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A STUDY ON METAL RESISTANCE AND LEAD DETOXIFICATION EFFICIENCY OF A GRAM POSITIVE BACTERIUM ISOLATED FROM EFFLUENTS OF TANNING INDUSTRY

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Abstract: A Gram positive lead resistant bacterial isolate was got form effluents of tanning industry. Maximum lead resistance of the isolate was determined. The strain CMBL17 tolerated maximum lead concentration at 2 mg/mL of the medium. Resistance of the isolate against cadmium, and chromium was also checked. Optimum growth conditions *i.e.*, incubation temperature, pH and inoculum size were noted for the growth of the isolate. The isolate was used for processing of lead in the medium. The bacterium showed fairly high efficiency in processing of lead present in liquid medium. Location of genes for lead processing was determined by detection of plasmids, curing of plasmids and determination of lead resistance after curing.

Key words: Bioremediation, lead resistant bacteria, industrial effluents, metal resistant bacteria.

INTRODUCTION

Lead gets entry into the environment as a result of a number of agricultural and industrial practices. Unfortunately it is a contaminant of soil, water and air. Contamination of soil by lead takes place as a consequence of the use of lead arsenate insecticides. High levels of lead in waste waters may originate from combustion of lead containing gasoline, coal burning or metal smelting (Trajanovska *et al.*, 1997). The practice of mining of lead for nuclear and metal industry and leaching from natural deposits are other sources of contamination. Certain transition metals like copper, cobalt, nickel and zinc, in trace amounts are essential for cellular metabolism. These usually act as cofactors of enzymes or part of certain complex organic molecules. However, higher concentrations of these metals becomes toxic for the cell. Other heavy metals like lead, cadmium, mercury, chromium and silver have no known beneficial functions inside the eukaryotic or bacterial cells. Rather these are toxic even at very low concentrations (Brown, 1992; Gadd, 1992).

A large number of bacteria have been isolated from metal contaminated sites receiving effluents of industrial wastes and from soil samples contaminated by battery manufacturing operations (Manovski *et al.*, 1992; Haq *et al.*, 1997). Environmental pollutants pose a high risk not only to human beings and other animals but also to bacterial populations in the area. However, the adaptive responses of bacteria are usually

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prompt and studied extensively. Various studies have indicated that bacteria which survive, and indeed, flourish in such environments have developed or acquired genetic systems which help bacteria resist and usually detoxify the metal ions in the environment. The genes coding cadmium, zinc, cobalt, chromium, copper, arsenic and nickel have been predominantly located on plasmids (Mergeay *et al.*, 1985; Chen *et al.*, 1986; Nucifora *et al.*, 1989; Cervantes *et al.*, 1990; Cooksey *et al.*, 1990; Nies *et al.*, 1990; Nies, 1992; Silver and Walderhaug, 1992; Liesegang *et al.*, 1993; Nies *et al.*, 1993; Williams *et al.*, 1993; Stoppel and Schlegel, 1995).

MATERIALS AND METHODS

Samples collection and isolation of lead resistant bacteria

Water samples were collected from effluents released by tanning industry. The samples were collected in sterile screw capped glass bottles, brought to the laboratory and stored at room temperature before spreading on plates. For selection of lead resistance bacteria LB agar plates with 1 mg/mL lead were used. Lead acetate was used as source of Pb^{2+}. Lead acetate solution and LB agar medium were autoclaved separately and allowed to cool down. When the temperature of the two solutions was slightly less than 60°C, the solutions were mixed and poured into plates. Industrial effluent (100 μl) was spread on the plates and the growth of colonies was observed after 24 hours. Colonies were picked and streaked for purification and for determination of maximum resistance of the strains against lead.

Resistance against cadmium and chromium

Resistance of the bacterial isolate against cadmium and chromium was checked by taking various concentration of chromium ($K_2Cr_2O_7$) and cadmium ($CdCl_2$), starting from 10 μL/mL of each metal ion in the medium. Minimum inhibitory concentration was recorded when the colonies failed to appear within 48 hours.

Estimation of lead in the medium

In order to estimate the amount of lead in the medium, dithizone method (E. Merck) was used. Reagent 1 (RI) was prepared by dissolving 10 mL of hydrazinium hydroxide in 70 mL of 1N HCl, then adding 20 g of NaCl and making the volume up to 100 mL with distilled water. Reagent 2 (R2) was prepared by dissolving 20 g potassium hydrogen carbonate, 5 g potassium cyanide, 5 g potassium sodium tartarate and 25 mL ammonium solution in 100 mL of distilled water. Dithizone solution was prepared by dissolving 15 mg of dithizone in 1000 mL of chloroform. Five mL of LB broth with lead concentration of 1 mg/mL was taken in test tubes. The tubes were inoculated with fresh bacterial cultures. After incubation for 24 hours 1 mL culture was taken from the tube aseptically. The culture was diluted to 25 times volume with distilled water. This mixture was taken in a separating funnel and 2.5 mL of R1 were added followed by addition of 2.5 mL of R2. After that 12.5 mL of dithizone solution was added. The mixture was shaken for 5 minutes and the pressure developed by shaking and reaction was released by removing the stopper or valve. The mixture was allowed to stand for a

few minutes. Two layers were formed. The lower layer containing chloroform was collected in a glass bottle and OD of the solution was taken at 515 nm against a blank which was prepared through a similar procedure by taking 25 mL distilled water without any culture. The same procedure was adopted to estimate lead at 0, 48 and 72 hours. Control of the lead processing was run using the medium without inoculation incubated at same conditions as that of the culture. Bacterial processing of lead was assessed by estimating the amount of lead in the medium after various time intervals. All the readings were taken in triplicate for statistical analysis.

Amount of lead was calculated by the following formula:

$$G = \frac{M \times 95.4 \times \text{dilution factor}}{a}$$

where G = amount of lead in mg/L, a = amount of water used in mL, M = absorbance.

Isolation of plasmid

A single colony of various lead resistant strains was selected and grown in LB liquid medium for plasmid isolation. The procedure adopted for isolation of plasmids was as described by Holmes (1984). Plasmid DNA isolated from the bacterial isolates was run on agarose gel and visualized under UV illuminator after staining with ethidium bromide. The isolation experiment was repeated three times to ascertain the presence of the plasmids.

Curing of the plasmid

Curing of the plasmid present in the strain was done by using ethidium bromide. The strain was grown in the medium containing ethidium at concentrations; 50, 100 and 150 μg/mL of the medium. After the growth, the culture was spread on LB agar plates. The colonies appearing on the plates were tooth picked on to the LB agar plates and the plates containing lead at a concentration of 1 mg/mL (both the plates had a grid for location of colonies. The colonies appearing on agar plates but not appearing on plates containing lead were taken as indication of the cells having the plasmid cured.

RESULTS

Isolation of lead resistant bacterial strain

For isolation of lead resistant bacterial strain, LB plates containing lead at 1 mg/mL concentration were used. Colonies appeared after 24 hours. A Gram positive bacterial isolate was selected for lead resistance and lead processing ability.

Metal and antibiotic resistance of the isolate

The bacterial isolate was grown on increasing concentrations of lead. The minimum inhibitory concentration of lead for the strains was 2 mg/mL. Metal resistance of the isolate against cadmium and chromium was also checked. The isolate showed resistance against these metals at a concentration of 70 mg/mL and 80 mg/mL respectively. The sensitivity of the isolate against various antibiotics was checked. The isolate showed resistance against septran and ceptazimide at a concentration of 30 μg/mL while it showed sensitivity against ampicillin, augmentin, amoxycillin, cefazolin, doxycyclin, enoxocin, erythromycine, gentamicin and minocyclinE.

Identification of the isolate

Gram staining of the bacterial culture showed Gram positive rods. The isolate formed circular white colonies with entire margins on LB agar plates. The strain showed positive activity for catalase, oxidase and Voges-Proskauer test and it was negative for coagulase test, starch utilization test, McConkey test, citrate utilization test and nitrate reduction test.

Optimum growth conditions and growth curve

The growth curve of the isolate was determined. It showed a lag phase of four hours after inoculation followed by an exponential phase of 10 hours and then a decline phase (Fig.1). The optimum pH for the growth of the bacterial strain was 7.0, temperature 37°C and inoculum size 20 μl/5 mL of the medium (Figs.2a,b,c).

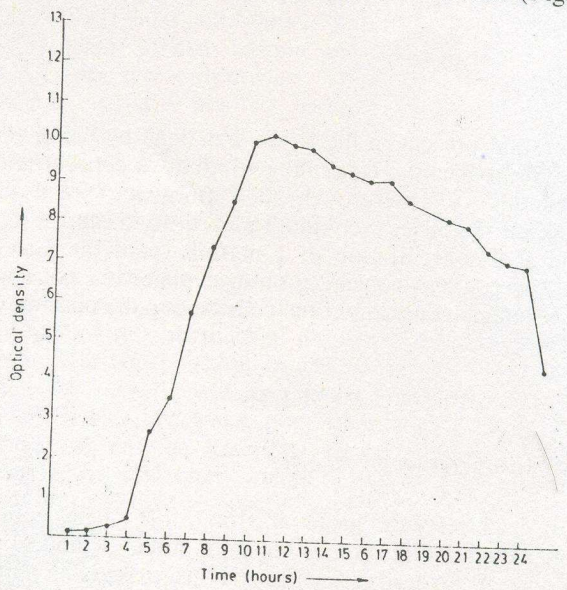

Fig. 1: Twenty four hours growth pattern of CMBL-17.

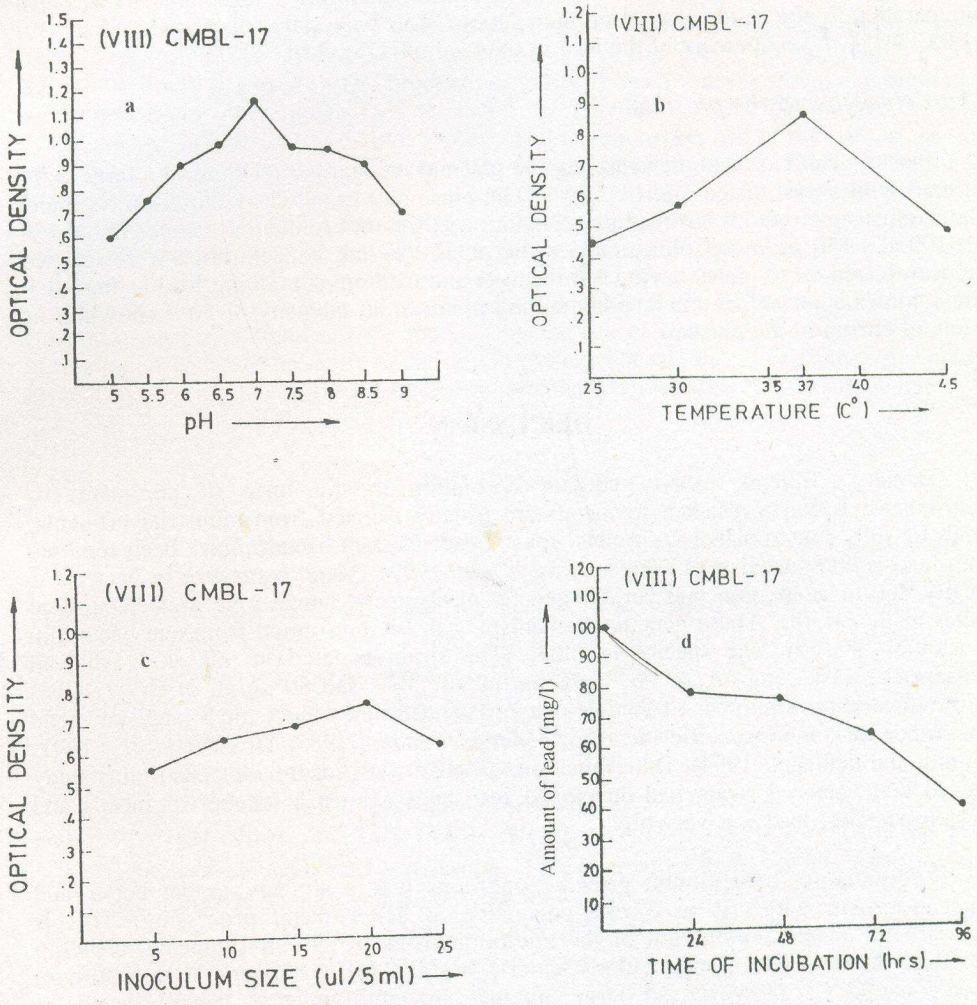

Fig. 2: Effect of: a, pH; b, temperature; c, inoculum size on the growth of bacterial isolates; and d, effect of bacterial activity on lead decontamination in the culture.

Processing of lead by the bacterial strain

A culture of the isolate was prepared in LB medium containing lead at a concentration of 1 mg/mL. The estimation of the amount of lead was done at 0, 24, 48, 72 and 96 hours of incubation. A control (medium containing 1 mg/mL lead) was also run parallel to the culture without inoculation. The bacterial isolate reduced 21.39, 23.05, 34.54, 60.63 percent of the lead in the medium (Fig.2d).

Characteristics of the plasmid

Plasmid isolation and then running the plasmid on agarose gel showed a large sized plasmid with an estimated size of 23 kb. The plasmid was cured by ethidium bromide. The strain was grown in LB medium containing ethidium bromide at a concentration of 50, 100 and 150 μg/mL. Colonies appearing on 150 μg/mL concentration were selected and transferred on to plates having LB medium and medium containing lead (1 mg/mL). The colonies appeared on the lead containing plates at an intensity of 50% showing the extent of curing of the plasmid.

DISCUSSION

Tannery effluents usually contain chromium in the form of chromates or dichromates. Bacteria resistant to metals are usually isolated from industrial effluents, ponds or soils contaminated with metal ions. Lead resistant bacteria have been reported from lead contaminated sites (Trajanovska *et al.*, 1997). Metal resistance in bacteria is usually due to adaptation and certain genetic mechanisms which give greater survival values to the strains. These genetic mechanisms can be transmitted from one bacterium to another or from one species to other. The strain isolated in this study showed resistance against 2 mg/mL of Pb^{2+}, 70 μg/mL of Cd^{2+} and 80 μg/ml of Cr^{6+}. It has been indicated by a number of studies that resistance often occurs for a range of metal ions rather than for a specific metal ion (Mergeay *et al.*, 1985; Dressler *et al.*, 1991; Schmidt and Schlegel, 1994). Determination of MIC values for three metals in this study showed that bacterial strain had developed resistance against a number of metals and resistance against lead was very high.

Determination of optimum growth conditions was a strategy to get better and efficient growth which is as a rule conducive to better metal processing. This is necessary for better exploitation of the microorganisms in the environmental clean-up operation. Many bacteria, particularly enteric bacteria and pathogens show optimum growth at 37°C. However, a large number of environmental bacteria such as *Pseudomonas* show optimum growth at 30°C. Many of the metal resistant bacteria can resist a number of heavy metal ions present in the environment (Collard *et al.*, 1994) possibly due to possession of common mechanisms of resistance. There are various external environmental conditions for better xenobiotic detoxification. In this regard, another important condition is the pH of the medium. Microorganisms are metal accumulator, and their ability is controlled by external pH (Morley and Gadd, 1995). They causes a change in valence or ionic state of metal which leads to effect the growth

of the microorganisms (Francis, 1990). Moreover, one or more types of processing become rate limiting at extreme temperatures (Innis and Ingraham, 1978).

The strain isolated in this study showed a considerable capability of processing lead. It reduced 21.39, 23.05, 34.54, 60.63 percent of the lead in the medium in 24, 48, 72 and 96 hours of growth. The processing ability showed that the strain can be used in metal processing operations. This indicated the potential of lead detoxification in the environmental clean-up operations. One of the conclusion from the results is that ethidium bromide proved an efficient agent for curing of plasmids. The presence of the plasmid in the strain indicated that the gene for processing of lead might be present on the plasmid. The failure of the growth of the isolate on lead containing medium after curing procedure indicated that the gene for processing of lead was possibly plasmid coded as it has been discovered in other studies that metal resistance and metal processing genes are usually plasmid borne. Another explanation would be that the chromosomal genes could also be mutated by the use of ethidium bromide, as ethidium bromide can cause frame shift mutations in genes. The antibiotic resistance of bacteria present in the industrial effluents and contaminated environments is well documented.

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