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Tolerance and Biosorption of Mercury by Microbial Consortia: Potential Use in Bioremediation of Wastewater

B. Muneer,[1] M. J. Iqbal,[4] F. R Shakoori[2] and A. R Shakoori[3]*

[1]*Institute of Industrial Biotechnology, GC University, Lahore 54000, Pakistan*
[2]*Department of Zoology, University of the Punjab, New Campus, Lahore 54590, Pakistan*
[3]*School of Biological Sciences, University of the Punjab, New Campus, Lahore 54590, Pakistan.*
[4]*The Centre of Research in Molecular Medicines (CRiMM),The University of Lahore, Pakistan.*

Abstract.- Mercury resistant microorganisms (bacteria, yeast and protozoa) were isolated from industrial effluents of tanneries and identified as *Bacillus licheniformis*, *Candida parapsilosis* and *Tetrahymena rostrata* on the basis of 16S rRNA and 18SrRNA gene sequence analysis. All microorganisms showed typical growth pattern except for the lag phase. The lag phase extended in the presence of mercury. Mercury processing ability of microorganisms was evaluated individually and in different combinations. *B. licheniformis*, *C. parapsilosis* and *T. rostrata* removed 73%, 80% and 40% of mercury, respectively, when used individually. *B. licheniformis* and *C. parapsilosis* reduced 85%, *C. parapsilosis* and *T. rostrata* removed, 77% and *B. licheniformis* and *T. rostrata* removed 73% mercury from the medium. Combination of three microorganisms *viz., B. licheniformis, C. parapsilosis* and *T. rostrata* when used simultaneously, removed 88% of Hg^{2+} after 96 h of incubation. It was concluded from this part of study that bacteria and yeast could make much more efficient inoculum for remediation of mercury-contaminated industrial waste water.

Key words: Heavy metal toxicity, mercury uptake, bioremediation, industrial wastewater.

INTRODUCTION

Mercury has been recognized as one of the most toxic heavy metals in the environment and has been released into environment in substantial quantities through natural events and anthropogenic activities (Kiyono and Hau, 2006). Industrial dumping of mercury into rivers and the consumption of coal and solid waste incineration has led to significant pollution of the environment (Von Canstein *et al.*, 2001). The toxicity of organic and inorganic mercury compounds is due to their strong affinity for sulfur containing organic compounds, such as enzymes and other proteins. Because of its high toxicity, mercury has no beneficial function. Mercury binds to the sulfhydryl groups of enzymes and proteins, thereby inactivating vital cell functions (Wagner-Dobler *et al*., 2000). Entrance of the most toxic species of mercury, methylmercury, into the human body results in different neurological disorder such as paresthensia and numbness in the fingers, which are common symptoms of Minamata disease (UNEP, 2003).

Even small amounts of mercury are toxic for all the organisms. However, some bacterial communities residing in the mercury-contaminated areas can exchange mercury resistance genes between each other, because of the continuous exposure to the toxic levels of mercury (Nascimento and Souza, 2003). The bacteria, yeast and protozoa play a major role in the global cycling of mercury in the natural environment. The microorganisms are able to resist heavy metal contamination through chemical transformation by reduction, oxidation, methylation and demethylation (Nascimento and Souza, 2003). Mercury resistant bacteria were first isolated from mercury contaminated soil in Japan (Robinson and Tuovinen, 1984). After this finding there were several reports of environmental bacteria, which were resistant to mercury compounds (De and Ramaiah, 2007; Chiu *et al.*, 2007). The mechanism of resistance to mercury in bacteria is mediated by a *merA* gene product which reduces Hg^{2+} compounds to metallic mercury Hg^0, which is obviously less toxic for them. The resistance to mercury is controlled by a set of genes organized in the *mer* operon. MerA has the key role in the removal of Hg (II) (Barkay *et al.*, 2003; Deckwer *et*

* Corresponding author: arshaksbs@yahoo.com, arshakoori.sbs@pu.edu.pk
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al., 2004; De *et al.,* 2006).

Mercury resistance ability has not only been reported in bacteria but also in different archea, protozoa and yeast (Schelert *et al.*, 2004; Vetriani *et al.,* 2004). Mercury resistant bacteria have high potential for the treatment of industrial effluents containing Hg(II) (Nascimento and Souza, 2003). Frequent occurrence of ciliates in wastewater or industrial effluents indicates that they are able to withstand the heavy metal contaminated environment. This property makes protozoa excellent candidate for exploitation in metal detoxification and bioremediation (Haq *et al.,* 2000; Shakoori *et al.*, 2004).

Bioremediation is an integrated management of polluted ecosystem where different microorganisms are employed which catalyze the natural processes in the polluted or in the contaminated aquatic or terrestrial ecosystem. Suitable, but high cost technologies have been identified for cleanup of heavy metal polluted soils (Iskandar and Adriano, 1997). Bioremediation generally utilizes microbes (bacteria, fungi, yeast, and algae), although higher plants are used in some applications. Although the bindings of metals to microorganisms have been described for many years, the commercial use of this procedure is slow. Microorganisms (bacteria, yeast and protozoa) showed remarkable ability to pick up heavy metals from the culture medium when they were used individually. In this study different combinations of microorganisms were used to evaluate the best combination for efficient removal of heavy metals.

MATERIALS AND METHODS

Sampling and growth medium

Water samples of the industrial effluents from ponds getting wastes of tanneries in Kasur (Pakistan) were collected in sterilized screw capped glass bottles. Physical parameters of wastewater viz., pH and temperature were also recoded. A large number of bacteria, yeast and protozoa were present in the wastewater.

Luria Bertani (LB) agar medium (1% NaCl, 1% tryptone, 0.5% yeast extract and 1.5% agar) was used for the growth of bacteria. YEPD (1% yeast extract, 0.5% peptone, 0.2% glucose and 1.5% agar) medium was used for culturing yeast. Bold basal medium [$NaNO_3$ (0.250 g/L), $CaCl_2.2H_2O$ (0.0250 g/L), $MgSO_4.7H_2O$ (0.0750 g/L), K_2HPO_4 (0.075 g/L), KH_2PO_4 (0.175 g/L), NaCl (0.025 g/L), EDTA (0.050 g/L), KOH (0.031 g/L), $FeSO_4$ $.7H_2O$ (0.0498 g/L), H_2SO_4 (0.001 ml/L), H_3PO_3 (0.01142 g/L), $ZnSO_4.7H_2O$ (0.00881 g/L), $MnCl_2.4H_2O$ (0.00144 g/L), MoO_3 (0.00071 g/L) $CuSO_4.5H_2O$ (0.00157 g/L) and $Co(NO_3)_2.6H_2O$ (0.00049 g/L)], diluted 1:1000 with distilled water was used for culturing protozoa.

Physical and biochemical characterization of the microorganisms

The isolates were tested and characterized by several physiological and several biochemical tests, besides Gram's staining such as Catalase, Voges Proskauer, tyrosine decomposition, citrate utilization, nitrate reduction, casein and starch hydrolysis, growth on media containing 7% NaCl, Sabouraud Dextrose agar and 0.001% lysozyme, and acid production from glucose was performed for identification of bacteria. Sporulation, formation of mycelium, carbon assimilation, acid production from different sugars, growth on 5% glucose and 10% NaCl containing medium, starch hydrolysis and ester production, Diazonium blue B (DBB) and urease tests were used for identification of yeast. Ciliates were identified on the basis of their shape and size (Cheesbrough, 1993; Collee *et al.*, 1989).

Ribotyping

For further identification of bacteria, genomic DNA was isolated and universal bacterial 16s rRNA primers

BF 5'-AGAGTTTGATCCTGGCTCAG-3'
BR 5'- ACGGCTACCTTGTTACGACTT -3'

were used to amplify the ~1.5 kb 16S rRNA gene fragment which was cloned in PTZ57R/T (Fermentas # K1214) and sequenced by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. The sequence was submitted in NCBI database with accession no. AB508839.

For identification of yeast, a pair of 18S rRNA primers

YR1 5′-GTTTCTAGGACCGCCGTA-3′ and
YR2 5′-CTCAAACTTCCATCGACTTG-3

were used to amplify the conserved region of 18S rDNA of yeast. A 581-bp fragment was cloned in PTZ57R/T vector. The sequence was submitted in NCBI database with accession no. AB509360.

For the identification of mercury resistant ciliates, the genomic DNA of the ciliate was isolated by a method described by Gaertig *et al*. (1994). A 250 bp fragment was amplified by using a pair of

PF (5´-AGGGTTCGATTCCGGAG-3´) and
PR (5´-GCTGCTGGCACCAGACT-3´) primer.

The fragment was cloned in PTZ57R/T vector. Sequencing was done. It was analyzed and then aligned with the 18S rDNA sequences of different ciliates in NCBI database for identification of ciliate species. The 18S rRNA gene sequences were compared with known sequences in the GenBank and ribosomal RNA database.

Growth curves of microorganisms

The effect of mercury on the growth of bacteria, yeast and protozoa was checked by counting the number of cells in the medium with the help of haemocytometer under microscope by taking 3 µl of culture. The cells were grown in their respective media with 100µg/mL of Hg^{2+}. The aliquots (2mL) of bacteria and yeast incubated medium were taken out at regular intervals of one hour for 48 hours. The growth of the protozoa was observed by counting the number of protozoan, every day for 5 days. The growth was compared with that of control culture, which contained no added mercury ions. Growth curves were prepared by plotting a graph between time (hours for bacteria and yeast and days for protozoa) of incubation along the X-axis and number of cells per ml along the Y-axis.

Estimation of Hg^{2+} processing ability of microorganisms

Metal processing capability of microorganisms was checked in single and in different combinations, such as bacteria and yeast, yeast and protozoa, bacteria and protozoa, bacteria, yeast and protozoa.

For determination of metal processing ability the calculated amount of bacteria and yeast cells (bacteria $10x10^7$ cells/mL and yeast $10x10^5$ cells/mL), yeast and protozoan ($10x10^5$ cells/mL of yeast and $10x10^3$ cells/mL of protozoa), bacteria and protozoan ($10x10^7$ cells/mL of bacteria and $10x10^3$ cells/mL of protozoa), bacteria, yeast and protozoa (bacteria $10x10^7$ cells/mL, yeast $10x10^5$ cells/mL and protozoa $10x10^3$ cells/mL) were added in water having glucose as a carbon source containing 100 µg/mL of Hg^{2+} and grown at optimum pH and temperature in culture flasks. A control was also run having 100 µg/mL of Hg^{2+} but without microorganisms. The culture samples were taken out of the flask after 0, 12, 24 and 48 h for estimation of mercury. The culture samples were centrifuged at 6000 rpm (4350 × g) for 15 min to spin down the cells. The absorbance was taken with the help of AA1275 atomic absorption spectrophotometer at wave length λ 253.7 nm and the concentration of metal in the supernatant was estimated. A graph was plotted between the time interval and the wavelength.

The experiment was performed in triplicate. The average of control and experimental groups were compared and significant differences evaluated by using Student's *"t"* test of significance (Sokal and Rohlf, 1984).

RESULTS

One of the goals of this study was to identify and characterize mercury-resistant microorganisms isolated from water contaminated with mercury ions. For this purpose, mercury resistant bacterium, yeast and ciliate were isolated from the wastewater samples. The temperature of the wastewater harboring the microorganisms was 30°C, pH was 8.6. On the basis of physical and biochemical tests bacterium was identified as *Bacillus* sp., yeast as *Candida* sp. and ciliate was identified microscopically as *Tetrahymena* sp.

The nucleotide sequence of ~1.5 kb amplified PCR product of 16 S rRNA of mercury resistant bacteria yielded 1516 bases. The blast analysis and alignment with different bacterium sequences in NCBI database showed 99% resemblance with

Bacillus licheniformis. The partly amplified (581 bp) PCR product of 18S rRNA from local yeast isolate was sequenced and blasted against similar sequences in the NCBI data. It showed 99% homology with *Candida parapsilosis* while the blast analysis of ciliate showed 97% resemblance with *Tetrahymena rostrata.*

Figure 1 shows the effect of Hg^{2+} on the growth of bacteria and yeast. It clearly shows the characteristic phases during the growth of culture.

It is clearly indicated that microorganisms without metal (control) treatment showed lag phase of 2-3 h. After this the organism showed accelerated growth rate for 12-21 h. The microorganisms with Hg^{2+} stress (treated) however, showed lag phase of 4-6 h and log phase of 16-18 h.

In the present study we used microorganisms bacteria, yeast and protozoa individually and then in different combinations to find out the best combination which could be used for removal of heavy metal contamination from the medium. When microorganisms used individually, it was observed that bacteria removed 73%, yeast 80% of chromium after 72 h of incubation and protozoa removed 40% of mercury after 96 h of incubation (Table I).

Table I.- Percentage removal of metals by microorganisms isolated from industrial wastewater.

Microorganisms	Time of incubation (Hours)	% age removal of metals
Bacillus licheniformis	72	73%
Candida parapsilosis	72	80%
Tetrahymena rostrata	96	40%

Figure 2 shows the ability of Hg^{2+}resistant microorganisms to reduce the level of mercury in different combinations. *B. licheniformis* and *C. parapsilosis* have ability to reduce 85% of Hg^{2+} from the medium after 48 h of incubation (Fig. 2a). Mercury resistant *C. parapsilosis* and *T. rostrata* when used simultaneously for removal of Hg^{2+} from the medium, it was observed that Hg^{2+} resistant microorganisms removed 77% Hg^{2+} after 96 h of incubation (Fig. 2b).

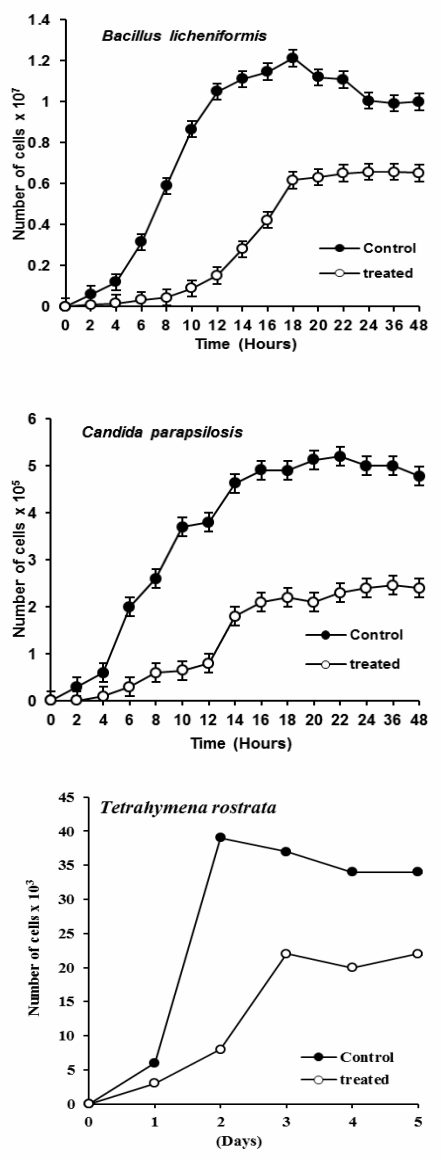

Fig. 1. Growth curves of *Bacillus licheniformis, Candida parapsilosis* and *Tetrahymena rostrata* in Hg^{2+} containing medium. Control cultures did not contain any metal ions.

Figure 2c shows the ability of Hg^{2+} resistant *B. licheniformis* and *T. rostrata* to reduce the level of Hg^{2+} from the medium after 96 hours of incubation. They removed 73% of Hg^{2+} after 96 h. The three microorganisms *B. licheniformis, C. parapsilosis* and *T. rostrata* when used simultaneously, removed 88% of Hg^{2+} after 96 h of

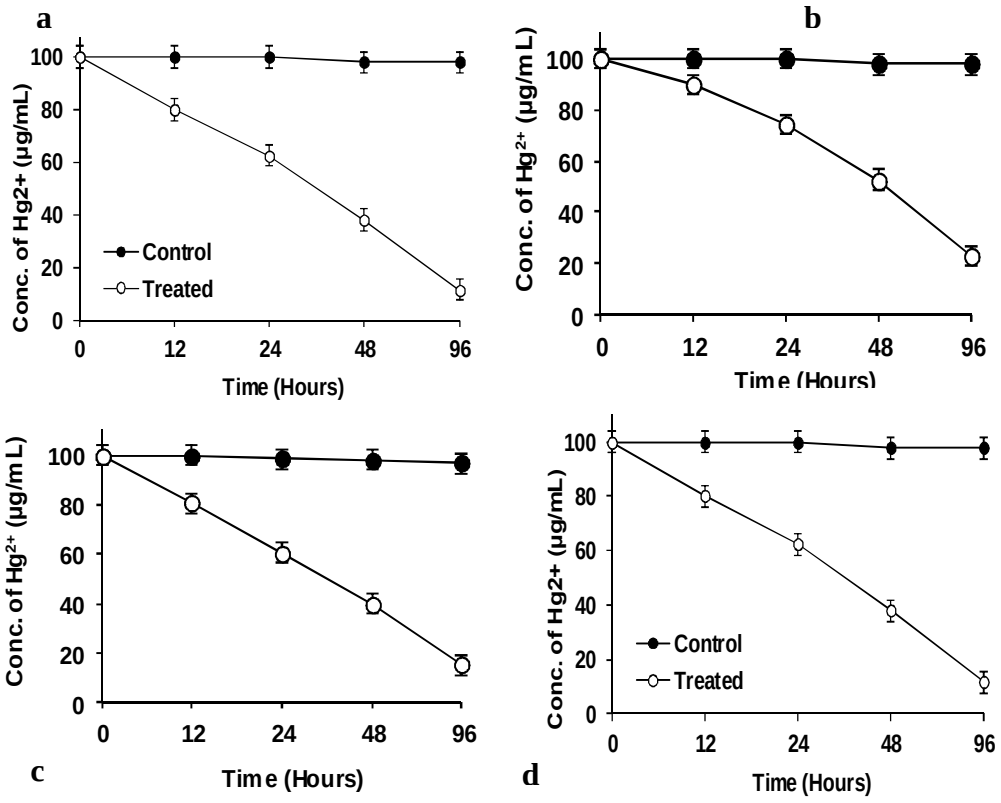

Fig. 2. The heavy metal processing ability of microorganisms a) *Bacillus licheniformis* and *Candida parapsilosisand* b) *Candida parapsilosisand* and *Tetrahymena rostrata* c) *Bacillus licheniformis* and *Tetrahymena rostrata* d) *Bacillus licheniformis*, *Candida parapsilosisand* and *Tetrahymena rostrata* isolates from industrial wastewater. The isolates were grown with 50 µg/mL of Hg^{2+}. The control culture medium contained heavy metal but no organism.

incubation (Fig. 2d). It was concluded from this part of study that bacteria and yeast provided the best combination of microorganisms for removal of Hg^{2+} from the medium. Simek *et al.* (1997, 2001) reported that protozoa used bacteria community as their food.

DISCUSSION

Resistance to mercury has been reported in different microorganisms. A number of bacteria including *Pseudomonas* sp., *Staphylococcus* sp., *Bacillus*, *E. coli*, *Proteus* sp., *Klebsiella* sp. and *Salmonella* sp. (Kiyono and Hau, 2006; Olukoya *et al.*, 1997; De *et al.*, 2006, Kargar *et al.*, 2012), *Saccharomyces cerevisiae* (Dar and Shakoori, 1999) and *Paramecium* sp. (Shuja and Shakoori, 2009; Shakoori *et al.*, 2004) have been found to be resistant to mercury. A large variety of microorganisms including bacteria, yeasts and protozoa are found in industrial wastewater (Haq and Shakoori, 2000; Rehman and Shakoori, 2001, 2003). Hansen *et al.* (1984) reported that growth in the presence of Hg results in prolongation of the lag phase of growth. Similar results were obtained in the present study. The metal removal abilities of various species of bacteria, algae, fungi and yeasts were investigated (Utigikar *et al.*, 2000). In the wastewater rich with metals only the heavy metal resistant strains can survive. They developed strategies to resist, tolerate, metabolize and to detoxify these substances (Shi *et al.*, 2002).

Microbial bioremediation using mercury-resistant microorganisms has been shown to be

useful (Deng and Wilson 2001; Essa *et al.,* 2002).Several studies have reported improvements in metal removal by immobilization of protozoa, yeast or bacterial cells (Zeroual *et al.,* 2001). Bacteria and yeast communities are central to the functioning of terrestrial ecosystem and consist of a large number of different bacterial and yeast type (O-Muter *et al.,* 2002). The bacterial population is heavily grazed by the protozoa (Hahn and Hofle, 1998, 2001; Pernthaler *et al.,* 2001; Simek *et al.,* 1997, 2001). Mercury reduction by mercury-resistant microorganisms is a good mechanism for mercury bioremediation, but the recovery of the metallic Hg^0 needs to be addressed, in order to avoid its escape into the atmosphere (Essa *et al.,* 2001).

The principal goal of bioremediation is to enhance the natural biological-chemical transformations that render pollutants harmless as minerals and thus to provide a relief and, if feasible, a permanent solution to the problem of contaminated environments. Remediation of sites contaminated with heavy metals is a complex problem (Sandrin *et al.,* 2000; De *et al.,* 2006). Bioremediation can be effective where environmental conditions permit microbial growth and activity (Vidali, 2001). Microorganisms in contaminated environments have developed resistance to mercury and are playing a major role in natural decontamination (Cursino *et al.,* 1999; De *et al.,* 2003). Microorganisms have important role in biogeochemical cycling of toxic metals (Lloyd and Lovley, 2001). Microorganisms including bacteria (*Salmonella* sp., *Legionella pneumophilia, Escherichia coli* and *Klebsiella pneumoniae*), protozoa (*Hartmenella vermiformis, Tetrahymena pyriformis, Paramecium* sp. and *Amoeba* sp.), yeast (*Candida albicans* and *Saccharomyces cerevisiae*), fungi (*Aspergillus* sp.) can remove metals individually and in consortia (Salunkhe *et al.,* 1998; Nies, 1999; Riggle and Kumamoto, 2000; Cervantes and Guiterrez-Corona, 1994; Congeevaram *et al.,* 2007). An enriched consortia of bacteria and yeast was reported to remove 99-100% of different metals Cr^{+6}, Pb^{+2}, Hg^{+2}, Ni^{+2} and Zn^{+2} from different heavy metals contaminated water (Lee *et al.,* 2008).

It was observed that protozoa may not be important in large scale processing of wastes containing heavy metals, but they share the capability of resisting this toxic metal ion with other microorganisms like bacteria and yeast. Mixed culture is considered to be important in an ecosystem due to cooperative actions. It would not be advisable to use a pure culture of a microorganism due to disturbances in population structures in an ecosystem.

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