*J. Mycopathol. Res.* ***53(1)*** *: 137-141, 2015;* (ISSN 0971-3719)


# *In vitro* study of plant growth promoting attributes of three cadmium bio-remediating *Pseudomonas* spp.

**ABHISHEK PAUL AND BEJOYSEKHAR DATTA**
*Department of Botany, Kalyani University, Kalyani 741 235, West Bengal*

Received : 25.04.2014 Accepted : 15.12.2014 Published : 27.04.2015

The present study deals with cadmium bioremediation potential and plant growth promoting activity of three strains of fluorescent *Pseudomonas* spp. isolated from different sources of industrial waste contaminated soil and water samples. All of them grow in Nutrient broth up to 500 µg/ml cadmium concentration and could also tolerate chromium, nickel and zinc. *In vitro* bio-remediation study showed that all the isolates were capable of removing approximately 50% of cadmium from the medium. Study of plant growth promoting features suggested that all the bacteria could solubilize inorganic phosphate (solubilization index varied from 1.8 to 2.5), and produce siderophore (diameter of orange halo on CAS plate varied from 12 to 20 mm) and showed catalase activity. Antagonism study of one of these strains against two fungal pathogens (*Rhizoctonia solani* and *Alternaria solani*) indicated positive result. These Cd bio-remediating strains might be exploited as biofertilizer in metal polluted agricultural field.

**Key words:** Cadmium, bio-remediation, phosphate solubilization, siderophore, catalase, antagonism, *Pseudomonas*

## INTRODUCTION

Heavy metal like cadmium became major anthropogenic pollutant due to its excess discharge into the environment as an industrial waste (Cunningham and Lundie, 1993). Soil acidity increases its mobilization and uptake by crops. In plants, Cd inhibits root and shoot growth, affects nutrient uptake and homeostasis, and frequently is accumulated in the edible portion of leaves, stem and food grains. Through food chain it enters into animals and human with their diet and can cause diseases such as itai-itai disease. Contamination of soil with Cd also negatively affects biodiversity and the activity of soil microbial communities (McGrath, 1994). Therefore, strategies should be developed to minimize the concentration of the metal in the soil.

In this context, application of metal-resistant microorganisms is a promising approach for remediation of heavy metal amended soils. Bacteria remove Cd from the environment through biosorption and intercellular uptake, converting it into insoluble form and making it unavailable for plants (Nies, 1999). In addition, bacteria producing siderophores and indole acetic acid, and solubilizing phosphate are capable of stimulating plant growth (Glick *et al.*, 1995) whereas indirect growth

promotion occurs through the elimination of pathogens by the production of antibiotics, siderophore, chitinase, and cyanide. Tripathi *et al.* (2005) have isolated and characterized a siderophore-producing lead and cadmium-resistant *Pseudomonas putida* KNP9 to improve plant growth.

The aim of the present *in vitro* investigations is to screen some Cd bio-remediating bacteria and also evaluate their plant growth promoting features, so that during application as Cd bio-remediating agents in metal polluted agricultural soil, they may act as bio-fertilizers also

## MATERIALS AND METHODS

### *Isolation of cadmium tolerant ($Cd^+$) bacteria*

Samples were collected from various heavy metal polluted soil and water sources. These bacteria were isolated on Nutrient agar medium containing 1mM of Cd as $CdCl_2$ by the dilution plating technique.

### *Study of heavy metal tolerance*

Maximum tolerance of the $Cd^+$ isolates against increasing concentrations of heavy metals *viz.*, cadmium ($CdCl_2$), chromium ($K_2CrO_4$), nickel ($NiCl_2$) and zinc ($ZnCl_2$) on agar medium was evaluated until the isolates would fail to grow on the medium supplemented with each of the metal. The concentration, above which no growth of the isolates occurred, considered as the highest tolerable concentration of the respective metal for the isolates.

### *Study of intrinsic antibiotics resistance*

Isolates were inoculated on Nutrient agar medium supplemented with antibiotics *viz.*, ampicilin, chloramphenicol, kanamycin, rifampicin, and tetracycline (50 µg/ml each) individually. Isolates were considered as resistant to the antibiotic if colony of the isolates would appear on the respective antibiotic supplemented medium.

### *Study of in vitro Cd bio-remediation efficiency*

Isolates were cultivated in Nutrient broth medium separately supplemented with 50 µg $mL^{-1}$ of Cd up to 96 hrs and an uninoculated medium was kept as control. To study the Cd removal efficiency, supernatants of Cd treated culture were collected, digested by treating with concentrated nitric acid at 80°C and Cd concentration was estimated by atomic absorption spectrometer (Agilent 240 AA Atomic Absorption Spectrometer, Agilent technologies). Percentage of Cd removal was calculated using the formula: $\{(C_0 - C_1) / C_0\} \times 100$; where $C_0$ = Cd concentration of control, $C_1$ = Cd concentration of supernatant after growth of the organisms.

### *Characterization plant growth promoting features Phosphate solubilisation test*

The phosphate solubilising activity was examined by inoculating the isolates on Pikovskaya's agar medium, incubated for 3 days at 28±2°C and the diameter of halo zone and bacterial colony were measured. The solubilization index [the ratio of the total diameter (colony + halo zone) to the colony diameter] was calculated by formula of Edi Premono *et al.* (1996).

### *Siderophore production test*

Siderophore production was determined as described by Schwayn and Neilands (1987) using blue indicator dye, chrome azurol sulfone. Bacterial isoates exhibiting an orange halo after 5 days of incubation at 28±2°C were considered positive for the production of siderophores.

### *Catalase test*

The culture of the isolates was added to appropriate amount of $H_2O_2$ on a glass slide. The catalase producing isolates showed bubble formation due to break down of $H_2O_2$ to $H_2O$ and $O_2$.

### *Assay of antifungal activity*

One of the isolates was tested for its antagonistic properties against two fungal pathogens (*Rhizoctonia solani* and *Alternaria solani*) by dual culture technique (Idris *et al.*, 2007). The antagonism effect was determined by observing the zone of inhibition and percentage of inhibition (I) was measured using the formula: $I = (R - r)/R \times 100$; where r = radius of the fungal colony opposite the bacterial colony and R = maximum radius of the fungal colony away from the bacterial colony.

## RESULTS AND DISCUSSION

### *Isolation of bacteria and characterization of their metal tolerance and antibiotic sensitivity*

The principal purpose of this study was to isolate

and characterize some Cd resistant bacteria from various heavy metal polluted soil and water sources. In our present investigation we characterized three cadmium-tolerant bacterial isolates putatively identified as *Pseudomonas* spp. capable of growing on 1mM Cd supplemented agar medium which were designated as KUCd2, KUCd3, and KUCd4 (Table 1). When they were exposed to increasing doses of Cd they could tolerate up to 5 mM concentration of Cd. Along with Cd resistant property, all three isolates could tolerate Zn up to 3 mM. Maximum tolerance level of Ni concentration was 2 mM for KUCd2, and 3 mM for the rest. Only KUCd3 could tolerate Cr up to 1 mM. Due to this multi-metal tolerance property these organisms could survive in environment polluted by Zn, Ni or Cr along with Cd and might perform as an effective Cd bio-remediating agent. Shamim and Rehman (2012) reported that another cadmium resistant bacterium, *Klebseilla pneumoniae* also showed tolerance to chromium, copper, lead, zinc, mercury and nickel. Among the isolates KUCd4 showed resistance against ampicillin and kanamycin, whereas KUCd2 had resistance against ampicillin only (Table 1).

### *Study of in vitro Cd bio-remediation efficiency*

When three isolates were grown in nutrient broth medium supplemented with 50 g/mL$^{-1}$ Cd, it was found that all of them were capable of removing around 50% Cd from the medium (Fig.1). The comparative order of Cd removal efficiency of the isolates was KUCd3(56.41%)>KUCd2 (52.39%)> KUCd4(46.31%). *Pseudomonas aeruginosa* BC15 was capable of absorbing 50% Cd within 48 hrs from the medium containing 100 mg of cadmium

**Table 1 :** Sources, metal tolerance and antibiotic sensitivity of the bacteria

| | | Maximum tolerance for heavy metal (mM) | | | | Antibiotics (50 µg mL$^{-1}$)* | |
|---|---|---|---|---|---|---|---|
| Isolates | Sources | Cd ($CdCl_2$) | Cr ($K_2CrO_4$) | Zn ($ZnCl_2$) | Ni ($NiCl_2$) | Ampicillin | Kanamycin |
| *Pseudomonas* KUCd2 | Soil sample from Rice-cultivated field, Nadia | 5 | - | 3 | 2 | + | - |
| *Pseudomonas* KUCd3 | Water sample from Kestopur cannel, Kolkata | 5 | 1 | 3 | 3 | - | _ |
| *Pseudomonas* KUCd4 | Water sample from Ganga river, Barrackpore | 5 | - | 3 | 3 | + | + |

'+' stands for resistant and '-' stands for sensitive.

*All the isolates were sensitive to chloramphenicol, tetracycline, rifampicin

**Table 2 :** Plant growth promoting attributes of the isolates

| Isolates | Calcium phosphate solubilisation index | Siderophore production diameter of orange halo (mm) | Catalase activity* | Antagonism assay % of inhibition | |
|---|---|---|---|---|---|
| | | | | *Rhizoctonia solani* | *Alternaria solani* |
| *Pseudomonas* KUCd2 | 2.5 | 15 | + | 60 | 75 |
| *Pseudomonas* KUCd3 | 1.8 | 20 | + | ND | ND |
| *Pseudomonas* KUCd4 | 2.2 | 12 | + | ND | ND |

*'+' stands for positive enzyme activity

'ND' not determined

per liter (Raja *et al.* 2006) and *P. aeruginosa* KUCd1 removed more than 75% soluble cadmium from the culture medium after 96 hrs (Sinha and Mulherjee, 2009).

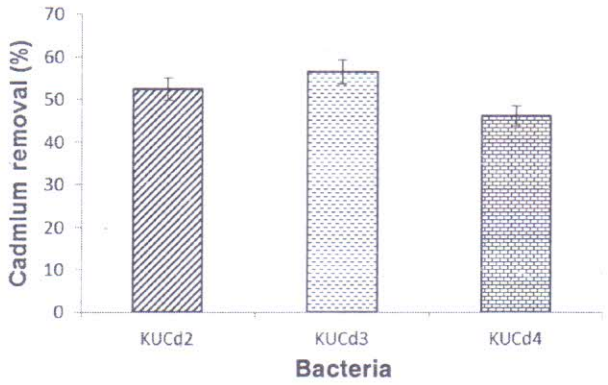


**Fig. 1 :** Cadmium removal (%) by KUCd2, KUCd3 and KUCd4 in Nutrient broth medium supplemented with 50 µg $mL^{-1}$ Cd. Data are the mean of three replications with error bars.

### *Characterization plant growth promoting features*

PGPR benefit plant by various mechanisms. In this study, all three isolates were identified as capable of solubilizing inorganic phosphate, producing siderophore and showing catalase activity (Table 2)

Phosphorus (P) is one of the major essential macronutrients for plant growth and development. But its availability in soil is very low, normally at levels of 1 ppm or less (Goldstein, 1994). Bacteria may contribute to plant nutrition by liberating P from insoluble phosphates (both organic and inorganic) compounds. Mineralization of most organic phosphorous compounds were carried out by production of enzymes, such as phosphatase, phytase, phosphonoacetate hydrolase, D-α-glycerophosphatase, C-P lyase by rhizobacteria. Solubilization of inorganic phosphates, such as tricalcium phosphate were found to be involved with acidification of the medium via biosynthesis and release of a wide variety of organic acids, such as gluconic acid, 2-ketogluconic acid, acetic acids, glycolic acid, oxalic acid, malonic acid, succinic acid, citric acid and propionic acid. De Werra *et al.* (2009) reported that the ability of *Pseudomonas fluorescens* CHA0 to solubilize mineral phosphate was due to production of gluconic acid. Solubilization Indexes [SI] measured for KUCd2, KUCd3 and KUCd4 colonies were 2.5, 1.8 and 2.2, respectively.

Iron is an essential growth element of all living organisms but under aerobic condition and at biological pH, it is very insoluble and scarcely available. PGPR produce siderophore(s) to sequester necessary iron and deprive pathogenic fungi of the element since the fungal siderophores have lower affinity for iron. Fluorescent pseudomonads produce siderophores such as, pyochelin, pyoverdin and pseudobactin. In our study all three bacteria were detected to produce high amount of

siderophore as evident from the diameter of the orange halo around the colonies of KUCd2, KUCd3 and KUCd4 were 15 mm, 20 mm and 12 mm, respectively. Sinha and Mukherjee (2008) reported a cadmium resistant *Pseudomonas aeruginosa* strain which showed Cd-induced siderophore production property to improve growth of mustard and pumpkin in Cd-contaminated soil through root colonization and protected the plants significantly from the toxic effects of Cd.

Catalase activity exhibited by all the isolates may be potentially very advantageous as reported by Samuel and Muthukkaruppan (2011).

Regarding antifungal activity, the bacterial isolate, KUCd2 showed antagonistic activity against *Rhizoctonia solani* [% of inhibition (I) measured as 60] and against *Alternaria solani* [I = 75]. Growth inhibition of the fungal pathogens by the bacterial isolate might be due to production of antibiotic compounds. Antibiotic compounds produced by fluorescent pseudomonads include phenazines, phloroglucinol, pyoluteorin, pyrrolnitrin, pyocyanine, hydrogen cyanide (a volatile compound).

From our study it can be proclaimed that the fluorescent *Pseudomonas* isolates are potential cadmium bio-remediating agents which can also confer their beneficial effects as bio-fertilizer as well as bio-control agents.

## ACKNOWLEDGEMENT

The work was supported by the grant received from University of Kalyani and also from DST-PURSE programme.

## REFERENCES

Cunningham, D.P. and Lundie, L.L. 1993. Precipitation of cadmium by *Clostridium thermoaceticum*. *Applied and Environmental Microbiology* **59**: 7–14.

De Werra, P., Pechy-Tarr, M., Keel, C and Maurhofer, M. 2009. Applied and *Environmental Microbiology*. **75**: 4162-4174.

De Werrs P., Pechy-Tarr m., Keel C., Mauhofer M. 2009. Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHAO Applied and Environmental Microbiology **75:** 4162-4174.

Edi Premono, J., Moawad, A.M. and Vlek, P.L.G. 1996. Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian Journal of Crop Science* **11**:13–23.

Glick, B.R., Karaturovic, D.M., Newell, P.C., 1995. A novel procedure for rapid isolation of plant growth promoting pseudomonads. *Cannadian Journal of Microbiology* **41**: 533–536.

Goldstein, A.H. 1994. *In*: Torriani-Gorni A, Yagil E, Silver S, ed. Phosphate in microorganisms: cellular and molecular biology, ASM Press, Washington, pp. 197–203.

Idris, H.A., Labuschagne, N. and Korsten, L. 2007. Screening rhizobacteria for biological control of fusarium root and crown rot of sorghum in Ethiopia. *Biological Control* **40**: 97–106

McGrath, S.P., 1994. Effects of heavy metals from sewage sludge on soil microbes in agricultural ecosystems, in: Ross, S.M. (Ed.), *Toxic Metals in Soil–Plant Systems*. Wiley, New York, pp. 247–273.

Nies, D. H. 1999. Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology* **51**, 730-750.

Raja, C.E., Anbazhagan. K. and Selvam, G.S. 2006. Isolation and characterization of a metal-resistant *Pseudomonas aeruginosa* strain. World *Journal of Microbial Biotechnology* **22**: 577-585.

Schwyn, B. and Neilands, J.B. 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**: 47–56.

Shamim, S. and Rehman, A. 2012. Cadmium resistance and accumulation potential of *Klebseilla pneumoniae* strain CBL-1 isolated from industrial wastewater. *Pakistan Journal of Zoology*, **44** : 203-208.

Sinha, S. and Mukherjee, S.K. 2009. *Pseudomonas aeruginosa* KUCd1, a possible candidate for cadmium bioremediation. *Brazilian Journal of Microbiology* **40**: 655-662.

Sinha, S. and Mukherjee, S.K. 2008. Cadmium–induced siderophore production by a high cd-resistant bacterial strain relieved Cd toxicity in plants through root colonization. *Current Microbiology*. **56**: 55–60.

Tripathi, M., Munot, H.P., Shouche, Y., Meyer, J.M. and Goel. R. 2005. Isolation and Functional Characterization of Siderophore-Producing Lead- and Cadmium-Resistant *Pseudomonas putida* KNP9. *Current Microbiology*. **50**: 233–237.

Samuel, S. and Muthukkaruppan, S.M. 2011. Characterization of plant growth promoting rhizobacteria and fungi associated with rice, mangrove and effluent contaminated soil. *Current Botany*. **2**: 22-25.