellulose agar plates by measuring hydrolysis zones (mm) visualized by flooding the plates with Lugol's iodine (Kasana *et al*., 2008). For this purpose, bacterial growth was streaked over the selective cellulose medium agar plates (pH 5.5). Zones of cellulose hydrolysis were observed and recorded after 72 hrs of incubations at 37 °C. On the basis of high MIC of chromium, five cellulolytic isolates were selected for further studies and characterized for their cellulolysis.

Assay of cellulase production: The cellulase activity (U/ml) was estimated by o-toluidine method described by Hartel *et al*. (1969). Cell-free culture fluid of an overnight grown bacterial isolate in cellulose medium was used as enzyme source. This method measures the release of glucose, produced in 60 min following incubation at 37°C from a mixture of enzyme solution (0.1 ml) from microcrystalline cellulose (0.1%) in acetate buffer (0.1 M pH 5, 0.4 ml). After the incubation of 2 ml of 300 mM TCA (Trichloroacetic acid), 2 ml of o-toluidine reagent were added in reaction mixture and boiled for 8 min. Reaction was terminated by placing in ice cold water and $O.D._{625nm}$ was then recorded immediately after harvesting the bacterial growth, 0.5 ml of the supernatant was processed similarly for estimation of glucose produced during growth. The cellulase activity was then calculated.

Seed germination assay for bacterial cultures: The seed germination promotion activity of the selected nitrogen fixing and cellulolytic bacterial isolates was determined following a modification of the method described by Belimov *et al*. (2001). Bacteria were grown in nutrient broth for 24 hrs at 30°C and then centrifuged. The cells were suspended in sterile dH_2O. Five ml of bacterial suspensions or sterile water (negative control) were added to petri dishes containing sterile filter paper. Chick pea seeds surface-sterilized with 10% hypochlorite solution for 15 min and then washed several times with sterile water were placed on the wetted filter papers. Germination of the seedlings was measured during 3 days after incubation at 28ºC. The assay was done in replica of two per assay (containing 6 seeds per dish).

Results

Soil analyses: Physicochemical parameters which influenced microbial ecological characteristics were recorded along the different depths of soils (Table 1). The Kasur tanneries' exposed soil was found basic in nature

with a pH range of 7.31-8.3. The alkalinity decreased gradually down the soil with maximum pH of 8.3 at 20 cm depth and a minimum of 7.3 at 60 cm depth. A similar trend down the soil depths was recorded for organic contents. The highest organic contents upto 18.66% were measured for the top layer (20 cm) which were significantly higher than the 40 and 60 cm deep soils. In contrary to organic contents and pH, electrical conductivity increased with increasing soil depths. Middle soil depth of 40 cm showed highest moisture contents around 15% while the lowest value (13%) was observed for the upper 20 cm soil layer.

Amongst the metal contents of the soil chromium was the prominent metal and next to the rank there were copper and lead. Lead could be detected ≤1 µg/ml in soils of all the three depths. Highest quantities of the metals appeared for the middle soil layer (20 to 40 cm depth) with 576 µg/ml of Cr and 4.9 µg/ml of Cu. Lowest levels of the metals were recorder from the uppermost soil layer (Table 1).

Isolation of cellulolytic and nitrogen fixing bacteria: A total of 28 cellulolytic bacteria were isolated from the sampled soils. Biochemical and morphological characteristics of the bacterial isolates are shown in Table 2. Maximum numbers of different cellulolytic bacterial isolates were found in upper soil from which 18 of the cellulolytic bacteria were isolated. From soils sampled from 40 and 60 cm depths four and seven different cellulolytic bacteriaisolates could be isolated, respectively. The soil was dominated with *Bacillus* sp., representing 89%, 100% and 71% of the total cellulolytic isolates from 20, 40 and 60 cm soil profiles, respectively. *Pseudomonas* was the second larger community representing 5.5% and 29% of cellulolytic bacterial community at 20 cm and 60 cm soil depths, respectively while a third bacterial genera *Listeria* with 5.5% contribution in top soil community was also observed.

Distribution pattern of nitrogen fixing bacteria among three soil layers appeared different from that of cellulolytic isolates. A total of twenty five nitrogen fixing bacteria were isolated with highest diversity from soils sampled from the surface. Five, twelve and eight colonies harbouring nitrogen fixing bacteria were isolated from 20, 40 and 60 cm soil depths, respectively. Their biochemical and morphological features are presented in Table 3. Nitrogen fixing bacterial community was also dominated with *Bacillus* sp. (56%) whereas *Corynebacterium* sp. (20%) *Paenibacillus* sp. (12%) were major genera at second and third places. Isolates belonging to genus *Pseudomonas* and *Azotobactor* were observed at 40 cm and 60 cm depths, respectively.

Minimum inhibitory concentrations (MICs) of heavy metals in nutrient broth and agar media: Minimum inhibitory concentration (MIC) of metals against different cellulolytic bacterial isolates ranged from 1600 to1900 µg/ml for chromium (Cr), 1100 to1250 µg/ml for lead (Pb), 40 to 65 µg/ml for mercury (Hg) and 600 to 800 µg/ml for copper (Cu). Cellulolytic bacteria isolated from 40 cm depth showed highest chromium resistance as compared to rest of the isolates whereas the bacteria isolated from 0−20 cm depth showed lowest MICs (1600 µg/ml) of chromium (Table 4).

MIC levels for chromium and lead against nitrogen fixing bacteria were lower than those observed for the cellulolytic bacteria. The MICs ranged from 700 to 1650 µg/ml for chromium and 850 to 1050 µg/ml for lead. On the contrary, MICs of mercury and copper appeared higher against nitrogen fixing bacteria, with ranges of 40 to 65 µg/ml for mercury and 700 to 850 µg/ml for copper, as compared to cellulolytic isolates (Table 5).

All the cellulolytic and nitrogen fixing bacterial isolates were found resistant against 100 µg/ml of Cr(VI) as K_2CrO_4 while majority (68%) of the isolates was resistant against 500 µg/ml of Cr(VI) as K_2CrO_4 concentration too. However, long incubation (10 days) was required for the bacterial isolates to grow in the presence of 500 µg/ml of Cr(VI) for cellulolytic bacteria as compared to the nitrogen fixing isolates which could manifest their growth at 4th day of incubation at the higher concentrations of the metal. Chromium resistance patterns, evidenced by vivid growth of cellulolytic and nitrogen fixing isolates on nutrient agar plates are shown in Tables 6 and 7, respectively.

As can be seen from these tables, both categories of the bacteria, in general, could resist a maximum of 500 µg/ml of K_2CrO_4 when cultivated on the metal amended nutrient agar. This limit is too below than MICs of the isolates revealed when they were grown in K_2CrO_4 containing nutrient broths. As in case of the fluid environment all the isolates, except ASNs9 grew well in the presence of higher 100 to 1650 µg/ml of Cr(VI).

Table 1. Physiochemical properties of soil sampled from different depths of the study area.

Soil depth (cm)	Site	Colour	pH	Moisture contents (%)	EC(mV)	Organic contents (%)	µg/ml of		
							Cu	Pb	Cr
20	A	Brown	8.1 ± 0.01	13.16 ± 5.4	89.66 ± 16.91	18.66 ± 0.54	1.4±	≤1	84.4 ± 2.1
	B		8.3 ± 0.01	12.96 ± 3.4	80.52 ± 15.53	16.76 ± 0.32	1.6±	//	80.12 ± 2.6
40	A	Grey	7.6 ± 0.01	15.33 ± 3.5	129.33 ± 0.88	6.0 ± 0.3	4.9±	//	576 ± 32.11
	B		7.7 ± 0.01	14.33 ± 0.5	139.16 ± 1.3	8.6 ± 0.45	5.5±	//	451 ± 21.2
60	A	Brown	7.5 ± 0.02	6.9 ± 7.65	148.66 ± 4.8	5.0 ± 0.23	0.4±	//	227 ± 10.60
	B		7.3 ± 0.02	5.2 ± 1.65	152.19 ± 5.3	4.4 ± 0.12	0.8±	//	223 ± 13.18

Values are means ± S.E. of three replicates; //, as above; EC, electrical conductivity

Table 2. Phenotypic and biochemical characterization of the cellulolytic bacteria from different localities and soil depths.

SD (cm)	Site	IC	Mp	GS	M	ES	Gr in NaCl (6.5%)	Biochemical tests				Carbohydrate utilization					Hydrolysis of			Identified as
								Cat	Oxi	VP	MR	Gl	Ga	La	Xy	Ar	St	Ge	Ca	
20	A	ASK1	Short rods	−ve	+ve	−ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	−ve	+ve	+ve	−ve	+ve	−ve	*Pseudomonas* sp.
		ASK2	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	*Bacillus* sp.
		ASK3	Rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK4	Rods (Diplobacilli)	+ve	+ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK5	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	*Bacillus* sp.
		ASK9	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK10	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
	B	ASK11	Rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	−ve	*Bacillus* sp.
		ASK12	Rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	−ve	*Bacillus* sp.
		ASK13	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	−ve	*Bacillus* sp.
		ASK14	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK15	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	−ve	+ve	+ve	+ve	+ve	−ve	*Bacillus* sp.
		ASK16	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	−ve	*Bacillus* sp.
		ASK17	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK18	Rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK20	Coccobacillus	+ve	+ve	−ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	*Listeria* sp.
		ASK21	Chains of rods	−ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK22	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
40	A	ASK23	Rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
	B	ASK24	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK25	Rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK26	Chains of rods	+ve	+ve	−ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
60	A	ASK27	Rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK28	Rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK29	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK30	Rods	−ve	+ve	−ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Pseudomonas* sp.
		ASK31	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
	B	ASK32	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASK33	Rods	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	*Bacillus* sp.

Abbreviations used: SD, Soil depth; IC, Isolation code; Mp, Morphology; GS, Gram's staining; M, Motility; ES, Endospore staining; Gr, Growth; Cat, Catalase; Oxi, Oxidase; VP, Voges-Proskauer test; MR, Methyl red; Gl, Glucose; Ga, Galactose; La, Lactose; Xy, Xylose; Ar, Arabinose; St, Starch; Ge, Gelatin; Ca, Casein; +ve, positive; −ve, negative

Table 3. Phenotypic and Biochemical Characterization of the nitrogen fixing bacteria isolated from different soil localities and depths.

SD (cm)	Site	IC	Mp	GS	M	ES	Gr in NaCl (6.5%)	Biochemical tests			Carbohydrate utilization					Hydrolysis of			Identified as
								Cat	Oxi	VP	Gl	Ga	La	Xy	Ar	St	Ge	Ca	
20	A	ASNt1	Rods	+ve	−ve	−ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	*Corynebacterium* sp.
		ASNt2	Rods (Diplobacilli)	+ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
	B	ASNt9	Rods	+ve	−ve	−ve	+ve	+ve	−ve	−ve	+ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	*Corynebacterium* sp.
		ASNt10	Rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	−ve	*Paenibacillus barcininencis*
		ASNt11	Rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	*Bacillus cereus*
40	A	ASNf1	Chains of rods	+ve	+ve	−ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASNf3	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASNf3A	Chains of rods	+ve	+ve	−ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	*Corynebacterium* sp.
		ASNf4	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASNf7	Chains of rods	+ve	−ve	−ve	+ve	+ve	−ve	−ve	+ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	*Corynebacterium* sp.
		ASNf9	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASNf10	Rods (Diplobacilli)	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve	+ve	*Bacillus* sp.
		ASNf14	Long rods (chains)	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
	B	ASNf15	Rods (Diplobacilli)	−ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve	*Pseudomonas* sp.
		ASNf16	Chains of rods	+ve	+ve	−ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASNf16A	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASNf18	Chains of rods	+ve	+ve	−ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
60	A	ASNs1	Chains of rods	+ve	−ve	−ve	+ve	+ve	−ve	−ve	+ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	*Corynebacterium* sp.
		ASNs1A	Rods (Diplobacilli)	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	*Paenibacillus* sp.
		ASNs2	Rods (Diplobacilli)	+ve	+ve	+ve	+ve	−ve	−ve	−ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	*Paenibacillus* sp.
		ASNs5	Short rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	−ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
	B	ASNs9	Chains of rods	−ve	−ve	−ve	+ve	+ve	+ve	−ve	+ve	−ve	−ve	+ve	−ve	+ve	+ve	+ve	*Azotobacter* sp.
		ASNs10	Chains of rods	+ve	+ve	−ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASNs12	Rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.
		ASNs13	Chains of rods	+ve	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	*Bacillus* sp.

Abbreviations used: SD, Soil depth; IC, Isolation code; Mp, Morphology; GS, Gram staining; M, Motility; ES, Endospore staining; Gr, Growth; Cat, Catalase; Oxi, Oxidase; VP, Voges-Proskauer test; Gl, Glucose; Ga, Galactose; La, Lactose; Xy, Xylose; Ar, Arabinose; St, Starch; Ge, Gelatin; Ca, Casein; ; +ve, positive;−ve, negative

Table 4. Minimum inhibitory concentrations (µg/ml) of different metals against cellulose degrading bacterial isolates cultivated in nutrient broth for 48 hrs at 37ºC.

Isolate code	Cr	Pb	Hg	Cu
ASK1	1750	1100	60	800
ASK2	1750	1100	40	800
ASK3	1750	1100	40	800
ASK4	1750	1100	40	800
ASK5	1750	1100	65	800
ASK9	1750	1150	40	650
ASK10	1600	1100	50	650
ASK11	1850	1100	60	650
ASK12	1800	1100	60	800
ASK13	1850	1250	60	800
ASK14	1750	1150	40	650
ASK15	1800	1250	60	800
ASK16	1800	1150	60	800
ASK17	1750	1200	40	600
ASK18	1750	1200	40	600
ASK20	1750	1250	60	600
ASK21	1750	1250	40	800
ASK22	1750	1250	40	800
ASK23	1850	1100	40	800
ASK24	1900	1150	40	800
ASK25	1850	1200	50	800
ASK26	1800	1100	50	850
ASK27	1700	1000	40	600
ASK28	1650	900	40	800
ASK29	1600	1050	40	800
ASK30	1500	1050	50	650
ASK31	1650	1000	40	600
ASK32	1700	1200	40	600
ASK33	1800	1200	40	600

Table 5. Minimum inhibitory concentrations (µg/ml) of different metals against free living nitrogen fixing bacterial isolates cultivated in nutrient broth for 48 hrs incubation.

Isolate code	Cr	Pb	Hg	Cu
ASNt1	1350	1050	50	750
ASNt2	1350	1000	65	700
ASNt10	1650	1000	65	700
ASNt9	1400	1050	50	750
ASNt11	1450	1050	65	700
ASNf1	1350	1050	65	750
ASNf3	1450	850	60	700
ASNf3A	1400	1000	50	700
ASNf4	1300	1000	50	700
ASNf7	1350	1050	65	700
ASNf9	1300	1050	60	700
ASNf10	1350	1050	65	700
ASNf14	1300	1000	50	700
ASNf15	1000	1000	65	700
ASNf16	1300	1000	65	700
ASNf16A	1300	1050	40	800
ASNf18	1000	950	55	700
ASNs1A	1400	1050	50	800
ASNs1	1350	1050	65	700
ASNs2	1350	1050	70	750
ASNs5	1500	1050	65	750
ASNs9	700	1000	40	700
ASNs10	1350	1050	65	750
ASNs12	1300	1050	60	700
ASNs13	1600	1200	40	850

Chromium reduction potential of the bacterial isolates: Cellulolytic bacterial isolates were found more efficient chromium reducers as compared to the nitrogen fixing bacterial isolates. However, all of the isolates of both categories did reduce chromium at varying degrees. Minimum reduction levels of 22.14% and 12.47% were expressed by cellulolytic isolate ASK20 and the nitrogen fixing isolate ASNf9, respectively while growing in the presence of 50 µg/ml of Cr(VI). Both of the isolates were habitants of 20 cm soil depth. However, the cellulolytic isolates which showed complete or nearly complete chromium reduction after an incubation period of 48 hrs also belonged to 20 cm depth. Among these isolates ASK12, ASK13, ASK15 and ASK16 showed 100% chromium reductions while ASK11 reduced the metal upto 99.73%. Chromium reduction potential of the cellulytic isolates of along with the description of soil zones from which they were isolated is presented in Fig. 1.

When the nitrogen fixing bacterial isolates were screened for their chromium reduction potential, efficient reducers represented all the three soil depths. The isolates ASNt10 and ASNt11 from 20 cm, ASNf3 and ASNf3A from 40 cm and ASNs13 from 60 cm soil depths showed 100% chromium reduction after an incubation period of 48 hrs while thriving in the presence of 50 µg/ml of Cr(VI). Fig. 2 presents profile of chromium reduction potential of the nitrogen fixing bacterial isolates.

The efficient cellulolytic (ASK15 and ASK16) and nitrogen fixing (ASNt10 and ASNs13) bacterial isolates which showed high resistance against metals as well as completely reduced Cr(VI) up to 100%, following exposure to 50 µg/ml of Cr(VI) were selected for further analyses of their specific biogeochemical roles and identified following 16Sr DNA sequencing.

Molecular characterization of the bacterial isolates: PCR amplification of 16SrRNA resulted in nucleotide sequence of upto 1480 bp. BLAST analysis of ASK15 showed highest 99% similarities with *Bacillus cereus* strain PR15 (accession number: JQ435675.1) and *Bacillus cereus* strain MSU AS (accession number: JF907013.1), respectively. While the isolate ASNt10 and ASNS13 showed 97% and 99% similarity with *Paenibacillus barcinonensis* strain BP-23 (accession number: NR_042272.1) and *Bacillus megaterium* strain TBA-R1-001 (accession number: DQ872156.1), respectively. Based on this information the two cellulolytic isolates (ASK15 and ASK16) were designated as *Bacillus cereus*-ASK15, *Bacillus cereus*-ASK16 while nitrogen fixing isolates (ASNt10 and ASNS13) were designated as and *Paenibacillus barcinonensis*-ASNt10 and *Bacillus megaterium*-ASNS13. The accession numbers allotted for the respective isolates are given in Table 8.

Table 6. Chromium resistance of the cellulolytic bacterial isolates cultivated on chromium amended nutrient agar media incubated at 37ºC for 10 days.

Isolate code	Cr(VI) (µg/ml)		
	100	250	500
ASK1	+(3)[a]	+(3)	-
ASK2	+(1)	+(3)	-
ASK3	+(1)	+(3)	+(10)
ASK4	+(1)	+(3)	+(10)
ASK5	+(3)	+(5)	-
ASK9	+(1)	+(3)	+(10)
ASK10	+(1)	+(3)	+(10)
ASK11	+(1)	+(5)	+(10)
ASK12	+(1)	+(3)	+(10)
ASK13	+(1)	+(5)	+(10)
ASK14	+(1)	+(3)	+(10)
ASK15	+(1)	+(5)	+(10)
ASK16	+(2)	+(5)	+(10)
ASK17	+(1)	+(5)	+(10)
ASK18	+(1)	-	-
ASK20	+(5)	-	-
ASK21	+(1)	+(5)	+(10)
ASK22	+(1)	+(5)	+(10)
ASK23	+(1)	+(3)	+(10)
ASK24	+(2)	+(5)	+(10)
ASK25	+(1)	+(5)	-
ASK26	+(2)	+(5)	+(10)
ASK27	+(1)	+(3)	+(10)
ASK28	+(3)	-	-
ASK29	+(2)	+(5)	+(10)
ASK30	+(1)	+(5)	+(10)
ASK31	+(1)	+(5)	+(10)
ASK32	+(1)	+(3)	+(10)
ASK33	+(2)	+(5)	+(10)

+: Growth appeared; - : no growth

a: Values in parenthesis indicate the day at which growth became observable.

Table 7. Chromium resistance of nitrogen fixing bacterial isolates cultivated on chromium amended nutrient agar media incubated at 37ºC for 10 days.

Isolate code	Cr(VI) (µg/ml)		
	100	250	500
ASNt1	+(3)[a]	-	-
ASNt2	+(3)	+(3)	+(4)
ASNt9	+(3)	-	-
ASNt10	+(3)	+(5)	+(10)
ASNt11	+(3)	+(5)	+(4)
ASNf1	+(3)	+(5)	+(10)
ASNf3	+(3)	+(4)	+(10)
ASNf3A	+(3)	+(3)	+(4)
ASNf4	+(3)	+(5)	+(10)
ASNf7	+(5)	+(10)	-
ASNf9	+(3)	+(4)	+(4)
ASNf10	+(3)	+(5)	+(10)
ASNf14	+(3)	+(5)	+(7)
ASNf15	+(3)	-	-
ASNf16	+(1)	-	-
ASNf16A	+(5)	-	-
ASNf18	+(5)	+(5)	-
ASNs1	+(1)	+(4)	+(4)
ASNs1A	+(1)	+(4)	-
ASNs2	+(1)	+(4)	+(4)
ASNs5	+(1)	+(4)	+(4)
ASNs9	+(2)	-	-
ASNs10	+(4)	-	-
ASNs12	+(3)	-	-
ASNs13	+(1)	+(4)	+(4)

+: Growth appeared; - : No growth

a: Values in parenthesis indicate the day when growth appeared

Characterization of selected cellulose degrading bacterial isolates for cellulolytic potential: Screening of cellulose degradation potential of cellulolytic bacteria was accomplished by growing them on cellulose selective medium and subsequently measuring the hydrolysis zones after 72 hrs of incubation. ASK15 and ASK16 produced hydrolysis zone of 13.9 and 11.96 mm diameter. When the cellulase was quantified the isolate ASK15 yielded 2.79 and 1.3 U/ml following 72 hrs of incubation in the cellulose selective medium (Table 8).

Characterization of selected bacterial isolates for nitrogen fixation and plant growth enhancing activities: To estimate nitrogen fixing ability of the isolates, nitrogenase activity was quantified. The isolates ASNt10 and ASNS13 represented nitrogenase levels of 0.46 and 0.80 nmolC_2H_4/ml/hr, respectively. When the isolates were assessed for ammonia production both isolates showed positive results of the nitrogen fixers (Table 8).

The bacterial isolates ASNt10 from top soil showed significantly higher levels upto 91.74 µg/ml of the plant growth promoting hormone (IAA) as compared to the isolate of deeper soil depth which could maximally yield IAA upto 14.08 µg/ml (Table 8).

Effect of bacterial isolates on seed germination: Bacterization of chick pea seeds revealed significant positive effects of nitrogen fixers' inoculations on seed germination as well as root growth as compared to uninoculated control (Table 8). Nitrogen fixing bacterial isolates were more potent inducers of seed germination as compared to the cellulolytic isolates, however, nearly similar effects on root growth were observed for both categories of the bacterial isolates while significantly higher roots' growths were obtained in case of bacterially treated seeds than the respective controls. The nitrogen fixing isolate, ASNt10 not only enhanced seed germination and root length but also promoted lateral root development (Table 8).

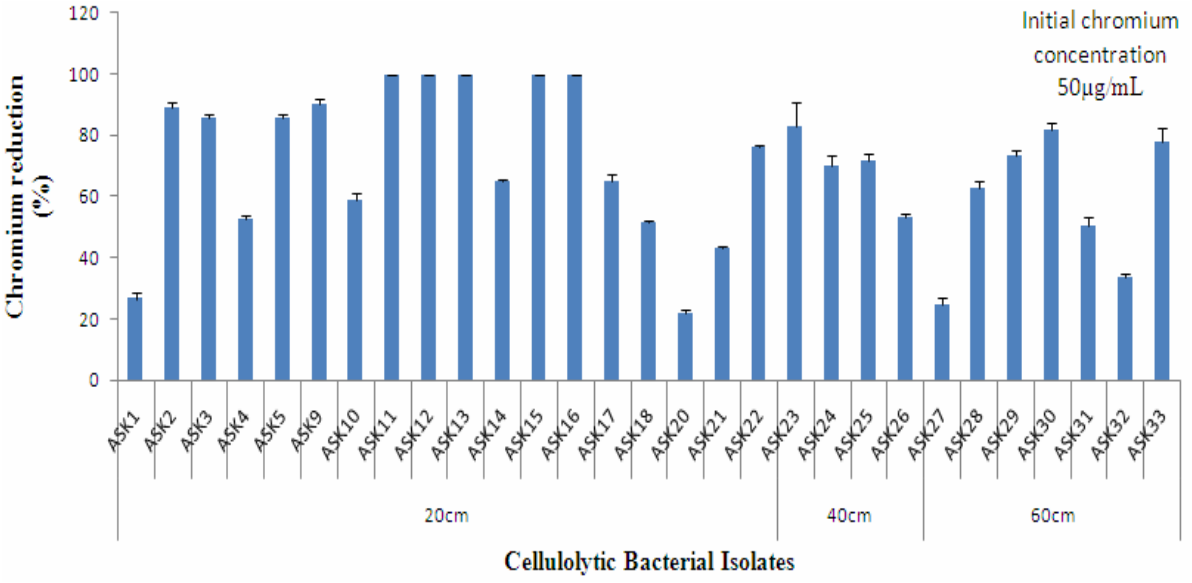

Fig. 1. Percent chromium reduction of cellulolytic bacteria representing different isolation soil depths incubated in nutrient broth ammended with 50 µg/ml of Cr(VI) following 48 hrs of incubation at 37°C; Values are means ± S.E. of triplicates.

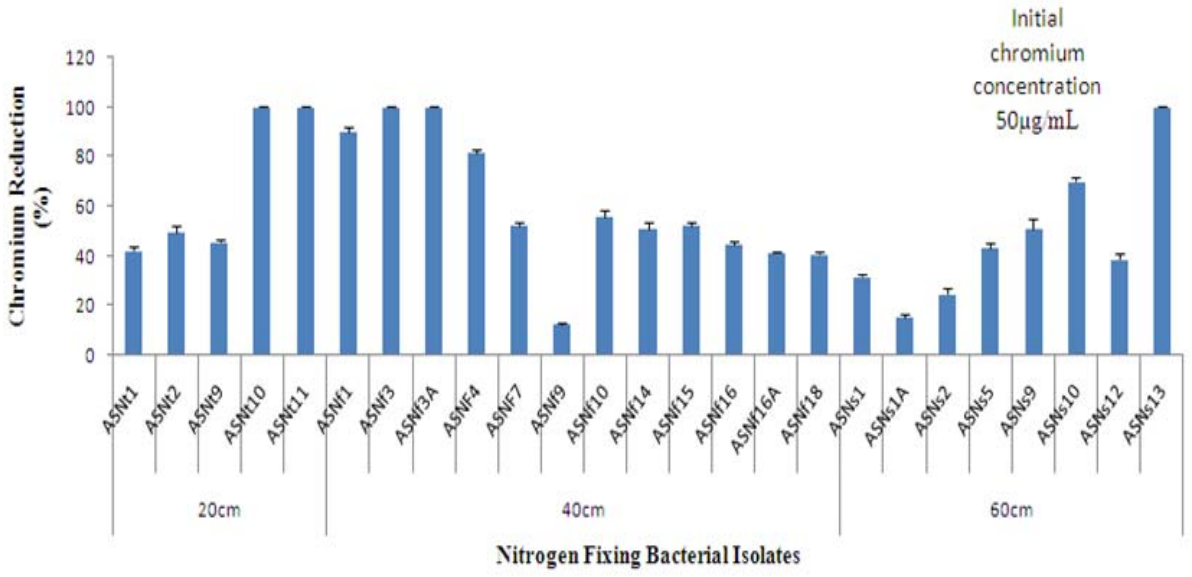

Fig. 2. Percent chromium reduction of nitrogen fixing bacteria representing different isolation soil depths incubated in nutrient broth ammended with 50 µg/ml of Cr(VI) following 48 hrs of incubation at 37°C; values are means ± S.E. of triplicates.

Discussion

Isolation of chromium reducing nitrogen fixing and cellulolytic bacteria from the metal contaminated soil yielded significant information about their potential of initiating / strengthening agricultural programmes in the pollution associated ruined land areas. Chromium stress, frequency of the isolations of cellulolytic as well as nitrogen fixing bacteria for different soil depths indicated effects of nutrients and oxygen availability etc. Dominance of aerobic cellulolytic microbiota in general cultivable lands (Saha *et al*., 2013) suggest their importance as biological augmentation agent for land recovery from soils ruined under long term exposure of heavy metals / other pollutants. More diversity of cellulose degrading bacterial isolates observed in uppermost soil layer, in the present study reflected cellulosic substrate availability. Plant as well as other organic wastes prevalence was evident in the study area. And cellulolytic bacteria maintain nutrient balance in such locations. Soil pH is another important factor for enrichment of specific microbial community. For the cellulolytic microbial groups, slightly alkaline soils facilitate their communities over the fungi for decomposition of cellulosic materials (Trujillo-Cabrera *et al*., 2013).

Table 8. Molecular identification and plant growth promoting activities of nitrogen fixing and cellulolytic bacterial isolates.

Bacterial isolates	Bacteria	Cellulose hydrolysis zone (mm)	Cellulase production (U/ml)	Indoleacetic acid (µg/ml)	Nitrogenase activity (nmolC_2H_4/ml/hr)	Ammonia production	% Seed germination (Control: 66.6[b])	Root length (cm) (Control: 0.4[c])	Accession No.
Cellulose degrading	*Bacillus cereus*-ASK15	13.9* ± 0.09	2.71* ± 0.21	ND	ND	ND	66.6[b]	1.9[b] ± 0.09	KF256130
	Bacillus cereus-ASK16	11.96 ± 0.11	1.31 ± 0.10	ND	ND	ND	50[c]	1.9[b] ± 0.06	KF256131
Nitrogen fixing	*Paenibacillus barcinonensi*-ASNt10	ND	ND	91.74 ± 6.22	0.46 ± 0.05	+ve	100[a]	2[a] ± 0.2	KC512769
	Bacillus megaterium-ASNs13	ND	ND	14.08 ±0.8	0.80* ± 0.04	+ve	100[a]	2.03[a] ± 0.1	KF256129

Values are means ± S.E. of three replicates and those with an esterics * or not sharing a common superscript are significantly different from others in a specific group at $p \leq 0.05$; +ve; ammonia produced; ND; not determined

These differences of cellulolytic bacteria isolated from different soil depths reflect variations of the soil level. In fact soil, a living heterogeneous matter, is under continuous pressure of changing environmental conditions due to biochemical, physical and anthropogenically imposed influences whose cumulative effect determines availability of specific ecological niches for specific organism (Liesack *et al*., 1997). Soil maturation and development depends upon the major biogeochemical cycles, organic matter decomposition and mineralization processes of soil microbiota (Swift & Anderson, 1993). An attempt made in the present study to evaluate the level of nutrient cycling in a chromium contaminated land area resulted in isolation of cellulolytic and nitrogen fixing bacteria but of varying potentials of nutrient cycling as well as chromium reduction from all the soil depths studied. Carbon and nitrogen are basic nutrients of soil and particularly important for plants growth. A balanced cycling of these elements is necessary to maintain soil health. Thus determination of functional microbial machinery responsible for nutrient homeostasis is very important.

Major diversity of nitrogen fixing isolates in this study was observed at deeper soil depths. These bacterial isolates were found to have a preference of their diversification at 40 cm soil depth. Since oxygen diffusion decreases with increases in soil depths, thus suitable soil depths may select and favour the bacterial groups for which facultative conditions are mandatory. Another possible reason could be the zone of plant root penetration where major nitrogen fixers are speculated to be present due to their particular role of nitrogen input (Johri *et al*., 2003). Thus it can be argued that to augment the soils with nitrogen fixers, specific depth(s) which might be defined with by the prevailing levels of O_2 be focused for successful results. It is noteworthy that besides the higher diversity of nitrogen fixers recovered from the depth soil, the nitrogen fixing efficiency of such isolates was also higher than those isolated from upper soils.

Analysis of chromium reduction potential of the bacterial isolates showed that they could overcome chromium toxicity of varying degrees by converting Cr(VI) to Cr(III). All of the isolates reduced µg/ml of Cr(VI) from 12 to 100% at varying levels. Biological chromium reduction has previously been reported in many bacterial genera under varying mechanisms involving intracellular metabolism, metabolic products or enzymatic processes. Understanding of such mechanisms has highlighted these microbes as amazing biotechnological tools for remediation of metals contaminated wastes (Cheung & Gu, 2007; Wani *et al*., 2007; Kathiravan *et al*., 2011). Metal tolerance of bacteria greatly varies with respect to biochemical and physicochemical properties of isolation source. The metal analysis of soil used in this study revealed that chromium was the dominant contaminant. Thus, the isolates accordingly showed higher levels of chromium resistance as compared to other metals. A variety of chromium of multiple metal resistant bacteria has previously been isolated from contaminated sites. Resistance mechanisms are either plasmid linked which encode membrane transporters to efflux metals while chromosome linked resistance is related to detoxification mechanisms involving reduction (Ramirez-Di'az *et al*., 2007). Detailed analysis in

this regard is required for the present isolates as the information of metals' reduction mechanisms is pivotal for practical applications and concerned predictions.

Higher nitrogen fixation potential (0.80 nmol C_2H_4/ml/hr) of the isolate ASNS13, of the present study than the other (ASNt10) dictates for importance of thorough screening of all the initial isolates from an environment to obtain the efficient microorganism(s) of desirable characteristics. The isolates from deeper soil layers, in general, showed even higher nitrogenase activities. Many metal resistant nitrogen fixing genera viz., *Bacillus*, *Paenibacillus, Azobacter* and *Rhizobia* have previously been reported (Wani *et al*., 2007 & 2008; Khan *et al*., 2010). Nitrogen fixing bacteria are more sensitive to heavy metals as compared to heterotrophic bacteria (Ahmed *et al*., 2005). Most of the nitrogen fixers also produce extra-cellular polysaccharides which help them withstand heavy metals toxicity (Geesey & Jang, 1990). A 30% reduction of nitrogen fixing activity of salt marsh sediments under high chromium concentration reported by Slater and Capone (1984) together with above information rendering the sensitivity of nitrogen fixers to heavy metals are suggestive of first determination of tolerable level of a given pollutant before the bacteria are adapted for N cycling under the stressed environments.

Rehabilitaion and re-vegetation of heavy metal polluted soils using metal reducing / resistant microbes with potential capabilities of plant growth hormone production may bioremediate the localities as well as enhance plant yields through stimulation of plant growth (Bashan & Holguin, 2002; Khan *et al*., 2009). Improved seed germination with indoleacetic acid (IAA) producing bacteria is a well known phenomenon (Idris *et al*., 2007). IAA enhances plant growth through cell enlargement, proliferation and differentiation. The hormone, a member of auxin family, not only work under metal stress when nitrogenase and phosphorous solubilization get inhibited but also stimulate plant uptake of nutrients and minerals from soil in conjunction with IAA of plant (Teale *et al*., 2006; Spaepen, 2007). Significant yields of IAA by the nitrogen fixing bacteria and prominent effects on chick pea seed germination as well as root lengths as compared to uninoculated controls in the present study permit application of the isolates for selected metal contaminated soils' remediation together with promising agricultural yields. It is admitted here that the present description is based on bacteria which were considered separate isolates based upon their colony morphologies, metals resistant levels and their cellulases and nitrogen fixing yields etc. Detailed biochemical characterization and molecular biological level identification of the selected isolates will give more insight for their potential applications.

Conclusions

Knowledge of habitat of microorganisms and information about the particular location(s) of a natural or anthropogenically created / modified environment might increase likelihood of isolating bugs of interest. Bacteria expressing cellulolytic and nitrogen up to 2.7 U/ml and 14 nmol/ml/hr, respectively have been isolated during the course of the present study. Nitrogen fixers could produce IAA upto 90 µg/ml. Isolation and preservation of the heavy metals' resistant cellulolytic and nitrogen fixing bacteria are promising for designing strategies addressing agricultural rehabilitation of metals, specifically chromium contaminated lands.

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