ISSN 0975 - 6299 Vol 2 / Issue 3 / Jul - Sept 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

MICROBIOLOGY

PRELIMINARY SCREENING OF SUBSURFACE SOILS FROM RADIONUCLIDES AND METAL CONTAMINATED AREA OF ORISSA COAST

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ABSTRACT

Radionuclides and metals have posed a great threat to the sustenance of life on earth. These are released by various anthropogenic activities and get accumulated without being eliminated. This environmental hazard can be alleviated by the microbes with the bioleaching property. The present study was designed to isolate microbes that help in the bioremediation of various metals and radionuclides from the sites of Indian Rare Earths Limited (IREL), Berhampur,Orissa. The isolated microbes ***Deinococcus radiodurans and Rhizopus*** have been shown to grow well in media supplemented with varied concentrations of different metals .***Deinococcus radiodurans*** was found to be the toughest bacterium to tolerate the radiations and is utilized for in situ bioremediation of radioactive waste by producing higher amount of alkaline phosphatase enzyme and the only fungus ***Rhizopus*** studied in this work was helpful in uranium precipitation.

KEY WORDS

Radio nuclides,Metals,Bioremediation ,IREL,Alkaline phosphatase,Radiations

INTRODUCTION

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.

The soil samples collected from the site of Indian rare earths limited, OSCOM, Gopalpur coast, Orissa a well known site of 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. The migration of uranium from uranium-mining and -processing operations as well as from the deep subsurface repositories of nuclear fuel and other radioactive wastes is of serious environmental concern. Therefore the biogeochemical composition of these extreme environments has been extensively studied during the last decade and it was observed that they are occupied by a large variety of bacteria [1] due to their capability to tolerate radioactivity to bioaccumulate uranium and biotransformation activities by the microbes thriving in and around the site need to be screened. Moreover it was demonstrated that these bacteria can be used for bioremediation of uranium-contaminated sites by *in situ* biostimulation [2-7] or for construction of biologically coated ceramic filters for cleaning of waters polluted with uranium. In the natural environments bacteria interact effectively with minerals and radiation.

MATERIALS & METHODOLOGY

Isolation and development of pure culture 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 & 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.

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 PTYA and NAM broth were prepared and autoclaved at 15 psi / 121˚C for 15 minutes followed by inoculation of pure cultures of *Deinococcus radiodurans*& *Rhizopus* and incubated at 37˚C. After an interval of 24 hours, the optical density of the broth was analyzed at 600nm for *Rhizopus,* the weight of the fungal mat was taken after 5 th and 10 th days respectively.

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˚C for 15 minutes. The media was inoculated by pure cultures of *Deinococcus radiodurans* by spread plate technique. Antibiotic discs (Streptomycin, Kanamycin, Tetracycline, and Erythromycin, Ampicillin, Penicillin and Polymyxin) were placed over the spread plates and placed in the incubator in upright position at 37˚C.After 24 hours of incubation; the zones of inhibition were measured.

Metal toxicity100mM 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˚C and analyzed by UV –Vis spectrophotometer at (600nm) at the time interval of 24 and 48 hours respectively for *Rhizopus,* the weight of the mat was taken on 5^{th} and 10^{th} day after inoculation.

U .V tolerance study Cell suspensions of *Deinococcus radiodurans* was inoculated on sterile Peptone tryptone yeast extract agar (PTYA) and Nutrient agar media and after incubation at 37˚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˚c to study the effect of UV radiation on growth and revival of the bacteria.

Radionuclide tolerance assay This 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 10minutes.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 were spread over their respective agar media and then incubated for over night. Again the pellets were mixed with 1 ml of 0.1N NaCl, and were then incubated at 37˚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˚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 .

P-Nitrophenyl phosphatase + H_2O → 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 Reagent 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 410 nm .

RESULTS

The study revealed that *Deinococcus radiodurans* is found to have the capacity of nitrate reduction, casein hydrolysis and gelatin hydrolysis activities (Table-1),and exhibited maximum growth after 96 hrs of incubation (Table-2),exhibited highest sensitivity to Kanamycin and least sensitivity to Polymyxin (Table-3),exhibited good growth even at 100mM concentrations of heavy metals and thus found to have resistance to metal toxicity (Table-4).The *Rhizopus* exhibited no growth at higher concentrations of heavy metals (Table-5 & 6) but exhibited tolerance to radionuclides and involved in the production of the enzyme alkaline phosphatase.(Table-7&8).

Fig 1
Pure culture of Deinococcus

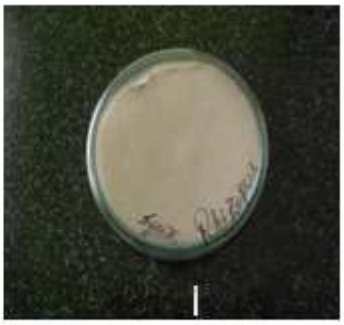

Fig 2
Pure culture of Rhizopus

Table 1
Biochemical tests of Deinococcus radiodurans

Biochemical tests	***Deinococcus radiodurans***
Glucose	-
Mannitol	-
Nitrate reduction	+
H_2S Production	-
MR-VP Test	-
Gelatin Hydrolysis	+
Casein Hydrolysis	+
Starch Hydrolysis	-
Gram Staining	+
Catalase	-

Table showing the biochemical reactivity of ***Deinococcus radiodurans***

Table 2
Spectrophotometric growth analysis of Deinococcus radiodurans at 600nm

Incubation time	**O.D at 600 nm**
24 hr	0.129
48 hr	0.378
72 hr	0.418
96 hr	0.462
120 hr	0.405

Table showing maximum growth of the strain at 96 hrs .

Antibiotic sensitivity test:

Table 3
Zone of inhibition (mm) of various antibiotics against Deinococcus radiodurans

Antibiotics	*D.radiodurans*
Streptomycin	9.5
Kanamycin	18.5
Tetracyclin	13
Erythromycin	6
Ampicillin	15
Penicillin	6
Polymyxin	5.5

Bacterial strain showing antibiotic potential agaist various antibiotics.

Table 4
Toxicity effect of various metals on Deinococcus radiodurans.

METALS	1Mm		2Mm		10 mM		100 Mm	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
CADMIUM	0.184	0.203	0.190	0.275	0.130	0.179	0.037	0.185
COBALT	0.133	0.291	0.162	0.232	0.147	0.255	0.088	0.139
CROMIUM	0.161	0.334	0.080	0.198	0.128	0.297	0.055	0.118
COPPER	0.128	0.257	0.188	0.284	0.130	0.297	0.181	0.239
MERCURY	0.123	0.241	0.099	0.127	0.085	0.176	0.143	0.193
SILVER	0.203	0.195	0.225	0.220	0.174	0.223	0.408	0.461
LEAD	0.262	0.273	0.252	0.174	0.245	0.323	0.071	0.197

Table showing metalotoxicity by the strain

Table 5
Toxicity effect of various metals on Rhizopus (5th day)

Growth of the fungus in gms after 5th day of incubation				
METALS	**1 mM**	**2 mM**	**10 mM**	**100 mM**
CADMIUM	0.211	0.105	No growth	No growth
COBALT	0.138	0.005	0.003	0.007
CROMIUM	0.002	0.002	No growth	No growth
COPPER	0.010	0.004	0.006	No growth
MERCURY	No growth	No growth	No growth	No growth
SILVER	0.001	0.012	0.001	No growth
LEAD	0.007	0.003	0.017	0.005

Table showing metalotoxicity by the strain

Table 6
Toxicity effect of various metals on Rhizopus (10th day)

Growth of the fungus in gms after 10th day of incubation				
METALS	**1 mM**	**2 mm**	**10 mM**	**100 mM**
CADMIUM	0.082	0.063	0.014	0.009
COBALT	0.001	0.005	No growth	No growth
CROMIUM	0.009	0.003	No growth	No growth
COPPER	0.019	0.005	0.012	No growth
MERCURY	No growth	No growth	No growth	No growth
SILVER	0.009	0.007	No growth	No growth
LEAD	0.018	0.010	0.005	No growth

Table showing metalotoxicity by the strain

Radionuclide and Alkaline phosphatase assay:

Table 7
Spectrophotometric growth analysis of Deinococcus radiodurans & Rhizopus after incubation with Radionuclides

Organisms in different conditions	***Deinococcus***	***Rhizopus***
Radionuclides with 0.1N NaCl	0.090	0.020
0.1 N NaCl and incubated for 1 hr	0.008	0.032
Normal saline	0.011	0.012

Table showing the growth results of the strain with radionuclides

Calculation for the assay:

Units of enzyme / ml = ($A_{410\ nm}$ test - $A_{410\ nm}$ blank) (11.1) D.F / (18.3) (0.1) (10)

Where 11.1 = Volume (in ml) of assay

D.F = Dilution factor

0.1 = Volume (in ml) of enzyme /culture mixed

18.3 = Milimolar extinction coefficient of PNPP

10 = Time of assay as per units definition.

Unit definition: One unit will hydrolyze 1.0µl of PNPP per minute at pH 8.8 at 37°c.

The data of the O.D of the microbes taken at the end of alkaline phosphatase assay were put on the above formula to get the results as following:

Table 8
Shows the Units of Alkaline phosphatase produced by Deinococcus radiodurans & Rhizopus

Organisms in different conditions	***Deinococcus***	***Rhizopus***
Radionuclides with 0.1N NaCl	0.054	0.0121
0.1 N NaCl and incubated for 1 hr	0.0048	0.0194
Normal saline	0.0066	0.0072

Table showing the results of Alkaline phosphatase assay

DISCUSSION

The soil sample was collected from the site of Indian rare earths limited, Berhampur Orissa. The major activity of this company is mining and extraction of various heavy metals and radionuclides for various commercial purposes. The soil samples contain micro-organisms tolerant to various metals and radionuclides which in turn may help in the process of bioremediation of various soils contaminated by these metals and radionuclides. By various biochemical tests, metal toxicity and growth studies with radionuclide containing soil revealed that the soil in and around the IREL contains *Deinococcus radiodurans* and *Rhizopus* those are known for their use as bioremediation agents.

ACKNOWLEDGEMENTS

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

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