IOSR Journal Of Pharmacy *www.iosrphr.org*
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
Volume 9, Issue 6 Series. I (June 2019), PP. 18-24

Isolation and Characterization of Heavy Metal Resistant Plant Growth Promoting Bacteria

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Abstract: Under metal stress, soil microorganisms including plant growth promoting bacteria (PGPB) develop many strategies to evade the toxicity generated by the various heavy metals. Such metal resistant PGPB, when used as bioinoculants or biofertilizers, can significantly improve the growth of plants in heavy metal contaminated/stressed soils. This paper focuses on the isolation and characterization of heavy metal resistant PGPB from soil sample obtained from banks of Meethi River. The isolation was carried out using the heavy metals lead, copper and zinc and five isolates i.e ZA, ZB, ZC, ZD and ZE were obtained which showed multiple resistance to all the three heavy metals. The MIC was performed by plate and tube dilution method. The plant growth promoting characteristics of the five isolates were studied. The production of Indole Acetic Acid (IAA) was determined and it was found that maximum IAA was produced by ZB(37mcg/ml) followed by ZC (32mcg/ml), ZE(14mcg/ml), ZA(9mcg/ml) and least was produced by ZD(8mcg/ml). All the five isolates showed phosphate solubilisation, ammonia production, catalase production and nitrogen fixation. It was also found that the isolates are cellulose negative, amylase negative and protease negative.In heavily contaminated soils where the metal content exceeds the limit of plant tolerance, it may be possible to treat plants with these PGPB thereby stabilizing, re-vegetating, and remediating metal-polluted soils. In addition, the application of the heavy metal resistant and plant-beneficial bacteria can be considered as bioremediating tools with great economical and ecological relevance.

Keywords: Bioinoculants**,** Bioremediation, Heavy Metal Resistance, PGPB.

Date of Submission: 24-05-2019 Date of acceptance: 10-06-2019

I. INTRODUCTION

Plant-microbe interactions in the rhizosphere are the determinants of plant health, productivity and soil fertility. Plant growth-promoting bacteria (PGPB) are bacteria that can enhance plant growth and protect plants from disease and abiotic stresses through a wide variety of mechanisms; those that establish close associations with plants, such as the endophytes, could be more successful in plant growth promotion. Several important bacterial characteristics, such as biological nitrogen fixation, phosphate solubilisation, ACC deaminase activity, and production of siderophores and phytohormones, can be assessed as plant growth promotion (PGP) trait **(Rocheli de Souza et al, 2015).** Plant growth promoting bacteria present in metalliferous soil (soil contaminated with heavy metals) may develop resistance to the heavy metals present in the soil.

Heavy metals are those elements with a molecular weight greater than 53, a density greater than 6 g cm^{-3} and an atomic number greater than 20. They occur naturally in rocks and soils but concentrations are frequently elevated as a result of pollution. They are also called trace elements, which are toxic to living organisms at excessive concentrations but some including Zn, Cu, Mn and so on, at low but critical concentrations are micronutrients used in the redox processes, regulation of the osmotic pressure and also enzyme components which are essential for the normal healthy growth and reproduction by living organisms. At high concentrations, these micronutrients damage DNA and membrane as well as loss of functions of enzyme. However, heavy metals like Ni, Co and Pb cause oxidative stress, lipid peroxidation, carcinogenesis, mutagenesis and neurotoxicity on humans, animals and plants at low concentrations.Elevated concentration of heavy metals are introduced into the environment through metalliferous mining, metal smelting, activities of metallurgical industries, waste disposals, corrosion of metalsin use and agriculture and petroleum exploration among others. The discharge of effluents containing heavy metals mounts pressures on the ecosystem and consequently Causes health hazards to plants, animals, aquatic life and humans. Upon surface contamination, the toxic metals are transported to groundwater and bio accumulated.**(Joshi B. H., Modi K. G,2013).** When present in agricultural soil these heavy metals may hinder the growth and development of plants. Also, the heavy metals present in soil may inhibit the growth and activity of the plant growth promoting bacteria if the bacteria are sensitive to the heavy metals.

Hence plant growth promoting bacteria which are resistant to these heavy metals could be used as bioinoculants in the metalliferous agricultural soil in order to improve plant growth and development in such soils. Bacterial inoculants can contribute to increase agronomic efficiency by reducing production costs and environmental pollution. The use of chemical fertilizers can be reduced or eliminated if the inoculants are efficient. This research focuses on the isolation and characterization of bacteria which are resistant to the heavy metals (Cu, Zn and Pb). The soil samples were taken from the banks of Meethi River which is the most heavily polluted river in Mumbai. The plant growth promoting activities of the isolated bacteria were studied and the bacteria could be used as bioinoculants in metalliferous agricultural soils.

II. MATERIALS AND METHODS

2.1Sample collection

Soil sample was collected in plastic bags from the banks of Meethi river and stored under dry conditions at R.T. **(Bhupindra K Pushkar et al., 2015)**

2.2Sample processing

Ten gm of soil sample was mixed with 20ml sterile distilled water and vortexed to mix the soil and water. The soil was allowed to settle down. The supernatant was collected and used as inoculant for isolation and screeningof the PGPB **(K. Geetha 2014.**)

2.3 Primary enrichment of sample

Two media i.e. nutrient broth and Luria broth were used for enrichment in order to optimize the medium to be used for further research.5ml of processed sample was inoculated in 50ml of Luria broth and nutrient broth respectively and incubated at room temperature under static conditions for 48hrs. **(Mohammed U. 2015)**

2.4 Secondary enrichment of sample

5ml of each primary enriched medium was inoculated into 50ml of fresh nutrient broth and Luria broth respectively and incubated at room temperature under static conditions for 48hrs**. (Pradyut Saikia 2015)**

2.5 Isolation of heavy metal resistant bacteria from the sample

Sterile media amended with heavy metal stock solutions i.e. 100mcg/ml of lead acetate, zinc sulphate and copper sulphate respectively was used. The enriched soil sample was serially diluted and the 10^{-6} dilution was used for performing pour plate technique. The colonies that appeared on the media were selected for further study. **(Adriana Giongo et al., 2010)**

2.6 Obtaining pure cultures

The isolated colonies were streaked on sterile LB agar and incubated at RT for 24hrs. The colonies obtained on these were again streaked on sterile LB agar and the colonies obtained were used as pure cultures.

2.7Determination of MIC

The minimum inhibitory concentration of the heavy metals was determined by tube dilution method as well as agar plate method. For the plate assay, L.B agar plates containing increasing concentration of heavy metals were used. The isolates were streaked on the plates and incubated at room temperature. In the tube dilution method, appropriate metal concentrations were prepared in tubes with a final volume of 10 ml of L.B broth and were inoculated with 0.1 ml of an 18-h-old culture of the studied bacterial strain. The lowest concentration of metal that completely prevents growth was termed as the 'minimal inhibitory concentration' (MIC).

2.8 Characterization of plant growth promoting activities

2.8.1 Phosphate solubilisation

Inoculate the culture (O.D adjusted to 0.1) on Pikovsky agar. Incubate at R.T for 72hrs. Halo zones around the colonies indicate phosphate solubilisation (**Elizabeth T. A. et al., 2017).**

2.8.2 IAA production

Inoculate the cultures (O.D adjusted to 0.1) in 10ml sterile L.B broth containing 0.05% tryptophan and incubate at R.T for 24hrs.Take 2ml aliquot from the tubes. Centrifuge at 6000 rpm for 30min. take 1ml of supernatant from this. Add 1 drop of orthophosphoric acid and 2ml of Salkowskii reagent to it. Incubate at R.T in dark for 30 min. Development of pink colour indicates IAA production. Take O.D at 530 nm using blank (1ml LB broth, 1 drop of orthophosphoric acid and 2 ml of Salkowskii reagent. The amount of IAA produced is determined by plotting a standard plot for IAA and extrapolating the points on the graph **(Dolf W. et al., 2018)**.

2.8.3 Ammonia production

Inoculate the cultures (O.D adjusted to 0.1) in 10 ml peptone water in each tube separately and incubate for 48-72 h at R.T. Add 0.5 ml Nessler's reagent in each tube. Development of brown to yellow colour indicates a positive test for ammonia production.

2.8.4 Catalase activity

Grow the culture on sterile trypticase soya agar. Add 3-4 drops of H_2O_2 to 48hr old bacterial colony. Effervescence indicates positive result for catalase test.

2.8.5 Amylase (starch hydrolysis) activity

Spot inoculate the cultures (O.D adjusted to 0.1) on sterile starch agar (Beef extract 3.0, Peptone 5.0, soluble starch 2.0, Agar 15.0, Distilled water 1 lit.).Incubate at R.T for 48hrs. Flood the plates with iodine solution. Colourless zone surrounding the colonies indicates production of amylase and starch hydrolysis. **(Gebreselema Gebreyohannes. 2015)**

2.8.6 Caseinase (protease) activity

Spot inoculate the cultures (O.D adjusted to 0.1) on sterile skim milk agar plates (Pancreatic. digest of casein 5.0, Yeast extract 2.5, Glucose 1.0, Agar 15.0, Distilled water 1000 ml, Skim milk 7% was added as inducer). Incubate at R.T for 24 hrs; zone of clearance around the colony indicates the enzymatic degradation of protease.

2.8.7 HCN production

Streak the bacterial isolates (O.D adjusted to 0.1) on Kings B agar medium amended with glycine (4.4 g/l). Place Whatman No.1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) in the lid of each Petri plate. Seal the plates air-tight with Parafilm and incubate at R.T for 48 h. A colour change of the filter paper from deep yellow to reddish-brown colour is considered as an indication of HCN production. **(A. Karmeel R. et. al., 2014).**

2.8.8 Cellulase activity

Spot inoculate the cultures (O.D adjusted to 0.1) on CMC (Carboxyl methyl cellulose) agar. Incubate at R.T for 24-48hrs. At the end of incubation, flood the agar medium with an aqueous solution of Congo red (1% w/v). Formation of clear zone indicates cellulose degradation. **(Arvinder Kauret al., 2014)**

2.8.9 Nitrogen fixation

Streak the bacterial isolates (O.D adjusted to 0.1) on sterile nitrogen free Jensen's agar medium. Incubate the plates at R.T for 5 days. Appearance of colonies indicates nitrogen fixation and nitrogenase activity.

III. RESULTS AND DISCUSSION

3.1 MIC of heavy metals

The MIC of copper, zinc and lead was determined using tube dilution method and agar plate method. The results are as tabulated in Table 1.and grafically represented in Fig.1, as per the MIC values obtained, among the three heavy metals copper was found to have the maximum toxicity followed by lead and the minimum toxicity was seen for zinc. Hence it could be said that the isolates show maximum resistance to zinc followed by lead and minimum resistance to copper.

Culture	MIC (mcg/ml)		
	COPPER	LEAD	ZINC
ZA	600	1000	1500
ZB	1000	1100	2500
ZC	800	1100	2500
ZD	800	1000	600
ZE	600	1000	1100

Table.1. The MIC of copper, zinc and lead

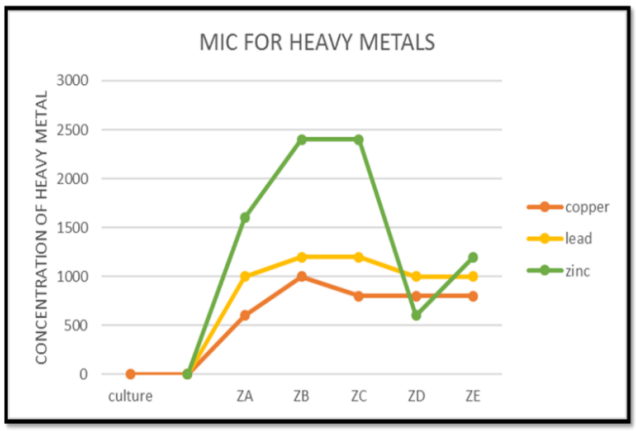


Fig.1. The MIC of copper, zinc and lead

Plant growth promoting activities are as shown in the figures below.

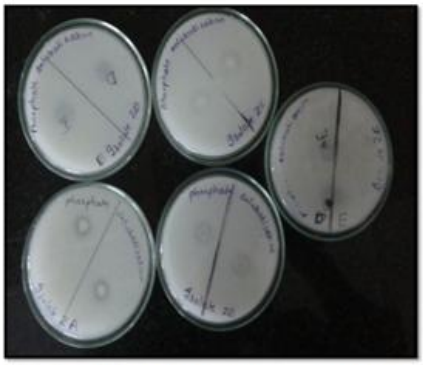


Fig.2.Zones of clearance around the Colonies indicating phposphate Solubilization

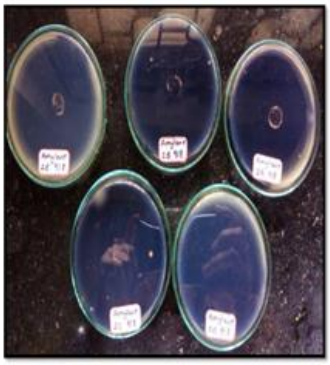


Fig.3.No zone of clearance indicating the absence of amylase activity

Fig.4.No colour change in the paper indicates absence of HCN production

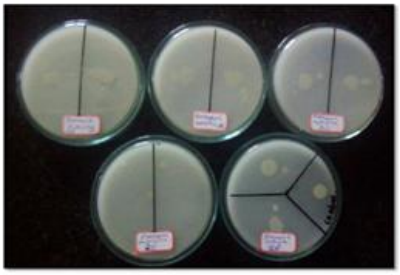
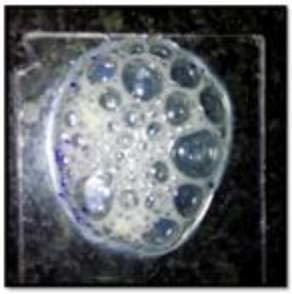


Fig.5.Absence of cellulase activity as there is no clearance aroung the colonies



Plant Growth Promoting Activity	ZA	ZB	ZC	ZD	ZE
Phosphate solubilisation	+	+	+	+	+
Ammonia production	+	+	+	+	+
Catalase production	+	+	+	+	+
Amylase production	-	-	-	-	-
Protease production	-	-	-	-	-
HCN production	-	-	-	-	-

Fig.6. Presence of effervescence indicates positive catalase test

Cellulase production	-	-	-	-	-
Nitrogen fixation	+	+	+	+	+

Table.2. Plant growth promoting activities of the five cultures under study

***3*.2 Characterization of plant growth promoting activities**

The isolates showed varied levels of PGPR traits such as phosphate solubilization, IAA, ammonia and HCN production and other PGPR traits . This is as shown in Table. 2.

3.2.1 Phosphate Solubilization

All the five strains exerted ability for phosphate solubilization on Pikovskaya medium with different efficacy. The phosphate-solubilizing activity was determined by the presence of halo zones around the colonies and this characterizes the microorganisms with ability to produce and release metabolites such as organic acids that chelate the cations bound to phosphate, converting them into soluble forms. The results are as depicted in fig. 2.

3.2.2 IAA production

Auxin is the most investigated hormone among plant growth regulators **(Dolf W. et al., 2018)**. The most common, best characterized and physiologically most active auxin in plant is indole-3-acetic acid (IAA). IAA is known to stimulate both a rapid response (e.g. increased cell elongation) and a long-term response (e.g. cell division anddifferentiation) in plants**.** In our study, all the 5 bacterial isolates were able to produce indole-3-acetic acid (IAA) growing in medium supplemented with tryptophan. Maximum IAA production was recorded in isolate ZB(37mcg/ml) followed by ZC(32 mcg/ml), ZE(14mcg/ml), ZA(9mcg/ml) and the least was produced by ZD(8mcg/ml). This is as presented grafically in Fig.7.

3.2.3 HCN Production

Ability for hydrogen cyanide synthesis was observed in none of the 5 isolates indicating that the isolates do not have any antagonistic properties. The results are as depicted in Fig.4.

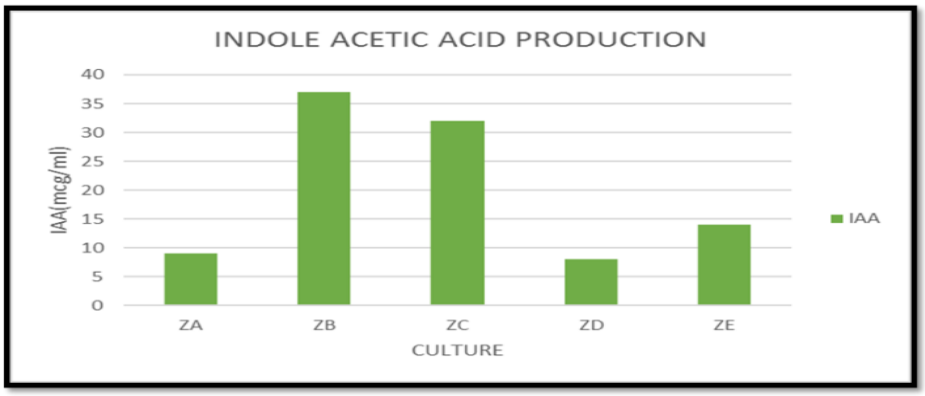


Fig.7. The amount of IAA produced by various strains of PGPB under study

3.2.4 Ammonia production

The production of ammonia observed in all the five isolates. The ammonia is useful for plant as directly or indirectly. Ammonia production by the plant growth promoting bacteria helps influence plant growth indirectly.

3.2.5 Nitrogen fixation

Nitrogen fixation activity of the isolates was tested using nitrogen free Jensens medium. Growth was observed on the plates indicating that all the five isolates carry out nitrogen fixation.

3.2.6 Proteolytic enzyme production

Proteolytic enzyme production was not detected in any of the isolates.

3.2.7 Cellulase activity
Cellulase activity was absent in all the five isolates as no zones of clearance were observed around the colonies on CMC agar after flooding with Congo red. This indicates the absence of cellulase activity in all the five isolates. Ref. Fig.5.

3.2.8 Amylase activity
Amylase activity was determined using starch agar medium. After 72 to 96 hr of incubation, the plates were flooded with Iodine solution, absence of halo zones around the colonies indicated absence of amylase activity in all the five isolates. **(Arvinder Kaur 2012).**The results are as shown in Fig.3.

3.2.9 Catalase activity
Catalase test was performed by adding three to four drops of H_2O_2on bacterial culture which was grown for 48hr on trypticase soy agar medium. The effervescence indicates Catalase activity. All the five isolates showed catalase activity. This is as shown in Fig.6.

IV. CONCLUSION

In order to overcome the heavy metal phytotoxicity, heavy metal resistant plant growth promoting bacteria must be used as bioinoculants in metalliferous soils. When subjected to heavy metal stress PGPB develop resistance mechanisms against the heavy metals and hence act as great biofertilizers when used as bioinoculants in agricultural soil contaminated with heavy metals. This study will help us develop a consortium of bacteria that are multi-resistant to three heavy metals i.e. Pb, Cu, and Zn and at the same time have plant growth promoting activities that will help better growth and development of plants. The MIC for three heavy metals copper, zinc and lead was determined as well as the plant growth promoting activities of the isolates from Meethi river were studied.

The developed consortium of heavy metal resistant plant growth promoting bacteria may be used as bioinoculant in the agricultural soil where the effluent water may be used for irrigation. Since the bacteria have plant growth promoting properties they will enhance the growth of the plants as well as help in getting a high-quality yield of the crop. At the same time, the heavy metal resistance will help the bacteria to survive in the soil and reduce the heavy metal stress in the soil. Waste disposal sites may also be used for agriculture if the developed consortium is used as bioinoculant. Therefore, this study aims in developing an alternative to the hazardous chemical fertilizers whose prolonged use can deteriorate the fertility of the agricultural soil.

Overall this research will help us develop a cost effective and environmental friendly approach for sustainable agriculture in heavy metal contaminated soil.This research is an effort to elucidate the concept of heavy metal resistant plant growth promoting bacteria in the current scenario. The latest paradigms of a wide range of applications of these beneficial bacteria in different agro-ecosystems will be presented explicitly to garner broad perspectives regarding their functioning and applicability.

ACKNOWLEDGEMENT

The authors thank the Principal and management of V.E.S college of Arts, Science and Commerce for financial support. They also thank to the non teaching staff of Microbiology department. Thanks to Prof. Amrin Gaonkar and Prof. Dona Joseph for the help they rendered during the research work.

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Zeenath Mohamed Mustafa.“ Isolation and Characterization of Heavy Metal Resistant Plant Growth Promoting Bacteria.”IOSR Journal of Pharmacy (IOSRPHR), vol. 9, no. 6, 2019, pp. 18-24.