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Isolation and Characterization of Lead Resistant Micro-organism from Industrial Effluent: (*A Bioremediation Approach)*

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The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. Microorganisms can be highly efficient bioaccumulators of soluble and particulate forms of metals. Microbe related technologies may provide an alternative or addition to conventional method of metal removal. The present study deals with isolation of lead resistant bacteria and their characterization from effluent water of Indian Iron and Steel Company (IISCO), Burnpur, West Bengal. Isolates were selected based on predominant growth on high level of heavy metal concentration. The basic characterization was done on the basis of morphological, physical, biochemical and antibiogram tests. Quantification of metal absorption was determined by Atomic absorption spectrophotometric analysis. Protein expressions of the isolates on metal stress were studied by SDS-PAGE. The identified heavy metal resistant bacteria could be useful for the bioremediation of heavy metal contaminated sewage and waste water.

Key words: Lead resistant Bacteria, Industrial effluent, SDS-PAGE, Bioremediation.

Microorganisms play an important role in the environmental fate of toxic heavy metals with a multiplicity of mechanisms effecting transformation between soluble and insoluble forms of the heavy metals (Gadd, 2000). Although some heavy metals are essential trace elements and toxic to all forms to life even in low concentration by forming complex compounds within the cells. Because heavy metals are increasingly found in microbial habitats due to natural and environmental processes, microbes have evolved several mechanisms to tolerate the presence of heavy metals. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of metal ions inside the cell and reduction of the heavy metal ions to a less toxic state. This is an important implication of microbial heavy metal tolerance because the oxidation state of a heavy metal relates to the solubility and toxicity of the metal itself (Lloyd, 2001). Thus the heavy metal resistant microorganisms have significant role in treatment of the metal containing soil. The detoxifying ability of these resistant microorganisms can be manipulated for bioremediation of heavy metals in wastewater, metal contaminated soil etc.

Effluents having heavy metals can be treated with these microorganisms by the processes like biosorption, bioaccumulation and bioprecipitation (Rajbanshi. 2008). Among all the heavy metal lead is the second most important metal of concern especially because lead poisoning of children is common and leads to retardation and semi permanent brain damage (Roane, 1999). Lead poisoning typically results from ingestion of food

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or water contaminated with lead; but may also occur after accidental ingestion of contaminated soil, dust, or lead based paint. Lead can affect almost every organ and system in the body. Once lead enters the body through ingestion, travels to the lungs then swiftly through the other part of the body like brain, spleen, muscle and heart. Not only to the human being, lead and other heavy metals also have severe toxic effects on microbial cells. Not only to the human being, lead and other heavy metals also have severe toxic effects on microbial cells. Lead cause toxicity by interacting nucleic acid, by binding to essential respiratory proteins, to oxidative damage by producing reactive oxygen species and displacing Ca ion and Zn ion in proteins. Microorganisms generally use specific transport pathways to bring essential metals across the cell membrane into cytoplasm; unfortunately toxic metal can also cross membrane, via diffusion or via pathways designed for other metals. Lead enters in microbial cells via transport systems for essential divalent cation such as Mn^{2+}.

In response to metals in the environment, micro organisms have evolved ingenious mechanisms of lead resistance and detoxification. Lead resistance in microbes is somewhat less studied but involvement of P-type ATPase and detoxification through sequestration is known as lead inducible specific mechanism. P-type ATPases transports lead from cell to periplasm using ATP (Nucifora, 1989; Rensing, 1997), Whereas CBA transporters are three-component transenvelope pumps of Gram negative bacteria that act as chemiosmotic antiporters (Nies, 1989; Hassan, 1999; Franke, 2003) efflux Pb from periplasm to outside the cell and cation diffusion facilitator (CDF) family transporters act as chemiosmotic ion-proton exchangers are also involved in lead efflux. (Xiong, 1998; Anton, 1999; Franke, 2001). Many bacteria have a cell wall or envelope that is capable of passively adsorbing high levels of dissolved metals, usually via a charge-mediated attraction (Mohamed, 2001). Binding of heavy metals by these organisms takes place mainly through exopolysaccharides (EPSs) (Loaec, 1997). Particular heavy metal binding capacity has been observed in uronic acid rich EPSs (Mohamed, 2001).

Thus lead resistance microorganisms can be used to remediate heavy metal containing soil, by a process known as bioremediation which is nothing but a pollution treatment technology that uses biological systems to catalyze the destruction or transformation of various chemicals to less harmful forms. Bioremediation is proven to be quite effective for the removal of metal ions from contaminated solution in a low cost and environment friendly manner. So, this study aimed to isolate and identify lead resistant bacteria from Indian Iron and Steel Company (IISCO) effluent water and characterizes them, so that they can be useful for the bioremediation of lead from the lead contaminated area.

MATERIALS AND METHODS

Sample Collection

Samples were collected from effluent water of Indian Iron and Steel Company (IISCO) located in Burnpur of Burdwan district of West Bengal, India. Physical and chemical parameters were different as in Table1.

Isolation of lead resistant bacteria from the collected effluent

Bacteria were isolated from the effluent using sterilized nutrient agar (NA) medium (pH-7.0±0.2) and were prepared using peptic digest of animal tissue (5 g/L), yeast extract (1.5g/L), beef extract (1.5 g/L), NaCl (5 g/L) and agar 20 g/L along with lead acetate in increasing concentrations in each plate from 1mM to 10mM with one control agar plate. 10^{-4} dilution was used and incubated at 37^0C for 48 hrs.

Morphological characterization

Morphological characteristics of the isolated bacterial colonies were observed under bright field microscopy (Olympus (OIC), GB 89954) of simple stained preparation of the isolates at 100 X magnifications. The detailed surface features were observed by SEM (Fig. 1 A,B,C,D).

Scanning electron microscopy

To determine the effect of metal on the surface feature of the cell, the metal treated as well as untreated cells were analyzed by Scanning Electron Microscopy (Hitachi Scanning Electron Microscope S-530). SEM images have a characteristic three-dimensional appearance and are useful for judging the surface structure of the sample. Both in control and treated cells, post growth, cells at a density of 10^8-10^9 cells mL^{-1} were harvested by centrifugation, stained and analyzed

using the software Quartz PCI version 8.0 image management system.

Biochemical characterization

Amylase Test was performed by growing the pure bacterial culture in sterilized Starch Agar plates containing both with lead acetate and without lead acetate followed by 48 hours incubation and Gram's iodine was used to indicate the presence of starch.

Catalase Test by adding a loopful appropriately grown (in medium containing lead acetate and without lead acetate) pure culture in the test tube containing substrate i.e. 3% H_2O_2. If Catalase produced by the bacteria; for the chemical reaction, $2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$ will liberate free O_2 gas. Bubbles of O_2 represent a positive Catalase test; the absence of bubble formation is a negative Catalase test.

Indole Test by growing pure bacterial culture in sterile tryptophan or peptone broth containing both with lead acetate and without lead acetate for 24-48 hours. Following incubation, 5 drops of Kovac's reagent (isoamyl alcohol, p-Dimethylaminobenzaldehyde and concentrated hydrochloric acid) was added to the culture broth. A positive result is shown by the presence of a red or red-violet color in the surface alcohol layer of the broth. A negative result appears yellow. A variable result can also occur, showing an orange color as a result. This is due to the presence of skatole, also known as methyl indole or methylated indole, another possible product of tryptophan degradation.

Gelatin hydrolysis test was performed by using Gelatin liquefaction and tested by stabbing the pure culture of the isolates in nutrient gelatin deep tubes containing both with lead acetate and without lead acetate. Following incubation at 4°C until the medium resolidifies. If gelatin has been hydrolyzed, the medium will remain liquid after refrigeration. If gelatin has not been hydrolyzed, the medium will resolidify during the time it is in the refrigerator.

Effect of Temperature on the growth of the isolates

Each microbial species requires a temperature growth range that is determined by the heat sensitivity of its particular enzymes, membranes, ribosome, and other components. So in order to get the optimum growth temperature (i.e. the temperature at which the rate of cellular reproduction is most rapid) of the isolates, test tubes containing Nutrient both with lead acetate and without lead acetate in were inoculated with the pure culture and incubated at different temperatures 10°C, 20°C, 30°C, 37°C, 45°C, 60°C for 15 mins and then incubated at 37°C for 48 hours. Optical density was measured to observe growth in terms of turbidity using a spectrophotometer.

Effect of pH on the growth of the isolates

Acidic and basic environment or pH of an environment dramatically affects bacterial growth. The pH affects the activity of enzymes especially those that are involved in biosynthesis and growth. Each microbial species possesses a definite pH growth range and a distinct pH growth optimum. Different microbial groups have characteristic pH optima. Thus to get the pH optima of the isolates this experiment was done. For this experiment separate test tubes containing Nutrient broth with Pb and without Pb were adjusted to different pH using 1(N) Hydrochloric acid and 1(N) Sodium hydroxide. pH adjusted to 2, 4, 6, 7, 8, and 10 and test tubes were incubated at 37°C for 48 hours and analyzed using spectrophotometer.

Effect of Osmotic pressure on the growth of the isolates

Since, bacteria were separated from their environment by a selectively permeable plasma membrane; they can be affected by changes in the osmotic pressure or water availability of their surroundings. The experiment of effect of osmotic pressure was done using Nutrient Broth containing Sodium chloride in different test tubes in varying concentration that are 2M, 4M, 6M, 8M and 10M for both the cases with lead acetate and without lead acetate in the medium and cultures were incubated for 48 hours at 37°C and analyzed using spectrophotometer.

Determination of growth profile

The four phases (lag, logarithmic, stationary, and death or decline) of growth of bacterial population was determined by measuring the turbidity in a broth culture. However, turbidity is not a direct measure of bacterial numbers but an indirect measure of biomass, which can be correlated with cell density during the log growth phase. The construction of a complete bacterial growth curve (increase and decrease in cell numbers versus time) was done by transferring aliquots of a 24 hrs incubated pure active culture

in freshly prepared nutrient broth medium containing both with lead acetate and without lead acetate contained in side arm flasks and kept in shaker incubator (at 180 rpm) for proper aeration at 30°C. Optical densities were measured after each 1 hour at OD660 nm (UV-1700 Pharma Spectrophotometer, Shimadzu).

Multiple sugar fermentation test

Fermentative degradation of various carbohydrates was detected by acid, gas production utilizing that particular carbohydrate. For this test fermentation medium (1% sugar solution with indicator Bromo thymol blue) were prepared, sterilized and poured in culture tubes with Durham tube, containing lead acetate and without lead acetate, incubated for 48hrs and observed.

Antibiotic resistance test

The agar cup assay method was used to determine antibiotic sensitivity of the isolates. Ampicillin, Chloramphenicol, Kanamycin, Rifampicin, Streptomycin and Tetracycline were used in a concentration of 5mg/ml by inoculating on the Mueller Hinton Agar plates (HiMedia). 0.5ml antibiotic solution were added and incubated at 37°C for 48 hours. Zone of inhibition were measured and were classified as resistance or sensitive strains.

Quantification of Lead accumulation

To quantify lead accumulated Atomic Absorption Spectroscopy (AAS) was used. For AAS, metal is determined by aspirating a metal solution into an air-acetylene flame to atomize the metal. A metal specific lamp is placed into the AAS and used to determine the difference in light absorbance between a reference source and the metal solution. For this purpose isolates are grown on media with lead acetate and a control were kept only with lead acetate and media, with no inoculum. After 72 hour the supernatant were collected by pelleting out cells by centrifugation at 6000 rpm for 10 minutes, the supernatant were analyzed by pollution control board, Govt of W. B.

Protein profiling of the isolates by SDS-PAGE

To compare the proteins expression in the stressed i.e. with lead acetate and normal condition, insoluble and soluble protein were isolated and SDS-PAGE was done. For protein isolation 48 hrs incubated pure culture (at 37°C) was used to harvest the cells at 6000gx 10 mins. The pellet was then resuspend in 150 µl of B-PER reagent (20mM Tris HCl, pH- 7.5, NaCl-0.08M) and again centrifuged at 13000gx 10 mins at 4°C. Supernatant contains soluble proteins and pellet which was dissolved in Phosphate buffer saline (PBS), contains the insoluble proteins. Both of these proteins are then separated by Sodium Dodecyl Sulphate-Poly-Acryl amide gel electrophoresis (SDS-PAGE).

RESULTS AND DISCUSSION

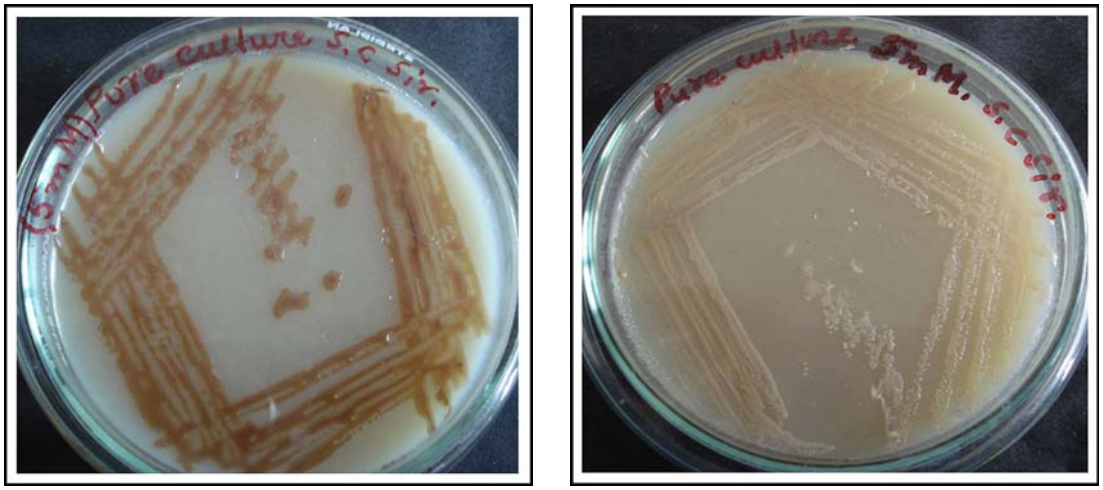
As the growing industry, oil refineries introduce lots of heavy metal in the environment, so in order to remediate the pollution by heavy metals by biological means is more eco-friendly, as it does not introduce any chemical for the remediation of the metals from the environment. The isolated bacteria can be use in lead bioremediation as they have ability to grow in presence of metals. Their ability to grow in minimal media shows the capacity to tolerate and grow on high concentrations of lead and they are able to use for large scale production.

Isolation of the LEAD (Pb) resistant organism

Two bacterial isolates namely Pb^rB1 and Pb^rB2 were selected and isolated from the industrial effluent of IISCO which are lead resistant. These bacterial strains are Resistant up to 6mM (Pb^rB1) [Fig.1- (A)] and 7mM (Pb^rB2) [Fig.1- (B)] concentration of lead. The code name of the isolates were assigned according to their isolation, 'Pb' stands for Lead, 'r' stands for resistance and 'B1' and 'B2' for Bacteria1 and Bacteria2.

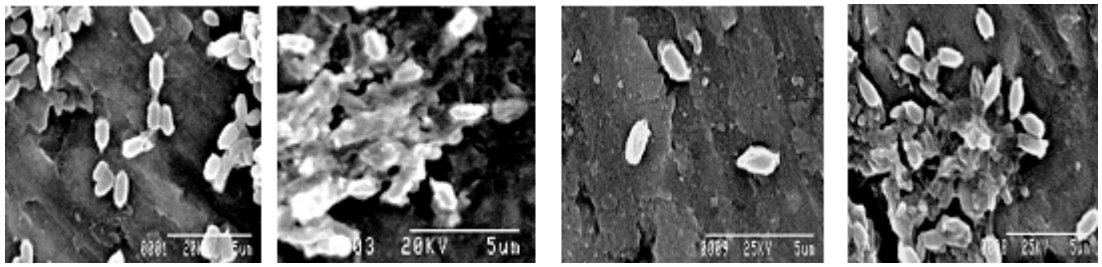
Colony and Morphological characterization of the isolates

The preliminary morphological characterizations of these isolates were done on the basis of simple staining which implies the short rod structure with spore forming nature of the isolates. The scanning electron micrograph confirms the rod shape of the bacteria and demonstrates the effect of metals on cell morphology and a slimy appearance surrounding the metal treated cells may be the extra cellular polymeric substances produced by the cells [(Fig. 2)]. The colony characteristics are summarized in Table 2.



(A) **(B)**

Fig. 1. Pure culture of the isolates A. Pb^{r}B1, B. Pb^{r}B2



A B C D

Fig. 2. SEM images of the isolates at different magnifications, (A.) Pb^{r}B1 at 6000X without metal; (B.) Pb^{r}B1 at 6000X with metals. (C.) Pb^{r}B2 at 8000X without metal; (D.) Pb^{r}B1 at 10000X with metals.

Table 1. Characteristics of industrial effluent used for the isolation of heavy metal-resistant bacteria

Physical characteristics of the sample water	
Color:	Brownish black.
Odor:	Pungent greasy smell.
Turbidity:	Fully turbid.
Chemical characteristics of the sample water	
pH:	6.0
BOD:	345 mg/L
COD:	720 mg/L

Table 2. Colony characteristics of isolated bacterial strains

Characteristics	Pb^{r}B1	Pb^{r}B2
Color	Brown	Off white
Shape	Round, Uniform edged	Round, Uniform edged
Nature	Mucoid, Slimy, Sticky	Slimy but not sticky
Size	Medium	Small

Table 3. Biochemical characteristics of the isolates, (+) Ve indicate positive result and (-) Ve indicate negative result

Tests	Pb^{r}B1(With Metal)	Pb^{r}B1 (Without Metal)	Pb^{r}B2(With Metal)	Pb^{r}B2 (Without Metal)
Amylase	(-) Ve	(+) Ve	(+) Ve	(+) Ve
Catalase	(+) Ve	(+) Ve	(+) Ve	(+) Ve
Indole	(-) Ve	(-) Ve	(-) Ve	(-) Ve
Protease	(-) Ve	(+) Ve	(+) Ve	(+) Ve

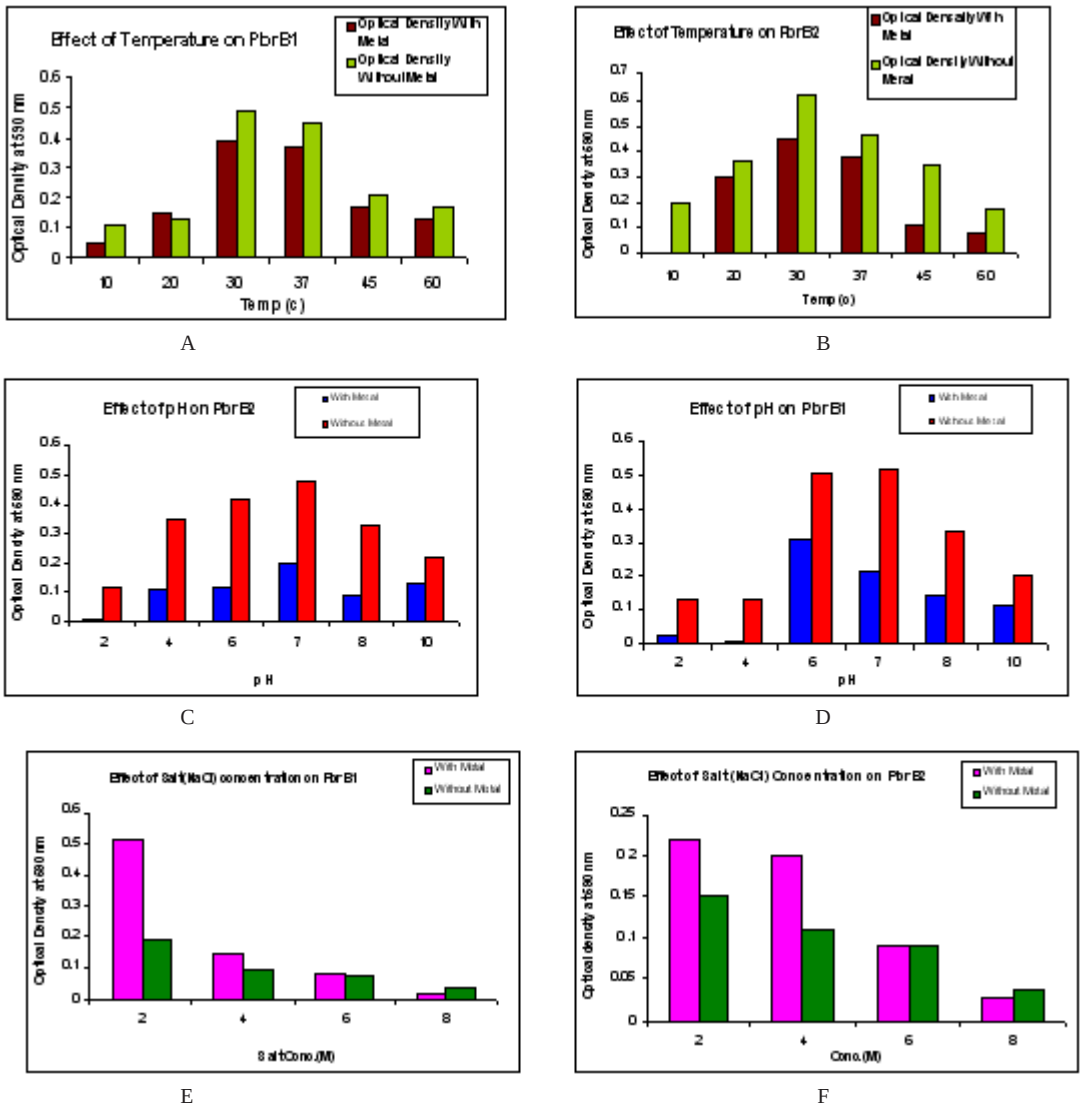


Fig. 3. Showing (A) & (B) Effect of temperature on the isolates, (C) & (D) Effect of pH on the isolates, (E) & (F) Effect of salt concentration on the isolates

Table 4. Antibiotic sensitivity pattern of the isolates

Antibiotic	Class	Spectrum of activity	Zone of inhibition (cm)		Effect on Isolates	
			Pb^rB1	Pb^rB2	Pb^rB1	Pb^rB2
Ampicillin	Beta-lactam	Broad	1.6	0	Resistant	Resistant
Chloram-phenicol	Chloram-phenicol	Broad	2.8	0	Sensitive	Resistant
Kanamycin	Aminogly-coside	Broad	4	2.8	Sensitive	Sensitive
Tetracycline	Tetra cycline	Broad	3.7	1.	Sensitive	Sensitive
Rifampcin	Semi-synthetic	Broad	3.2	0	Sensitive	Resistant
Streptomycin	Aminogly-coside	Broad	0	0	Resistant	Resistant
Cassia alata (Water extract)	Water extract of *C. alata* leaf	NA	1.6	0	Resistant	Resistant
Cassia alata (Ethanol)	Ethanol extract of *C. alata* leaf	NA	0	0	Resistant	Resistant
Cassia alata (Methanol)	Methanol extract of *C. alata* leaf	NA	3.8	0	Sensitive	Resistant

Biochemical characterization of the isolates

From the biochemical characterization it can be demonstrated that both the isolates being strong catalase producer are obligate aerobes. The presence of Catalase provides a defense mechanism against the reactive oxygen species and thus ability to exist in aerobic conditions and thereby facilitates the metabolic efficiency. Both the bacterial isolates lacks tyrptophanase enzyme, as gives negative result for the Indole test. Pb^rB2 produce a significant amount of amylase and protease both in presence and absent of lead, where as Pb^rB1 produce significant amount of amylase and protease without metal, but metal stress inhibit production of the enzymes.

Effect of Temperature, pH, Salt concentration on the growth of the isolates

Optimum temperature and pH for the growth of Pb^rB1 is 30°C and pH 7 and can grow well on 37°C and pH 6, but metal stress minimize growth in both the cases. In case of Pb^rB2 growth is optimum on temperature 30°C and pH 7, and growth also affected by the metal stress. Both the isolate grows well on 2mM salt concentration and Pb^rB2 can tolerate well the mM concentration of the salt [Fig 3- (E) and (F)].

Bacterial growth curve determination

The growth profiles (Optical density Vs Time graph) of both the isolates are as follows. Both the isolates follow a sigmoid pattern of growth in presence as well as absence of Pb. But the growth pattern of Pb^rB1 shows difference in the log and exponential phase in presence or absent of metal, except that in case with metal it shows a short lag phase, and slight long exponential phase. It may be due to that in presence of metal it may takes some more time for duplication. Where as in case of Pb^rB2, with metal the cell enters in to stationary phase more rapidly i.e. have a short exponential phase than that of without metal, it may be due to the presence of metal the nutrients present in the medium utilized rapidly to duplicate than in the normal condition.

Determination of Antibiotic resistance

Antibiotic sensitivity pattern shows (Table 4) that the Pb^rB1 is resistant to Ampicillin, Streptomycin, *Cassia alata* extract (water and ethanol) and Pb^rB2 shows the resistance toAmpicillin, Chloramphenicol, Rifampicin, Streptomycin, *Cassia alata* extract (Water, Ethanol

Table 5. Acid-gas production of Pb^{r}B1 utilizing given sugars
(+) Ve indicate positive result and (-) Ve indicate negative result

Sugar	Without metal		With metal (2 mM)		With metal (4mM)	
Dextrose	+	+	-	-	-	-
Galactose	+	-	-	-	-	-
Lactose	+	-	-	-	-	-
Maltose	+	-	-	-	-	-
Sucrose	+	-	-	-	-	-
Xylose	+	+	-	-	-	-

Table 6. Acid-gas production of Pb^{r}B1 utilizing given sugars
(+) Ve indicate positive result and (-) Ve indicate negative result

Sugar	Without metal		With metal (2 mM)		With metal (4mM)	
Dextrose	+	+	-	-	-	-
Galactose	+	+	-	-	-	-
Lactose	+	+	+	+	-	-
Maltose	+	+	-	-	-	-
Sucrose	+	+	+	+	-	-
Xylose	+	+	-	-	-	-

Table 7. Table showing the results of atomic absorption spectroscopy

Isolates	Lead concentration in supernatant at 0 hr (mg/L)	Lead in supernatant after 72 hr (mg/L)	Amount of Lead absorbed (mg/L)	Percentage of lead abosorption (%)
Pb^{r}B1	1517.32	896.5	620.82	40.92
Pb^{r}B2	1517.32	618.3	899.02	59.25

and Methanol). Resistance to multiple drugs may involve the plasmid encoded mdr genes.

Multiple sugar fermentation test

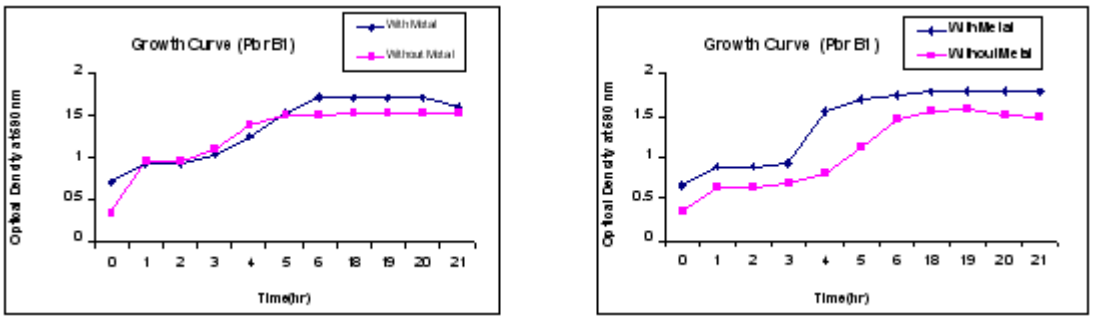
Multiple sugar fermentation tests were done with the isolates with respect to the sugars Dextrose, Galactose, Lactose, Maltose, Sucrose, Xylose and the acid-gas production by the isolates utilizing these sugars was observed which are given as follows in the tabulated form. From the observation it can be conclude that both the isolates can utilize the easily available sugar with and without presence of metal (2mM), but as the metal concentration increases up to 4mM they lose the ability to utilize the metals, it may be due to the reason that at the presence of metal the cell stops the expression of the proteins that can utilize the provided carbon source to save the energy for expression for the essential proteins that can help to exist in the metal stressed environment.

Quantification of lead accumulation

Quantification of lead accumulation was done using Atomic absorption spectroscopy. From this study, it can be concluded that the isolates are able to absorb metal by adsorption in EPS or any other mechanism as there is a decrease in metal concentration after 72 hrs from the initial amount of metal present in the solution.

Protein profiling of the isolates by SDS-PAGE

Soluble and insoluble protein of the isolates with and without metal were isolated and



A B

Fig. 4. Growth profile of the isolates (A) Pb^{r}B1, (B) Pb^{r}B2

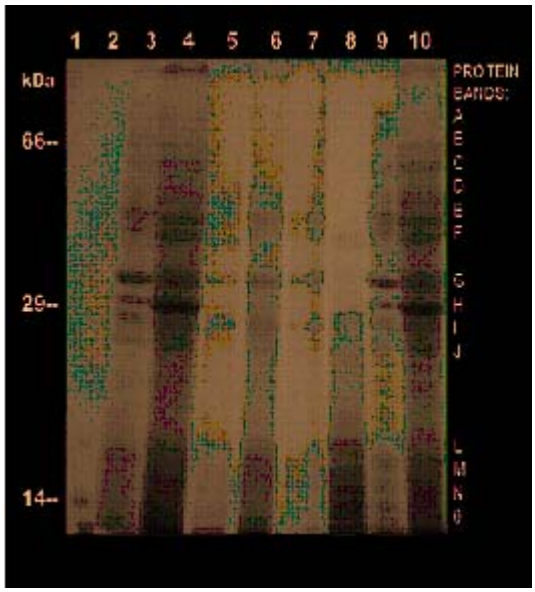


Fig. 5. SDS-PAGE of the proteins of the isolates showing protein bands (A,B,C,D,E,F,G,H, I,J,L,M,N,O). Lane3 and lane 9 is the insoluble protein of Pb^{r}B1 and Pb^{r}B2 repectively without metal, Lane 4 and lane10 is the isoluble protein of Pb^{r}B1 and Pb^{r}B2 with metal, lane 5 an lane 7 is the soluble protein of Pb^{r}B1 and Pb^{r}B2 without metal,lane 6 and lane 8 is the soluble protein of Pb^{r}B1 and Pb^{r}B2 with metal

run on SDS-PAGE. The band pattern in SDS-PAGE shows the expression of proteins in presence and absence of lead. As for the insoluble protein the thick band shows the high expression of proteins in presence of metals. Whereas protein expression is high without metal in case of soluble proteins. From this it can be demonstrated that metal stress can regulate the expression of soluble protein, and insoluble proteins are more involved in the lead detoxification. From the detailed study of banding pattern it can be said that in case of insoluble proteins with metal stress H protein (Approximately of 29 kDa) expressed well where as in normal condition G protein expressed well, so it may possible that protein have some important role on lead resistance.

CONCLUSION

At the end it can be concluded that the entire study can head towards an application mode, main utilization being in bioremediation of lead. Further study can proceed towards designing of a bioremedial package constituting either of single pure culture or as a consortium with microbes in varying proportion after observing the field application. One of the main applications can be in economical recovery of metals like Pb from ores and minerals or the further studies from other lead contaminated area can specify the use isolated strains as biosensor. On the other hand the lead accumulation process can be further investigated for the study of molecular mechanisms underlying the bioremediation phenomenon and the study of differential expression of genes in response to the metal stress can give an interesting area of research.

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