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EXPLORING COMPETENCE OF FLUORESCENT *PSEUDOMONAS* SPP. FOR HEAVY METAL TOLERANCE

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ABSTRACT

During the current investigations in the laboratory, some potential strains of fluorescent nature were isolated from the rhizospheric soil of plants viz., Tomato, Brinjal and Sugarcane from different field sites of vidarbha region. These isolates were subsequently identified as fluorescent *Pseudomonas spp.* by performing standard microbiological and biochemical tests. The present studies deal with exploration of the heavy metal tolerance capability of these isolates against various concentrations of some selected heavy metals. In preliminary investigations, total 6 heavy metal compounds – Zinc Sulphate ($ZnSO_4$), Potassium dichromate ($K_2Cr_2O_7$), Copper sulphate ($CuSO_4$), Arsenic chloride ($AsCl_3$), Mercuric chloride ($HgCl_2$), Lead nitrate [$Pb(NO_3)_2$] were screened against these isolates to generate a sensitivity profile. Further the growth and fluorescence pattern of the said isolates were verified by incorporating different concentration of heavy metals while performing the standard tests and through spectrophotometric analysis. The effect of heavy metals on growth and PGPR activity was analyzed in this study. The heavy metal resistant isolates could be interned in solid or liquid bioinoculants and could be effectively used in agricultural soil contaminated with heavy metals.

KEYWORDS: Heavy metal compounds, fluorescent *Pseudomonas*, contaminants, MIC, PGPR.

1. INTRODUCTION

The sewage sludge, industrial waste and long term use of chemical fertilizer in agriculture contaminated the agricultural soil due to toxicity rendered by the heavy metals such as Cadmium (Cd), Copper (Cu), Zinc (Zn), Nickel (Ni), Cobalt (Co), Mercury (Hg), Chromium (Cr), Lead (Pb) and Arsenic (As).[1,2,3] The soil microorganism suffer from direct or indirect impacts of the heavy metals. However, these heavy metals, in traces, are requisite for soil biota as co-factor for enzymatic reaction.[4] Heavy metal toxicity in soil and water causes dreadful effects on plants, animals and humans.[5] The bioremediation of these heavy metals remains the major problem till today. Bioremediation of heavy metals by using microorganisms has drawn a great attention globally. Some fluorescent *Pseudomonas spp.* showed key role in degradation of pesticides as well as heavy metals.[6] According to literature, few microorganisms are capable of surviving in high concentration of heavy metal including *Pseudomonas spp.* In addition to heavy metal tolerance ability, they also have plant growth promotional and phytopathogenic activity and hence could be effectively used in agricultural practices.

Pseudomonas spp. are the significant bacteria in agriculture soil and the fluorescent *Pseudomonas sp.* is one of the most prominent strains of genus *Pseudomonas.*[7] The traits that make fluorescent *Pseudomonas spp.* helpful for use in agriculture include, production of phytoharmones, siderophore, antibiotics, lytic enzymes and hydrogen cyanide (HCN). The competitive root colonization and induction of systemic resistance against plant pathogen are other important properties of these spp. Many workers focus on heavy metal tolerance capacity of these spp. because heavy metals reduce soil fertility and harm soil beneficial microorganisms.[7]

The present study deals with probable isolation and characterization of heavy metal tolerant strains of fluorescent *Pseudomonas.* Six different heavy metals Zinc sulphate ($ZnSO_4$), Potassium dichromate ($K_2Cr_2O_7$), Copper sulphate ($CuSO_4$), Arsenic chloride ($AsCl_3$), Mercuric chloride ($HgCl_2$), Lead nitrate [$Pb(NO_3)_2$] were used for this study. The plant growth promotional activity of these isolates, like phosphate solubilization, Indol acetic acid production, was analyzed by incorporating different concentration of each heavy metals.

The changes in fluorescence pattern of isolates against the use of heavy metals were analyzed on king's B medium. The heavy metal tolerance ability of these isolates makes them reliable and prospective candidates for impound in liquid bioinoculent.

2. MATERIALS AND METHODS

2.1. Sampling sites and collection

The soil samples were collected from rhizospheric region of Sugercane,Tomato and Brinjal plants from villages Bazargaon, Mohapa, Kalmesher and Kuhi respectively, Dist. Nagpur. The samples were collected in polythene bags for transportation in the laboratory and kept in refrigerator till use.

2.2. Isolation of fluorescent *Pseudomonas* isolates

A soil suspension was prepared by shaking 1g of soil having 2-3 cm undamaged root pieces with tightly adhered soil in 100 ml of sterile distilled water and kept for 24hrs on a rotary shaker to release the rhizoplane bacteria. The processed samples were serially diluted from 10^{-1} to 10^{-6} and 0.1 ml of the suspension was spread on to King's medium B (KMB) agar plate and incubated at 28ºC for 48h. The occurrence of fluorescent *Pseudomonas* was examined under UV light (356 nm) by using Spectroline Ultravoilet Transilluminator.

2.3. The heavy metals used for study

The study was carried out by using six heavy metal compounds given in Table 1.

Table 1 - Heavy metals used in the study

Sr. No.	Metal	Salt used
1.	Zinc	$ZnSO_4$
2.	Copper	$CuSO_4$
3.	Chromium	$K_2Cr_2O_7$
4.	Arsenic	$Ascl_3$
5.	Mercury	$HgCl_2$
6.	Lead	$Pb(No_3)_2$

2.4. Heavy metal sensitivity profile

In preliminary studies, these heavy metal compounds were tested against the isolates to generate a toxicity sensitivity profile by Kirby-Bauer test method. The overnight grown cultures of fluorescent *Pseudomonas* isolates were utilized for preparing King's B agar spreadplates & accordingly, incubated with selected heavy metal compounds for 24 hrs at optimum growth temperature of isolate. The zone of inhibition was noted for standardizing the sensitivity profile of test organisms.

2.5. Determination of effect of heavy metal compounds on bacterial growth

The growth curve of isolates were determined by incorporating 50ug/ml, 100ug/ml, 150ug/ml, 200ug/ml, 250ug/ml and 300ug/ml concentration respectively of each heavy metal and measuring the optical density at 600nm by spectrophotometer (Dynamica Halo DB - 20S).

2.6. Determination of MIC

The bacterial cultures were streaked onto the Nutrient Agar (NA) medium, containing different concentration of heavy metal salts and then plates were incubated at 37°C for 24-48 hrs, checking everyday for bacterial growth. The concentration of heavy metals at which no bacterial growth was observed, considered as the MIC of that metal for that strain.

2.7. Visual Quantization of fluorescence

King's B agar plates were streaked with the King's B broth containing test organism treated by different concentration of heavy metal. The streaked plates were kept in incubator for 24-48 hrs and were photographed. The bacterial fluorescence was observed visually under UV-Transilluminator.

2.8. Determination of effect of heavy metal on the activity of isolate

Different concentrations of heavy metal were used to perform following tests.

2.8.1. IAA production

Heavy metal of different concentration was added to King's B broth and transferred into test tubes. No Heavy metal was added in control tubes. All the tubes were inoculated with test bacterial culture. Determination of indol acetic acid production (IAA) was performed according to method described by Ramyasmruthi S. *et al.* Briefly, bacterial cultures were grown in metal containing KMB broth and supplemented with Tryptophan (5mg/ml) for 24 hrs at 28^0 C. Fully grown bacterial cultures were centrifuged for 30 min at 3000 rpm. Later, 2 ml of culture filtrate was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl3 solution).

2.8.2. Phosphate solubilization

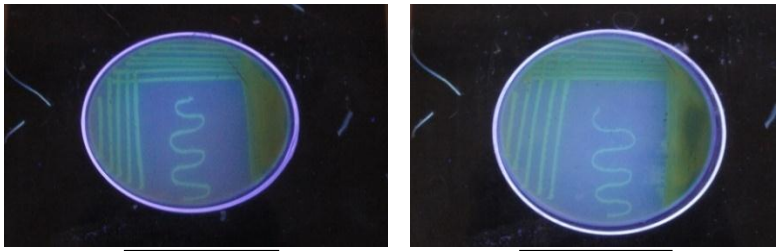
Phosphate solubilization activities of the isolates were checked on metal-Pikovskaya Agar plates and Pikovskaya Agar plates by agar well diffusion method. Bacterial cultures were grown in KMB broth and 50 ul were poured in well and incubated the plates at 28^0 C.

Phosphate solubilization was determined by clear halo zone formation after 3 days of incubation.

3. RESULTS AND DISCUSSION

3.1. Isolation of fluorescent Pseudomonas bacteria

The test organisms used in this study were found to be the fluorescent *Pseudomonas* sp. These strains were isolated from the rhizosphere region of crop plant Tomato and Sugarcane from different field sites of Vidarbha region while no any strain was found from the rhizosphere region of Brinjal plant. On the basis of standard microbiological and biochemical tests, the test organisms were identified as the *Pseudomonas sp. PcFRB039* (FP 3) and *Pseudomonas aeruginosa* (FP 4) a fluorescent bacterial strains [Fig.1 (A&B)].

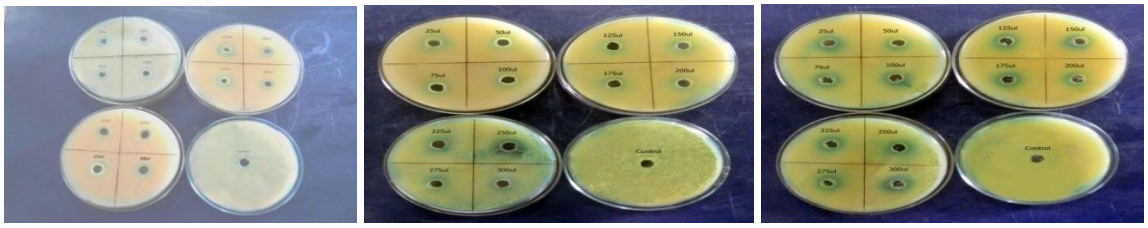


A : FP 3 B : FP 4

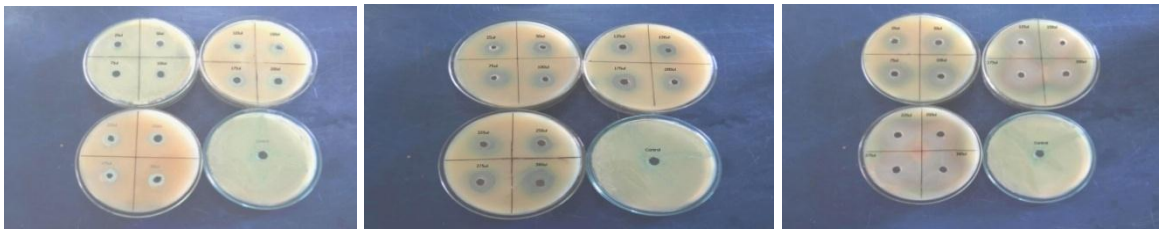
Fig.1 (A&B) : Fluorescent *Pseudomonas* isolates under UV-light

3.2. Preliminary screening of the test organisms against heavy metals

The isolates were tested for their sensitivity towards six different heavy metals by Kirby-Bauer test method. The bacterial isolates were treated with the six heavy metal – ZnSO4, CuSO4, K2Cr2O7, $AsCl_3$, $HgCl_2$, $Pb(NO_3)_2$. The growth of bacterial isolates was analyzed on the basis of zone of inhibition against different concentration of heavy metals used. When the isolate FP3 and FP4 were tested against heavy metals- ZnSO4, CuSO4, K2Cr2O7, $AsCl_3$, no zone or slightest zone was found as compared to the control up to the conc. 300 ug/ml, indicating good growth of isolates even after addition of contaminants. While greater zone of inhibition for heavy metal $HgCl_2$, $Pb(NO_3)_2$ indicated sensitivity of the isolates for these heavy metals{ [fig. 2 (a-f) & Table 2] and [Fig. 3 (g - l) & Table 3]}.



a. ZnSO | b. $CuSO_4$ | c. $K2Cr_2O_7$

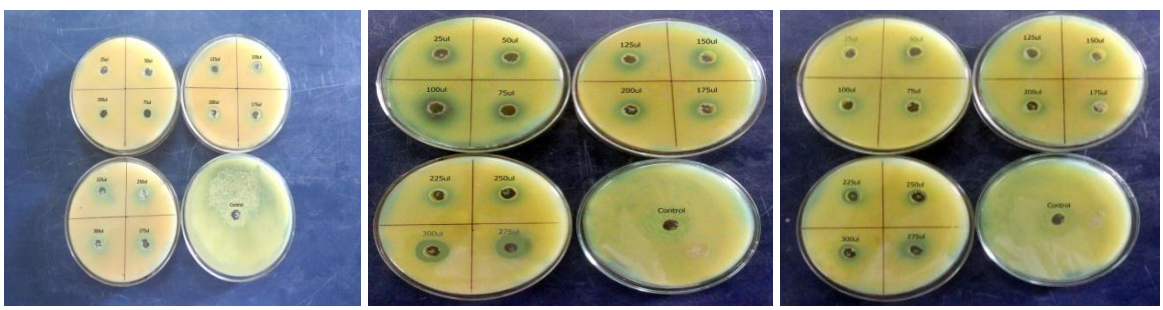


d. $AsCl_3$ | e. $HgCl_2$ | f. $Pb(NO_3)_2$

Fig. 2 (a - f): Zone of inhibition shown by isolate FP3 against different heavy metals

Table 2 - Interpretation of zone given by isolate FP3 against different conc. of heavy metals

Sr. No.	Heavy metal compounds	Zone diameter in (mm) for FP3											
	Concentration of heavy metal (ug/ml)	25	50	75	100	125	150	175	200	225	250	275	300
	Control(without heavy metals)	No zone											
1.	ZnSO4	-	-	8	10	11	11	13	14	13	14	15	16
2.	K2Cr2O7	8	8	8	10	10	12	12	10	13	14	14	14
3.	CuSO4	8	8	10	10	12	12	11	12	13	13	14	17
4.	$AsCl_3$	-	-	8	8	10	10	12	12	13	14	16	18
5.	HgCl2	12	15	16	16	16	15	15	17	16	16	18	22
6.	$Pb(NO_3)_2$	17	20	20	24	27	28	30	38	37	39	40	40



g. $ZnSO_4$ | h. $CuSO_4$ | i. $K2Cr_2O_7$

j. $AsCl_3$ k. $HgCl_2$ l. $Pb(NO_3)_2$

Fig. 3 (g - l) : Zone of inhibition shown by isolate FP4 against different heavy metals

Table 3 - Interpretation of zone given by isolate FP4 against different conc. of heavy metals

Sr. No.	Heavy metal compounds	Zone diameter in (mm) for FP4											
	Concentration of heavy metal (ug/ml)	25	50	75	100	125	150	175	200	225	250	275	300
	Control(without heavy metals)	No zone											
1.	ZnSO4	-	-	8	8	10	12	12	13	15	14	15	16
2.	K2Cr2O7	-	9	9	11	11	10	10	12	12	12	14	15
3.	CuSO4	10	12	12	14	14	15	15	15	15	16	19	20
4.	$AsCl_3$	12	13	13	15	14	15	16	17	16	17	18	19
5.	HgCl2	16	17	17	18	20	19	21	21	22	21	21	22
6.	$Pb(NO_3)_2$	15	20	25	25	28	28	34	35	34	36	36	38

3.4. Spectrophotometric analysis

The effect of heavy metal compounds on cell density of *P.* fluorecens strains FP3 and FP4 was analysed by spectrophotometer. Heavy metal compounds $ZnSO_4$, $K_2Cr_2O_7$, $CuSO_4$ and $AsCl_3$ had little or no effect, while $HgCl_2$ and $Pb(NO_3)_2$ had adverse effect on the cell density, after 24 hrs to 48 hrs of incubation with heavy metal compounds. These results showed that the bacterial candidates under study was found to be resistant to heavy metals $ZnSO_4$, $K_2Cr_2O_7$, $CuSO_4$ and $AsCl_3$ while sensitive to $HgCl_2$ and $Pb(NO_3)_2$ (Figs. 4 & 5).

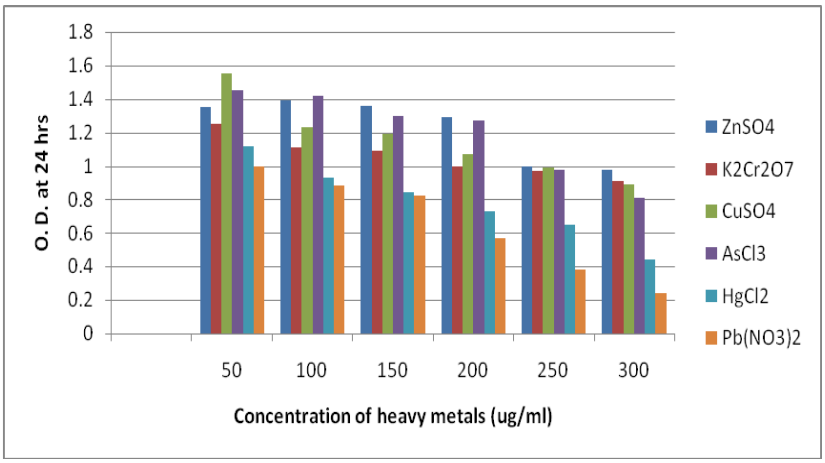


Fig. 4 : Effect of different concentration of heavy metal on cell density of *Pseudomonas sp.*

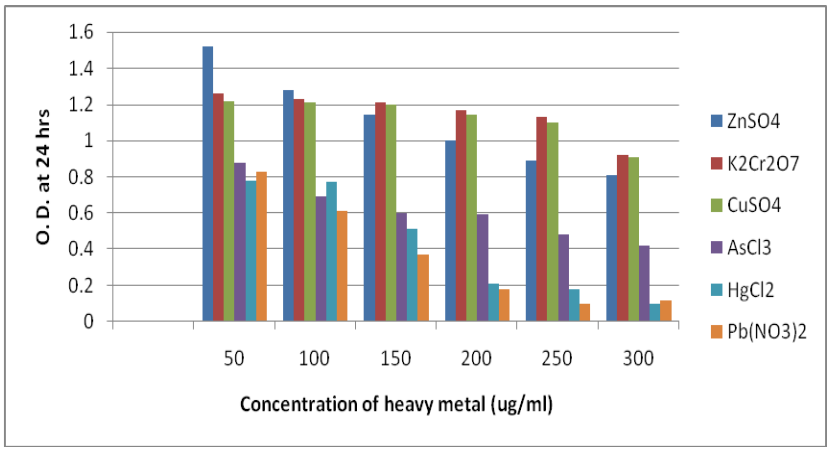


Fig. 5: Effect of different concentration of heavy metal on cell density of *Pseudomonas aeruginosa PcFRB039*

3.5. Determination of MIC

The minimal inhibitory concentration of heavy metal compounds were evaluated against selected heavy metals for heavy metal tolerance study.[8] The MIC of each heavy metals was shown in Table 4.

Table 4 - Resistance of bacteria to heavy metals.

Bacteria	Heavy metal	MIC
FP- 3	ZnSO4	270 ug/ml
	CuSO4	280 ug/ml
	K2Cr2O7	260 ug/ml
	$AsCl_3$	270 ug/ml
	$HgCl_2$	200 ug/ml
	$Pb(NO_3)_2$	140 ug/ml
FP- 4	ZnSO4	220 ug/ml
	CuSO4	270 ug/ml

	K2Cr2O7	250 ug/ml
	$AsCl_3$	180 ug/ml
	$HgCl_2$	140 ug/ml
	$Pb(NO_3)_2$	110 ug/ml

(FP = Fluorescent *Pseudomonas* isolate, MIC = Minimum inhibitory concentration)

3.6. Digital photographic data

The fluorescent *Pseudomonas* strains under study, showed fluorescence under UV light when observed under UV-Transilluminator. The overnight grown isolates in King's B broth inoculated with different concentrations of heavy metals were used for streak plating and plates were observed for fluorescence under UV-light. The bacterial cultures got fluorescence after 24 hrs of incubation for all concentrations of heavy metals (100ug/ml to 300ug/ml), while decrease in the fluorescence was found after 48 hrs of incubation [fig. 6 (a-h)].

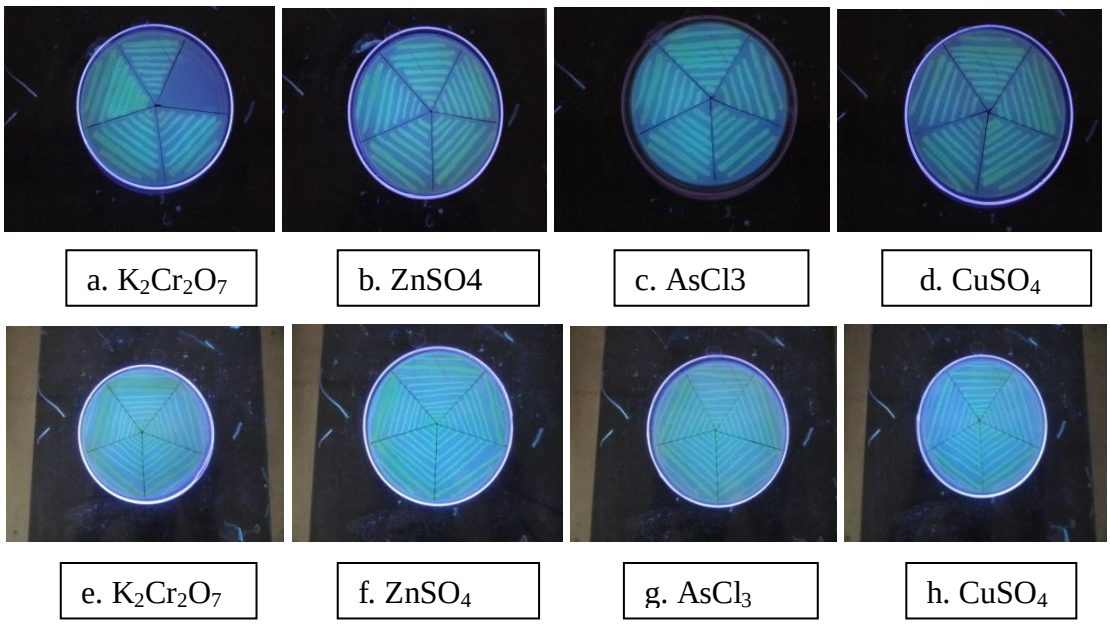


Fig. 6 (a-h): Qualitative measurement of fluorescence of FP3 and FP4 through digital photography of plates under UV-light

3.7. Effect of heavy metals on PGPR activity

The plant growth promotion activity of the isolates FP3 and FP4 was analyzed by incorporating the heavy metals for Indol acetic acid (IAA) production and phosphate solubilization tests and it is found that both the isolates showed pink colour development that is IAA production as well as halo zone formation after 24 hrs of incubation at 28^0 C. However, activity of these isolates to produce halo zone on Pikovskaya's media and indole acetic acid production was reduced as compared to control. It clearly indicated that the

isolates under study were found to be resistant to the heavy metals ZnSO4, CuSO4, K2Cr2O7and $AsCl_3$.

The presence of higher concentration of heavy metals in the soil disturbs the soil beneficial microflora. The heavy metal contamination in the soil reduce the growth as well as plant growth promotional activity of the soil bacteria. However, metal resistant plant growth promoting bacteria improved the plant growth and yield. Many workers suggested that the soil bacteria enhanced the plant growth and mimics the toxic effects of heavy metals on plants.[10] Osborne *et al*., (2010) studied the metal tolerance concentration of plant growth promoting rhizobacteria strains and found that they were able to survive till 300 mg/l on cadmium amended minimal medium.

In the present study, metal resistant PGPR strains FP3 and FP4 were isolated and studied for their heavy metal tolerance capacity. According to Cazorla *et al.* 2002, *Pseudomonas* strains were found resistant to Cu. These results were found to be similar to this study. Filali *et al.*, (1999) studied bacteria isolated from wastewater such as, *Psuedomonas aeroginosa, Klebsiella pneumoniae, Proteus mirabilis* and *Staphylococcus* and found to be resistant to heavy metals and antibiotics.[13] Microbial tolerance to heavy metal is due to detoxifying mechanism developed by resistant microorganisms such as metal reduction, metal efflux etc., but presence of very high concentration of heavy metals, exert inhibitory action on microorganisms by blocking the essential functional groups.

According to Carlot *et al*., (2002) the *Pseudomonas* sp. (SP1), isolated from hydrocarbon contaminated soil, was found to be a potential heavy metals tolerant bacteria and efficient in producing plant growth promoting (PGP) compounds. This result was found to be similar to this study, as the isolate FP3 and FP4 were found resistant to the heavy metals - Zinc sulphate ($ZnSO_4$), Potassium dichromate ($K_2Cr_2O_7$), Copper sulphate ($CuSO_4$) and Arsenic chloride ($AsCl_3$), as well as competent in producing plant growth promoting (PGP) compounds. According to literature few microorganisms are capable to survive in high concentration of heavy metal including *Pseudomonas spp.* In addition to heavy metal tolerance ability, they also have plant growth promotional and phytopathogenic activity and hence can be effectively used in agricultural practices.

4. CONCLUSION

The sewage sludge, industrial waste and long term use of chemical fertilizers in agriculture, contaminated the agricultural soil by heavy metal toxicity which cause threats to the environment and health because of their toxicity and non-biodegrability. The soil microorganism suffer from direct or indirect impacts of the heavy metals. Microorganisms with the ability to tolerate and reduce heavy metal compounds can be used for detoxification of contaminated environment. The heavy metal resistant bacteria could be a potential representative for reducing heavy metal toxicity.

Remediation of heavy metal compounds by microorganisms has drawn great attention today for its potential applications in industry. Metal resistant soil bacteria also enhance the plant growth as well reduced the heavy metal toxicity. The selection of such microorganisms, having both traits viz., metal tolerant capacity and competent in producing plant growth promoting (PGP) compounds could be proved useful to speed up agriculture productivity. Hence, these competent isolates under study could be used efficiently in metal containing soil also for increasing crop yield.

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