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Uptake of Heavy Metals by a Ciliate, *Tachysoma pellionella*, Isolated from Industrial Effluents and Its Potential Use in Bioremediation of Toxic Wastewater

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Extensive industrialization and unplanned disposal of industrial effluents have led to increase the emission of pollutants into ecosystems (Diagomanolin et al. 2004). Heavy metals in wastewater come from industries and municipal sewage, and they are one of the main causes of water and soil pollution. Heavy metal contamination and the problems that it poses to the biota have been well documented (Raskin and Ensley 2000; Meagher 2000). Accumulation of toxic metals, e.g., Hg, Cu, Cd, Cr, and Zn, in humans has several consequences such as growth and developmental abnormalities, carcinogenesis, neuromuscular control defects, mental retardation, renal malfunction and wide range of other illnesses (Thiele 1995).

Cr is carcinogenic, embryotoxic, teratogenic and mutagenic in animals (Nair and Krishnamurthi 1991). It has been found that teratogenic effects of Cr^{6+} are more severe than Cr^{3+}. Cr compounds have been shown to cross the placenta and to induce abnormalities and lethality in mice (Junaid et al. 1995), abnormalities in embryos (Asmatullah et al. 1999) and lethality in human embryos (Bona et al. 1992) as well as in adults (Kurosaki et al. 1995). The toxicity, mobility and bioavailability of Cr depend fundamentally on its chemical form. Cr^{6+} is highly soluble and about 300 times more toxic than Cr^{3+}. On the other hand, Cr^{3+} precipitates at the average pH of natural waters. The nature and behavior of Cr in wastewater depends on the physicochemical conditions of the effluents originating from various industrial sources (Kotas and Stasicka 2000, Myriam et al. 2005).

Lead is a ubiquitous toxic metal which have mutagenic, carcinogenic, genotoxic, anthropogenic and phytotoxic effects (Alvarez et al. 2003; Zelikoff et al. 1988). Severe lead toxicity has been known to cause sterility, abortions and neonatal mortality and morbidity. The most serious effects of lead are related to central nervous system (Goyer 1993). Lead is not used in any way in human metabolism, so there is no tolerable amount. It is considered as non-essential metal with no biological role in microorganisms, animals and plants (Bruins et al. 2000).

Microbial metal removal has received much attention in the past years due to the potential use of microorganisms for cleaning metal-polluted water (Ledin 2000).

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Several studies have indicated biological reduction of metals by microorganisms (Pas et al. 2004; Campos et al. 2005; Sannasi et al. 2006). 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).

One of the objectives of this study was to evaluate the survival of protozoa in media containing heavy metals such as Cd^{2+}, Pb^{2+}, Cu^{2+}, and Cr^{6+} and determine the uptake of chromium and lead by these organisms.

MATERIALS AND METHODS

Wastewater samples from a tannery effluent were collected in screw capped sterilized bottles from Kasur (Pakistan). Some physicochemical parameters of wastewater *viz.*, temperature (°C), pH, dissolved oxygen (mg/l), chromium (µg/ml) and lead (µg/ml) were measured (APHA 1992). 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 used 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 *Tachysoma* was observed in the cultures by counting the number of ciliates at regular intervals.

The growth curves of *Tachysoma* 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/), 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 (0.001,L/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 *Tachysoma* to four metal ions i.e. Cr^{6+}, Cu^{2+}, Pb^{2+} and Cd^{2+} was

checked by addition of the respective metal salts ($K_2Cr_2O_7$, $CuSO_4.5H_2O$, $Pb(NO_3)_2$ 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 Cr^{6+}, Cu^{2+}, Pb^{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 40 days for Cr^{6+} and Pb^{2+}, 20 days for Cu^{2+}, and 23 days for Cd^{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 cells 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 *Tachysoma* was checked by adding separately $Cr^{6+}$and Pb^{2+} at a concentration of 10 µg/ml in the culture medium. The control culture medium was also run for each metal containing the same concentration as in the treated one (10 µg/ml) but without the ciliates. The cultures were incubated for 6 days and from each medium (control and treated) 5 mL culture was taken out under aseptic 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 Cr^{6+} and Pb^{2+} by atomic absorption spectrophotometer (Varian, U.S.A) at wavelength 357.9 and 217.0 nm respectively. The amount of metals in the supernatants was determined using standard curves. The percentage reduction in the amount of Cr^{6+} and Pb^{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 each treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

RESULTS AND DISCUSSION

Table 1 shows physicochemical characteristics of industrial wastewater in five different ponds, from where *Tachysoma* was isolated. *Tachysoma* 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 17.66°C to 24.0°C, pH ranged between 7.46 and 8.9, dissolved between 0.46 ±0.01 and 1.87 ±0.03 mg/L. These ponds had Cr^{6+} ranging between 1.30 ±0.04 and 2.50 ±0.08 µg/mL and Pb^{2+} ranging between 0.21 ±0.04 and 0.70 ±0.004 µg/mL.

The growth curve pattern of *Tachysoma* was obtained by counting the number of

Table 1. Physicochemical parameters of wastewater collected from five different ponds receiving tannery effluents in Kasur district.

Parameters	Pond no.1	Pond no.2	Pond no.3	Pond no.4	Pond no.5
Temperature (°C)	23.66±0.47*	17.66±0.47	24.00±0.47	21.00±0.81	19.33±0.47
pH	8.86 ±0.04	8.63 ±0.12	8.93 ±0.04	8.94 ±0.04	7.46.±0.12
Dissolved oxygen (mg/mL)	1.58 ±0.01	0.46 ±0.01	1.87 ±0.03	1.20 ±0.02	1.48 ±0.01
Chromium (µg/mL)	2.50 ±0.08	1.50 ±0.08	1.70 ±0.08	1.30 ±0.04	1.40 ±0.04
Lead (µg/mL)	0.70 ±0.04	0.35 ±0.08	0.42±0.008	0.51±0.004	0.21±0.008

*Means±standard deviation; n=3.

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 was observed on day7, when the cell counts in Bold-basal, Wheat and Rice, LB and Molasses media were respectively 1690, 1360, 940 and 670 cells/mL. The number of cells increased from 80 to 820 cells/mL in LB medium, from 82 to 580 cells/mL in 1% Molasses medium, from 84 to1185 cells/mL in Wheat and Rice medium and from 78 to 1445 cells/mL in Bold-basal salt medium (Fig.1). This laboratory has already reported the growth of *Vorticella microstoma* and *Stylonychia mytilus* in Bold-basal salt medium (Shakoori et al. 2004; Rehman et al. 2005). In the present study, *Tachysoma* has been successfully grown in the Bold-basal salt medium.

Mitotic activity, which is indicated by cell population, was adversely affected by the presence of metal ions in culture media. The control culture contained $1.52x10^3$ cells/ml on day 1, which decreased to $1.35x10^3$ cells/ml after 40 days. However, when Cu^{2+} (20µg/ml) was added the number decreased from $1.35x10^3$ ±2.18 cells/mL to $0.84x10^3$ ±3.48 cells/ml ($P<0.001$) in 20 days. In the presence of Pb^{2+} (40 µg/ml) the number of cells decreased from $1.48x10^3$ ±3.50 to $1.06x10^3$ ±2.28 cells/ml ($P<0.001$), $1.34x10^3$ ±3.54 to $0.94x10^3$ ±4.18 cells/ml ($P<0.001$) in Cr^{6+} (40 µg/ml) after 40 days, whereas the number of cells decreased from 0.85 $x10^3$ ±2.38 to 0.47 $x10^3$ ±2.78 cells/ml in the presence of Cd^{2+} (23 µg /ml) in 23 days ($P<0.001$). The reduction in the cell population was 38% (Cu^{2+}), 28% (Pb^{2+}), 30% (Cr^{6+}) and 45% (Cd^{2+}), respectively. The order of resistance regarding the reduction in the number of the cells was, therefore, $Pb^{2+}>Cr^{6+}>Cu^{2+}>Cd^{2+}$.

The *Tachysoma pellionella* was found to be resistant to Cr^{6+} at a concentration of 40 µg/mL. The ciliate was also found to tolerate Cu^{2+}, Pb^{2+} and Cd^{2+} at a concentration of 20 µg/mL, 40µg/mL and 23µg/mL, respectively. There was apparently no reduction in the size of *Tachysoma pellionella* 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 $CuSO_4$ and $CdCl_2$. The presence of Pb $(NO_3)_2$ did not make any significant effect on the movement of ciliates. The order of resistance on the basis

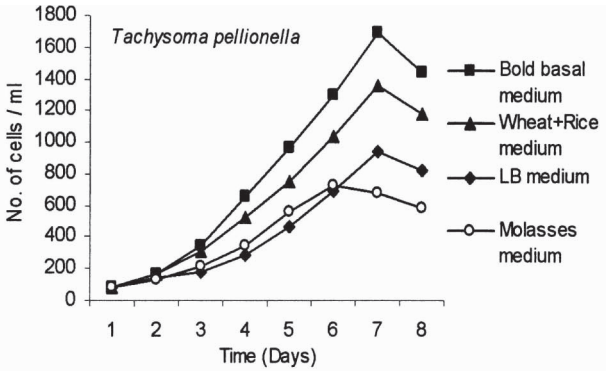

Figure 1. Growth curves of *Tachysoma pellionella* in different media containing no metal ions.

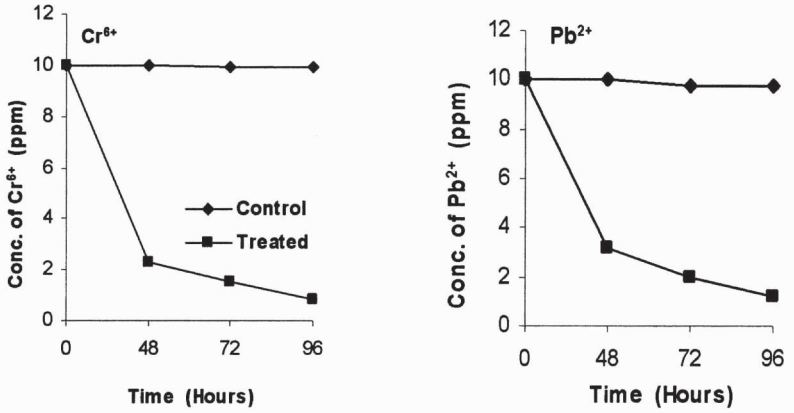

Figure 2. Uptake of Cr^{6+} and Pb^{2+} by *Tachysoma pellionella* growing in Cr^{6+} and Pb^{2+} containing medium. The controls did not contain cells of the isolate.

of motility was $Pb^{2+}>Cr^{6+}>Cd^{2+}>Cu^{2+}$.

Tachysoma could efficiently process Cr^{6+} and Pb^{2+} from the medium. The ciliate culture grown in the medium containing Cr^{6+} (10 µg/ml) could reduce 77% from the medium after 48 hours, 85% after 72 hours and 92% after 96 hours, respectively. It could also reduce 68% Pb^{2+} after 48 hours, 80% after 72 hours, and 88% after 96 hours from the medium containing Pb^{2+} at a concentration of 10 µg/ml (Fig. 2).

Madoni *et al.* (1996) found that 26.2 mg/L of Cr^{6+} caused the disappearance of only 2 out of 16 species and lowered the protozoan density by 12.5%, whilst 68.8 mg/L of Cr^{6+} caused disappearance of 4 out of 16 species and raised the protozoa mortality to 25%. The very low toxicity of chromium could be ascribed to the

change in the valence state of Cr from the hexavalent to the less toxic and soluble trivalent form. Imai and Gloyna (1990) reported that this process can occur during the activated sludge treatment. This highlights the capability of the activated sludge microbiota (bacteria and protozoa) to survive and operate also when atypical concentrations of heavy metals enter the plant.

Madoni et al. (1996) found that *Aspidisca cicada* showed a 70% survival in the presence of 6.98 mg/L of Pb. Shakoori et al. (2004) reported a very high level of metal resistance in *Vorticella microstoma*. The ciliate was found to tolerate Pb^{2+} at a concentration of 550 µg/mL and this concentration did not make any significant effect on the movement of ciliate. The order of resistance on the basis of motility was $Pb^{2+} > Zn^{2+} > Cr^{6+} > Cd^{2+} > Cu^{2+}$.

The ciliate, *Vorticella microstoma*, was found to be resistant to Cr^{6+} at a concentration of 260 µg/mL. The ciliate showed remarkable potential to remove metal ions from the culture medium. The concentration of Cr^{6+} was reduced 48% after 192 hour in a culture medium containing Cr^{6+} (100 µg/ml). 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. 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 (Shakoori et al. 2004; Rehman et al. 2005).

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). Microorganisms have a high affinity for metals and can accumulate both heavy and toxic metals by a variety of mechanisms (Shakoori and Muneer 2002; Yilmaz 2003, Pas *et al.* 2004). These have been used to remove metals from polluted industrial and domestic effluents on a large scale. 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).

The presence of metal resistant ciliate in industrial effluents carrying highly toxic metal ions has indicated adaptation of these organisms to environment containing toxic metals. In this study we have reported the isolation of *Tachysoma pellionella* which is resistant to highly toxic metal ions. This capability of the organism can be exploited for metal detoxification operations.

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