*RESEARCH PAPER*:

# Isolation of microbes from radionuclides and metal contaminated sub-surface soils for bioremediation of radioactive waste management

B. PRAVEEN, AMRITA PANDA, SISIR DAS AND S.P.S. BISHT

*Asian Journal of Environmental Science,* (June, 2011) Vol. 6 No. 1 : 53-57

See end of the article for authors' affiliations

Correspondence to :

**S.P.S. BISHT**
Department of Biotechnology, Roland Institute of Pharmaceutical Sciences, BERHAMPUR ORISSA, INDIA
sps_bisht@yahoo.com

## SUMMARY

**Radionuclides and metals have posed a great threat to the sustenance of life on this earth. These are released by various human activities and get accumulated without being eliminated. This environmental hazard can be alleviated by the utilization of metal assimilating microbes.The main objective of this study is to isolate microbes that help in the bioremediation of various metals and radionuclides from the sites of Indian Rare Earths Limited (IREL),Berhampur, Orissa, India.The isolated strains RIP-1 and RIP-2 *have* been shown to grow well in media supplemented with varied concentrations of different metals and exhibited maximum tolerance to UV radiations.**

Praveen, B., Panda, Amrita, Das, Sisir and Bisht, S.P.S. (2011). Isolation of microbes from radionuclides and metal contaminated sub-surface soils for bioremediation of radioactive waste management . *Asian J. Environ. Sci.*, **6**(1): 53-57.

**Key words** : Radio nuclides, Bioremediation, IREL, Radiations

In India, rare-earth compounds are produced from the beach sand mineral monazite. Caustic digestion of the mineral followed by selective acid extraction is the method used to separate composite rare-earth fraction. The composite rare-earth chloride contains low levels of natural radionuclide and is the starting material for individual rare-earth compounds which have wide applications.

One major research effort focuses on the stabilization of metal contaminants, such as uranium and chromium, through *in situ* stimulation of natural microbial communities to reduce the metals to less soluble forms. These communities in the subsurface have the capacity to immobilize several metal contaminants but are often limited by the lack of suitable electron donors. As electron donors (*e.g.*, methanol, ethanol, pyruvate, glucose) are added, oxygen is consumed through aerobic respiration. Then, alternate electron acceptors are used by bacteria, typically in a sequence determined by community metabolic potential, electron donor bioavailability, and decreasing energy yield of reaction. Oxidized soluble metals, such as U(VI) and Cr(VI), may be transformed to reduced, insoluble forms through various metabolic and coupled biogeochemical processes.

Previous studies, summarized in (Suzuki and Suko 2006) and (Wall and Krumholz 2006), have shown that although uranium bioremediation through subsurface electron donor addition is possible, significant challenges persist e.g electron donors shown to stimulate aqueous uranium reduction upto 51% or less sediment-bound uranium (Ortiz-Bernad *et al*., 2004 and Wu *et al*., 2006). Often, the electron donor is chosen to target organisms known to rapidly reduce uranium in laboratory settings. Nonlinear relationships between microbial community structure and subsurface biogeochemistry have been demonstrated for immobilization of metals (Palumbo *et al*., 2004), suggesting that, although enrichment of specific target organisms has been documented in field experiments (Holmes *et al*., 2002; Nevin *et al*., 2003; Anderson *et al*., 2003; Istok *et al*., 2004, North *et al.,* 2004; Chang *et al*., 2005; Wu *et al*., 2006), the prevalence of individual taxonomic groups may not be sufficient to describe the potential for long-term uranium immobilization. Sorbed or mineral forms may continue to release uranium to

**Received:** *February, 2011*
**Accepted :** *April, 2011*

ground water after cessation of electron donor addition, especially as carbonate complexes.

During the present investigation the soil samples collected from the outskirts of Indian rare earths limited, Gopalpur ,Orissa.This site is well known which for the radionuclides in eastern coast of India.The major radionuclides and heavy minerals that are present in the site are Ilmenite, Rutile, Zircon, Sillimanite, Garnet and Monazite from beach sand and producer of rare earth (Lanthanides) chemicals, thorium nitrate, etc. Hazardous waste sites require remediation to contain and prevent the further spread of toxic chemicals into the environment. Following removal of the contaminant source, natural attenuation and bioremediation are two components of cleanup strategies. It is particularly true for such industrial metals as lead, cadmium, zinc, and nickel. It is also true for such important radio nuclides that are present in radioactive waste as $^{90}Sr$, $^{137}Cs$, and isotopes of Pu (plutonium) and U (uranium).

## MATERIALS AND METHODS

**Isolation of microbes from soil by serial dilution:**

Micro organisms are ubiquitous in their occurrence and the common sources for their isolation are soils, lakes and river beds. Common techniques used for the isolation of industrially useful micro organisms include soil dilution and plating on suitable media. Bacteria can reproduce until they reach population densities of approximately $10^9$ (one billion) /ml. Thus, it becomes necessary to dilute them in order to isolate discrete colonies.

**Biochemical tests:**

Several biochemical tests were performed *viz.,* Gram staining, fungal staining, endospore staining, Catalase, carbohydrate fermentation, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, hydrogen sulphide production, nitrate reduction and VP .

**Bacterial growth analysis:**

The growth of micro organisms is a highly complex and coordinated process, ultimately expressed by increase in cell number or cell mass. The required media Peptone tryptone yeast extract agar (PTYA) and Nutrient broth were prepared and autoclaved at 15 psi / 121$^0$C for 15 minutes, followed by inoculation and incubation at 37$^0$C.

**Antibiotic sensitivity assay:**

The agar diffusion assay is one method for quantifying the ability of antibiotics to inhibit bacterial growth. The required agar media Peptone tryptone yeast extract agar (PTYA) and Nutrient agar media were prepared and autoclaved at 15 psi / 121$^0$C for 15 minutes. The media was inoculated with the pure cultures of *RIP-1*and *RIP-2* by spread plate technique. The standard antibiotic discs (Streptomycin, Kanamycin, Tetracycline, and Erythromycin, Ampicillin, Penicillin and Polymyxin) were placed over the spreaded plates and incubated at 37$^0$C .

**Metal toxicity:**

100mM of test metals (Cadmium, Cobalt, Chromium, Copper, Mercury, Silver and Lead) was prepared and kept as stock. These metals further diluted to 1mM, 2mM, 10mM, and 100 mM followed by 1ml of metals of different concentrations and 1ml culture was added and mixed and incubated at 37$^0$C and analyzed by UV –Vis spectrophotometer at (600nm) at the time interval of 24 and 48 hours, respectively.

**U .V. tolerance study:**

Cell suspensions of *RIP-1* and *RIP-2* were inoculated on sterile Peptone tryptone yeast extract agar (PTYA), Nutrient agar media and after incubation at 37$^0$C exposed to U.V light for 10 minutes, 20minutes, 1 hour and 2 hours. 100 µL of the U.V exposed culture was spreaded over agar plates and incubated at 37$^0$C to study the effect of UV radiation on growth and revival of bacteria.

**Radionuclide tolerance assay:**

This assay was performed to know the affinity of the isolated organisms towards radionuclides. The cells from mid log phase were taken and then diluted with 100ml of sterile broth. And 1ml of these cultures was centrifuged at 6000 rpm for 10 minutes.The supernatant was discarded. The cell pellets were dissolved in 0.1N NaCl (pH 4.0). The pellets were diluted in normal saline.100µl of this suspension was spread over their respective agar media and incubated over night.Again the pellets were mixed with 1 ml of 0.1N NaCl, and incubated at 37$^0$C. After 1 hr incubation 100µl of this suspension was taken and spread over their respective agar media.Finally the pellets were dissolved with 0.5 ml of uranium acetate solution and 0.5 ml of 0.1N NaCl (pH 4.0) followed by 1 hr incubation. After 1 hr incubation, 100µl of this suspension was taken and spread over their respective agar media.This became the first incubation step.At this step, the cultures were serially diluted in sterile saline.This serially diluted $10^{-6}$ samples were again spread over their respective agar media. These spread plates were kept in the incubator for over night growth. This 2$^{nd}$ incubation cultures were inoculated into sterile broth and incubated at 37$^0$C for 24 hours .These broth cultures were then used for the study of alkaline phosphatase assay.

**Alkaline phosphatase assay:**

This enzyme is responsible for the bioprecipitation of radionuclides.So, this enzyme was taken into account to know their response towards radionuclides.

P-Nitrophenyl phosphatase + $H_2O \rightarrow$ P-Nitrophenyl + Pi

**Method:** Stopped spectrophotometric rate determination

**Reagent A:** 100mM Glycine buffer with 1mM $MgCl_2$ , pH-8.8 at 37°C

**Reagent B:** 15.2mM p-nitrophenyl phosphate solution (PNPP)

**Reagent C:** 20mM NaOH solution

0.5 ml of reagents A and B were mixed in a sterile test tube and incubated for 10 minutes. After incubation, 0.1 ml of distilled water was added to the blank test tubes. In the other test tubes, 0.1ml of broth cultures was added and was again incubated for 10 minutes. Then 10 ml of reagent C was added to all the test tubes including blank and the absorbance was taken at 410nm.

## RESULTS AND DISCUSSION

The study revealed that one isolate RIP-1 was a gram +ve strain and found to have glucose utilization,nitrate reduction and catalase activities (Table 1), exhibited maximum growth after 210 min of incubation (Table 2), exhibited highest sensitivity to Ampicillin and least sensitivity to streptomycin (Table 4), exhibited good growth even at 100mM concentrations of heavy metals and thus found to have resistance to metal toxicity with biochemical characterization and its growth on radionuclide containing media indicates its potential for bioremediation. It has shown very good growth in PTYA medium which is specific to Listeria dentrificans and is concluded this strain may be the representative of same group which is known for its bioremediational capability (Table 5). The another isolate RIP-2 was found to be gram +ve and exhibited caseinhydrolysis ,gelatin hydrolysis and catalase activities (Table 1), exhibited maximum growth after 72 hrs of incubation (Table 3), exhibited high sensitivity to Tetracycline and insensitivity to Pencillin (Table 4), exhibited growth at higher concentrations of heavy metals (Table 6). Both the strains exhibited tolerance to radionuclides and involved in the production of the enzyme alkaline phosphatase (Table 7 and 8).

The collection site is active zone of Indian Rare Earths Limited, Berhampur Orissa and the major activities of this company is mining and extraction of various heavy metals. The soil sample was thought to ascentain n micro-organisms that may be tolerant to various metals and radionuclides which in turn may help in the bioremediation of various soils that have been contaminated by these metals and radionuclides. Screening of microbes from the soil samples gave strong indications that the soil contains many nicrobes of interest with special reference to

**Table 1: Biochemical tests of strain RIP-1 and IP-2**

| Biochemical tests | RIP-1 | RIP-2 |
|---|---|---|
| Glucose | + | - |
| Mannitol | - | - |
| Nitrate reduction | + | - |
| $H_2S$ production | - | - |
| MR-VP Test | - | - |
| Gelatin hydrolysis | - | + |
| Casein hydrolysis | - | + |
| Starch hydrolysis | - | - |
| Gram staining | + | + |
| Catalase | + | + |

**Table 2: Spectrophotometric growth analysis of strain RIP-1 at 600nm**

| Incubation time | O. D at 600 nm |
|---|---|
| 120 minutes | 0.062 |
| 150 minutes | 0.052 |
| 180 minutes | 0.045 |
| 210 minutes | 0.203 |
| 240 minutes | 0.050 |

**Table 3: Spectrophotometric growth analysis of strain RIP-2 at 600nm**

| Incubation time | O.D at 600 nm |
|---|---|
| 24 hr | 0.150 |
| 48 hr | 0.164 |
| 72 hr | 0.360 |
| 96 hr | 0.195 |

**Table 4: Zone of inhibition (mm) of various antibiotics against the strains RIP-1 and P-2**

| Antibiotics | Zone of inhibition in mm | |
|---|---|---|
| | RIP-1 | RIP-2 |
| Streptomycin | 8 | 15 |
| Kanamycin | 20 | 20 |
| Tetracyclin | 12 | 14 |
| Erythromycin | 14 | 13 |
| Ampicillin | 27 | 2 |
| Penicillin | 17 | No zone |
| Polymyxin | 14 | 11 |

**Table 5: Toxicity effect of various metals on strain RIP-1**

| Metals | 1 mM | | 2Mm | | 10mM | | 100mM | |
|---|---|---|---|---|---|---|---|---|
| | 24hrs | 48 hrs | 24 hrs | 48 hrs | 24 hrs | 48 hrs | 24 hrs | 48 hrs |
| Cadmium | 0.092 | 0.072 | 0.071 | 0.090 | 0.103 | 0.228 | 0.063 | 0.242 |
| Cobalt | 0.213 | 0.114 | 0.109 | 0.072 | 0.072 | 0.152 | 0.099 | 0.073 |
| Cromium | 0.060 | 0.231 | 0.138 | 0.297 | 0.013 | 0.042 | 0.112 | 0.099 |
| Copper | 0.141 | 0.163 | 0.149 | 0.333 | 0.097 | 0.139 | 0.273 | 0.258 |
| Mercury | 0.274 | 0.326 | 0.054 | 0.047 | 0.095 | 0.230 | 0.263 | 0.280 |
| Silver | 0.220 | 0.284 | 0.138 | 0.182 | 0.102 | 0.222 | 0.345 | 0.301 |
| Lead | 0.256 | 0.441 | 0.128 | 0.148 | 0.099 | 0.301 | 0.270 | 0.294 |

**Table 6 : Toxicity effect of various metals on strain RIP-2**

| Metals | 1Mm | | 2 Mm | | 10 Mm | | 100 mM | |
|---|---|---|---|---|---|---|---|---|
| | 24 hrs | 48 hrs | 24 hrs | 48 hrs | 24 hrs | 48 hrs | 24 hrs | 48 hrs |
| Cadmium | 0.120 | 0.180 | 0.140 | 0.150 | 0.108 | 0.134 | 0.113 | 0.358 |
| Cobalt | 0.121 | 0.137 | 0.112 | 0.097 | 0.166 | 0.082 | 0.124 | 0.202 |
| Cromium | 0.114 | 0.224 | 0.095 | 0.130 | 0.150 | 0.076 | 0.076 | 0.138 |
| Copper | 0.179 | 0.409 | 0.210 | 0.142 | 0.186 | 0.113 | 0.199 | 0.203 |
| Mercury | 0.033 | 0.054 | 0.137 | 0.030 | 0.164 | 0.126 | 0.078 | 0.198 |
| Silver | 0.112 | 0.152 | 0.228 | 0.250 | 0.241 | 0.185 | 0.292 | 0.593 |
| Lead | 0.139 | 0.220 | 0.900 | 0.096 | 0.243 | 0.207 | 0.204 | 0.358 |

**Table 7 : Spectrophotometric growth analysis of RIP-1 and RIP-2 after incubation with Uranium at 410nm**

| Organisms in different conditions | RIP-1 | RIP-2 |
|---|---|---|
| Uranium with 0.1N NaCl | 0.012 | 0.008 |
| 0.1 N NaCl and incubated for 1 hr | 0.016 | 0.011 |
| Normal saline | 0.021 | 0.010 |

**Table 8: Shows the Units of Alkaline phosphatase produced by strains RIP-1 & RIP-2**

| Organisms in different conditions | RIP-1 | RIP-2 |
|---|---|---|
| Uranium with 0.1N NaCl | 0.0072 | 0.0048 |
| 0.1 N NaCl and incubated for 1 hr | 0.0097 | 0.0066 |
| Normal saline | 0.012 | 0.0060 |

microbes for bioremediation of radionuclides and radioactive waste management practices.

**Acknowledgement:**

The authors are thankful to Administration and Management of Roland Institute of Pharmaceutical Sciences, Berhampur, Orissa, India for financial support.

**Authors' affiliations:**

**B. PRAVEEN, AMRITA PANDA AND SISIR DAS,** Department of Biotechnology, Roland Institute of Pharmaceutical Sciences, BERHAMPUR (ORISSA) INDIA

## REFERENCES

**Anderson, R.T.,** Vrioni, H.A., Ortiz-Bernad, Resch, C.T, Long, P.E., Dayvault, R., Karp, K., Marutzky, S., Metzler, D.R., Peacock, A., White, D.C., Lowe, M. and Lovley, D.R. ( 2003). Stimulating the *in situ* activity of Geobacter species to remove uranium from the groundwater of a uranium-contaminated aquifer. *Appl. Environ. Microbiol.,* **69**:5884-5891.

**Chang. Y.J.,** Long, P.E., Geyer, R., Peacock, A.D., Resch, C.T., Sublette, K., Pfiffner, S., Mithgall, A.S., Anderson, R.T., Vrionis, H.A., Stephen, J.R., Dayvault, R, Ortiz-Bernad, I., Lovley, D.R. and White, D.C. (2005). Microbial incorporation of C-13-labeled acetate at the field scale: Detection of microbes responsible for reduction of U(VI). *Environ. Sci. Technol.,* **39** : 9039-9048.

**Holmes, D.E.,** Finneran, K.T., O'Neil and Lovley, D.R. (2002). Enrichment of members of the family Geobacteraceae associated with stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. *Appl. Environ. Microbiol.*, **68**:2300–2306.

**Istok, J.D.**, Senko, J.M., Krumholz, L.R., Watson, D. , Bogle, M.A., Peacock, A., Chang, Y.J. and White, D.C. ( 2004). In situ bioreduction of technetium and uranium in a nitrate-contaminated aquifer. *Environ. Sci. Technol.*, **38**:468–475.

**Nevin, K.P.,** Finneran, K.T. and Lovley, D.R. (2003). Microorganisms associated with uranium bioremediation in a high-salinity subsurface sediment. *Appl. Environ. Microbiol.,* **69**:3672–3675.

**North, N.N.,** Dollhopf, S.L., Petrie, L., Istok, J.D., Balkwill, D.L. and Kostka, J.E. ( 2004). Change in bacterial community structure during *in situ* biostimulation of subsurface sediment cocontaminated with uranium and nitrate. *Appl. Environ. Microbiol*., **70**:4911–4920.

**Ortiz-Bernad, I.,** Anderson, R.T., Vrionis, H.A. and Lovley, D.R. ( 2004). Resistance of solid-phase U(VI) to microbial reduction during in situ bioremediation of uranium-contaminated groundwater. *Appl. Environ. Microbiol*., **70**:7558–7560.

**Palumbo, A.V.**, Schryver, J.C., Fields, M.W., Bagwell, C.E., Zhou, J.Z., Yan, T., Liu, X. and Brandt, C.C. (2004). Coupling of functional gene diversity and geochemical data from environmental samples. *Appl. Environ. Microbiol.,* **70** :6525–6534.

**Suzuki, Y.** and Suko, T. ( 2006). Geomicrobiological factors that control uranium mobility in the environment: Update on recent advances in the bioremediation of uranium-contaminated sites. *J. Miner. Petrol. Sci.,* **101**:299–307.

**Wall, J.D.** and Krumholz, L.R. (2006). *Uranium reduction*. *Annu. Rev. Microbiol.,* **60**:149–166.

**Wu, W.M.**, Carley,J., Gentry,T., Ginder-Vogel, M.A., Fienen,M., Mehlhorn,T., Yan,H., Caroll,S., Pace, M.N., Nyman, J., Luo, J., Gentile, M.E., Fields, M.W., Hickey, R.F., Gu, B.H., Watson, D., Cirpka, O.A, Zhou, J.Z., Fendorf, S., Kitanidis, P.K., Jardine, P.M. and Criddle, C.S. ( 2006). Pilot-scale *in situ* bioremedation of uranium in a highly contaminated aquifer: II. Reduction of U (VI) and geochemical control of U (VI) bioavailability. *Environ. Sci. Technol*., **40**: 3986–3995.