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Lead Resistant Yeast from Industrial Waste Water Capable of Decontaminating it of Heavy Metals

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Abstract.- Five lead resistant yeasts CMBLY Pb 1-5 isolated from industrial waste water tolerated 2 to 2.5 mg Pb/ml. CMBLY Pb 4 and CMBLY Pb 5 could also tolerate cadmium upto 1 mg/ml, whereas rest of the isolates could grow in a medium containing 500 µg cadmium/ml. Most of the strains could resist chromium upto 500 µg/mL except for CMBLY Pb 1 which tolerated only upto 20 µg/ml. When concentration of heavy metal was increased, colony size was reduced and the growth period was delayed. Three strains CMBLY Pb 1, 2, 5 grew best in alkaline medium pH 7.5-8.0, while CMBLY Pb 3 and 4 showed optimum growth at pH 6.0. The optimum temperature for all the yeast strains was 30°C. The total proteins of lead resistant yeast isolates were analyzed through polyacrylamide gel electrophoresis to identify lead induced protein fractions. All the yeast isolates were capable of processing lead effectively, when grown in media containing lead. The yeast strains CMBLY Pb 2 was found to be the most efficient one causing 62% reduction in the medium within a period of 96 hours.

Key words: Heavy metal resistant yeast, bioremediation, lead and yeast.

INTRODUCTION

Industrial wastes are the highest source of pollution causing severe damage to biosphere. In Pakistan, the industries dump their wastes in the environment without any proper treatment. One of the heavy metals in industrial effluents is lead, which is accumulated in tissues and is carcinogenic, teratogenic and mutagenic (Baker *et al*., 1980; IARC, 1980; Lilis, 1981; Abbott, 1985; Miestrich, 1989). Lead causes cerebral edema and damage to endothelial cells in infants and children, affects intellectual levels of children as well as animals (Rutter and Jones, 1983) short life span of erythrocyte (Pagila *et al*., 1975) and impairment of haeme synthesis due to inhibition of enzymes such as ALA-dehydrase and haeme synthetase (FPA, 1980). The multi-system toxicity of lead is mediated by inhibition of enzyme processes or pathological alteration in cellular and mitochondrial membrane, and interference in heme synthesis and nucleotide metabolism (Friberg and Nelson, 1981).

One of the strategies to decontaminate the waste water of heavy metals is the use of microorganisms which are adapted to resist metal and keep low intracellular concentration of toxic pollutants by (i) inherited ability to resist high concentration of toxic pollutants through evolution under extreme environmental conditions (Brock, 1978a) (ii) acquiring transferred resistance to polluted environment through plasmids (Silver, 1983).

Dancis *et al*. (1994) found that *Saccharomyces cerevisiae* encodes a protein required for high affinity uptake of heavy metals in some microbes, small chelating agent are excreted for binding and transporting metal ions (Williams, 1981; Neilands, 1979). Membranes are very important in case of metal ion uptake. Wiliams (1981) determined a number of chemical parameters that influence the uptake of metal ion by living cell, including charge, ionic radius, preference of metal for coordination to certain organic ligand etc. Summer and Silver (1978) found that microorganisms, which are adapted to polluted environment, convert metals to less toxic forms or more inert forms, which in turn reduce the stress of metals in immediate vicinity.

Yeasts synthesize metallothioneins (MT), which sequester metal ions such as lead, copper and cadmium and are biosynthetically regulated at the level of gene transcription in response to heavy metals, hormones, cytokines, other physiological and environmental stresses (Radtke *et al*., 1995; Zhou and Thiele, 1993; Lu and Thiele, 1996; Tamai *et al*., 1994). Tohoyama *et al*. (1995) isolated MTs

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with 53 amino acids residues. MTs in yeast are controlled by (i) amplification of MT gene and its transcriptional regulation and (ii) its constitutive expression (Tohoyama *et al*., 1992). Cysteine residues are important for chelation. Cismowski and Huang (1991) reported that structural rearrangement in mutants containing alteration from cysteine 50 to tyrosine resulted in disrupting the normally tight protein clusters. MTs cause changes in metal speciation and are intracellular binding compounds, which control metal movement and toxicity (Kushner, 1993). Yu *et al*. (1994) found that yeast produces isopeptidase (phytochelations) which appear to function in metal buffering when MT genes are not present or expressed. According to Schnell and Entian (1991) yeast encodes a highly acidic protein with mostly hydrophobic character in which some repetitive amino acids regions (such as Cys-Ser-Glu) may act as metal binding sites.

The aim of the present study was to isolate and characterize yeast strains from industrial effluents having property of resistance to heavy metals especially lead. The efficiency of yeast isolates in bioremediation of lead was also evaluated.

MATERIALS AND METHODS

Isolation of lead-resistant yeast

Water samples were collected in sterilized glass bottles from industrial effluents of Kala Shah Kaku and Kasur industrial areas. Soil samples were collected from petrol pumps of Gulberg area in Lahore city. To isolate lead resistant yeast strains, YEPD (yeast extract 1 g, Peptone 0.5 g, Dextrose 0.2 g) agar medium containing lead (100 µg/ml) was used (pH 7.2-7.4). Two antibiotics, Gentamicin (0.025 µg/ml) and Chloramphenicol (0.05 µg/ml) were added to avoid bacterial contamination. Then 50 µl of sample was spreaded on the Petri plates and incubated at 30°C. To obtain pure culture streaking was done 4-5 times. The colonies of the yeast strains were identified after microscopic examination.

Lead resistance of yeast strains

The concentration of lead in agar was increased gradually from 100 µg/ml to 2500 µg/ml. The yeast strains were streaked and incubated at 30°C for eight days to check the lead resistance of particular yeast strain.

Resistance of lead resistant yeast to other heavy metals

Resistance of yeast isolates to cadmium and chromium was also analyzed by growing them on YEPD plates containing various concentrations of Cd and Cr. Salts used were $CdCl_2$ and $K_2Cr_2O_7$. Initial concentration of cadmium and chromium was 10 µg/ml which was gradually increased. Yeast culture was streaked on the sterilized Petri plates containing specific medium and incubated at 30°C upto 8 days.

Determination of optimum growth conditions

For determination of optimum pH, the pH YEPD liquid medium (5 ml) added in three sets, each of eight test tubes, were adjusted at 5.0, 5.5, 6.0, 7.0, 7.5, 8.0, 8.5 and 9.0 autoclaved, inoculated with 20 µl inocula and incubated for ten hours at 30°C in a shaking water bath. The growth was measured at 600 nm with a spectrophotometer.

For determination of optimum temperature the isolated yeast were grown in YEPD liquid medium at 25, 30, 35 and 45°C to check the effect of temperature on the growth. Experiment was run in triplicate and absorbance was noted at 600 nm after 10 hours of incubation.

For determination of optimum amount of inoculum, 5 ml YEPD liquid medium, pH adjusted according to optimum pH of the respective strain, three sets each of six tubes was autoclaved, inoculated with different concentrations *i.e.* 10, 20, 30, 40, 50 and 100 µl of pure culture of isolated strain and incubation for ten hours at 30°C shaking water bath. O.D. was taken at 600 nm.

Growth curve

YEPD medium (5 ml) with optimum pH of respective strain was added in 24 sterilized test tubes and autoclaved. After inoculation tubes were incubated at 30°C in shaking water bath and were taken out one by one every hour. Absorbance was recorded at 600 nm and graph was plotted.

Morphological and biochemical characterization

The shape, colour and size of colony and individual cells were recorded using yeast morphology agar. The size of cells was measured by ocular micrometer. The mode of vegetative

reproduction was also studied. To check whether the particular strain produces spores or not three types of media *viz.* GordoKowa Agar medium (0.1 glucose, 1 g peptone, 0.5 g sodium chloride and 2 g agar in 100 mL distilled water), 2% glucose agar medium (0.5 g yeast extract, 2 g glucose and 2 agar in 100 mL of distilled water) and aqueous agar medium (2 g agar in 100 mL distilled water) were used. The pure culture of isolated yeast strains were streaked on the three media and incubated at 30°C for ten days. Then colony of the yeast strain was removed and smear was prepared. After heat fixation, stained with malachite green (5% aqueous), counter stained with safranine (0.5% aqueous) and observed under microscope. Spores were green in colour while vegetative cells were red.

The yeast isolates were also tested for oxidase activity, catalase activity, motility, citrate utilization, starch hydrolysis, Voges Proskauer test, nitrate reduction, carbon assimilation test (xylose, fructose, lactose, glucose etc).

Protein isolation and analysis

In order to isolate proteins of yeast strains, YEPD liquid medium (100 ml) in two 250 ml flasks, control and experimental was inoculated with fresh cultures of isolates and cells were harvested for 48 hours. In the experimental flask the cells were given stress of 500 µg Pb/ml Pb for one hour. Cells were then pelleted in a refrigerated centrifuge at 4000 rpm and then washed with Z buffer (16.1 g $Na_2HPO_4.7H_2O$, 2.7 ml 2-merceptoethanol in 1 L of distilled water). The pellet was transferred in eppendorf tube and 400 µl Z-buffer and 500 µl glass beads were added into it. Then tubes were vortexed for 2 minutes with pauses of 30 seconds. Samples were again centrifuged and supernatant was saved for gel electrophoresis.

The protein estimation was done with a spectrophotometer at 280 nm and then run on 12% non-SDS gel at 120 mAmp for overnight. The gel was stained with silver nitrate. For this the gel was fixed with 50% methanol and 12% acetic acid for one hour and rehydrated with 10% methanol and 5% acetic acid for 20 minutes. Then 0.0034 M $K_2Cr_2O_7$ and 0.0032 $NHNO_3$ was added for five minutes. Gel was washed with water and stained with 0.004 M $AgNO_3$. Gel was kept at 25°C on light box for 5 minutes, then in the normal light with constant swirling for 25 minutes. The gel was rinsed with water and developed with 0.28 M Na_2CO_3 and 0.05% formaline solution in excess. The reaction was stopped with 1% glacial acetic acid, which was also used as gel preservative.

Lead processing ability

Yeast isolates were analyzed for their ability to accumulate lead from the YEPD broth at a concentration of 1 mg/ml lead. Readings were recorded at 24, 48, 72 and 96 hours of incubation. Control with no yeast was also run in parallel. Amount of lead in the medium was estimated using dithizone method.

For estimation of lead, sample was diluted 25 times by adding 24 ml distilled water in one ml sample. For each 25 ml of sample, 2.5 ml of reagent 1 (20 g NaCl, 10 ml hydrazinium hydroxide in 70 ml of 1 N HCl, volume made upto 100 mL by adding distilled water) and reagent 2 (20 g potassium hydrogen carbonate, 5 g potassium cyanide, 5 g potassium sodium tartarate, and 25 ml ammonia solution and final volume made upto 100 ml with distilled water) taken in separating funnel and 12.5 ml dithizone solution (15 mg dithizone in 1000 ml of chloroform) was added. Mixture was shaken for five minutes. Two layers were separated. The upper layer contained water was discarded whereas lower layer of chloroform was collected in glass stopper bottle. Absorbance was taken at 515 nm and amount of lead was calculated by following formula:

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

Where G is the amount of lead in mg/L, a is the amount of water used in ml and m is absorbance.

RESULTS

Lead resistant yeast

CBLY1 was isolated from water sample of industrial effluents of Kala Shah Kaku industrial area. CBLY2 and CBLY3 were isolated from soil

Table I.- Minimum inhibitory concentrations of lead, cadmium and chromium on the growth of yeast isolated from industrial water.

Heavy metals	CMBLY 1	CMBLY 2	CMBLY 3	CMBLY 4	CMBLY 5
Pb^{2+} (mg/ml)	2.0	2.0	2.0	2.5	2.5
Cd^{2+} (µg/ml)	500	500	500	1000	1000
Cr^{6+} (µg/ml)	20	500	500	500	500

sample of petrol pumps of Gulberg area in Lahore city, while two strains CBLY4 and CBLY5 were isolated from industrial effluents of Kasur region. They could grow in media containing Pb^{+2} concentration as high as 2.0-2.5 mg/ml (Table I).

At 100 to 500 µg/ml lead, the growth appeared after 24 hours. When the concentration was gradually increased, the growth was delayed and the size of the colonies became smaller. At a concentration of 1000 µg/ml the growth appeared after 48 hours. At 2000 µg/ml small colonies appeared after 4-7 days, while only CBLY4 and CBLY5 showed growth at 2500 µg/ml but colonies were minute and appeared after 10 days.

Table I shows MICs of cadmium and chromium against yeast isolates. All the strains showed resistance upto 500 µg/ml cadmium but colonies appeared after 7 days. Only CBLY4 and CBLY5 showed resistance at 1000 µg/ml and very small colonies appeared after 7-14 days. CBLY1 was least resistant to chromium as it showed growth only uptill 20 µg/ml and growth appeared after 7 days, while other strains showed resistance to chromium at a concentration of 500 µg/ml and growth was observed after 7-14 days.

Optimum growth conditions

Figures 1-3 show effect of pH, temperature and size of inoculum on the growth of yeast isolates. CMBLY Pb 1, CMBLY Pb 2 and CMBLY Pb 5 showed optimum growth at alkaline pH (7.5, 7.5 and 8 respectively). CMBLY Pb 3 and 4 grew better in acidic pH with optimum optical density at a pH of 6.

All the isolated yeast showed best growth at 30°C and further increase in temperature resulted in decrease in growth. The growth of isolates was increased with increasing inoculum size.

Growth curve

Figure 4 shows growth curves of various yeast isolates. CMBLY Pb 1 showed lag phase of 5 hours, which was followed by log phase of 9 hours. CMBLY Pb 2 showed lag phase of 6 hours and log phase was of 10 hours. In case of CMBLY Pb 3 lag phase of 6 hours, followed by log phase of 7 hours. CMBLY Pb 4 showed lag phase of 12 hours and log phase of 7 hours. CMBLY Pb 5 showed lag phase of 12 hours, followed by log phase of 6 hours. In all the above cases stationary phase followed and a typical sigmoid type curve was observed.

Morphological and biochemical characteristics

Table II shows morphological and biochemical characteristics of the isolates. All the strains reproduce by budding. Mostly single bud appeared some times or three buds were also observed. The individual cells were oval in shape and the size ranged between 0.376-0.452 µm.

All the five isolates were spore producing. All isolates were Gram positive and showed catalase activity, but were negative for oxidase activity and Voges-Proskauer test. They all could assimilate sugars tested, but could not hydrolyze starch. Nitrate reduction test was positive for CMBLY Pb 1 and 3 while negative for other isolates.

Total yeast proteins

The proteins isolated from control as well as heavy metal treated samples were analyzed by polyacrylamide gel electrophoresis (Fig. 5).

In CMBLY Pb 1 five bands were present in control and seven in treated, two bands from control disappeared in treated and three new bands could be seen. When comparing CMBLY Pb 2 have 7 bands in control and 8 in treated. Three bands were absent in treated and four new one appeared. CMBLY Pb 3

Fig. 1. Effect of pH on the growth of yeast isolates as indicated by optical density of the culture.

possesses 8 bans in control and nine in treated, three bands disappeared and 4 new bands could be seen. In CMBLY Pb 4, there were 9 bands in control and 8 in treated but 5 of the proteins were not present in the treated and 4 new had appeared. The total number of bands in CMBLY Pb 5 in control and treated were the same but difference lies in the position of two bands.

Fig. 2. Effect of temperature on the growth of yeast isolates as indicated by optical density of the culture.

Removal of lead

Figure 6 shows the efficiency of removal of Pb^{2+} from the culture medium by different yeast isolates. CMBLY Pb 2 was the most efficient strain in removing lead y 62% after 96 hours of incubation (Table III). CMBLY Pb 4 removed 57% of lead from the medium after 96 hours of incubation.

Fig. 3. Effect of size of inoculum on the growth of yeast isolates as indicated by optical density of the culture.

DISCUSSION

Extensive industrialization has brought about huge changes in the distribution of elements at the surface of the earth. The conventional methods for the treatment of industrial effluents containing heavy metals consume large amount of energy and chemicals. Therefore more practical economic methods are being explored. One of these methods is to isolate heavy metal resistant microorganisms as these have evolved strategies to cope up with stressed conditions (Wood and Wong, 1983). Metal

Fig. 4. Growth curves of yeast isolates.

resistant microorganisms become dominant in habitats contaminated with relevant metals. Bredicevisky *et al*. (1993) have reported three yeast species showing heavy metal resistance. Lead was to found to be the third toxic substance with mercury being first, cadmium second and selenium being least toxic element.

In order to check the effect of lead on the

Fig. 5. Polyacrylamide gel electrophoresis pattern of proteins isolated from the lead resistant yeast isolates: Lane 1: CBLY 1 in medium without lead; Lane 2: CBLY 1 in medium with lead (500 µg/ml); Lane 3: CBLY 2 in medium without lead; Lane 4: CBLY 2 in medium with lead (500 µg/ml); Lane 5: CBLY 3 in medium without lead; Lane 6: CBLY 3 in medium with lead (500 µg/ml); Lane 7: CBLY 4 in medium without lead; Lane 8: CBLY 4 in medium with lead (500 µg/ml); Lane 9: CBLY 5 in medium without lead; Lane 10: CBLY 5 in medium with lead (500 µg/ml).

Table II.- Morphological and biochemical characteristics of yeast isolates.

Test	CMBLY Pb 1	CMBLY Pb 2	CMBLY Pb 3	CMBLY Pb 4	CMBLY Pb 5
Colony colour	Light pink	White	White	Creamy white	Creamy white
Colony shape	Round	Round	Round	Round	Round
Colony margins	Smooth and convex	Smooth and convex	Smooth and convex	Wavy	Wavy
Reproduction	Budding	Budding	Budding	Budding	Budding
Cell shape	Oval	Oval	Oval	Oval	Oval
Cell size	0.413	0.426	0.376	0.452	0.359
Catalase test	+	+	+	+	+
Oxidase test	-	-	-	-	-
Motility	-	-	-	-	-
Voges-Proskauer test	-	-	-	-	-
Nitrate reduction test	+	-	+	-	-
Citrate utilization	-	+	+	-	-
Starch hydrolysis	-	-	-	-	-
Glucose assimilation	+	+	+	+	+
Xylose assimilation	+	+	+	+	+
Fructose assimilation	+	+	+	+	+
Lactose assimilation	+	+	+	+	+

Table III.- Percentage reduction of the lead present in medium due to activity of yeast isolates.

Time in hours	CMBLY Pb 1	CMBLY Pb 2	CMBLY Pb 3	CMBLY Pb 4	CMBLY Pb 5
24	14.39	15.35	19.39	9.59	13.43
48	27.95	30.46	23.73	13.14	31.07
72	28.84	41.72	43.65	36.34	36.72
96	45.3	61.79	51.31	56.75	52.67

Fig. 6. Capability of yeast isolates to process lead in YEPD medium.

production of proteins a comparison was carried out among control and lead treated strains through polyacrylamide gel electrophoresis. It was found that new bands appeared in the treated yeast indicating that new proteins were induced under stress conditions. Beside the production of specific proteins, there was also reduction of bands in the treated as compared with the control, indicating toxic effect of metal on protein machinery of resistant strain. Faber and Bolan (1992) reported decrease in the synthesis of proteins in response to metal stress and seems to be more universal stress parameter than induction of proteins.

Bioaccumulation of lead by yeast isolates indicates that their use would prove highly effective in detoxification of wastewater containing lead. The yeast strains isolated in the present study are adaptable to local conditions of environment. So they can be successfully applied in biochemical plants to reduce pollutants in the nature and industrial effluents can be converted into valuable manure.

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