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Heavy metal tolerant bacterial isolates with potential PGP from eastern states of India

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Abstract:

Heavy metal contamination in soil and water poses a threat to food chain safety and human health being toxic to humans and other living beings even at low concentrations.Heavy metal-resistant bacteria possessingplant growth-promoting (PGP) propertiesare used in heavy metal-contaminated soils to ameliorate heavy metals and improve plant growth. These microorganisms utilize a variety of coping mechanismsto deal with heavy metal toxicity.This study aimed to identify multi-heavy metal-tolerant (multi-HMT) bacterial isolates from soil and groundwater samples across Bihar and Uttar Pradesh, India. The preliminary screeningshowed that 46 isolates were capable of tolerating six heavy metals at 1.5 mM concentration. The PGP traits of these multi-HMT bacterial isolates were evaluated and all isolates showed ammonia production. Many soil and water isolates (18% and 11%), exhibited five out of six direct PGP traits. The isolate CDS4, displayed all four indirect PGP traits, indicating its potential as a phytostimulator, biofertilizer and stress alleviator. Further studies to determine the agricultural and environmental application of multi-HMT isolates for promoting growth of different plantsmay indicate its application for sustainable agriculture.

Keywords: Bihar**,** multi-HMT, PGP, Uttar Pradesh

Introduction

Heavy metals are present in the environment due to a number of geogenic and anthropogenic process(Dœlschet al., 2005),(Rodríguez-Hernández et al., 2022). As heavy metals are stable, they cannot be degraded and are difficult to remove, resulting in persistent environmental hazards. Reports indicate that the permissible limit of heavy metal in soil for Zinc, Chromium, Cadmium, Lead, Arsenic are 50, 100, 0.8, 85, 20 mg/kg respectively (Osobamiroet al., 2019).

Although, excessive concentrations of heavy metals are toxic to all organisms, microorganisms and plants, living creatures need trace amounts of some heavy metals to survive. Elevated concentrations of heavy metal causes oxidative stress in plants which can be extremely detrimental to them(Pandey et al., 2017), (Georgiadouet al., 2018).Studies have indicated that plants grown in heavy metal contaminated soils may absorb and accumulate heavy metals in vegetables, fruits, and crops, posing health risks to humans and animals through the food chain(Singh and Ghosh, 2014), (Jaishankaret al., 2014).

There are numerous acute and long-term negative consequences of heavy metal that have an impact on various human organs. The harmful effects of heavy metals include complications with the gastrointestinal and renal system, neurological disorder, skin lesion, vascular damage, immune system dysfunction and cancer (Rahmanet al., 2019),(Kumar et al., 2021).Compared with traditional remediation technologies such as chemical or physical remediation, bioremediation is a sustainable and cost-effective way to remediate heavy metal contaminated environments (Brbootiet al., 2011). It has been demonstrated that soil microbes have an impact on metal bioavailability and mobility through redox changes, acidification, production of iron chelators, biotransformation of metals into less toxic forms, bioaccumulation of these metals within their cells, and binding of metals within their cell walls (Biosorption)(Banerjee et al., 2011 and Dey et al., 2016). Metal-resistant PGP bacteria are among the microbes that could potentially be used in bioremediation (Mesa-Marín et al., 2020), (Oubohssaineet al., 2022)

Plant growth-promoting rhizobacteria (PGPR) are soil-borne microorganisms that can aggressively colonise the rhizosphere and improve plant growth and yield. The processes that directly promote growth are: (i) nitrogen fixation; (ii) solubilization of phosphorus; (iii) generation of phytohormones like auxins (indole acetic acid (IAA), (iv) sequestering of iron by producing siderophores; and (v) lowering of ethylene concentration (Majeed et al., 2015), (Sharma et al., 2021).Indirect effects of PGPR on plants include inhibiting pathogen-produced enzymes *viz*. chitinase, protease, lipase or toxins, antibiosis and the stimulation of plant defence

systems which may work together to stop plant infection(Kumar et al., 2012), (Bhattacharyya et al., 2020).

In the eastern Indian states, Bihar and Uttar Pradesh, both of which are situated in the Ganga-Meghna-Brahmaputra (GMB) basin, groundwater contamination by heavy metals is a serious problem (Singh and Ghosh, 2014, Kumar et al., 2015 and Kumar et al., 2017).Several studies have revealed Arsenic, Cadmium, Chromium, Copper, Lead, and Mercury, nearly 3000 times greater than the safe limit prescribed by the World Health Organization (WHO, 2011). The water quality of river Ganga has steadily deteriorated because of growing industrialisation and urbanisation. The vast majority of businesses along the banks discharges all or some of their untreated wastewater into the river Ganga (Arya et al., 2013), (Shrivastavet al., 2016). Besides this, tributaries of river Ganga further increases the river's pollution burden.

Around 65% and 76% of the population of Uttar Pradesh and Bihar, directly depends on agriculture for their livelihood[*1,*2]. Rainwater is the primary source of irrigation in Bihar and Uttar Pradesh, however due to irregular rainsdependability on groundwater sources for irrigation increases. Due to groundwater contamination, not only soil but food items are also contaminated with heavymetals. In recent studies, significant heavy metal accumulation has been reported in the crops, vegetables and fruit growing in soil contaminated/irrigated with heavy metal (Raychaudhuri,2016). A recent study in 2021,detected that the amount of arsenic in food was substantially higher than that in groundwater(Mondal et al., 2021) which may be due to bioaccumulation, water loss due to cooking.However, in locations with high levels of heavy metal in groundwater, the fact that heavy metal exposure through food is as common as that from drinking water is not widely understood. Heavy metal mitigations are mostly focused on reducing exposure through groundwater rather than from contaminated food (Kumar et al.,2021).

Despite the fact that multiple studies and a government report have identified other heavy metal contamination in the Ganga besides arsenic. The research studies have primarily focused on arsenic and the identification of arsenic resistant bacteria to remediate heavy metals from groundwater.There is a lack of focus on research into other heavy metal contamination in groundwater, soil, and plant accumulation. Bacteria that can remediate/mitigate multiple heavy metals rather than just arsenic and reduce the accumulation of these heavy metals in plants should also be screened and a characterised consortium could be applied as biofertilizer to mitigate the accumulation of heavy metals in crops.

With this point of view, this preliminary studyisolated multi-HMT bacteria from the rhizospheric soil and underground water in arsenic - affected districts of Uttar Pradesh and Bihar

and screened them for PGP properties. The findings of this study could potentially help us to get knowledge about the ability of the indigenous multi-HMT bacteria from Bihar and U.P to promote plant growth, which could be further applied as single or as consortium to bioremediate metal pollution.

Materials and methods

The chemicals used in this study were all of analytical grade. All of the media used in the study was obtained from HiMedia Laboratories Pvt. Ltd. India and the heavy metal salts were procured from Molychem. The metal stock solution was sterilised using 0.20µm(Minigen) syringe filters, and the growth medium was sterilised by autoclaving at 121^0C for 15 minutes. All spectrophotometric analysis was performed on UV spectrophotometer (Bio-Era).

Study area

Groundwater and rhizospheric soil samples were collected from 17 different arsenic-contaminated locations in Bihar and Uttar Pradesh, spread across four districts (Figure1,2 andTable1) based on Central Water Commission (CWC-2019) report. Locations with arsenic contamination greater than 0.02mg/L were considered for the study.

Table 1. Location of sampling cites for soil and groundwater samples.

Uttar Pradesh					
Sample	**District**	**Block**	**Sampling location**	**Coordinates(Latitude, Longitude)**	**As Content (**mg/L)
Sample 1	Azamgarh	Palhana	Palhana Devi Mandir	25° 46' 54.174" N, 83° 5' 37.8528" E	0.03
Sample 2	Azamgarh	Palhana	Union Mini Bank	25° 47' 0.4344" N, 83° 5' 37.8528" E	0.03
Sample 3	Azamgarh	Rani Ki Sarai	Sri Durgaji Children City Inter College	26° 0' 16.2936" N, 83° 6' 47.8476" E	0.04
Sample 4	Azamgarh	Rani Ki Sarai	Union Bank Of India	26° 0' 16.2936" N, 83° 6' 47.8476" E	0.04
Sample 5	MaunathBhanjan	Ghosi	Police Station, Ghosi	26° 6' 56.214" N, 83° 32' 38.58" E	0.05
Bihar					
Sample6	Bhojpur	Baghakol	SelampurMathiya Shiv Mandir	25° 39' 12.9492" N, 84° 32' 4.4124" E	0.05
Sample7	Bhojpur	Baghakol	Shiv Mandir Baghakol	25°39' 12.9492" N,84° 32' 19.2804" E	0.05
Sample8	Bhojpur	BarkiSinghi	GangiBrambh Temple	25° 34' 33.312" N, 84° 40' 27.9084" E	0.04
Sample9	Bhojpur	BarkiSinghi	The Excel Institute BarkiSinghi	25° 36' 39.978" N, 84° 33' 1.0476" E	0.04
Sample10	Bhojpur	Tenua	Shiv Temple Tenua	25° 36' 40.5" N, 84° 32' 39.4188" E	0.04
Sample11	Bhojpur	Tenua	Om Kanta Medical Hall	25° 36' 39.7692" N, 84° 33' 1.083" E	0.04

Sample12	Bhojpur	Simaria	Shree Lakshmi Narayana Temple	25° 40' 59.9484" N, 84° 43' 34.82" E	0.03
Sample13	Bhojpur	Giddha	State Bank Of India	25° 34' 33.2004" N, 84° 47' 29.374" E	0.03
Sample14	Bhojpur	Inglishpur	Englishpur Primary School	25° 22' 52.2156" N, 84° 31' 29.708" E	0.03
Sample15	Buxar	ManikpurSimri	Digital SevaCenter(CSC)	25° 40' 17.5548" N, 84° 5' 56.9688" E	0.04
Sample16	Buxar	Garhani	Bank of Baroda Csp. Garhani(Dhamaniya)	25° 24' 33.4944" N, 84° 33' 30.488" E	0.02
Sample17	Buxar	Parasiya	Shiv Mandir	25° 17' 16.296" N, 83° 59' 38.4" E	0.02

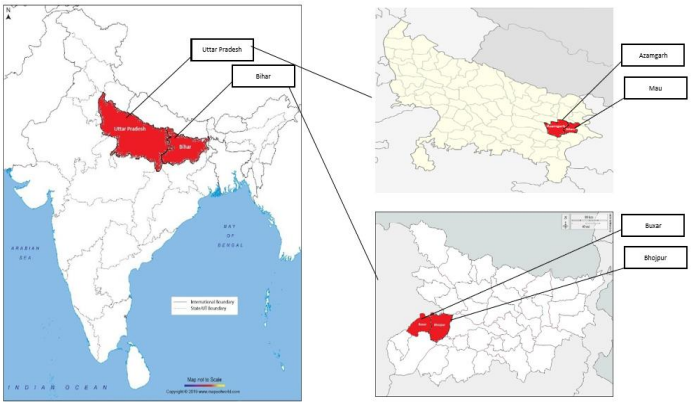

Figure 1(a) Sampling locations from the states of U.P, Bihar

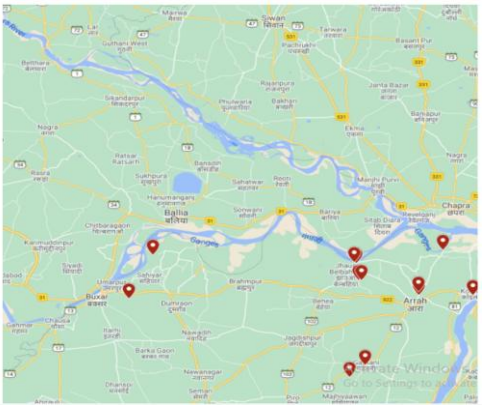

Figure 1(b): Images of the Sampling location sampling locations via Google maps.

Collection of samples

Water samples were collected in two different sets of clean, dried HDPE plastic bottles. To prevent metal precipitation, adsorption, and degradation, one set of samples was treated with 2-3 drops of nitric acid. The other set of samples was used for bacteria isolation and was kept in an ice box.The rhizospheric soil (approx. 50g) from the agriculture fields of the selected sites was collected in the month of December in sterile polythene bags with a sterile spatula. The samples were stored at 4^0Celsius and were used for isolating bacteria.

Enrichment and Isolation of Cadmium Tolerant bacteria

Cadmium tolerant bacteria were isolated by inoculating 1 g of soil or 500µl of water in 10 ml of LB Broth medium supplemented with 0.1 mM cadmium (Cadmium nitrate) and incubated for 72 hours at 28 ± 2^0C under static condition. Each sample was enriched for cadmium resistance by transferring 500µl of the enriched media to fresh media having a cadmium concentration increased from 0.5 mMuptoto 1.5 mM. Following enrichment, the cell suspension was plated on LB agar media (1.5 mM Cadmium) and incubated for 72 hours at 28 ± 2^0C. Bacterial colonies with various morphologies were further purified (Pawaret al., 2016) and used for characterization.The pure culture was maintained in Nutrient broth with 50 % (v/v) of glycerol and stored at -80^0C.

Multi metal tolerance and phenotypic characterization

All Cadmium tolerant bacterial isolates were tested inLB agar amended with 1.5 mM of each of the five heavy metals for tolerance *viz*. arsenic (As), zinc (Zn), lead (Pb), chromium (Cr), and cobalt (Co). The salts of heavy metal were sodium arsenite ($NaAsO_2$), zinc sulphate ($ZnSO_4$), potassium dichromate ($K_2Cr_20_7$), cobaltous chloride($CoCl_2$), and lead acetate($Pb(C_2H_30_2)_2$ metal salts. The plates were incubated for 72 hours at 28 ± 2^0C and growth was measured at 560 nm to (Ahirwa and N.K,2016).The isolates exhibiting tolerance to all heavy metal were further characterized for Gram's nature and the morphological characteristics.

Assessment of Direct PGP traits

Multi-HMT bacterial isolates (24 hrs old) were screened for their inorganic phosphate solubilizing activity on Pikovskaya's medium by spot inoculating (10µl) and incubating at 28 ± 2^0C for 7 days (Pandey et al., 2017).The appearance of a clear zone (solubilization area) around the bacterial growth indicated positive result. Phosphate solubilization index was calculated by using the formula:

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

The positive isolates were further quantified for their ability of phosphate solubilisation inPikovskaya's broth medium at 28 ± 2^0C for 7 days(Fiske and Subbarow, 1925). After incubation, the culture was centrifuged for 15 minutes at 10,000 rpm. The phosphate content of the supernatant was measured at 650 nm after adding ammonium molybdate and ANSA reagent. All experiments were done in triplicate.Bacterial isolates were cultured in LB medium supplemented with 1mg/ml of tryptophan and incubated at 28 ± 2^0C for 72 hours. Following a 15 minute centrifugation at 10,000 rpm of the cultures, 2ml of Salkowski reagent (70% of perchloric acid, 2ml 0.5 M FeCl3 solution) was added to 1ml of the supernatant. The detection of a pink colour indicates the isolates ability to produce IAA. The absorbance at 530 nm was measured with a spectrophotometer and the IAA concentration was calculated using a standard curve in the 10-100 µg/ml range (Gordon and Weber, 1951), (Patten and Glick, 2002).Multi-HMT bacterial isolates (24 hrs) were tested for ammonia production using 5 ml of peptone water and incubated at 28 ± 2^0C for 48 hrs. After incubation, 1ml of Nessler's reagent was added. The development

of a brown to yellow coloration indicates the isolates to be positive for ammonia production (Cappuccino and Sherman, 1999).

The ability for multi-HMT bacterial isolates to fix atmospheric nitrogen was examined on Jensen's agar medium by spot inoculating the bacterial culture (10µl) followed by incubation at 28 ± 2^{0}C for 5 days. Bacterial growth was utilised to demonstrate the atmospheric nitrogen fixing process in a qualitative manner (Kifle and Laing, 2016). 24 hrs old culture were inoculated on Nutrient agar supplemented with 4.4g / L of Glycine. Whattman Filter paper no 1 soaked in 2% sodium carbonate and 0.5% picric acid was placed inside the lid of petri plate and sealed with paraffin wax. The plates were incubated at 28 ± 2^{0}C for 96 hrs. Development of yellow to brown colour on filter paper indicated HCN production (Alstrom and Burns, 1989). Catalase test was performed as per Kumar et al., (2012).

Assessment of Indirect PGP traits

The ability of the multi-HMT bacterial isolates to produce amylase and pectinase was assessed by spot inoculating (10µl) bacterial cultures on starch agar plates and pectin agar plates, respectively. In order to confirm the secretion of amylase and pectinase the plates were flooded with Iodine solution and 1% Lugol solution, respectively, after 4 -7 days of incubation at 28 ± 2 ^{0}C. A clear halo surrounding the colonies, indicated isolate to be amylase and prctinasepositivde(Anand et al., 2010).Cellulase activity of isolates was determined by inoculating (10µl) bacterial isolate on Carboxymethylcellulose (CMC) agar plate(4 -7 days' incubation at 28 ± 2 ^{0}C). Positive cellulase producers were colonies that were surrounded by clear halos after adding 1% Congo red (15 minutes) and de-stained with 1% NaCl solution (5 minutes)(Cattelanet al., 1999).Caseinase activity of isolates was determined by inoculating the bacterial isolates on a Skim Milk agar plate (72-hour incubation at 28 ± 2 ^{0}C) and examined for a distinct halo zone(Kaur et al., 1988).

Results and Discussion

The Indo-Gangetic plains of Bihar and Uttar Pradesh are severely impacted by heavy metal contamination. The Central Water Commission published a report in 2019 that highlighted various arsenic hotspots across India. Bihar has the most arsenic-affected districts, with levels ranging from 0.01 to 0.05 mg/litre, followed by Uttar Pradesh. The Central Water Commission also issued reports identifying heavy metal contamination in the Ganga River. The choice of the study location was based on these reports and the impact of the metal contamination. In the present study, rhizospheric soil samples and underground water samples of U.P and Bihar were

used to first enrichandisolate cadmium tolerant bacteria (1.5mM). A total of 50 morphologically distinct cadmium tolerant bacteria were isolated of which 29 bacterial isolates were from soil and 21 bacterial isolates were from water.Forty-sixof the cadmium-tolerant bacteria showed tolerance tofive heavy metals (arsenic, zinc, lead, cobalt, and chromium) at 1.5 mM concentrations (Figure 2). Except for zinc, CDS18 and CDW2, CDW7, and CDW11 were not tolerant to any of the four heavy metals (Ahirwar and Narula,2016).

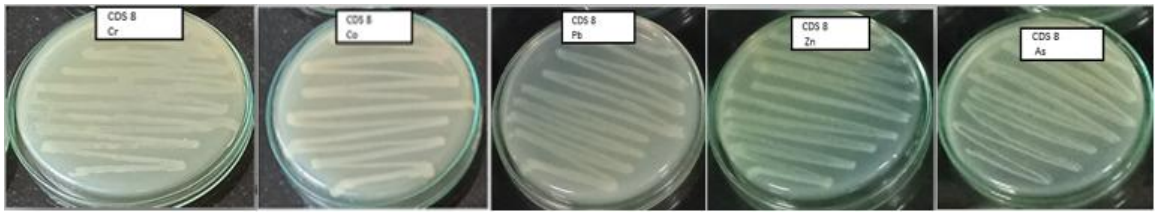

Figure 2: Multi heavy metal tolerance in soil isolate CDS 8

Previous researchers isolated arsenic-tolerant bacteria in arsenic-contaminated areas of Bihar and Uttar Pradesh, but to the best of our knowledge, few studies have been conducted to isolate multi-HMT bacteria from Bihar and Uttar Pradesh (Kushwaha et al., 2023).Kumari and Shardenduet al (2017) isolated an arsenite-tolerant bacterium (30 mM) and two arsenate-tolerant bacteria (150 mM) in surface water in Begusari, Bihar. Shardenduet al, (2017) also isolated three arsenite tolerant bacteria (25-35 mM) and three arsenate tolerant bacteria (280-310mM) in agricultural field ofSemariojjhapatti, Bihar. Biswas and Sarkar (2019) discovered two arsenic-tolerant bacteria in the shallow aquifers of Bihar's Bhojpur district that could withstand arsenite (AsIII) concentrations up to 70 mM and arsenate (As^{5+}) concentrations up to 1000 mM.In Uttar Pradesh, Tantryet al. (2015) reported 7 arsenic resistant bacteria (0.1%-0.6%) from the upper Gangetic plains. Similarly, in Baracich, Uttar Pradesh, Hare et al. (2017), reported three bacteria that could tolerate arsenic concentrations ranging from 500ppm to 600ppm.According to Aksornchuet al, (2008), bacteria growing in heavy metal contaminated sites exhibit high levels of tolerance to heavy metals in order to protect a cell component and their tolerance development could be linked to the amount and length of heavy metal exposure. In the current study, the approach used was to enrich for cadmium tolerance among the bacterial isolates and to evaluate their tolerance to multi heavy metals. However, this is a preliminary study, and further research will provide us with a better understanding of the bacterial isolate's heavy metal tolerance level and thee mechanisms.

Most of the multi-HMT bacterial isolates from soil and water were found to be gram negative rods 39.3% and 66.7% respectively (Figure 3). Both gram positive and negative bacteria

have distinct mechanisms for heavy metal resistance. Although this study did not embark on the metagenomics analysis, the microbiome analysis of the soil and the water from these areas will indicate the microbial diversity. Such studies of rhizospheric microbiome analysis will be helpful in indicating the presence of agricultural important microorganism.

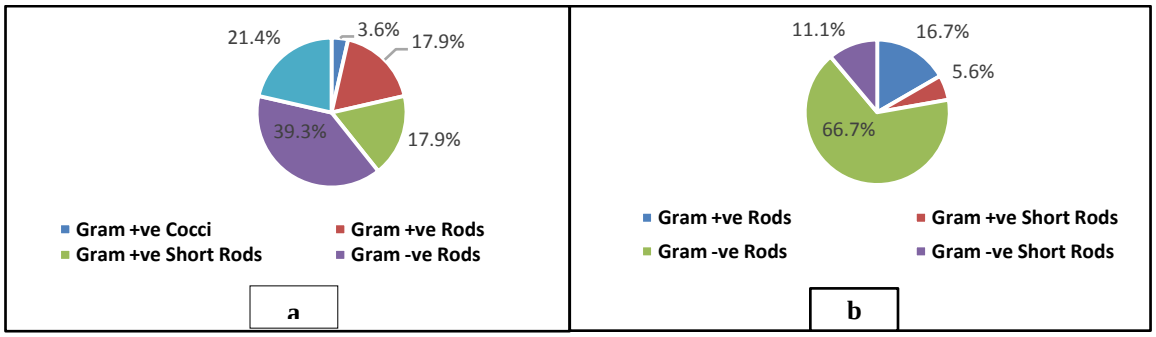

Figure 3: (a)Gram nature of soil isolates and(b) water isolates

Direct PGP traits

Previous studies have demonstrated the benefits associated with PGPRin the rhizospheric niche by reducing abiotic and biotic stress thereby promoting better plant development, health and increasing nutrient accessibility. To effectively apply bacterial-assisted bioremediation techniques, the selection of heavy metal tolerant PGPR strains is used as a strategy. The present study embarked on assessing the PGP properties of multi-HMT bacteria from soil and water and to the best of our knowledge,Kushwaha et al (2023)indicated multi- HMT isolated in U.P and no studies are reported in Bihar until now. Five multi-HMT isolates from soil i.e. CDS6, CDS12, CDS16, CDS22, CDS27 and 2 multi-HMT isolates from water i.e. CDW4, CDW8 showed 5 out of 6 direct plant growth promoting properties. Based on the preliminary screening for various direct PGP traits, 18% soil isolates and 11 % of water isolates showed traits like phosphate solubilization, nitrogen fixation IAA, ammonia, and catalase production (Table2).

Eleven soil and 7 water isolates showed the ability to solubilize inorganic phosphate by forming a halo around the inoculum (Figure7a). The isolates CDS20, CDS9 and CDW3 showed high phosphate solubilisation*viz.* 730, 587and 587 µg/ml respectively. All the isolates showed phosphate solubilization in the range from 79- 444 µg/ml, while two of the isolates CDS15, CDS26 did not show any detectable phosphate solubilization (Figure 4).

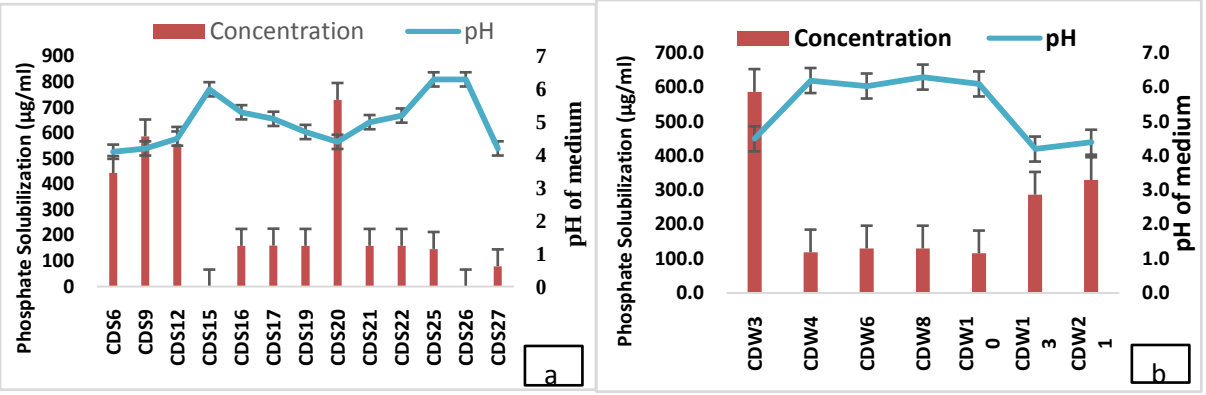

Figure 4: A representative of Multi-HMT soil (a)and water isolates (b) exhibiting phosphate solubilization inPikovskaya's broth medium (28 ± 2^0C for 7 days).

Although phosphate is a vital element required for plant growth, it is mostly unavailable to plants because the majority of phosphate in soil is present in insoluble forms and plants can only assimilate it in either monobasic or dibasic ions. The solubilization of phosphate and the subsequent availability of it to the plant through a range of solubilization events, including acidification, chelation, exchange reactions, and the generation of acids (Gluconic, Citric, etc.), is one of the key roles that PGPRs play in plant growth and development. A notable pH decrease from 7 (at the time of inoculation) to 4.0 was observed in the culture filtrate. The highest phosphate solubilization was observed for CDS20 with a significant decrease in the pH level of the media (4.4). Interestingly, the decrease in the pH synchronised with the amount of phosphate solubilized (Figure 4) namely in the other isolates *viz*. CDS6, CDS9, CDS12, CDW13, CDW21. The pH reduction is caused by the production of organic acids to solubilize insoluble phosphate. The inverse relationship between the pH of the culture broth and the amount of phosphate solubilized by the isolates suggests that these organic acids are involved in the medium's acidification and subsequent release of phosphate. The released phosphate can bind to other heavy metals and cause their mineralization, which renders them immobile in addition to providing phosphorus for plant growth (Jiang et al., 2022).

In Nadia district, West Bengal similar results were observed by Lahaet al. (2019), where ansoil arsenic tolerant bacteria was reported to solubilize inorganic phosphate up to 2500 µg/mL.Sowmya et al (2020)reported 7 Uranium tolerant bacteria in Karnataka capable of solubilizing phosphate in the range 252.1 ± 14.8 to 1,312.9 ± 53.2 µg/mL. Rajendran and Sundaram (2020)reported a cadmium tolerant phosphate solubilizing bacteria from the contaminated soil of Salem District, Tamil Nadu.

Table 2: Direct plant growth promotion activities in multi-HMT bacterial isolates from soil.

Isolates	**P**	**C**	**H**	**A**	**I**	**N**	**Isolates**	**P**	**C**	**H**	**A**	**I**	**N**
CDS1	-	+	-	+	+	+	CDW1	+	+	-	+	-	+
CDS2	-	-	-	+	+	-	CDW3	+	-	-	+	+	-
CDS3	-	+	-	+	-	+	CDW4	+	+	-	+	+	+
CDS4	-	+	-	+	+	-	CDW5	-	+	-	+	+	+
CDS5	-	+	-	+	+	-	CDW6	+	+	-	+	+	-
CDS6	+	+	-	+	+	+	CDW8	+	+	-	+	+	+
CDS7	-	+	-	+	+	-	CDW9	-	+	-	+	+	+
CDS8	-	-	-	+	+	-	CDW10	+	+	-	+	+	-
CDS9	+	+	-	+	-	+	CDW12	-	+	-	+	+	-
CDS10	-	+	-	+	-	-	CDW13	+	-	-	+	+	+
CDS11	-	+	-	+	+	-	CDW14	-	-	-	+	+	+
CDS12	+	+	-	+	+	+	CDW15	-	-	-	+	-	+
CDS13	-	+	-	+	+	-	CDW16	-	+	-	+	-	-
CDS14	-	+	-	+	+	-	CDW17	-	+	-	+	+	+
CDS15	+	+	-	+	+	-	CDW18	-	-	-	+	+	-
CDS16	+	+	-	+	+	+	CDW19	-	-	-	+	+	-
CDS17	+	+	-	+	-	-	CDW20	-	-	-	+	-	-
CDS19	+	+	-	+	-	+	CDW21	+	+	-	+	+	-
CDS20	+	+	-	+	-	-	CDS25	+	+	-	+	-	+
CDS21	+	+	-	+	-	+	CDS26	+	+	-	+	-	-
CDS22	+	+	-	+	+	+	CDS27	+	+	-	+	+	+
CDS23	+	+	-	+	-	-	CDS28	-	+	-	+	-	-
CDS24	-	+	-	+	+	-	CDS29						

+: Positive -: Negative :P: Phosphate solubilization, I: IAA production, C: Catalase production, H: HCN production, N: Nitrogen Fixation, A: Ammonia production.

IAA is one of the most potent phytohormone produced by the PGPR and it affects a number of physiological processes in plants, such as cell expansion, cell division, root initiation, growth rate, phototropism, geotropism, and apical dominance. The IAA secretion propertied was shown by 13 and 8 soil and water isolates respectively (Figure 5 and 7e). The IAA production ranged from 1 to 47.4 µg/mL and 0.2 to 38.9 µg/mL in isolates from soil and water (Figure 5). The highest IAA was produced by isolate CDS27 (47.4 µg/mL), followed by CDW14 (38.9 µg/mL). CDS6 and CDW18 produced the least amount of IAA (0.1 µg/mL and 0.2 µg/mL).Patel et al. (2022), reported Cadmium tolerant isolates showing IAA production of 183.66 ± 1.52 µg/mL from Daman Ganga riverside, Gujrat. Mohan et al. (2014), reported two chromium tolerant isolates showing IAA production of (46µg/ml) and (30µg/ml) in Dindugal, TamilNadu.Nayak et al. (2016), found *Stenotrophomonas maltophila* strain BN1 from Sundarban capable of producing IAA in range of 3-5.7 µg/ml.

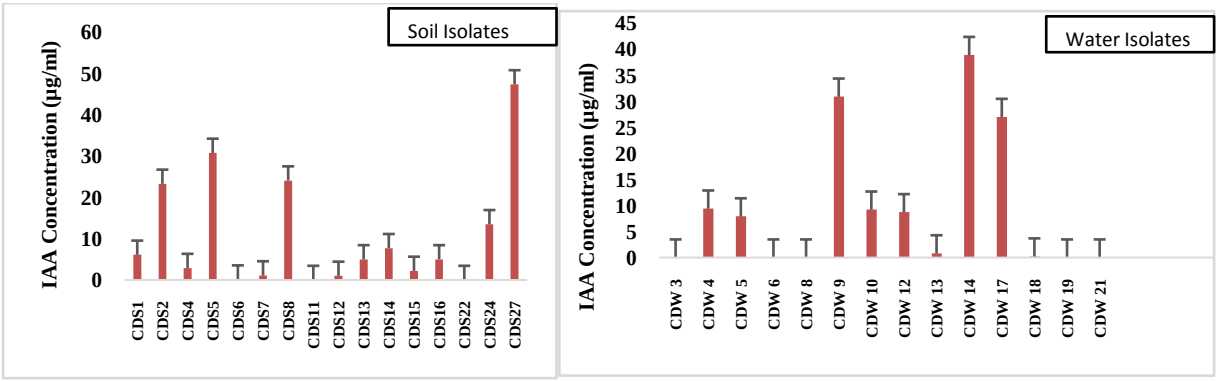

Figure 5 Multi-HMT soil and water isolates exhibiting IAA production.

Ammonia production and nitrogen fixation are important PGPR characteristics that influence plant growth and significantly improve crop quality and yields. The nitrogen needs of the host plant are met by microbial cells hydrolysing urea to generate ammonia and carbon dioxide, which promotes biomass and root and shoot elongation. Because a large portion of atmospheric nitrogen remains unavailable to plants, atmospheric nitrogen fixation by rhizospheric isolates is critical.A qualitative examination of ammonia synthesis by multi-HMT bacterial isolates revealed that all bacterial isolates (100%) could produce ammonia (Figure 6, Figure7d).Of all the multi-HMT bacterial isolates, 39% of soil isolates and 50% of water isolates could grow by fixing nitrogen (Figure 6).Twenty of the multi-HMT bacterial isolates demonstrated both ammonia production and atmospheric nitrogen fixation.

In order to stop the growth of pathogens, microbes produce HCN, a volatile secondary metabolite that specifically inhibits the oxidation of cytochrome C in an electron transport system. By inhibiting the growth of soil-borne phytopathogens and blocking the electron chain in pathogens, these PGPR's promotes plant growth. HCN production was not observed in any of the multi HMT isolates as they were unable to change the colour of Whattman filter paper no. 1 to brown (Figure 6, Figure 7b).Among the multi-HMT isolates,93% of soil and 61% of water isolates showed production of catalase enzyme (Figure6).

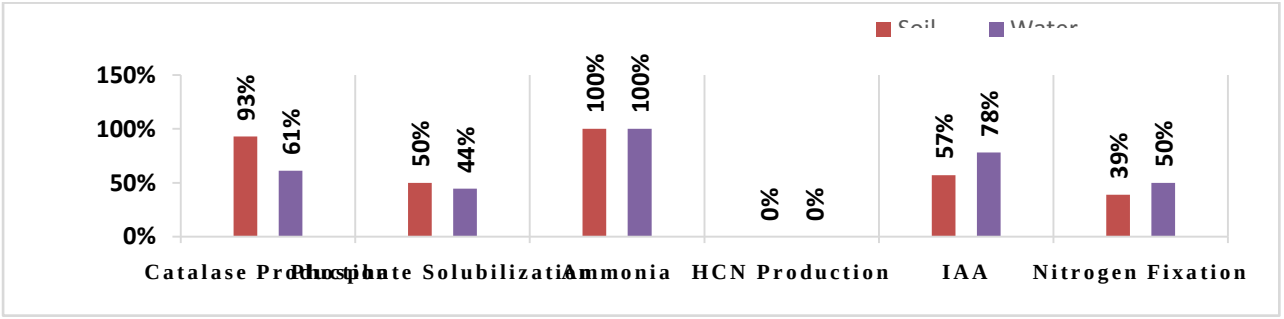

Figure 6: Direct plant growth promoting properties in multi-HMT isolates from soil and water

Evaluation of Indirect GP traits

The hydrolytic enzymes such as amylase, cellulase, pectinase and protease are well-known for their antagonistic effects on several fungal phytopathogens, by hydrolysing their cell wall. They also promote the degradation of organic matter in soil. In the current study, the soil isolate CDS4 was able to produce all four enzymes (Table 3). Caseinase activity was observed in 9 soil and 2 water isolates (32% and 11%), respectively, while amylase activity (Figure 9a) was observed in 3 soil and 3 water isolates (11% and 17%).

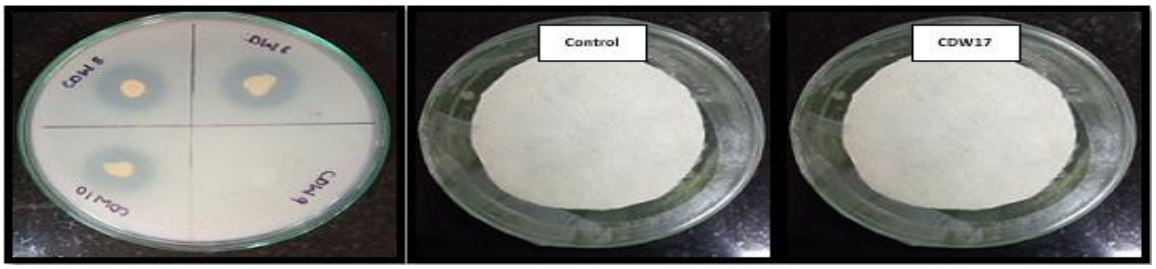

Figure7(a): Phosphate Solubilization **Figure 7(b):** HCN Production

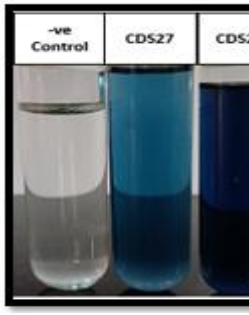

Figure 7(c): Phosphate Solubilization

Figure7(d): Ammonia Production

Figure 7(e): IAA Production

Table 3: Indirect plant growth promotion activities in multi-HMT tolerant bacterial isolates.

Isolates	A	C	P	Ca	Isolates	A	C	P	Ca
CDS1	-	+	+	-	CDW1	-	+	-	-
CDS2	-	-	+	-	CDW3	-	+	-	+
CDS3	-	+	+	-	CDW4	-	+	-	-
CDS4	+	+	+	+	CDW5	-	+	-	-
CDS5	-	+	+	-	CDW6	-	+	-	-
CDS6	-	+	-	+	CDW8	+	+	-	-
CDS7	-	+	-	-	CDW9	+	+	-	-
CDS8	-	-	+	-	CDW10	-	+	+	-
CDS9	-	+	+	+	CDW12	-	+	+	-
CDS10	-	+	-	+	CDW13	-	+	+	-
CDS11	-	+	-	+	CDW14	-	-	-	-
CDS12	-	+	+	+	CDW15	-	-	-	-
CDS13	-	+	+	-	CDW16	-	+	-	-

CDS14	-	+	-	-	CDW17	-	+	-	-
CDS15	-	+	-	-	CDW18	+	-	-	-
CDS16	-	+	+	-	CDW19	-	-	-	-
CDS17	-	+	+	+	CDW20	-	-	-	+
CDS19	+	+	-	-	CDW21	-	-	-	-
CDS20	+	+	-	-	CDS25	-	+	-	-
CDS21	-	+	+	-	CDS26	-	+	-	-
CDS22	-	+	+	+	CDS27	-	+	+	-
CDS23	-	-	-	-	CDS28	-	+	+	+
CDS24	-	+	-	-	CDS29	-	+	-	-

+: Positive -: Negative, A: Amylase Activity, C: Cellulase activity, P: Pectinase activity, Ca: Caseinase activity.

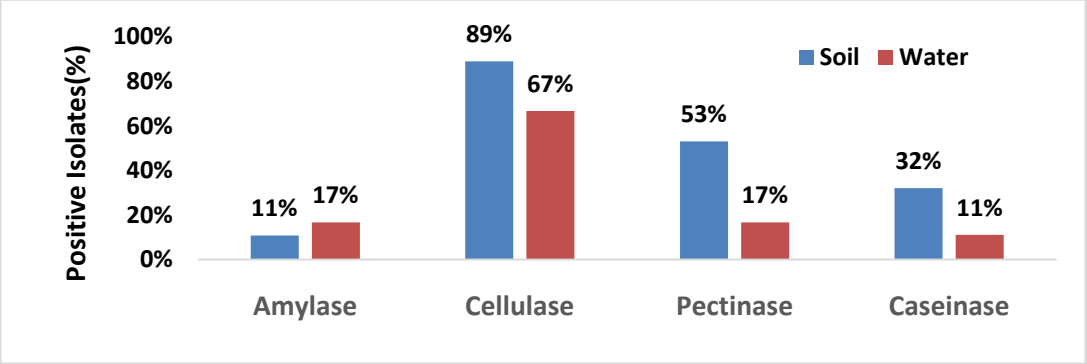

Figure 8: Indirect plant growth promoting properties in multi-HMT isolates from soil and water

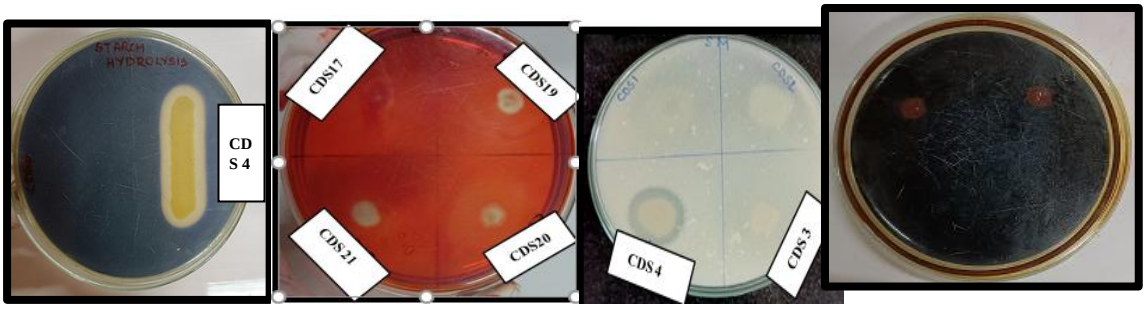

Figure 9(a): Amylase activity of CDS4, **(b):** Cellulase activity of CDS17,19,20,21, **(c):** Cellulase activity of CDS4, **(d):** Pectinase activity of CDS 3, 4

Cellulase enzyme (Figure 9b, c) was produced by 25 soil and 12 water isolates (89% and 67%), while 15 soil and 3 water isolates (53%, 17%)showed pectinase enzyme (Figure 9d) secretion. All of the indirect PGP features were negative in soil isolate CDS23 and water isolates CDW14, CDW15, CDW19, and CDW21 (Table 3). The isolates from soil (44%) and water (32%) were positive for at least one indirect PGP trait.The use of rhizospheric microorganisms with the innate potential for hydrolytic enzyme production can be a potent alternative to

chemical fungicides for control of fungal phytopathogens due to their non-toxic and ecologically benign nature.

Conclusion

The current study demonstrated the isolation of indigenous multi-HMT bacterial isolates from different sites in Uttar Pradesh and Bihar that possess multiple plant growth-promoting traits. The preliminary screeningindicated that 46 multi-HMT bacterial isolates were able to tolerate 1.5 mM concentrations of six heavy metals (Arsenic, Zinc, Lead, Cadmium, Cobalt, and Chromium).Analysis of direct PGP trait, indicated that a number of isolates showed presence of 5 of the 6 traits, whereas for indirect PGP trait, only one soil isolate and six water isolates showed 2 of the 4 indirect PGP traits. These isolates can be used as a consortium to alleviate the toxic effects of heavy metals. However, further research to better understand the heavy metal tolerance level of the bacterial isolates and to demonstrate the potential effect of these bacterial isolates in green house and field conditions will add a value towards environmental susitanability.

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