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Heavy Metal Resistant Ciliate, *Euplotes mutabilis,* Isolated from Industrial Effluents Can Decontaminate Wastewater of Heavy Metals

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Pollution due to chemicals, including heavy metals, is a problem that may have negative consequences on the biosphere. Heavy metals, particularly in industrial effluents, are constantly contaminating our environments, and pose serious threat to human life. Mercury is one such metal which has been reported to produce metabolic disorders in variety of animals such as fish (Company et al. 2004), rat (Reinhardt and Pelli 1986), rabbit (Shakoori et al. 2002) and man (Miwa et al. 1987). Various health problems such as pneumonitis, abnormal cramps, bloody diarrhea and suppression of urine, cancer, and hypersecretion of sweat glands are caused by mercurial and mercuric forms of mercury. Romero et al. (2004) studied the toxic effects of mercury chloride in two cell lines of renal origin. The most notable findings in treated cells were the presence of intracytoplasmic inclusion bodies and apoptotic bodies.

Copper is rarely found in natural water but is found in man-polluted environments (Andrews and Sutherland 2004). Any copper present normally originates from industrial effluents, seepage, water from refuse dumps, pesticides or corrosive water that has come into contact with fitting and pipes containing copper. Copper ions inhibit macromolecules synthesis and other enzymatic reactions (Company et al. 2004), affect bacterial growth (Kozdroj 1995), and dramatically decrease cell numbers (Gordon et al. 1993). Nitrogenase activity was decreased at higher concentrations of copper viz. 5 ppm, 10 ppm and 25 ppm (Vasundhara et al. 2004). Gross photosynthesis rate was decreased when treated with 250 and 500μg Cu/L (Andrade et al. 2004).

At present, chemical processes are commonly used to remove heavy metals from wastes. Such methods have several disadvantages, for instance, unpredictable metal ion removal, high reagent requirements, and the generation of toxic sludges (Ciba et al. 1999). Recently, microbial bioremediation has emerged as an alternative technique to such traditional chemical treatments (Brierley 1990). The long-term survival of protozoa in media containing relatively high concentrations of heavy metal ions shows that these organisms have evolved strategies to tolerate, resist or detoxicate organic substances and heavy metals (Haq et al. 2000; Shakoori et al. 2004; Rehman et al. 2005). A number of authors have already emphasized the role of protozoa in wastewater treatment plants (Madoni et al. 1996; Shakoori et al. 2004; Rehman et al. 2005).

One of the objectives of this study was to evaluate the survival of protozoa in media containing heavy metals such as Cd^{2+}, Hg^{2+}, Cu^{2+}, Pb^{2+}, and Cr^{6+} and determine their uptake of copper and mercury.

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MATERIALS AND METHODS

Wastewater samples were collected in screw capped sterilized bottles from five different ponds in industrial area of Sialkot (Pakistan). Some physicochemical parameters of wastewater viz., temperature (°C), pH, dissolved oxygen (mg/L), mercury (µg/mL) and copper (µg/mL) were measured. The samples were inoculated in Bold-basal salt medium in 100 mL conical flasks (Haq et al. 1998). A large number of bacteria, yeast, algae, and various protozoa were present in the original wastewater sample. For isolation of protozoa, antibiotics, i.e. ampicillin (25 µg/mL), chloramphenicol (35 µg/mL) and gentamicin (25 µg/mL) were added in the culture media to prevent growth of bacteria. Algae were excluded by keeping the culture in semidarkness. Yeast was excluded by absence of any organic substance in the medium. Culture was plated on YEPD medium and no growth appeared on the fungal medium.

Axenic culture of protozoa was made according to Shakoori et al. (2004). One hundred milliliter of different media, in 250 mL conical flask, was inoculated under aseptic conditions with 10µL of inoculum containing 40-50 ciliates. The cultures were maintained in the laboratory for one week at room temperature (25-27°C). The growth of *Euplotes* was observed in the cultures by counting the number of ciliates at regular intervals.

The growth curves of *Euplotes* were determined in different media i.e. LB (2 % (w/v) proteose peptone and 0.1% Bacto yeast extract), Molasses medium (1% aqueous solution of molasses), wheat and rice grain medium (1 boiled rice and wheat grain in 10mL of distilled water) and Bold-basal salt medium [$NaNO_3$ (0.25g/L), $CaCl_2.H_2O$ (0.025g/L), $MgSO_4.7H_2O$ (0.075g/L), K_2HPO_4 (0.075g/L), KH_2PO_4 (0.175g/L), NaCl (0.0025g/L), EDTA (0.05g/L), KOH (0.031g/L), $FeSO_4$.$7H_2O$ (0.04g/L), H_2SO_4 (0.001µL/L), H_3BO_3 (0.01142g/L), $ZnSO_4.7H_2O$ (0.00881g/L), $MnCl_2.4H_2O$ (0.00144g/L), MoO_3 (0.00071g/L), $CuSO_4.5H_2O$ (0.00157g/L) and $Co(NO_3).6H_2O$ (0.00049g/L)], diluted 1:1000 with distilled water, for 8 days. Glucose as carbon source was only added as 1g/L in Bold-basal salt medium. The pH of each medium was adjusted at 7.5. No metal ions were added in these media. The growth of culture was checked by counting number of protozoan cells in the medium as described earlier (Haq et al. 1998).

Resistance of *Euplotes* to five metal ions i.e. Cr^{6+}, Cu^{2+}, Pb^{2+} Hg^{2+} and Cd^{2+} was checked by addition of the respective metal salts ($K_2Cr_2O_7$, $CuSO_4.5H_2O$, $Pb(NO_3)_2$ $HgCl_2$ and $CdCl_2$) in the Bold-basal salt medium. Metal ions were sterilized separately and added to the medium when the temperature of the salt medium was slightly less than 50°C. For Cu^{2+}, $Hg^{2+,}$ and Cd^{2+} the concentration in the medium on the first day was 1µg/mL with an increase of 1µg/mL every day for 22 days. For $Cr^{6+}$and Pb^{2+} the concentration in the medium on the first day was 2 µg/mL with an increase of 2 µg/mL every day for 30 days (Cr^{6+}) and 37 days(Pb^{2+}). Although the death of protozoa is confirmed by the lysis of the cell but the movement is considered to be a vital sign of life. When the protozoan became inactive, no more metal was added.

The effect of different metal ions on growth of culture was checked by counting number of protozoan cells in the medium. At least three counts were taken to get a mean of every reading. The growth was compared with that of the control culture, which contained no metal ions added. The activity, shape and size of the protozoans were also noted. The size was measured with an ocular micrometer

after restricting the movement of the ciliates by putting the culture in methylcellulose and staining with 1% neutral red.

The metal processing capability of *Euplotes* was checked by adding separately $Hg^{2+}$and Cu^{2+} at a concentration of 10 and 5 µg/mL respectively in the culture medium. The separate control culture medium was also run for each metal containing the same concentration as in treated one i.e. 10 µg/mL for Hg^{2+} and 5 µg/mL for Cu^{2+} but was without the ciliates. The cultures were incubated for 6 days and from each medium (control and treated) 5 mL culture was taken out under sterilized conditions after 0, 48, 72 and 96 hours, respectively. The cultures were spun down at 3000 rpm for 15 minutes and the supernatants were used for the estimation of $Hg^{2+}$and Cu^{2+} by atomic absorption spectrophotometer (Varian, U.S.A) at wavelength 253.7nm and 324.7nm, respectively. The amount of metals in the supernatants was determined using standard curves. The percentage reduction in the amount of $Hg^{2+}$and Cu^{2+} in the medium was calculated.

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

RESULTS AND DISCUSSION

Table I shows physicochemical characteristics of industrial wastewater in five different ponds, from where *Euplotes* was isolated. *Euplotes* was present in all the ponds. The appearance of various metal resistant micro-organisms in ponds constantly receiving toxic industrial effluents showed a high capacity to evolve in response to xenobiotic stress. The temperature of ponds harboring the ciliates ranged between 24.5°C to 26.5°C, pH ranged between 7.52 and 8.6, dissolved oxygen between 0.36 ±0.01 and 1.77 ±0.03 mg/L. These ponds had Cu^{2+} ranging between 0.30 ±0.04 and 2.10 ±0.08 µg/mL and Hg^{2+} ranging between 0.01 ±0.04 and 0.20 ±0.004 µg/mL.

Table 1. Physicochemical parameters of wastewater collected from five different ponds in the industrial area of Sialkot, Pakistan.

Parameters	Pond no.1	Pond no.2	Pond no.3	Pond no.4	Pond no.5
Temperature (°C)	24.45±0.47	25.66±0.47	25.00±0.47	25.00±0.81	26.45±0.47
pH	8.47 ±0.04	7.52 ±0.12	8.38 ±0.04	8.64 ±0.04	8.05.±0.12
Dissolved oxygen (mg/mL)	1.68 ±0.01	0.36 ±0.01	1.77 ±0.03	1.30 ±0.02	0.98 ±0.01
Copper (µg/mL)	2.10 ±0.08	0.50 ±0.08	0.70 ±0.08	0.30 ±0.04	0.40 ±0.04
Mercury (µg/mL)	0.20 ±0.04	0.15 ±0.08	0.02±0.008	0.01±0.004	0.01±0.008

*Means±standard deviation

The growth curve pattern of *Euplotes* was obtained by counting the number of cells in the culture every day for 8 days. There was a gradual increase in the number of cells in each culturing medium. The maximum growth of the protozoan

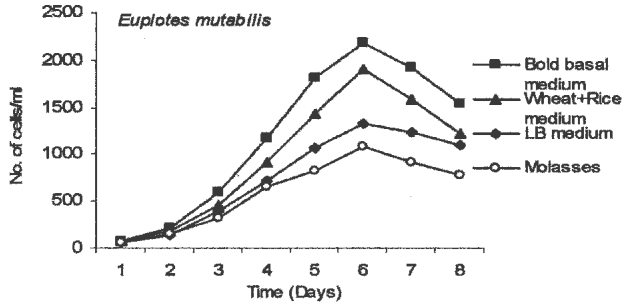

Figure 1. Growth curves of *Euplotes mutabilis* in different media containing no metal ions.

was observed on day 6, when the cell counts in Bold-basal, Wheat and Rice, LB and Molasses media were respectively 2180, 1900, 1320 and 1080 cells/mL. The number of cells after 8 days of growth period increased from 60 to 110 cells/mL in LB medium, from 60 to 780 cells/mL in 1% Molasses medium, from 80 to to1220 cells/mL in Wheat and Rice medium and from 80 to 1540 cells/mL in Bold-basal salt medium (Fig.1). Generally it is tedious to grow protozoa in the laboratory due to special organic supplements needed in the medium for their growth (Weekers and Vogels 1994). This laboratory has already reported the growth of two protozoan species in a medium containing salts only (Haq et al. 1998). In this study *Euplote*s has been successfully grown in the Bold-basal salt medium. This finding would elucidate new trends in culturing protozoa.

Mitotic activity, which is indicated by cell population, has been adversely affected by the presence of metal ions in culture media. The control culture contained $1.42x10^3$ cells/mL on day 1, which decreased to $1.25x10^3$ cells/mL after 30 days. However, when Cu^{2+} (22 µg/mL) was added the number decreased from $1.47x10^3$ ±1.53 to $1.01x10^3$ ±2.08 cells/mL ($P<0.001$), whereas the number of cells decreased from $1.45x10^3$ ±2.51 to $0.28\ x10^3$ ±4.00 cells/mL in the presence of Hg^{2+} (22 µg /mL) in 22 days ($P<0.001$). In the presence of Pb^{2+} (74 µg/mL) the number of cells decreased from $1.34x10^3$ ±5.03 to $0.262x10^3$ ±4.58 cells/mL ($P<0.001$), $1.69x10^3$ ±4.51 to $0.90x10^3$ ±2.64 cells/mL ($P<0.001$) in Cr^{6+} (60 µg/mL) after 30 days, whereas the number of cells decreased from $0.94x10^3$ ±0.51 to $0.15\ x10^3$ ±2.00 cells/mL in the presence of Cd^{2+} (22 µg /mL) in 22 days($P<0.001$). The reduction in the cell population was 92% for Cu^{2+}, 87% for Cd^{2+}, 79% for Pb^{2+}, 77% for $Hg^{2+}$and 72% for Cr^{6+} after 22 days of metal stress. The order of resistance regarding the reduction in the number of the cells was, therefore, $Cr^{6+}>Hg^{2+}>Pb^{2+}>Cd^{2+}>Cu^{2+}$.

Euplotes was found to be resistant to Cu^{2+} at a concentration of 22 µg/mL. The Cu-resistant ciliate was also found to tolerate Cd^{2+}, Pb^{2+}, Cr^{6+} and Hg^{2+} at a concentration of 22 µg/mL, 74 µg/mL, 60 µg/mL and 22 µg/mL, respectively. There was apparently no reduction in the size of *Euplotes* cells. Movement, which is a vital sign of life, was taken as a parameter of metal toxicity. The movements of ciliate slowed down in the presence of $K_2Cr_2O_7$ but almost stopped in the presence of $HgCl_2$, $CuSO_4$ and $CdCl_2$. The presence of Pb $(NO_3)_2$ did not make any significant effect on the movement of ciliates.

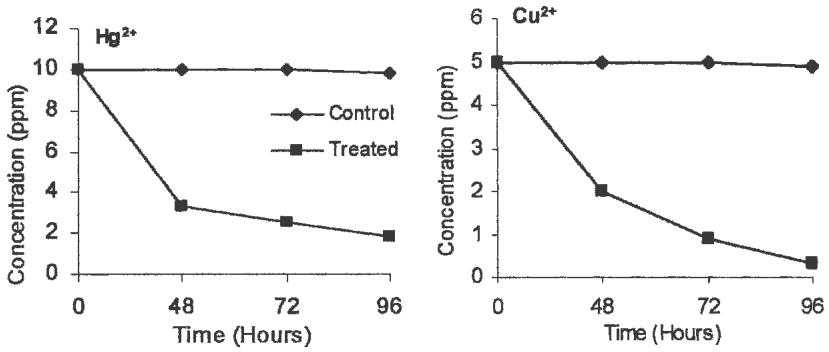

Figure 2. Uptake of Hg^{2+} and Cu^{2+} by *Euplotes mutabilis* growing in Hg^{2+} and Cu^{2+} containing media. The controls did not contain cells of the isolate.

Euplotes could efficiently process Cu^{2+} and Hg^{2+} from the medium. The ciliate culture grown in the medium containing Cu^{2+} (5 µg/mL) could reduce 60% of copper from the medium after 48 hours, 82% after 72 hours and 95% after 96 hours. It could also reduce 67% Hg^{2+} after 48 hours, 75% after 72 hours, and 82% after 96 hours from the medium containing Hg^{2+} at a concentration of 10 µg/mL (Fig. 2).

Microorganisms have a high surface area-to-volume ratio because of their small size and therefore provide a large contact area that can interact with metals in the surrounding environment (Ledin 2000). It is well known that bioremediation of toxic pollutants has advantages over other techniques as it is cheap, non-destructive and contamination remains localized (Riser-Roberts 1998).

Copper was generally more toxic to ciliate populations than Cd, Hg or Zn. For five of the seven tested species, the 24-h LC_{50} values ranged from 10 to 21µg/L. *Euplotes affinis* showed low sensitivity of 64µg/L for Cu^{2+} (Madoni et al. 1992). Simanov (1987) reported a 24-h LC_{50} of 500 µg Cu/L for *C. campylum* using copper nitrate. Madoni et al. (1996) reported that no mortality was registered for *Opercularia coarctata* when treated with copper, and *Opercularia minima* showed a 56% of mortality at the highest concentration of copper (6.12 mg/L). *Vorticella convallaria* showed a 95% survival even in the presence of the highest Cu concentration (6.12 mg/L).

For the freshwater ciliate *Tetrahymena pyriformis*, the LC_{50} was 3.3 mg Hg/L after 96 h exposure to mercuric chloride (Carter and Cameron 1973). Madoni et al. (1994) reported that some higher values were observed for tested ciliates (17.5 to 64 µg/ L), with the exception of *Uronema nigricans* which showed an LC_{50} of 4.3 µg/ L. In comparison with *Daphnia magna*, the two *Euplotes* species were more tolerant to Cd, Hg and Zn.

Environmental contamination with toxic heavy metal ions in the industrial wastes

is one of the major concerns of developing countries like Pakistan. Industrial effluents loaded with toxic metal ions are released in the environment without any treatment. Clean-up of mercury containing wastewater by mercury resistant microbes is a simple, environmental friendly and cost effective alternative to current treatment technologies (Wanger 2003).

Rehman et al. (2005) reported that *Stylonychia* could efficiently process Pb^{2+} from the medium. The protozoan culture grown in medium containing lead (10.0 µg/mL) could reduce 80% of lead from the medium after 48 hours, 82% after 72 hours and 86% after 96 hours, respectively. Bioaccumulation of lead by ciliates indicates that their use would prove highly effective in detoxification of wastewaters containing lead. The resistance and accumulation of metal ions such as Cr^{6+}, Pb^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+} is well documented. The interest in these processes is the potential use of such microorganisms in biological wastewater treatment (Rehman et al. 2005; Shakoori et al. 2004; Madoni 2000).

Ciliates present in the toxic wastewater are the indication that they are adaptable to local conditions of environment and can be used successfully in wastewater treatment plants. In this study we have reported the isolation of *Euplotes* which is resistant to highly toxic metal ions, and this organism may be employed for metal removal from wastewaters.

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