

BIOTECHNOLOGICAL IMPLICATION OF FLAGELLATE PROTOZOANS IN CLEANING INDUSTRIAL EFFLUENTS CONTAINING HEAVY METAL Cr⁺⁶

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Abstract: A flagellate protozoan was isolated from tanneries' effluent collected from the vicinity of city Kasur. Axenic culture of the protozoans was established in 0.1% aqueous solution of molasses supplemented with antibiotic chloramphenicol. The medium supported a healthy growth of the microorganisms both in terms of number as well as size, however, these parameters decreased after one week and few days, respectively. Following Cr⁺⁶ exposure it was found that up to 4 µg of the metal/ml of the medium, the protozoans showed, but a significantly decreased growth throughout the study period. In case of higher concentrations: 6, 8 and 10 µg of Cr⁺⁶/ml the protozoans showed a further delay of one-day in the manifestation of growth. Following 9, 7 and 6 days of incubation the protozoans were not observed in case of the culture media containing 6, 8 and 10 µg of Cr⁺⁶/ml, respectively. Cultures of the flagellates exposed to chromium were found invariably devoid of dividing cells, throughout the observation period. The flagellates were found to reduce Cr⁺⁶, up to a level of 6 µg/ml of the medium. Beyond this level the protozoans remained unsuccessful to reduce the metal.

Key words: Protozoan and Cr⁺⁶, biological reduction of Cr⁺⁶, bioremediation of heavy metals.

INTRODUCTION

In Pakistan the industrial effluents are generally discharged directly or through adjoining land areas into nearby streamlets/rivers. Besides a large variety of industrial effluents representing various kinds of pollutants, effluents from tanneries must be treated on priority basis because the waste contains heavy metal chromium and is converting cultivable land areas to highly polluted shallow ponds. A vivid example of the problem is being represented by city Kasur. The heavy metal chromium is used in the tanning process and the waste water of the study area has been found to contain 1.93 to 6.45 µg/ml of Cr⁺⁶ with pH values ranging from 8 to 9. (Qazi *et al.*, 1997).

Apart from very low levels of chromium, necessary for carbohydrate and fat metabolism (Anderson *et al.*, 1983; Chakraborty and Mishra, 1992), its higher amounts are highly toxic and express carcinogenic, teratogenic and mutagenic effects in a variety of organisms (Nieto *et al.*, 1989; Nair and Krishnamurthi, 1991; Lansdown, 1995). Valency of chromium determines its cytotoxic effects. In living system, chromium is usually found in trivalent state (Ohtake and Silver, 1992). Hexavalent forms of chromium

are known to be more toxic than CrIII. Because CrVI is easily taken up by the cells as the chromate anion via the sulphate anion transport system. Following entry within living system the CrVI is reduced intracellularly through reactive intermediates such as CrV and IV to more stable and less toxic CrIII by cellular reductants such as glutathione, vitamin C and B₂, as well as flavo-enzymes. The intracellular CrIII forms tight complexes with biological ligands such as DNA and proteins (DeFlora and Wetterhahn, 1989; DeFlora *et al.*, 1990; Campos *et al.*, 1995). Regarding the mechanism of the cytotoxicity including carcinogenic and mutagenic effects of chromium, it has been explained that during the intracellular reduction of CrVI to CrIII reactive oxygen species are generated, through reactions such as CrV with H₂O₂ in a Fenton-like manner to produce hydroxyl radicals (Aiyar *et al.*, 1990; Coudray *et al.*, 1992). The reactive oxygen species including superoxide ion, hydrogen peroxide and hydroxyl radicals produced as a result of redox cycling of chromium and other transition metal ions, have been reported for their oxidative deterioration of biological macromolecules including DNA damage and lipid peroxidation (Stohs and Bagchi, 1995; Susa *et al.*, 1996). Sugden and Wetterhahn (1996) have suggested that CrIV formed upon disproportionation of CrV oxidizes (oxygen-dependent oxidation) the nucleotide deoxyribose sugar moiety at the C-4' position via a phosphate bond intermediate. These workers found that the nucleoside thymidine show no reaction, while extent of CrV induced nucleotide oxidation was greater for thymidine diphosphates.

For environmentally sound strategies to protect our environment from various pollutants, bioremediation is probably the best choice. Bioremediation is the technology of using biological agent particularly microorganism to remove toxic pollutants from the environment especially soil and water. The pollutants are decomposed to non-toxic substances through microbial metabolism. Either microorganism indigenous to the environment, improved strains, or genetically engineered species are used for bioremediation. Microbe-enhancing substances are added to promote growth and metabolic activity of microorganisms already present in the environment (Pelczar *et al.*, 1993; Campos *et al.*, 1995).

Presence of protozoan species in industrial effluents containing heavy metals has allured many workers to study these microorganisms for their metal detoxification abilities and possible role in bioremediation processes (Madoni *et al.*, 1996; Qazi *et al.*, 1997; Haq *et al.*, 1998). In this regard axenic culturing of protozoa is required to study effect(s) and/or fate of a pollutant in a particular species (Lessard, 1993; Wecker and Vogels, 1994; Qazi *et al.*, 1997). The flagellate protozoans reported here, isolated from waste-water ponds getting tanneries' effluents, have been found to tolerate and reduce Cr⁺⁶ up to 6 µg/ml of the medium. These protists as well as other microbial community of the habitat can be exploited for the bioremediation of industrial effluents containing the heavy metal.

MATERIALS AND METHODS

Axenic culturing of flagellates

Samples of the industrial effluents from ponds getting waste water of tanneries in Kasur city, about 54 km south-east of Lahore, were collected in sterilized containers. From one sample, most abundant protozoan flagellate was isolated mechanically by micropipette to collect individual cells directly from water sample, as described originally by Lessard (1993). For this purpose the sample was filtered through a clean, Whatman filter paper to render the filtrate free from particulate material, and one drop of it was taken on a clean, sterile slide with the help of sterile inoculating loop. While observing under microscope at 125X, the flagellates were transferred to a drop of distilled water on the same slide with the help of a narrow-nosed Pasteur pipette. The diluted sample was sucked up in a capillary tube that was gently put and lifted on the slide to make six droplets. These droplets were diluted with distilled water, to avoid rapid evaporation. The droplet(s) containing either only one flagellate or multiples of the same was picked up with Pasteur pipette and transferred to a 250 ml conical flask containing 50 ml of autoclaved 0.1% molasses solution (w/v). About 40 flagellates were introduced in this manner and the flask was kept at room temperature. To inhibit bacterial growth 1% solution of chloramphenicol (Sigma) in methanol was used to provide 20 μg of the drug per ml of the culture. Stock culture of the flagellates was maintained in the laboratory, at room temperature. Fresh stock culture was made by inoculating 1.0 ml of a previous culture into 100 ml of the medium within 15 days.

Assessment and optimization of growth

Growth of the protozoans under different experimental conditions was assessed daily, by measuring size and number of motile and dividing cells/ml of culture. The microorganisms were counted using haemocytometer at 125X. Their number excluding the dividing cells was counted in total of the sixteen squares, represented by four squares at each corner of the chamber including those crossing the right and below bordering lines of each quadrant of the squares. The corner-squares were selected to minimize the chance of recounting a motile protozoan. Relative growth was assessed by dividing number of cells/ml at a given stage by the value of the same parameter at time of inoculation. Diameter of the protozoans, was measured at 500X with the help of ocular and stage micrometers.

All cultures were grown in 250 ml conical flasks except mentioned otherwise by inoculating 0.1 ml of a stock culture to 19.9 ml medium. Various growth conditions were optimized. Oxygen requirement was assessed by growth of the flagellates in test tubes and the conical flasks at room temperature. The protozoans, were grown also in 12 hours light/dark cycle and complete darkness, provided by covering the culture flasks with black paper. The protists' growth was studied at 10°C (refrigerator), 30°C (room temperature)

and 37°C (incubator), pH of freshly prepared medium ranged from 7.5 to 8.0. The cultures were also grown in medium having pH of 10 attained by adding 1% aqueous solution of NaHCO_3 .

Microscopic observations

Living flagellates were immobilized with 1% methylcellulose for microscopic observations as described by Cox (1981). The protozoans were also processed for chemical fixation and staining. Filtered albumen (albumen + glycerine; 1:1 ratio) was applied on one side of cover slip and a drop of the flagellate's culture was dropped from a height of about 1" on it. The drop was subsequently spread to make a thin smear, which was allowed near to air dry and then fixed in Bouin's fixative for 20 minutes. The smears were stained as described by Cox (1981), for the staining of parasitic protozoans except for few modifications. Smears were dipped in 70% ethanol and subsequently stained in Ehrlich's haematoxylin till the protozoans appeared as dark blue-black. After a washing of 5 minutes in 70% alcohol, the stained smears were differentiated in acid alcohol (1.5% HCl in 95% ethanol), till the stain left the cytoplasm and the process was soon stopped by washing the cover slips and slides in 70% alcohol. After passing through 50% alcohol for 5 minutes, the smears were washed in tap water, till the specimens appeared blue in colour. They were then passed through 70% and 90% alcohols and stained in 1% alcoholic eosin for about two minutes. After washing the stain in 95% alcohol the smears were dehydrated, cleared in xylene and mounted in Canada balsam. Photomicrograph of the flagellates were taken on a camera-fitted microscope.

Chromium VI tolerance and its reduction by the flagellates

$\text{K}_2\text{Cr}_2\text{O}_7$ was used as source of Cr^{+6} (Bianchi and Levis, 1985) and stock solution of this salt was prepared by dissolving 0.707 gm of $\text{K}_2\text{Cr}_2\text{O}_7$ in 500 ml of glass distilled water. Culture media containing 2, 4, 6, 8 and 10 μg of Cr^{+6} /ml in 0.1% (w/v) molasses were then prepared by adding appropriate amounts of sterile chromium stock solution, tap water and 10% solution of molasses.

Cr^{+6} was measured in uninoculated culture media containing different concentrations of the heavy metal as well as in cell free 12-days old cultures by the Petrilli and DeFlora method (1977), 7.0 ml of a sample was taken in 250 ml conical flasks and mixed with 43.0 ml of glass distilled water. pH was then adjusted between 0.81 and 0.95, with concentrated H_2SO_4 and the volume was made up to 100 ml with the water. 2.0 ml of diphenyl carbazide reagent, freshly prepared by dissolving 250 mg/50 ml of acetone was added and after 10 minutes optical density of each sample was measured at 540 nm, taking glass distilled water as reference. Statistical analyses between different parameters of control and experimental cultures were made by employing Student's 't' test.

RESULTS

Axenic culturing of flagellate protozoans

At inoculation cultures contained $0.21 \pm 0.01 \times 10^4$ cells/ml ($n=4$) growth of the flagellate protozoan was detectable on day-2 following inoculation. Maximum relative growth of the protozoans in control cultures was observed at day-3rd, while from the 7th day the number of flagellates decreased retrogressively up to the end of experimental period (Table I). At the initial stages the cells in control cultures attained average diameter of about 10 μm . The size of the flagellate decreased continuously so that at day-12th following the inoculation the figure turned out to be as 6.18 μm (Table II).

Microscopically various details of the protist such as presence of vacuoles, flagella and shape of the cell were observable, even in living stage, while observing under the microscope (Fig.1). The cells fixed in Bouin's fixative and subsequently stained with haematoxylin and eosin, also revealed structural details such as size of the flagellum, shape of the cell, location of chromatin material and relative size of the protozoans (Fig.1).

Optimization of growth conditions

Relative growth of the protozoan grown in test tubes (little dissolved O_2) remained significantly lower than the values for the cultures in conical flasks (Fig.2). Similarly, the number of dividing cells $\times 10^4/\text{ml}$ was found significantly less in cultures grown in test tubes and at day 7th and 9th the values turned out to be 0.67 ± 0.57 and 1.00 ± 0.00 as compared to the control figures of 2.67 ± 0.57 and 2.33 ± 0.57 , respectively. However, size of the cells did not show difference between two types of the cultures (Fig.2).

The protozoans growth increased significantly in cultures kept away from light, sparing the initial phases (Fig.2). At days 6th and 7th post-inoculation values indicating relative growth of the protozoans cultured under darkness were 404.93 ± 5.67 and 485.22 ± 11.11 as compared to the figures 324.69 ± 20.41 and 329.63 ± 22.56 , respectively for the cultures grown in usual light/dark cycle. Similarly, numbers of dividing cells in cultures kept in darkness for the said stages, 1.33 ± 0.57 and $2.67 \pm 0.57 \times 10^4/\text{ml}$ were significantly higher than the value 1.00 ± 0.00 for each of the corresponding study periods for the protists grown in usual light/dark cycle. Regarding the size of the cell at day 4th cells' diameter of cultures grown under darkness decreased significantly than the values for the cultures kept under normal light/dark cycle (Fig.2).

The flagellates did not show growth at all in inoculated medium kept at 10°C , grew well at room temperature, while at 37°C significant reduction in growth as well as cells' size was observed (Fig.2). Concerning the number of dividing cells, except at day-2 with a figure of 1.00 ± 0.00 no dividing cells were observed in cultures incubated at 37°C .

Table I:
Effect of Cr^{+6} on relative growth of the flagellates, grown in control (A) containing 2 (B), 4 (C), 6 (D), 8 (E) and 10 μg (F) of Cr^{+6} /ml of medium.

Treat- ment	DAYS AFTER INOCULATION											
	2 ^a	3	4	5	6	7	8	9	10	11	12	
Control ^f	131.71 ^b ±9.23	186.86 [•] ±8.09	152.09 ±9.23	143.62 ±9.23	161.90 [•] ±37.90	126.19 ±22.04	88.09 ±5.23	71.04 [•] ±7.23	69.04 ±10.76	34.47 ±3.23	26.90 ±3.23	
2 µg	6.72 ^{***} ±2.38	7.58 ^{•***} ±3.77	5.96 ^{***} ±2.38	4.38 ^{***} ±0.77	5.53 ^{•***} ±2.77	7.14 ^{***} ±4.76	6.33 ^{***} ±3.19	4.76 ^{•***} ±0.00	4.38 ^{***} ±0.77	4.38 ^{***} ±0.76	4.00 ^{***} 1.57	
4 µg	1.19 ^{***} ±2.38	6.33 ^{•***} ±1.80	5.52 ^{***} ±0.90	4.76 ^{***} ±0.00	4.38 ^{•***} ±0.81	1.19 ^{***} ±2.38	4.76 ^{***} ±0.00	4.00 ^{•***} ±0.90	4.76 ^{***} ±0.00	4.00 ^{***} ±0.90	2.38 ^{***} ±2.76	
6 µg	N.D.	7.93 ^{•***} ±2.85	7.14 ^{***} ±2.04	4.42 ^{***} ±0.76	4.76 ^{•***} ±0.00	2.38 ^{***} ±2.71	3.61 ^{***} ±2.50	3.19 ^{•***} ±1.28	N.D.	N.D.	N.D.	
8 µg	N.D.	8.76 ^{•***} ±4.00	2.38 ^{***} ±0.90	1.19 ^{***} ±2.38	3.57 ^{•***} ±2.38	4.76 ^{***} ±0.00	N.D.	N.D.	N.D.	N.D.	N.D.	
10 µg	N.D.	3.57 ^{•***} ±2.38	N.D.	N.D.	1.57 ^{•***} ±3.14	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	

a. Protozoans were detectable on day-2; b. Mean of four replicates \pm S.D.; N.D.: Not detected; •: Chloramphenicol supplied (20 $\mu\text{g}/\text{ml}$); Values with asterisks are significantly different from those in A; Student's 't' test ***: $P < 0.001$.

Table II: Diameter of the flagellates in μm grown in media containing various concentrations of Cr⁶⁺/ml.

Treat- ment	DAYS AFTER INOCULATION											
	2 ^a	3	4	5	6	7	8	9	10	11	12	
Control ^c	10.23 ^b ±0.36	9.42 ±0.70	9.67 ±0.44	7.65 ±0.30	7.65 ±0.32	7.42 ±0.00	6.47 ±0.74	6.78 ±0.47	7.32 ±0.07	7.32 ±0.07	6.18 ±0.81	
2 µg	10.91 ±0.07	10.98 ±0.07	11.27 ±0.10	11.16 ^{***} ±0.21	10.54 ^{**} ±0.74	10.65 ^{***} ±0.74	11.05 [*] ±0.03	11.05 ±0.03	10.98 ±0.07	10.95 ±0.03	10.95 ^{**} ±0.10	
4 µg	10.98 ±0.03	11.05 ±0.10	11.08 ±0.07	10.98 ±0.14	11.27 ^{***} ±0.43	11.05 [*] ±1.94	11.01 ±0.03	11.01 ±0.03	10.32 ±1.24	10.66 ±0.59	11.14 ±6.40	
6 µg	N.D.	10.98 ±0.03	11.08 ±0.07	10.94 ±0.10	11.05 ^{***} ±0.07	11.09 ±6.40	11.05 ±5.49	10.98 ±0.03	N.D.	N.D.	N.D.	
8 µg	N.D.	10.95 ±0.10	11.01 ±0.18	11.09 ^{***} ±0.54	10.25 [*] ±5.23	10.76 ^{***} ±0.58	N.D.	N.D.	N.D.	N.D.	N.D.	
10 µg	N.D.	10.24 ±5.34	N.D.	N.D.	11.35 ±6.51	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	

^a Protozoans were detectable on day-2; ^b Mean of four replicates \pm S.D. N.D.: Not detected; Values with asterisk(s) are significantly different from those in A. Student's t test *: P 0.05; **: P 0.01; ***: P 0.001.

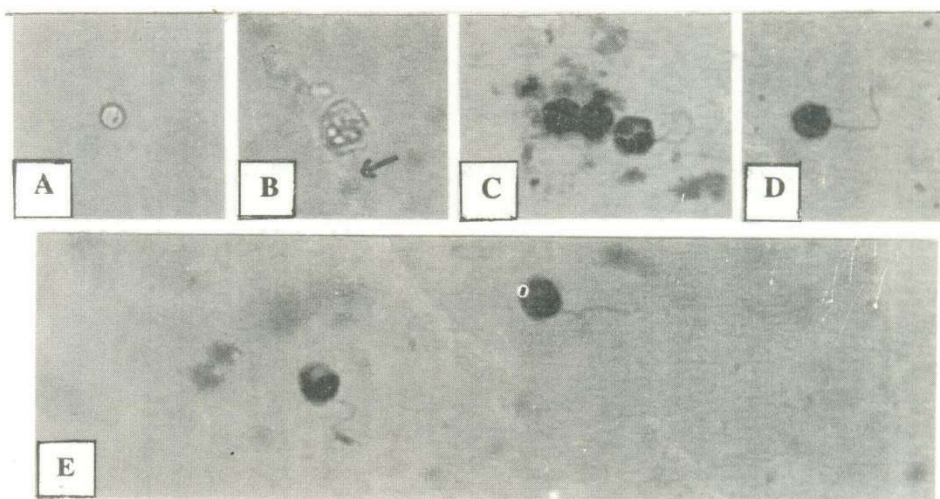


Fig. 1: Photomicrograph of unstained, living flagellates, immobilized with 1% methyl cellulose at 400X (A), at 1000X (B). Note various vacuoles within the cell, a flagellum (arrow), round bottom and flattened area at the base of flagellum are evident. The flagellates from a 10-day old culture (C): chromatin material and a flagellum are prominent. A flagellate from a 3-day old culture kept at usual light/dark cycle (D). Another view of the protist (E). Photomicrograph from C to E (1000X) represent Haematoxylin and eosin stained preparations.

Table III: Reduction of Cr^{+6} in the culture medium after 12 days of the flagellate's growth.

Cr^{+6} $\mu\text{g/ml}$ of culture fluid		% reduction of Cr^{+6}
At inoculation	After 12-days	
2	$0.08 \pm 0.17(4)^a$	95.82 ± 8.36
4	$0.92 \pm 1.26(4)$	77.25 ± 31.15
6	$2.74 \pm 0.47(4)$	54.43 ± 7.79
8	8.89 ± 0.65	- ^b
10	$10.10 \pm 0.20(3)$	-

^a: Mean \pm S.D; ^b: No decrease was observed. Number in parenthesis indicates sample size.

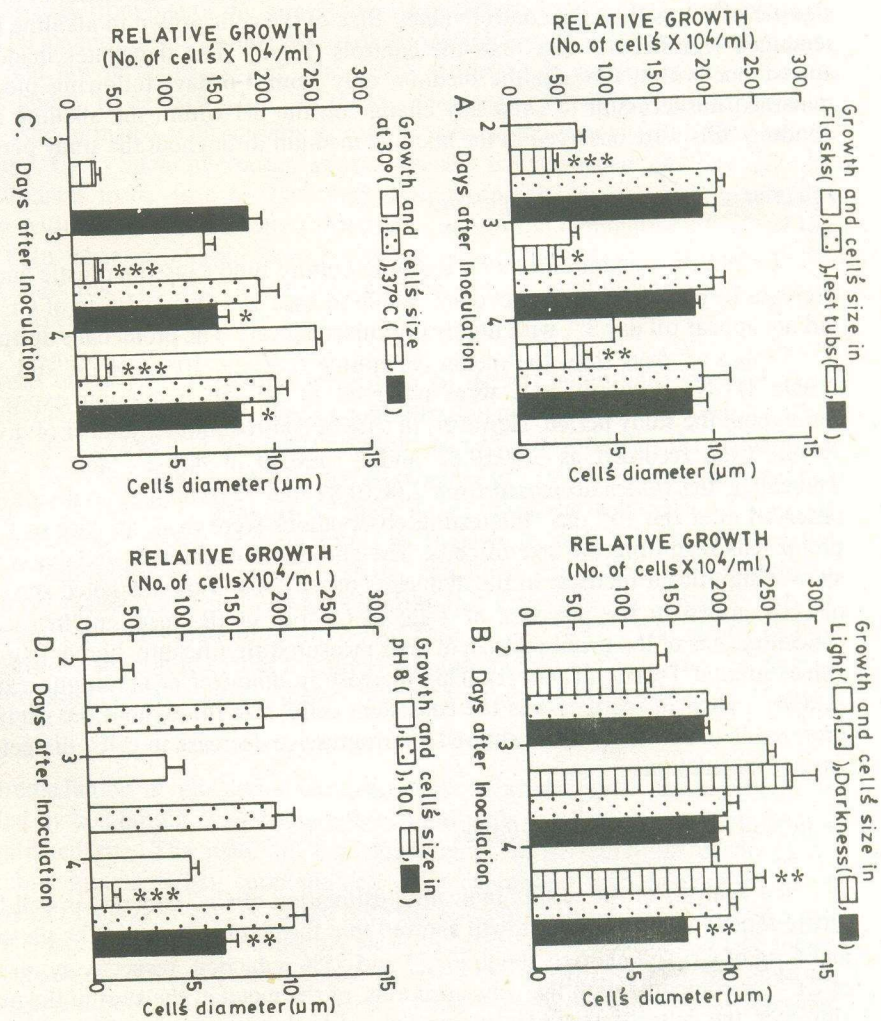


Fig. 2: Optimization of growth conditions of the flagellates: Effect of amount of dissolved oxygen (A), light (B), temperature (C) and pH (D). Values with asterisks (*) are significantly different from respective stage of first treatment. Student's 't' test *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

pH of freshly prepared medium was found to be about 8. Growth of the protozoans turned down this pH to a value around 7 after two days of inoculation, which then remained almost consistent for rest of the period. Appearance of growth was delayed in the alkaline medium (pH 10) till the day 4 after inoculation with number of the protozoans significantly less than the control values. Size of the cells grown in alkaline medium also remained significantly less than the controls (Fig.2). The flagellates made their weak appearance within the alkaline medium only from 4-6 days following inoculation and remained unsuccessful to exert any change on the pH within the alkaline medium. No dividing cells were observed in the alkaline medium throughout the study period.

Effect of Cr^{+6} on growth of flagellates

Cr^{+6} in an amount of 2 and 4 $\mu\text{g/ml}$ of culture fluid caused a drastic and significant decrease in growth of the protozoans. While in case of 6, 8 and 10 μg of Cr^{+6}/ml growth did not appear till day 3rd, with highly diminished levels. The protozoans disappeared after 9th, 7th and 6th days from the media containing 6, 8 and 10 μg of Cr^{+6}/ml , respectively (Table I). No dividing cells were observed in all the chromium exposed cultures, throughout the study period. However, in case of control culture number of dividing cell $\times 10^4/\text{ml}$ were recorded as 3.00 ± 0.82 and 3.75 ± 0.50 at day 2nd and 3rd, respectively. Thereafter, the values decreased from 2.00 ± 0.82 to 0.75 ± 0.50 and no dividing cells were observed after the 10th day. Interesting observations were made in case of Cr^{+6} exposed protozoans regarding the size of cells. The chromium exposed flagellates were found to show a significant increase in the diameters of the cells. This was noted specially in case of cells grown in the presence of 2 μg of Cr^{+6}/ml of the medium. In case of higher concentrations of the metal the size of cells measured significantly higher than the control values around 1-week (Table II). This increase in diameter of chromium exposed cells, actually indicated more or less the consistent cells' size throughout the study, while the protozoans in control cultures showed a retrogressive decrease in cells' diameter after few days of inoculation.

Reduction of Cr^{+6} by the flagellates

An analysis of the results indicating estimation of Cr^{+6} contents in cell free culture media following 12-days of growth showed that flagellates grown in the presence of 2, 4 and 6 μg of Cr^{+6}/ml showed about 96, 77 and 55% reduction, respectively, in the amount of Cr^{+6} , as compared with the concentrations, of the metal at the start of the experiments. However, the cultures grown in the presence of higher amounts of the heavy metal i.e., 8 and 10 $\mu\text{g/ml}$ were found unable to exert any reducing effect. Rather, some slightly higher amounts of the metal possibly due to death of the protists and subsequent release of Cr^{+6} into the culture fluids, were recorded (Table III).

DISCUSSION

Effluents of the study area were found to harbour a number of microorganisms. A flagellate protozoan from one of the samples was successfully grown under the laboratory conditions. The axenic culture of the protozoan responded negatively to alkaline nature of the medium and low levels of oxygen. The axenic culturing of the protist was aimed at exploring its ability to detoxify Cr^{+6} . As these microorganisms were obtained from an environment contaminated with varying concentrations of the heavy metal, they indeed were tolerating Cr^{+6} . Such microorganisms from the contaminated environment are potential candidates to be used for bioremediation processes. An organism essentially plays its role within its natural habitat differently, due to the cumulative effects of the community, than what is inferred from its axenic/pure culture study. However, if the purpose is to study role played by a particular microorganism, establishing axenic culture then becomes necessary.

The results of the present study indicate that the heterotrophic flagellate protozoan was able to tolerate and detoxify Cr^{+6} upto a concentration of 6 $\mu\text{g/ml}$ in the medium. This tolerance level corresponded to the concentrations of Cr^{+6} prevailing in the effluents of study area, that have been found from 3-6 $\mu\text{g/ml}$ (Qazi *et al.*, 1997). But in case of higher concentrations of the metal i.e., 8 and 10 $\mu\text{g/ml}$ of the medium, the isolated protozoans did not show considerable growth, both in terms of number as well as size of the cells. Likewise, the protozoans, in the present study, remained completely unsuccessful to reduce the heavy metal (Cr^{+6}) in higher concentrations. Chromium is known to inhibit ciliate protozoans' growth (Madoni *et al.*, 1996; Qazi *et al.*, 1997). The results of this investigation bring a support to earlier findings that higher levels of chromium are toxic to a variety of organisms (Nieto *et al.*, 1989; Nair and Krishnamurthi, 1991; Lansdown, 1995).

For bioremediation of metal-contaminated sites, microbial mats' construction has been suggested by combining cyanobacteria inoculum with a sediment inoculum from a metal contaminated site. The mats are held together by slimy secretions produced by various microbial groups. When contaminated water containing high concentrations of metals such as Cd, Pb and Cr etc., is passed over the microbial mats immobilized on glass wool, there is rapid removal of the metals from the water due to deposition of metal compounds outside the cell surfaces as well as chemical modification of the aqueous environment surrounding the mats (Bender *et al.*, 1995). The flagellate protozoan reported here can be employed for the development of such strategies to control the water pollution through bioremediation. This notion is supported from previous studies on the same area, which have indicated many microbes resisting varying levels of concentrations of Cr^{+6} (Qazi *et al.*, 1997; Haq *et al.*, 1998). Further work is needed to optimize growth conditions and thus to enhance the efficiency of the microorganisms, in term of detoxification (reduction) of Cr^{+6} , inhabitants of the tanneries' effluents, including bacteria and protozoa.

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(Received: June 24, 2000)