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Microbiological characterization of plant growth promoting bacteria isolated from vermiwash and their possible utilization in chromium contaminated fields

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ABSTRACT

Microbial scavenging of soil contaminants is one of the benign ways of turning such soils into cultivation. In this study , as many fifty isolates were isolated from vermiwash and screened for plant growth promotion (PGP), biocontrol attributes and hexavalent chromium Cr (VI) tolerance. Out of 50 bacterial isolates, three isolates were identified as Pseudomonas luteola (Vi1), Pseudomonas aeruginosa (Vi2) and Bacillus tequilensis (Vi4) which showed strong PGP properties such as phosphorus, potassium and zinc solubilization. The isolates Vi1 and Vi2 exhibited higher phosphorous solubilization quantified in range of 317-487 µg/ml. Zinc solubilization estimates of Vi1 and Vi2 were 14- 75 µg/ml, respectively .The isolates, Vi2and Vi4 displayed the antagonistic property against two pathogens viz., Ceratocystis fimbriata (MTCC 2281) and Fusarium oxysporum (MTCC 284), evident from their respective reduced growth by 51% and 46%. The other two test strains, Vi1and Vi2 showed consistent growth in nutrient agar amended with 200 ppm Cr (VI). These observations indicate that vermiwash has beneficial microbes and it can be used for polluted Cr (VI) environment or bioremediation in addition to PGP and biocontrol action.

Keywords: Vermi-wash, Plant growth promotion, Antagonistic property, Hexavalent Chromium tolerance

INTRODUCTION

In the pursuit of sustainable agricultural practices, the exploration and utilization of organic alternatives over conventional fertilizers have gained significant attention (Keditsu and Srivastava, 2014). Vermiwash, a liquid extract derived from the process of vermicomposting, emerges as a promising solution in this context. This eco-friendly elixir is generated as water permeates through vermicompost, carrying with it a rich blend of water-soluble nutrients, enzymes and beneficial microorganisms (Nayak *et al.,* 2019). As agriculture grapples with the challenges of soil degradation, nutrient depletion, and environmental concerns associated with chemical inputs, vermiwash stands out as a potential game-changer (Srivastava and Bora, 2023). The multi-dimensional properties of vermiwash and its diverse agricultural applications range from soil enrichment to its impact on seed germination to pest control and disease suppression, offering a holistic approach to sustainable farming (Gudeta *et al.*, 2021). Ansari, *et al.* (2010) reported improvement in soil health and increased productivity of okra (*Abelmoschus esculentus*) following vermiwash treatment. Upscaling of vermiwash is, therefore, required to isolate beneficial microbes and their utilization for PGP, biocontrol and heavy metal remediation. As issues of low soil fertility, decline in crop productivity, reduced microbial activity prevails in Indian context, utilization of vermiwash isolates serve many purposes ranging from phosphorous, potassium, zinc solubilization potential and antagonistic ability against phytopathogens to tolerance against heavy metal polluted environments (Srivastava and Malhotra, 2014).

In this background, the present study aimed to isolate and identify the beneficial microorganisms present in vermiwash and examine their potential for PGP attributes, heavy metal tolerance and biocontrol efficacy against pathogens. Such an attempt would provide valuable insights pertaining to wider use perspective of vermicompost-derived vermiwash as an alternative to chemicals-based inputs as a part of renewed organic agriculture.

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MATERIALS AND METHODS

Vermiwash sample collection for enumeration, isolation and characterization plant growth promoting bacteria

As many four vermiwash samples were collected in a pre- sterilized falcon tube aseptically from vermiwash producing units of ICAR-CISH, Lucknow, India, located geographically at a latitude of 26.5334 °N and longitude of 80.4651 °E. Vermi wash samples were examined for microbial population using specific media such as actinomycetes isolation agar, king's B Agar and nutrient agar by serial dilution method using following formula: No. of bacteria per ml of the original suspension (CFU/ml) =No of colonies x dilution factor/amount plated. One ml of suspension was serially diluted against nine ml of sterile autoclaved water. A 100 µl aqueous sample from 8^{th} dilution factor test tube was spread on nutrient agar, King's B agar, Cryema agar and actinomycetes isolation agar plate. The colonies appeared on media were counted in one square of the petri plate and average of total count was finally considered. The colonies were further purified on nutrient agar plate. After enumeration of bacterial isolates, they were screened for PGP activity (Kumar *et al.*, 2021).

Plant growth promoting attributes

Bacterial isolates were screened for plant growth promoting properties, such as: i. phosphate solubilization using Pikovskaya agar (Pikovskaya, 1948); ii. screening for zinc solubilization on zinc-solubilizing agar (Saravanan *et al.*, 2004); iii. siderophore production on chrome azurol-S-agar media (Meyer and Abdallah, 1978); iv. production of indole acetic acid using the salkovaskaya reagent (Meudt and Gaines, 1967); v. peptone water to produce ammonia and vi. production of hydrogen cyanide using King's B medium supplemented with glycine (Baker and Schippers 1987). The isolates that tested positive for PGP properties were selected for further characterization.

Morphological and biochemical characterization

Morphological properties were identified according to Bergey's Manual of Determinative Bacteriology (1994). The selected bacterial isolates were tested for biochemical activities. IMViC test (Edmund, 1980) and utilization of different sugars (kit-based) were performed (Mac Faddin, 2000).

Identification of bacterial isolates using16SrRNA Sequencing

Selected microbes (Vi1, Vi2, and Vi 4) were identified through ribotyping by amplifying the 16S rRNA gene for bacterial strains. The Zymo Research Isolation Kit (ZR Fungal/Bacterial DNA Mini Prep TM) was used to isolate genomic DNA according to the specified protocol using the universal primers described by Edwards *et al.* (1989) following gDNA isolation. Partial 16S rRNA gene PCR products were sent to Xcelaris Pvt. India Ltd. Different microbial isolates were identified based on the prototype strain sequence in GenBank and the percentage of maximum sequence similarity.

Studies on antagonistic properties of isolates

Antagonistic properties of bacterial isolates were recorded by dual culture plate assay method as described by Kumar *et al.* (2021) against phytopathogenic fungi *Ceratocystis fimbriata* (MTCC 2281) and *F. oxysporum* (MTCC 284). The reduction in the radial mycelium growth of mycelium growth of pathogens over control was determined using formula, which assessed fungal mycelium growth with bacterial treatment as per formula: % inhibition = C-T/C x100, where C and T stand for control of fungal mycelium growth (in mm) and the fungal mycelium's radial growth (in mm) in the presence of an antagonist bacterial isolate, respectively.

Screening of bacterial isolates for heavy metal tolerance

Chromium heavy metal tolerance capacity of selected vermiwash strains was assessed using agar plate dilution method (Holt *et al.*, 1994). Stock solution of Potassium dichromate($K_2Cr_2O_7$) salt concentration 1000 mg/l was used for preparing different concentration of Cr (VI) on agar media plates with as many seven different concentrations viz., 0, 50, 100, 150, 200,250, 300 ppm maintained in nutrient agar media plates. Selected strains were streaked on chromium -amended plates and incubated at 30± 2 °C for 4 -5 days (Farag and Zaki, 2010). The bacterial isolates surviving at highest chromium concentration was designated as maximum tolerance and beyond which no growth existed.

RESULTS AND DISCUSSION

Microbial distribution and their characterization for PGP potential

Total of 50 (Vi_1 to Vi_{50}) morphologically distinct bacteria were isolated from four different vermiwash samples. The maximum numbers of bacterial colonies were observed on actinomycetes agar (32×10^6 and 18×10^6) on cryema media, marked by abundance of actinomycetes in vermiwash (Figure 1). All bacterial isolates were screened for PGP properties. Out of 50 strains, three isolates viz., Vi1, Vi2 and Vi4 showed good plant growth promoting activity, which were selected for onward characterization.

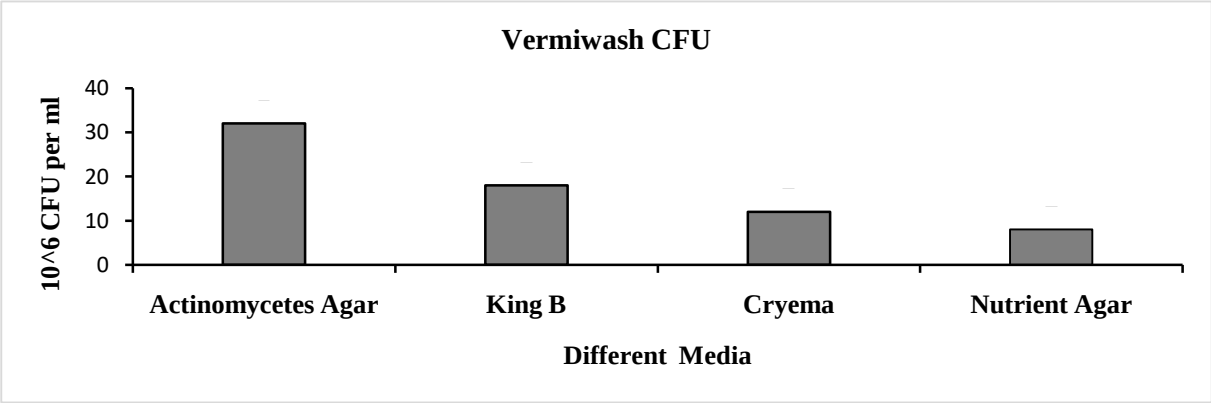

Figure 1: Variation in microbial count of vermi -wash obtained on different growth media

Plant growth promoting traits

The bacterial isolates, *Pseudomonas luteola* (Vi1), *Pseudomonas aeruginosa* (Vi2) and *Bacillus tequilensis* (Vi4) were screened for PGP activity. Phosphorus was solubilized by three isolates, Vi1, Vi2 and Vi4; while potassium was solubilized by Vi1 and Vi2. Only Vi2 and Vi4 solubilized zinc (Figure 2) The test strain Vi2 showed positive results for zinc, phosphorous, potassium solubilization, HCN, siderophore, starch, and indole acetic acid (IAA). However, Vi1 and Vi4, shows negative results for HCN and siderophore production (Table 1). Further, quantification of phosphate solubilization were observed in the range of 317-487 µg/ml in phosphate broth by three potential isolates. In addition, Vi2 and Vi4 exhibited 14 – 75 ug/ml zinc solubilization capacity; while Vi_1 showed negative results (Figure 3). Bacteria isolated from vermiwash showing good phosphorous, potassium and zinc solubilizing properties suggested involvement of different sets of enzymes to solubilize complex forms into simpler forms to release bioavailability of nutrients accessible to uptake by plant. These results partially corroborated with results of Ravichandran *et al.* (2021) reporting the presence of enzymes like as proteases, amylases, urease and phosphatase in the process of nutrient cycling.

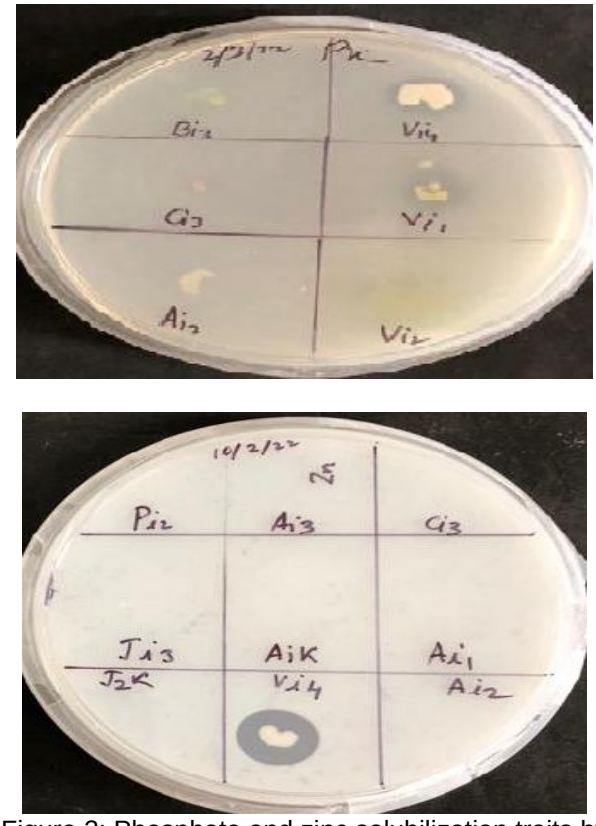

Figure 2: Phosphate and zinc solubilization traits by bacterial isolates from vermiwash

Table 1: Plant growth promoting traits of different bacterial isolates obtained from vermiwash

Isolate	Zinc solubilization	Phosphorous solubilization	Potassium solubilization	HCN	Siderophores	NH_3	Starch	Indole acetic acid
Vi1	-	+++	++++	-	-	+++	-	-
Vi2	++++	++	++	++++	+++++	++++	+++++	++++
Vi4	+++++	+++	-	-	-	-	-	-

Heat map analysis of phosphate and zinc solubilization by selected strains

A heat map with clustal correlation analysis was performed to analyze the quantification pattern with different treatments. Results revealed three different groups (Vi4, Vi2 and Vi1) were constructed representing each treatment with different media such as phosphate solubilizing broth (PSB) and zinc solubilizing broth (ZSB) at different incubation periods (3, 5 ,7, 9, 13 and 15 -days). The isolates, Vi4 and Vi1 showed highest phosphorous solubilization at the end of 15-days. Phosphorous solubilization pattern was observed as Vi4>Vi1>Vi2 in decreasing order. Zn solubilization on the other hand was shown by two groups Vi4 and Vi2 only. Vi4 group was observed most effective in both zinc and phosphate solubilization in PSB and ZSB treatments after 15-days period (Figure 3).

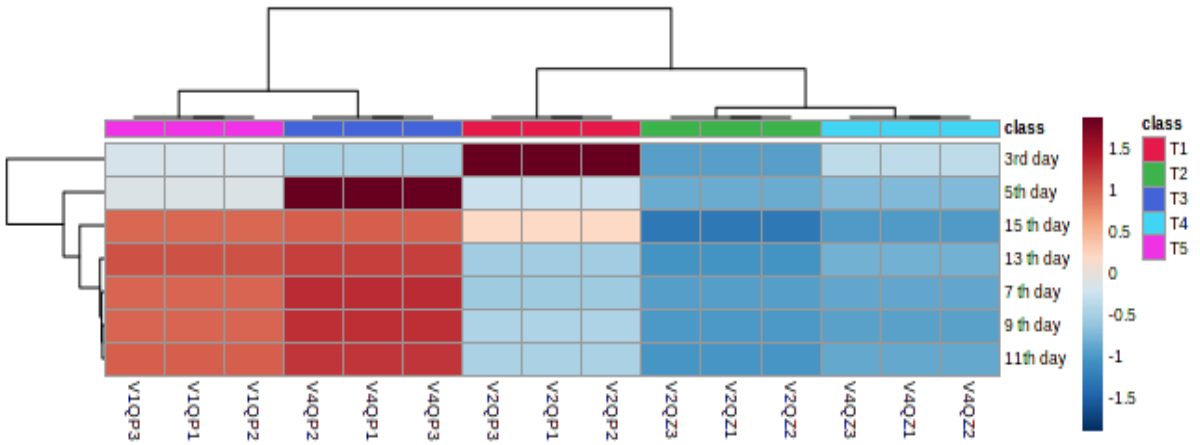

Figure 3: Heat map study of phosphorous and zinc quantification by three bacterial isolates (Vi1, Vi2 and Vi4)

Morphological and biochemical characteristics

Colony morphological features revealed isolate Vi1 colonies as milky yellow, flat, mucoid and irregular; while Vi2 was characterized as green, flat, smooth round with dense growth. In contrast to above two strains, Vi4 isolate displayed colonies as white, flat, smooth , circular, viscous and punctiform (Table 2).

The biochemical characteristics results further showed all the three isolates performed positive response against methyl red (MR), lysine and ornithine . Two test isolates (Vi1& Vi4) showed urease and esculin positive response ;while one isolate (Vi4) displayed positive result for nitrate reduction , coupled with negative response against indole, VP (Voges Proskauer), ONPG, phenylalanine deamination, H_2S, arabinose and xylose (Table 3). Based on the morphology and biochemical tests, the isolates were taxonomically categorized into two genera identified as Pseudomonas and *Bacillus* (Kumar *et al.,* 2021).

Table 2: Morphological characteristics of three screened bacterial isolates from vermiwash

Isolates	Gram Stain	Colony morphological characteristics
Vi1	Gram -	Milky yellow, flat, dry mucoid and irregular
Vi2	Gram -	Green, flat, smooth, moist and round
Vi4	Gram +	White, flat, smooth, circular, viscous and punctiform

Table 3: Biochemical characterization of selected bacterial isolates, displaying a range of different nutrient solubilizing and plant growth promotion traits

		Isolates		
Test name	Principle	Vi1	Vi2	Vi4
ONPG	Detects beta galactosidase activity	-	-	-
Lysine	Detects lysine decarboxylation	+	++++	+++
Ornithine Utilization	Detects ornithine decarboxylation	+	++++	+++
Urease	Detects urease activity	+	-	+++
Phenylalanine deamination	Detects phenyl deamination activity	-	-	-
Nitrate reduction	Detects nitrate reduction	-	-	+++++
H_2S production	Detects H_2S production	-	-	-
Citrate utilization	Detects capability of organism to utilize citrate as sole organic carbon source	-	++++	+++++
Malonate utilization	Detects capability of organism to utilize sodium malonate as sole organic carbon source	-	++++	+++
Esculin hydrolysis	Esculin hydrolysis	++++	-	++++
Arabinose	Arabinose utilization	-	-	-
Xylose	Xylose utilization	-	-	-
Adonitol	Adonitol utilization	-	++	-
Saccharose	Saccharose utilization	+	-	-
Raffinose	Raffinose utilization	++++	-	-
Trehalose	Trehalose utilization	-	-	-
Glucose	Glucose utilization	-	++	+++
Lactose	Lactose utilization	-	-	-

Molecular identification of bacterial isolates

Blast analysis using 16S rRNA gene sequencing revealed that the isolated strains Vi1, Vi2, Vi4 belong to *Pseudomonas luteola* (Vi1)*, Pseudomonas aeruginosa* (Vi2), *Bacillus tequilensis* (Vi4) and exhibited 100% similarities. 16S rRNA gene sequence of Vi1, Vi2, Vi4 have been submitted to the NCBI Genbank under accession numbers OR044073, OR037751, OR394057 respectively. A phylogenetic tree was built using the neighbor joining method to determine evolutionary relationships (Figure 4).

Antagonistic ability of bacterial isolates against fungal pat hogen

Only two isolates, Vi2 and Vi4, showed antagonistic activity against the fungal pathogens *F. oxysporum* (MTCC 284) and *Ceratocystis fimbriata* (MTCC 2281). Isolate Vi2showed a high percentage inhibition of up to 50 percent against MTCC 2281, and 46 percent inhibition against MTCC 284 after 5 days of time interval (Fig.5, 6& 7). Ram *et al.* (2020) earlier reported antagonistic traits of microbes isolated from indigenous cow- based organic suspensions. Different secretions of antibiotics accounted for its antagonistic ability to disrupt fungal cell walls or interference with other cellular process (Boulahouat et *al*, 2023). Another possible mechanism is accountable to production of volatile organic compounds like hydrogen cyanamide, ammonia etc. Above all, VOCs can disrupt fungal cell membranes, inhibit spore germination, or induce oxidative stress, ultimately suppressing the fungal growth and development (Yang *et al.*, 2023).

Figure 4: Phylogenetic tree based on sequences of PCR amplified 16S rRNA genes of vermiwash bacterial isolates

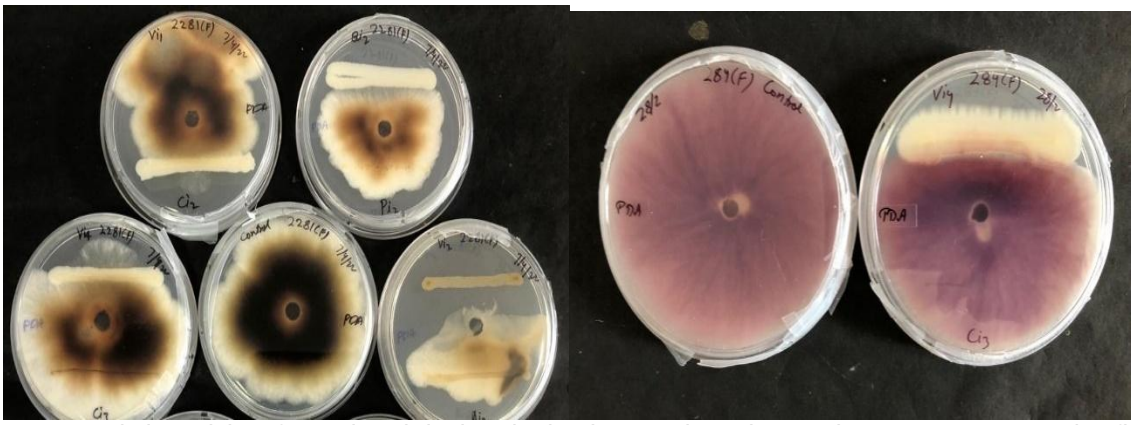

Figure 5: Antagonistic activity of vermiwash isolated microbes against phytopathogen MTCC 2281 (*C. fimbriata*) and MTCC 284 (*F. oxysporum)*

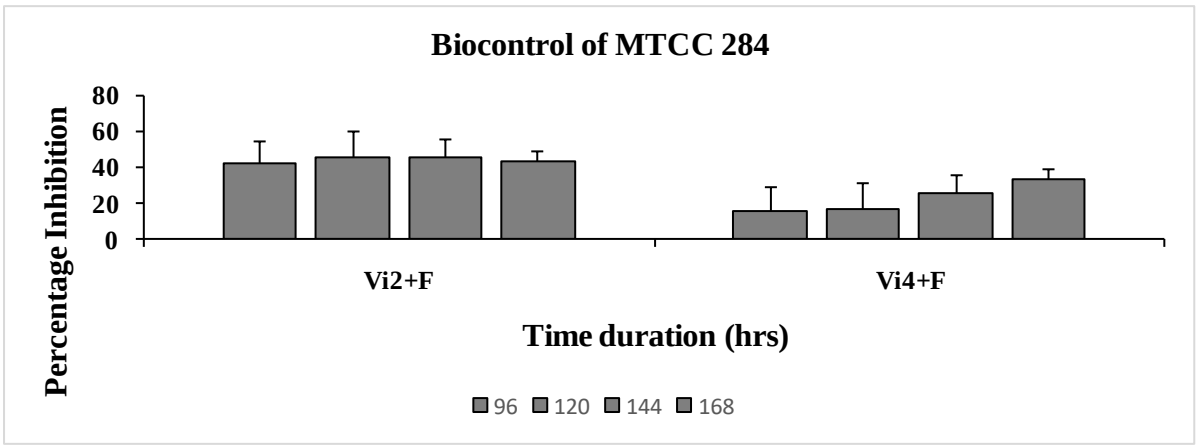

Figure 6: Antagonistic properties of bacterial isolates from vermi-wash against MTCC 284 (*F. oxysporum*)

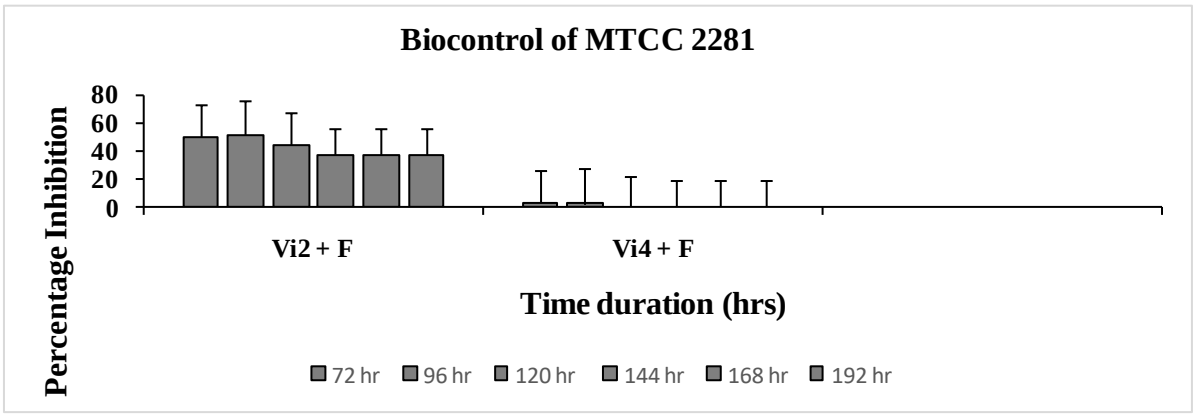

Figure 7: Antagonistic properties of bacterial isolates from vermiwash against MTCC 2281 (*Ceratocystis fimbriata)*

Heavy metal tolerance screened isolates

Amongst all the isolates, *Pseudomonas aeruginosa* (Vi2) showed maximum Cr (VI) heavy metal tolerance up- to 200 ppm. While *Pseudomonas luteola* (Vi1) exhibited tolerance up -to 100 ppm and *Bacillus tequilensis* (Vi_4) showed no growth in chromium -amended nutrient agar .These bacterial isolates reduced the chromium Cr(VI) to Cr (III) enzymatically ,

possessing specific enzymes to modify or detoxify the chromium (Pushkar *et al.*, 2021). Our results indicated that cow-based ingredients added in vermicompost were enriched with heavy metal tolerant bacteria and well adapted to grow in heavy metal stressed conditions. These observations established these bacterial strains as an effective tool for plant growth promotion in chromium contaminated environment (Figure 8 and Table 4).

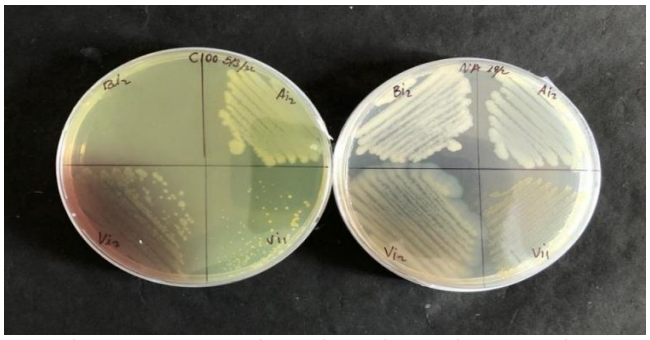

Figure 8: Hexavalent chromium tolerances by selected isolates

Table 4: Cr (VI) Heavy metal tolerance of selected isolates from vermiwash

	Heavy metal (Cr) concentration (ppm)				
Isolate	50	100	150	200	300
Vi_1	++++	+++	-	-	-
Vi_2	++++	++++	+++	+++	-
Vi_4	-	-	-	-	-

CONCLUSION

Bacterial isolates from vermiwash showed data based evidence in favor of its PGP characteristics, effective biochemical utilization, good hexavalent chromium heavy metal tolerance in addition to biocontrol action. These isolates can be used as a tool for application in agricultural field for plant growth or as a biocontrol agent against plant pathogen or as a consortium displaying such twin roles.

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