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Isolation and characterization of an endophytic bacterium, *Bacillus megaterium* KHDEB4, associated with root-nodules of green gram cv.OUM-11-15 (*Vigna radiata* L.) and its role in Chromium (VI) tolerance

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Abstract
Vigna radiata L. (Green gram) is an important legume crop in India due to its high nutritive value and proteinaceous nature. Soil bacteria exist in root or root-nodules of *V. radiata* in either symbiotic relationships or in associations. In the current study, the endophytic bacterium *Bacillus megaterium*strain KHDEB4 was isolated from surface-sterilized rootnodules of *V. radiata*cv.OUM-11-15and characterized phenotypically and genotypically. The results indicated that KHDEB4 consumed a variety of sugars as sole carbon source, and produced catalase, amylase, urease but not nitrite as well as nitrate reductase. KHDEB4 exhibited some plant growth-promoting traits, such as production of indole acetic acid and acetoin (in MR VP test). In addition, comparative sequence analysis of the 16S rRNA gene showed that KHDEB4 exhibited 99 % homology with *Bacillus megaterium.*The 16S rRNA sequence of *Bacillus megaterium*strain KHDEB4was submitted to NCBI gene bank and catalogued the accession number as KY679149. *Bacillus megaterium*strain KHDEB4 was found to be pH tolerant (up to 8.5) and also could tolerate heavy metal Chromium (VI) in the form of potassium dichromate (K_2 Cr_2 O_7)salt up to concentration of 130ppm. In conclusion, *Bacillus megaterium*strain KHDEB4belongs to the group of plant growth promoting rhizobacteria and could have significant applications on Cr (VI) tolerance and its rhizoremediation along with crops.

Keywords: *Bacillus megaterium*, *Vigna radiata*, endophyte, Chromium

Introduction
Endophytes are group of microorganisms having the ability to enter inside the plant hosts colonizing the intercellular spaces. They exist in a range of tissues types within a broad range of plants, colonizing the plant systemically with bacterial/fungal colonies and biofilms (Jalgaonwala, 2011).They are ubiquitous, colonize most of the plants, and have been isolated from almost all plants examined till date. Endophytes are to live symbiotically within the plants. While growing inside the plant the endophytes shows no visible symptoms of infection and disease. The close association of endophytes with internal tissues of host plant has increasingly gained them scientific and commercial interest due to their potential to improve plant quality and growth.They exhibit complex interactions with their hosts which involves mutualism and antagonism. Their association can be obligate or facultative. Plants strictly limit the growth of endophytes, and these endophytes use many mechanisms to gradually adapt to their living environments. In order to maintain stable symbiosis, endophytes produce several compounds that promote growth of plants and help them adapt better to the environment (Ulrich *et al*, 2008).Some of the endophytes are known to protect their host from being attacked by fungi, insect and mammals by producing secondary metabolites.Among them, endophytic bacteria are thought to interact closely with their host plants, and therefore could be used as biological control agents in sustainable crop production.Endophytes are known to supply nutrients to plant by fixing atmospheric nitrogen and solubilizing ion. This ultimately leads to increase in plant immune system as well as protects plant from infection by plant pathogens.studies have also shown role of endophytes in removal of soil contaminants. Studies on endophytes their significance and role in plant metabolism is an important area to explore.

Green gram [*Vigna radiata* (L.) Wilczek], is an important pulse crop of India. It is also commonly known as mung bean, which is an ancient and well known leguminous crop of Asia. It is quite versatile crop grown for seeds, green manure and forage and it is also considered as Golden Bean because of its nutritional values and suitability for increasing the

fertility of the soil, by the way of addition of nitrogen to the soil. On an average, pulse crops add upto 30 kg nitrogen per hectare per year. Because of its short duration, it fits well in crop rotation and mixed cropping systems. Presently, the per capita share of pulses in nutrition supply in India with respect to energy, protein and fat is 117.4 K cal, 6.9 g and 1.0 g per day respectively. An adult male and female requires 80 and 70 g per capita per day, respectively for balanced diet.Green gram crop covers a total world area of 5 m ha with a total production of 3 mt. It is widely cultivated throughout the South Asia including India, Pakistan, Bangladesh, Sri Lanka, Thailand, Cambodia, Vietnam, Indonesia, Malaysia and South China. India is an important pulse growing country contributing 28 per cent to the global pulse basket from an area of about 37 per cent (Masood Ali*et al*. 2000).

Soil pollution is the primary source that transmits pollutants like heavy metals from environment to living organisms. From soil, plants adsorb and accumulate heavy metals. Through the food chain, heavy metals enter the animal kingdom including humans and cause health risks. Few physicochemical and phytoremediation approaches have been proved effective in removing heavy metals from contaminated soils. The toxicity of plants due to heavy metals, particularly on agricultural economic crops, presents a challenge to plant scientists concerned with yield and quality in crop production (Bishehkolaei *et al.* 2011). Some heavy metals such as chromium (Cr), lead (Pb), mercury (Hg), and cadmium (Cd), especially in large amounts, could affect growth and productivity of plants (Najafian *et al.* 2012). They are usually accumulated due to unplanned municipal waste disposal, mining, use of extensive pesticides, and chemical fertilizers (Pandey *et al.* 2008).

Nowadays, contamination of the environment by Cr has become a major concern and its toxicity to plants depends on its valence state with Cr(VI) being highly toxic and mobile than Cr(III) (Gupta *et al* 2009). Cr(III) occurs naturally in the environment and is an essential nutrient, whereas Cr(VI) is generally produced by industrial processes (Shanker *et al* 2005). Chromium (VI) is considered the most toxic form which usually occurs, associated with oxygen as chromate ($CrO4^{-2}$) or dichromate ($Cr2O7^{-2}$) oxyanions.According to Dixit *et al*., 2002 Cr is toxic to plants and interferes with several metabolic processes as exhibited by reduced seed germination or early seedling development, induced chlorosis in young leaves, reduced pigment content, damaged root cells, impaired photosynthesis, altered enzymatic function, stunted growth, and plant death (Panda *et al.*2003). In India, Cr (VI) contamination is a major problem around various industries using Cr compounds, which causes considerable negative impact on crop production (Chandra and Kulshreshtha 2004).

The application of plant–microbe interaction for the remediation of contaminated soil is an important and well-adapted technology (Hansda *et al*. 2014).. Rhizobacteria generally colonize root and enhance plant growth in degraded soils, as they can alleviate biotic and abiotic stress by releasing phytohormones (e.g., IAA, ethylene), solubilizing minerals and producing iron chelating compounds (e.g., siderophores) (Ahemad, 2015). Several studies have reported the utilization of plant growth promoting (PGP) rhizobacteria for the bioremediation of heavy metals including Bacillus sp., Pseudomonas sp. etc. (Samuel *et al*. 2013; Sobariu *et al*. 2016).

The present study is an attempt to reveal the role of isolated strain *Bacillus megaterium* strain KHDEB4 in tolerance of heavy metal chromium on leguminous plant, *Vigna radiata* (green gram)..

Materials and Methods

Isolation of Bacillus megaterium strain KHDEB4 from root-nodules

*Bacillus megaterium*strain KHDEB4 was isolated from Green gram (cv OUM-11-15) nodule samples were collected from Balarampur village ($N20^{0}11.881'$, $E85^{0}27.827'$) of Khordha district of Odisha, India. Nodules were dipped in 0.1% sodium hypochloride (NaOCl) solution for 30 s and washed successively ten times with sterile distilled water to remove the traces of toxic NaOCl. Surface sterilized nodules were then mixed with 5 ml sterile distilled water and crushed to obtain a milky suspension of bacteriods.The suspension was serially diluted and plated using the spread plate technique onto Nutrient Agar (Hi-Media, India) plates. The plates were incubated at 35 ± 2 °C for 48 h and observed for bacterial growth on agar surface. KHDEB4 were transferred regularlyto new agar slants to keep them viable.The morphological characteristics; color, diameter, and edgeof the colony of strain KHDEB4 was determined.

Biochemical characterization of the isolate

Morphological characteristics, viz. shape, size, motility and Gram's stain of the endophytic isolates were examined under phase contrast light microscope (×100 objective). Various physiological and biochemical tests such as triple sugar iron, mannitol, motility, methyl red (MR), voges-proskauer (VP) (acetoin production), citrate utilization (Simmon's Citrate Agar), anaerobic growth, amino acid decarboxylase, indole production, nitrate reduction, carbohydrate oxidation and fermentation and enzyme activities, i.e. urease, oxidase, catalase, cellulose, amylase, chitinase, lipase (Tributyrin), caseinase, Dnase, nitrate reductase, nitrite reductase, gelatinase were tested following standard methods (Bergey's manual) (Patel *et al*. 2013).The isolates were further screened for antibiotic resistance by disc diffusion method on Mueller-Hinton agar plate (Bauer*et al.* 1966).

Molecular characterization (16s rRNA)

The genomic DNA was isolated by using (PureLink Genomic DNA kit, Invitrogen) genomic DNA isolation kit. Amplicon was electrophoresed in a 1% Agarose gel and visualized under UV-VIS gel doc system. The 16S rDNA was PCR amplified using the forward primer (5'AGAAAGGAGGTGATCCAGCC3') and reverse primer (5'AGAGTTTGATCMTGGCTCAG3') at 94°C for 4 min, 94°C for 1 min, 58°C for 1 min, 72°C for 1.30 s for 30 cycles, and then 72°C for 8 min. The PCR reaction mixture consisted of template DNA (150 ng), enzyme: *Taq* polymerase (1.5 U/µl), 10 X *Taq* polymerase buffer (100 mM Tris (pH 9), 500 mMKCl,15 mM $MgCl_2$, 0.1% gelatin), dNTP mix (10 mM), 10 µm each primers. The amplified full length products (1.4 kb) were sequenced using ABI 3130xl analyzer following Sangers dideoxy termination method. The sequences were BLAST at ncbi.nlm.nih.gov and the Phylogenetic tree was constructed after multiple sequence alignment in MEGA4 software. The Phylogenetic tree was constructed after multiple sequence alignment using cluster algorithm (Yushmanov & Chumakov 1988).The phylogenetic tree was built on the matrix of pair distances between sequences. In the boot strap a multiple alignment was resembled 100 times.

Abiotic stress tolerance

pH tolerance

The identified isolate was inoculated to YMB maintained at pH 4.0, 5.0, 7.0, 8.5 and 10 respectively and incubated (30 °C, 72h). The turbidity of the medium was measured at an interval of 24 h for 3 days at 660nm wavelength using visible spectrophotometer.

Chromium tolerance (MIC)

The minimum inhibitory concentration (MIC) is the lowest concentration that prevented thebacterial growth; in this study, MIC was determined through turbidimetric analysis (Rajesh *et al*. 2014). The stock solution of Chromium[Cr (VI)] as Potassium dichromate ($K_2 Cr_2 O_7$) salt was prepared in double-distilled water and added to the nutrient broth in various concentrations (30 ppm to 150 ppm). Each tube was inoculated with approximately 1.5 * 10^6 colony forming units/mL cells. The optical density (600 nm) was measured after 24 h incubation at 37°C. in various concentrations (30 ppm to 150 ppm).

Results and Discussions

Isolation of endophytic bacterium

The endophytic bacterial colonies were isolated on YMA (Yeast mannitol agar) medium from the root nodule of green gram (cv OUM-11-15). Cell sizes of these bacterial isolates varied from small to medium but were rod shaped. Cells also appeared gummy, flat to raised, rough, colony size varied from small to very large and had a pinkishwhite colour. Isolate was found to be gram positive. These findings are synonymous with Hussain *et al*. 2002 and Sobti *et al*. 2015.

Biochemical and molecular characterization of the efficient isolate

The isolate KHDEB4 was a gram positive, aerobic, spore forming, motile, rod shaped bacterium.It showed medium growth on medium with 10% NaCl while growth was luxuriant on medium with 10 % lactose and glucose. The isolate was found positive for MR,VP and indole. The isolate was positive to urease, catalase, oxidase, caseinase, and urease but was negative to nitrate reductase, nitrite reductase and lipase (Table 4). The isolate was observed to be capable of utilizing fructose, dextrose, galactose and sorbitol as C source through both oxidative and fermentative pathways (Table 5). Antibiotic screening showed that, KHDEB4 was sensitive to erythromycin, ciprofloxacin, neomycin, amikacin, and streptomycin but resistant to amphotericin (Table 6).

Table 1: Biochemical tests of isolate KHDEB4

Tests	Results	Tests	Results
Oxidase	+ve	Nitrite Reductase	-ve
Catalase	+ve	H_2S production	+ve
Lipase	-ve	Mannitol motility	+ve
Caseinase	+ve	Methyl red	+ve
Dnase	-ve	Voges–proskauer	+ve
Chitinase	-ve	Citrate	-ve
Urease	+ve	Indole	+ve
Amylase	-ve	Growth on anaerobic agar	-ve
Gelatinase	-ve	ONPG	+ve
Nitrate Reductase	-ve	Esculin hydrolysis	+ve
Growth on YEMA with bromothymol blue	+ve		

Table 2: Sugar utilization pattern of isolate KHDEB4

Sugar discs	Oxidation	Fermentation	Sugar discs	Oxidation	Fermentation
Sucrose	-ve	-ve	Galactose	+ve	+ve
Dextrose	+ve	+ve	Raffinose	-ve	-ve
Mellibiose	+ve	-ve	Lactose	-ve	-ve
Inulin	+ve	-ve	Sorbitol	+ve	+ve
Inositol	-ve	-ve	Adonitol	+ve	-ve
Fructose	+ve	+ve	Cellobiose	-ve	-ve
Salicin	+ve	-ve	Rhamnose	-ve	-ve

Table 3: Antibiogram profile of isolate KHDEB4

Antibiotic discs	Sensitivity Tests
Amikacin (Ak30)	S
Ciprofloxacin (CIP5)	S
Bacitracin (B10)	MS
Streptomycin (S10)	S
Polymyxin-B (PB100)	MS
Tetracycline (T30)	MS
Erythromycin (E15)	S
Amphotericin-B (Ap50)	R
Penicillin-G (P10)	MS
Neomycin (N30)	S

*R = Resistant (< 5 mm), MS= Moderately Susceptible (> 10 to 20 mm), S = (> 20 mm)

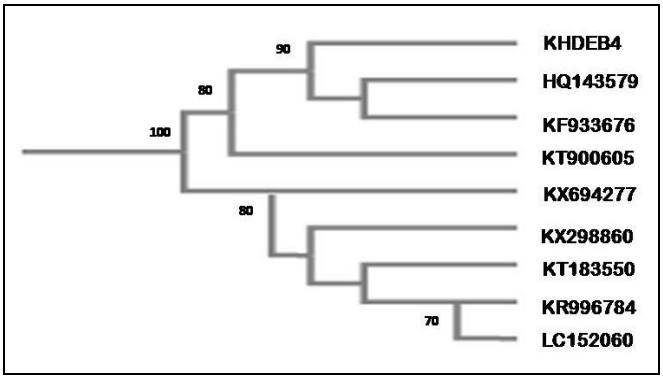

Fig 1: Phylogram based on 16S rRNA sequence of different species of *Rhizobium*. NCBI phylogram showed the identity (99%) of 16S rRNA gene of our native *Bacillus megaterium* strain KHDEB4 with the different strain of *Bacillus megaterium.*

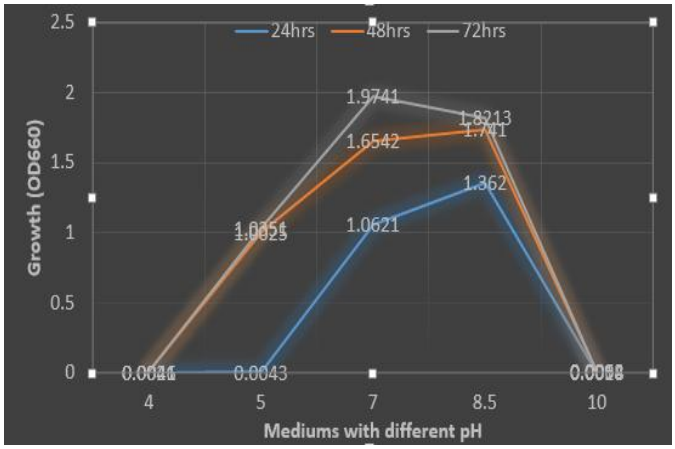

Fig 2: Tolerance of *Bacillus megaterium*to different ranges of pH

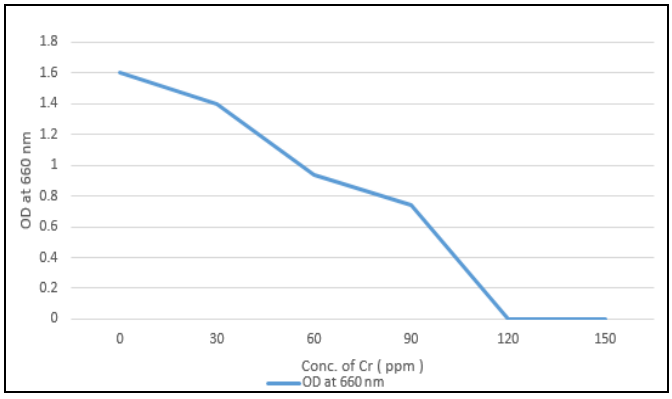

Fig 3: Minimum Inhibitory Concentration (MIC) of *Bacillus megaterium* to ranges (0 to 150 ppm) of Chromium concentration

16S rRNA gene sequencing and analysis of phylogeny for the novel *Bacillus megaterium*strain KHDEB4

16S rRNA sequencing of *B.megaterium* strain KHDEB4isolated from root nodule was performed. The amplified fragment of 16S rRNA of *B.megaterium*(KHDEB4) was sequenced, and boot strap analysis revealed that the sequences matched 99 % with the 16S rRNA sequence of *B.megaterium*. NCBI results showed that *B. megaterium* (KHDEB4) from green gram field is novel. The 16S rRNA sequence of *B. megaterium*was submitted to NCBI gene bank and catalogued the accession number as KY679149 (Fig. 1). The 16S rRNA gene phylogeny has been widely used for differentiation of diverse diazotrophic microorganisms (Young 1992; Zehr *et al*. 2003).

Abiotic stress tolerance

B. megaterium strain KHDEB4 was further tested for abiotic stress tolerance with wide range of pH (4.0, 5.0, 7.0, 8.5 and 10.0) (Figure 2). and different concentrations of Chromium (K_2 Cr_2 O_7) as heavy metal (0,30,60,9,120, and 150ppm). Results were recorded through optical density reading with spectrophotometer (at 660 nm) at 24, 48 and 72 h of incubation.

As per the observations with different pH ranges KHDEB4 strain has been recorded maximum growth at pH 8.5 where as it has been recorded minimum growth at pH 10 followedby pH 4 at 24, 48 and 72 h of incubation. Growth strain KHDEB4was stopped at 120ppm of Cr(VI), thus this concentration was considered as MIC as shown in Figure 3.MIC determination in microorganisms for a particular heavy metal reveals its extent of tolerance to that particular heavy metal (Dadrasnia*et al*. 2015).

Conclusions

Heavy metal toxicity especially Cr (VI) toxicity is one of the most serious issue faced by many crops throughout the world.However, our results indicate that *B. megaterium*strain KHDEB4 could be promising in bioremediation of Cr (VI) toxicity. The findings of present investigation suggests that*B. megaterium*strain KHDEB4maintains physiological traits that could benefitmicrobial survival and maintenance in contaminated environments.

Reference

1. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. 1966; 45:493-496.
2. Bergey, David H, John G, Holt, Noel R Krieg, Peter HA *et al*. Bergey'sManual of Determinative Bacteriology (9th ed.). Lippincott Williams &Wilkins. 1994; ISBNO-683-00603-7.
3. Bishehkolaei R, Fahimi H, Saadatmand S, Nejadsattari T, Lahouti M, Yazdi FT. Ultrastructurallocalisation of chromiumin *Ocimumbasilicum*, Turkish Journal of Botany. 2011; 35(3):261-268.
4. Chandra P, Kulshreshtha K. Chromiumaccumulation and toxicity in aquatic vascular plants, Botanical Review 2004; 70(3):313-327.
5. Dadrasnia A, Chuan Wei KS, Shahsavari N, Azirun MS, Ismail S. Biosorption Potential of Bacillus salmalaya Strain 139SI for Removal of Cr (VI) from Aqueous Solution. International Journal of Environmental Research and Public Health 2015; 12:15321-15338.
6. Dixit V, Pandey V, Shyam R. Chromium ions inactivate electron transport and enhance superoxide generation *in vivo* in pea (*Pisumsativum* L. cv. Azad) root mitochondria," Plant Cell and Environment 2002; 25(5):687-693.
7. Gupta S, Srivastava S, Pardha Saradhi P. Chromium increases photosystem 2 activity in Brassica juncea, Biologia Plantarum 2009; 53(1):100-104.
8. Hansda A, Kumar V, Anshumali A, Usmani Z. Phytoremediation of heavy metals contaminated soil using plant growth promoting rhizobacteria (PGPR): A current perspective. Recent Res. Sci. Technol 2014; 6:131-134.
9. Hussain M, Ashraf M, Saleem M, Hafeez FY. Isolation and Characterization ofRhizobial Strains From Alfalfa. Pakisthan Journal of Agricultural Science. 2002; 39:32-34.
10. Masood Ali, Shivkumar. Problems and prospects of pulsesresearch in India. Indian Farming, 2000, 4-13.
11. Najafian M, Kafilzadeh F, Azad HN Tahery Y. Toxicity of chromium (Cr6+) on growth, ions and some biochemical parameters of *Brassica napus*L" World Applied Sciences Journal. 2012; 16(8):1104-1109.
12. Panda SK, Chaudhury I, Khan MH. Heavy metals induce lipid peroxidation and affect antioxidants in wheat leaves, Biologia Plantarum. 2003; 46(2):289-294.
13. Pandey SK, Pandey M, Town Z. Germination and seedling growth of field pea *Pisumsativum* Malviya Matar-15 (HUDP-15) and Pusa Prabhat (DDR-23) under varying level of copper and Cr, Journal of American Science. 2008; 4:28-40.
14. Patel PH, Patel JP, Bhatt SA. Characterization and phylogenetic relatedness of Azotobactersalinestris. Journal of Microbiology and Biotechnology Research. 2013; 3:65-70.
15. Rajesh V, Kumar ASK, Rajesh N. Biosorption of cadmium using a novel bacterium isolated from an electronic industry effluent. Chem. Eng J. 2014; 235:176-185.
16. Ruby E Jalgaonwala. International Journal of Pharma and Bio Sciences, 2011, 2(1).
17. Samuel J, Paul ML, Ravishankar H, Mathur A, Saha DP, Natarajan C *et al*. The differential stress response of adapted chromite mine isolates Bacillus subtilis and Escherichia coli and its impact on bioremediation potential. Biodegradation. 2013; 24:829-842. doi: 10.1007/s10532-013-9631-8
18. Shanker AK, Cervantes C, Loza-Tavera H, Avudainayagam S. Chromium toxicity in plants, Environment International. 2005; 31(5):739-753.
19. Sobariu DL, Fertu DIT, Diaconu M, Pavel LV, Hlihor RM, Drãgoi EN *et al*. Rhizobacteria and plant symbiosis

in heavy metal uptake and its implications for soil bioremediation. N. Biotechnol, 2016. doi: 10.1016/j.nbt.2016.09. 002 [Epub ahead of print].
20. Sobti S, Belhadj HA, Djaghoubi A. Isolation and Characterization of the Native Rhizobia under Hyper-Salt Edaphic Conditions in Ouargla. Science direct. 2015; 74:1434-1439.
21. Ulrich K, Ulrich A, Ewald D. FEMS Microbial Ecol. 2008; 63:169-180.
22. Young JPW. Phylogenetic Classification of Nitrogen-fixing Organisms. In Biological Nitrogen Fixation. New York: Chapman & Hall, 1992, 43-86.
23. Yushmanov SV, Chumakov KM. Algorithms for building maximum topological similarity phylogenetic trees. *Molekularnaya Genetika Mikrobiologiyai Virusologiya*, 1988; 3:9-l 5 (in Russian).
24. Zehr JP, Jenkins BD, Short SM, Steward GF. Nitrogenase Gene Diversity and Microbial Community Structure: A Cross-system Comparison. Environmental Microbiology. 2003; 7:539-554