Isolation of Heavy Metal Resistant Bacteria for Sustainable Crop Production

Soumitra Nath[1*], Indu Sharma[2], Bibhas Deb[1] and Virender Singh[3]

[1]Bioinformatics Centre, Gurucharan College, Silchar, Assam (788 004), India
[2]Department of Microbiology, Assam University, Silchar, Assam (788 011), India
[3]Himachal Institute of Life Sciences, Ponta Sahib, H.P (173 025), India

Article History

Manuscript No. c103
Received in 30th August, 2012
Received in revised form 14th May, 2013
Accepted in final form 6th June, 2013

Correspondence to

E-mail: nath.soumitra1@gmail.com

Keywords

Heavy metal, MIC, antibiotic tolerance, rhizosphere.

Abstract

Thirty five heavy metal resistant bacteria were isolated from contaminated crop field of Southern Assam, India, against copper, zinc, cadmium and lead. The predominant isolates were identified as *Proteus* sp., *Klebsiella* sp., *Staphylococcus* sp., *Bacillus* sp. *and Pseudomonas* sp.. Some isolates exhibited high resistance to heavy metals with minimum inhibitory concentration (MIC) for heavy metals as 60 µg ml^{-1} (for copper), 180 µg ml^{-1} (for lead) 130 µg ml^{-1} (for cadmium) and 1800 µg ml^{-1} (for zinc). They also showed multiple heavy metal tolerance and were multi-antibiotic resistant. There was decrease in total count and microbial population diversity with increasing metal concentrations. The present study showed a correlation between heavy metal resistance and antibiotic tolerance among bacterial isolates. The effect of heavy metal resistant strains on Oryza sativa inoculated in contaminated soil showed a remarkable increase in the shoot length when compared with control pots.

1. Introduction

The quality of life on earth is inextricably linked to overall quality in the environment. The pollution of the ecosystem by heavy metals is a real threat to the environment because metals cannot be degraded like organic pollutants and persist in the ecosystem having accumulated in different parts of the food chain (Igwe et al., 2005). The use of domestic and industrial effluents, which may contain high concentrations of heavy metals on agricultural lands, is a common practice in some parts of the world. These toxic metals, when concentrated on plant tissues can have damaging effects on the plants themselves and may also pose health hazards to man and animals (Kumar et al., 2010). Heavy metals become toxic when they are not metabolized by the body and accumulate in the soft tissues.

It is important to study the indigenous microorganisms in heavy metal polluted sites. Excessive accumulation of heavy metals in agricultural soils through wastewater irrigation, may not only result in soil contamination, but also lead to elevated heavy metal uptake by crops, and thus affect food quality and safety (Muchuweti et al., 2006). Microorganisms are generally the first to be affected by the discharges of heavy metals into the environment. Microbial ecosystem can drastically alter the fate of the metal entering into aquatic or soil environments (Brown, 1996). Yeast, fungi, algae, bacteria and some aquatic plants have been reported to have the capacity to concentrate metals from dilute aqueous solutions and to accumulate them inside the cell structure (Modak et al., 1996). In order to survive in heavy metal polluted environments, many microorganisms have developed resistance to toxic metal ions. These mechanisms include: metal exclusion by permeability barriers, active transport of the metal away from the cell organism, intracellular sequestration of the metal by protein binding, extracellular sequestration, enzymatic detoxification of the metal to a less toxic form and reduction in metal sensitivity of cellular targets (Bruins et al., 2000). The detoxification mechanisms may be directed against one metal or a group of chemically related metals. Furthermore, the detoxification mechanisms may vary depending on the type of microorganism. This transformation of the contaminant is an incidental reaction catalyzed by enzymes present in the cell's metabolic system (Prasenjit and Sumathi, 2005).

The objectives of this study were to investigate heavy metal stress on bacteria, isolated from contaminated sites of Cachar district of Assam, India and to evaluate the bioremediation potential of the strains for better crop improvement.

2. Materials and Methods

2.1. Isolation and identification of bacteria

Soil sample were collected from industrial effluents, sewages, garages and petrol pumps of Cachar district of Assam, India in sterilized polyethylene bags and were immediately brought to the laboratory. Samples were then streaked on selective media with the help of calibrated loop and incubated at 37^0C for 24 hrs for recovery of potent isolates. Morphological characteristics of recovered isolates viz., Colony morphology (colour, shape, margin, elevation and surface) and cell morphology (shape, arrangement, gram reaction) were studied. Various biochemical tests were also performed for identification of isolates (Holt et al., 1994; Cappuccino and Sherman, 2005).

2.2. Screening and determination of minimum inhibitory concentration (MIC) of HMRB

Bacterial isolates were screened by growing them on heavy metal incorporated nutrient agar media and MIC of heavy metal resistant bacteria (HMRB) were determined by gradual increasing the concentration of heavy metals in the media. Heavy metals used were Cd^{2+} ($CdCl_2$), Cu^{2+} ($CuSO_4.5H_2O$), Pb^{2+} [$(CH_3COO)_2Pb.3H_2O$] and Zn^{2+} (Zinc metal powder), with starting concentration of 50 µg ml^{-1}. The concentration of heavy metals on NA plates were increased each time until the strains failed to grow on the plates. The culture growing on last concentration was transferred to the higher concentration by streaking on the plates. MIC was noted when the isolates failed to grow on plates.

2.3. Antibiogram pattern of recovered isolates

All recovered isolates were characterised for their resistant pattern by Kirby-Bauer disc diffusion method and Double disc diffusion method. The commonly used antibiotics were Ampicillin, Amikacin, Amoxycillin, Chloramphenicol, Cefixime, Gentamicin, Kanamycin, Cefalexin, Methicillin, Ofloxacin, Tetracycline and Ceftriaxone. The recovered isolates were inoculated in Muller Hinton Broth and antibiotic disc were placed equidistantly on surface the Mueller Hinton Agar (MHA) plates. After incubation at 37ºC for 24 hrs, the zone of inhibition was measured in mm and the results were interpreted using standard chart that relates the zone diameter to degree of microbial resistance (Bauer et al., 1966).

2.4. Pot experiment

Pot experiments were performed to determine the bioremediation potential of HMRB isolates. Heavy metal contaminated soil collected from paddy field nearby industrial sites and garages are put in 2 different sets, each set containing 6 pots. Pots were coded according to the inoculums added (Ps, St, Pr, Kl and All) and compared them with a control (C) set (Table 1). The bacteria showing the highest MIC was taken and inoculated in nutrient broth, for the formation of biofertilizers. The broth was kept in rotator shaker incubator at 37^0 C for 4-5 days. Seeds of *Oryza sativa* were soaked in petriplates containing sterile water for 24hrs and sown on all 12 pots. The bacterial broth serving as biofertilizers and distilled water was added to each pot every day. The shoot length was measured at 5 days interval upto 15^{th} day.

3. Results and Discussion

The turbidity of microbial enrichment broth was taken as a primary indicator for microbial growth. Enriched culture was streaked with the help of calibrated loop on Nutrient agar and further on different selective media such as Phenylalanine Agar (PAA), Manitol Salt Agar (MSA), Starch Agar, Macconkey Agar and Pseudomonas Isolation Agar (PIA) for isolation of different bacteria from different soil samples. A total of 62 isolates were recovered from 10 soil samples.

3.1. Morphological and biochemical characterization of recovered isolates

62 types of bacteria were recovered from samples and identified by their colony characterization and gram staining and biochemical testing (Holt et al., 1994; Sneath, 1986) and were identified as *Proteus* sp., *Klebsiellasp*., *Staphylococcus* sp., *Bacillus* sp. *and Pseudomonas* sp.. The highest prevalence was observed by *Pseudomonas* sp. (19%) and *Klebsiella* sp. (17%*)*, about 23% of the total isolates remains unidentified.

3.2. MIC of the bacterial isolates against some selected heavy metals

All the bacterial isolates were screened for heavy metal tolerance by growing them on heavy metal incorporated media. Out of which, 35 isolates showed heavy metal tolerance against Cu, Cd, Pb and Zn with MIC ranging from 50 to 1800 µg ml^{-1} for different heavy metals (Table 2), confirms the emerge of highly heavy metal resistant bacteria in the polluted sites. A better opportunity for bioaccumulation of heavy metals by bacteria was observed when compared with the work done by Rajbanshi, 2008, where MIC for heavy metals ranges from 150 µg ml^{-1} to 500 µg ml^{-1}. Heavy metal tolerance test indicated highest tolerance to Zinc by Ps-3 (1800 µg ml^{-1}), Copper by Kl-1 and Ps-5 (60 µg ml^{-1}), Lead by Pr-2 (180 µg ml^{-1}) and Cadmium by Ps-3 (130 µg ml^{-1}). It was observed that, none of the *Proteus* sp., *Bacillus* sp. and *Staphylococcus* sp. can tolerate the copper stress even at 50 µg ml^{-1}. The present study also fails to recover cadmium resistant *Bacillus* sp. and *Klebsiella* sp.

3.3. Antibiotic tolerance and sensitivity

Most of the isolates in the present study showed multiple antibiotic resistance to atleast three heavy metals. 6 strains of *Pseudomonas* sp. and 5 strains of *Staphylococcus* sp. showed high resistance towards a group of antibiotics and were multi-metal resistant. The present study showed some resemblance with the long back work of Calomiris et al., (1984), Rasheed et al., (2009), who found a correlation between the resistance

Table 1: Pots are marked according to the inoculums used; to each pots broth and distilled water were added on routine basis

A	B	C	D	E
C	Control	No	50 ml	50 ml
Ps	*Pseudomonas* sp.	5 ml	45 ml	50 ml
St	*Staphylococcus* sp.	5 ml	45 ml	50 ml
Pr	*Proteus* sp.	5 ml	45 ml	50ml
Kl	*Klebsiella* sp.	5 ml	45 ml	50ml
All	Mixture of Ps, Pr, Kl and St	(5+5+5+5) = 20ml	30 ml	50ml

A=Pot Labelling (codes); B=Bacterial strains inoculated; C= Broth added; D=Distilled water added; E=Total volume.

Table 2: Minimum inhibitory concentration of potent isolates on respective heavy metals

A	Strain	Copper (Cu)	Cadmium (Cd)	Lead (Pb)	Zinc (Zn)
B	Pr-2	*NG	50 µg ml^{-1}	180 µg ml^{-1}	1500 µg ml^{-1}
C	Bc-4	NG	NG	150 µg ml^{-1}	1450 µg ml^{-1}
D	Ps-3	50 µg ml^{-1}	120 µg ml^{-1}	130 µg ml^{-1}	1800 µg ml^{-1}
E	Kl-1	60 µg ml^{-1}	NG	150 µg ml^{-1}	1500 µg ml^{-1}
F	St-5	NG	110 µg ml^{-1}	150 µg ml^{-1}	1500 µg ml^{-1}

A=Bacterial Isolates; B=*Proteus* sp.; C=*Bacillus* sp.; D=*Pseudomonas* sp.; E=*Klebsellia* sp.; F=*Staphylococcus* sp.

*NG, No growth

to high level of heavy metals and antibiotic in the bacterial species. This fact was also established by other researchers that multiple metal resistance bacterial isolates exhibits high resistance towards a group of antibiotics (Vajiheh et al., 2003). Filali et al., (1999) studied bacteria isolates *Pseudomonas aeruginosa, Klebsiella pneumonia*, *Proteus mirabilis* and *Staphylococcus* sp. resistant to heavy metals and antibiotics.

3.4. Effect of HMRB on the shoot growth of oryza sativa inoculated in industrial soil

The effects of HMRB on shoot elongation of *Oryza sativa* in industrial soil, collected from paddy field nearby paper industry is shown in Figure 1. No growth was observed for the first two days, but after 3rd day the shoots began to develop in some pots. On 5th day, growth pattern was observed except the pots marked as Kl, Pr and C. It was found that the pots marked as Ps had a remarkable shoot growth of 34 cm when compared with control pot with shoot length of 23 cm.

3.5. Effect of HMRB on the shoot growth of oryza sativa inoculated in garrage soil

The efficacy of potent isolates was also tested in heavy metal contaminated garage soil (Figure 2). Significant shoot growth was observed in all the pots including control after 3rd day of inoculation. Overall, the control pot without any bacterial

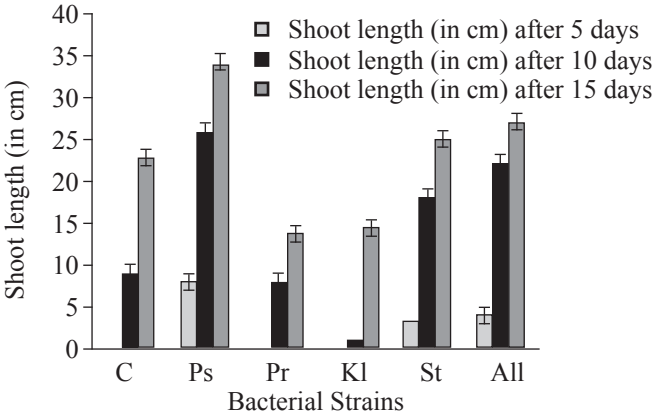

Figure 1: Shoot length (in cm) of *Oryza sativa* seedlings in heavy metal contaminated industrial soil and inoculated with HMRB and control

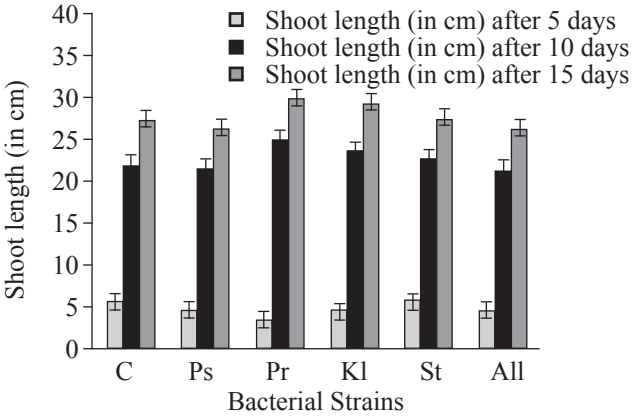

Figure 2: Shoot length of *Oryza sativa* seedlings in heavy metal contaminated garrage soil and inoculated with HMRB and control

inoculums added, showed a negligible difference inferring the incompetence of HMRB as biofertilizer in garage soil. On 15th day, the highest growth of *Oryza sativa* was observed in pots marked as Pr (25 cm) and Kl (24.5 cm) whereas the control pot grows upto 23 cm.

Although a number of studies have demonstrated the importance of bacterial inoculation for plant growth and heavy metal accumulation in heavy metal polluted environments (Abou-Shanab et al., 2003; Idris et al., 2004; Khan, 2005; Sheng and Xia, 2006). It is evident from the present study that the application of HMRB specifically adapted to high concentrations of heavy metals will increase the ability to remediate heavy metal contaminated soils.

4. Conclusion

Based on the present study, it could be concluded that it is possible to develop new bioremediation strategies with the inoculation of HMRB with co-cropping or intercropping systems in order to enhance biological-extraction of metals

from contaminated soils. A better understanding of the soil physio-chemical properties and plant-bacteria interaction is needed to optimize bioremediation potential followed by proper field investigations. The long term effect of pollutants has led to emergence of multi-metal and multi-antibiotic resistant bacteria in the study areas. Pot experiment study demonstrated that isolated strains of *Pseudomonas* sp., *Proteus* sp. and *Klebsellia* sp. could increase the growth of *Oryza sativa* in contaminated field, dedicating sites which are set aside for long term research purpose. Further research is still required to expand the knowledge of the microbial genetics to increase capabilities to degrade heavy metals from contaminated crop fields. In addition, we need to understand the mechanisms involved in mobilization and transfer of metals in order to develop future strategies and optimize the bioextraction process.

5. Acknowledgements

The authors wish to extend their grateful thanks to Department of Biotechnology, Govt. of India, New Delhi for the establishment of Bioinformatics Centre and Institutional Level Biotech Hub in Gurucharan College, Silchar, India. The authors are also thankful to Himachal Institute of Life Sciences, Ponta Sahib, (H.P), India for providing laboratory facility to carry out initial research work.

6. References

Abou Shanab, R.A.I., Angle, J.S., Delorme, T.A., Chaney, R.I., van Berkum, P., Moawad, H., Ghanem, K., Ghozlan, H.A., 2003. Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. New Phytologist 158, 219-224.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disc method. American Journal of Clinical Pathology 45(4), 493-496.

Brown, L.M., 1996. Removal of Heavy Metals from Water with Microalgal Resins 1: Process Development. Water Treatment Technology Program Report No. 74. US Department of the Interior Bureau of Reclamation.

Bruins, M.R., Kapil, S., Oehme, F.W., 2000. Microbial resistance to metals in the environment. Ecotoxicology and Environmental Safety 45, 198-207.

Calomiris, J.J., Armstrong, J.L., Seidler, R.J., 1984. Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Applied and Environmental Microbiology 47(6), 238-1242.

Cappuccino, J.G., Sherman, N., 2005. Microbiology: A Laboratory Manual, 7th Ed, Pearson Benjamin Cummings, San Francisco. ISBN 978-81-317-1437-9.

Filali, B.K., Taoufik, J., Zeroual, Y., Dzairi, F.A.Z., Talbi, M., Blaghen, M., 1999. Wastewater bacterial isolates resistant to heavy metals and antibiotics. Current Microbiology 41,151-156.

Holt, J.G., Krieg, R.N., Sneath, A.H.P., Staley, T.J., Williams, T.S., 1994. Bergey's Manual of Determinative Bacteriology, 9th Edition. (International Edition).

Idris, R., Trifonova. R., Puschenreiter, M., Wenzel, W.W., Sessitsch, A., 2004. Bacterial communities associated with flowering plants of the Ni hyperaccumulator Thaspi goesingense. Applied and Environmental Microbiology 70, 2667-2677.

Igwe, J.C., Nnorom, I.C., Gbaruk, B.C.G., 2005. Kinetics of radionuclides and heavy metals behaviour in soils: Implications for plant growth. African Journal of Biotechnology 4(B), 1541-1547.

Khan, A.G., 2005. Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in Phytoremediation. Journal of Trace Elements in Medicine and Biology 18, 355-364.

Kumar, A., Bisht, B.S., Talwar, A., Chandel, D., 2010. Physico-Chemical and Microbial Analysis of Ground Water from Different Regions of Doon Valley. International Journal of Applied Environmental Sciences 5(3), 433-440.

Modak, J.M., Natarajan, K.A., Saha, B., 1996. Biosorption of Copper and Zinc Using Waste *Aspergillus niger* Biomass. Minerals and Metallurgical Processing 13(2), 52-57.

Muchuweti, M., Birkett, J.W., Chinyanga, E., Zvauya, R., Scrimshaw, M.D., Lester, J.N., 2006. Heavy metal content of vegetables irrigated with mixture of wastewater and sewage sludge in Zimbabwe: implications for human health. Agriculture. Ecosystem and Environment 112, 41-48.

Prasenjit, B., Sumathi, S., 2005. Uptake of Chromium by *Aspergillus foetidus*. The Journal of Material Cycles and Waste Management 7, 88-92.

Rajbanshi, A., 2008. Study on Heavy Metal Resistant Bacteria in GOheswori Sewage Treatment Plant. Our Nature 6, 52-57

Rasheed, F., Khan, A., Kazmi, S.U., 2009. Bacteriological analysis, antimicrobial susceptibility and detection of 16S rRNA gene of *Helicobacter pylori* by PCR in drinking water samples of earthquake affected areas and other parts of Pakistan. Malaysian Journal of Microbiology 5(2), 123-127.

Sheng, X.F., Xia, J.J., 2006. Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. Chemosphere 64, 1036-1042.

Sneath, P.H., 1986. Bergey's Manual of Systematic Bacteriology 2, 999-1006.

Vajiheh, K., Naser, B., Giti, E., 2003. Antimicrobial, heavy metal resistance and plasmid profile of coliforms isolated from nosocomial infections in a hospital in Isfahan, Iran. African Journal of Biotechnology 2(10), 379-383.