African Journal of Biotechnology Vol. 10(45), pp. 9101-9113, 17 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2405
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Characterization of copper resistant ciliates: Potential candidates for consortia of organisms used in bioremediation of wastewater

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Accepted 25 May, 2011

Metals are environmental pollutants of major concern due to their ecological, sanitary and even economic consequences. Both prokaryotes and eukaryotes inhabiting such environments carry cellular systems that maintain the metal homeostasis. The ciliate protists tolerate elevated concentrations of metals, which are accumulated, bound to metallothioneins (MTs) peculiar to these organisms. Copper is one of such contaminant found in the wastewater of local industries. The concentrations of copper which caused 50% reduction (LC_{50}) in the cell population of *Tetrahymena* sp RT1, and two *Euplotes* spp. RE-1 and RE-2, isolated from the industrial waste, were found to be 60, 48 and 49 ppm, respectively, compared to those of the cultures without copper in the media. RT-1 showed significantly high tolerance to copper ions and could uptake 52.66% of the copper ions from the medium. The axenic culture of RT-1 could uptake 61.2% of copper from the medium compared to 68.41 and 59.16% by the ATCC culture of *Tetrahymena thermophila* and *T. pyriformis*, respectively. RT-1 tolerated about 500 µM copper in the medium without affecting its movement. This ciliate showed promise as a member of the consortium used for bioremediation of copper contaminated wastewater.

Key words: Copper toxicity, metallothionein, growth curve of ciliates, metal uptake, bioremediation.

INTRODUCTION

Industrialization has led to increased emission of pollutants into the ecosystems (Diagomanolin et al., 2004). Heavy metals in wastewater come from industries and municipal sewage, and are one of the main causes of water and soil pollution. Accumulation of these metals in wastewater depends on the type of industries in the region, and careless disposal of wastes (Shakoori et al., 2004; Chipasa, 2003). Usually, no vegetation and consequently no animal life exist in areas where there is frequent dumping of industrial effluents and wastes. The organisms, if present, employ a variety of strategies to reduce heavy metal toxicity depending on the nature of the heavy metals and the organism under stress. They have developed tactics to resist, tolerate, metabolize and detoxify these substances (Nagel et al., 1996; Prasad et al., 1998; Haq and Shakoori, 2000; Silver and Phung, 2005). They remove toxic metal ions via adsorption to cell surfaces (Mullen et al., 1989), binding with cell envelopes (Flatau et al., 1987), intracellular accumulation (Laddaga and Silver, 1985), complex formation by exopolysaccharides (Scott and Palmer, 1988), biosynthesis of metallothioneins and other proteins (Robinson et al., 2001) and transformation to more volatile forms (Robinson and Tuovinen, 1984).

In contrast to conventional chemical and physical methods, such as precipitation, adsorption, electrodialysis and reverse osmosis, biological methods of heavy metal removal using bacteria (Branco et al., 2004), algae (Rehman and Shakoori, 2001), yeast (Shakoori et al., 2005), actinomycetes (Wong and So, 1993) and plant tissues (Chen et al., 1996) have several potential advantages (Wilde and Benemann, 1993). Most studies have been done using fungi and bacteria (Pas et al., 2004; Campos et al., 2005; Sannasi et al., 2006), but relatively few studies (Rehman et al., 2006) are available on metal interactions in protist ciliates. Ciliated protozoa

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are very common in aquatic environments and in all types of aerobic and anaerobic biological treatment systems (Pauli et al., 2001). The long term survival of protozoa in media containing relatively high concentration of heavy metal ions shows that these organisms have strategies to tolerate, resist or detoxify organic substances and heavy metals (Shakoori et al., 2004). Along with all other organisms, protozoa must also be contributing in the consortium to either accumulate or efflux the metals (Haq and Shakoori, 2000; Muneer et al., 2009).

Among protozoa, ciliates are good candidates for use as whole cell biosensors to detect the presence and determine the bio-available concentrations of heavy metal ions in natural samples (Martin-Gonzalez et al., 2006). Keeping in mind the importance of ciliates in the bioremediation of industrial waste (Haq and Shakoori, 2000; Muneer et al., 2009), and as model eukaryotic organisms, this study was undertaken to determine the efficiency of ciliate isolates in processing and or uptake of copper ions from growth medium. This efficiency qualifies isolates to be part of the consortium of organisms used for remediation of copper contaminated wastewater.

MATERIALS AND METHODS

Culture collection

Cultures of ciliate protozoa were taken from Cell and Molecular Biology Laboratory (CMBL) of Departrment of Zoology, University of the Punjab, Lahore. Two of these ciliates, *Tetrahymena* RT-1 and *Euplotes* RE-1, were isolated from the tannery effluents of Kasur, and one, *Euplotes* RE-2, from industrial effluents of Sialkot. Initial identification of protozoa was done by observing their body shape, morphological features, movements and behavior (Edmondson, 1966; APHA, 1989; Curds, 1982; Curds et al., 1983) using a ZEISS Axiostar plus microscope. Axenic cultures of *Tetrahymena thermophila* B2-7a (Tt, ATCC No. 30307) and *T. pyriformis* phenoset A (Tp, ATCC No. 30327) were obtained from American Type Culture Collection (ATCC).

Culture maintenance

The ciliate cultures RT-1, RE-1 and RE-2 (a drop of 5 µl), were introduced separately in 250 ml sterilized Erlenmeyer flasks containing 50 ml Bold-basal salt medium ($NaNO_3$ 0.25 g/l, $CaCl_2.H_2O$ 0.025 g/l, $MgSO_4.7H_2O$ 0.075 g/l, K_2HPO_4 0.075 g/l, KH_2PO_4 0.175 g/l, NaCl 0.025 g/l, EDTA 0.05 g/l, KOH 0.031 g/l, $FeSO_4.7H_2O$ 0.04 g/l, H_2SO_4 0.001M, H_3BO_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, with 5 to 7 wheat grains (Shakoori et al., 2004). The pH of the medium was adjusted at 7.3 to 7.6 and kept at room temperature (27±2°C) in ambient light. The growth of cultures was observed daily by counting the number of protozoan cells in the medium under a light microscope, using a haemocytometer. The medium was renewed regularly after a week in the case of RT-1, and after two weeks in the case of RE-1 and RE-2.

The ATCC cultures were maintained in *Tetrahymena* specific medium, ATCC medium 357 (proteose peptone 0.5%, tryptone 0.5%, K_2HPO_4 0.02%). Their growth was observed periodically by counting the number of organisms in the medium under a light microscope. The cultures were aseptically inoculated (inoculum's size = 10 µl) to a fresh flask containing 50 ml of sterilized ATCC 357 medium (pH 7.2, 27±2°C).

Determination of growth

The growth curves of all three ciliates (RT-1, RE-1 and RE-2) were determined in different media, *viz*., wheat and rice grain medium (50 ml tap water containing a few boiled wheat and rice grains), LB medium (tryptone 1%, yeast extract 0.5%, NaCl 0.5%), PY medium (proteose peptone 2%, yeast extract 0.1%), ATCC medium 357 and Bold-basal salt medium (five to seven wheat grains were added per 50 ml). The pH of each medium was adjusted at 7.2 to 7.5. Three sterilized 250 ml flasks with 50 ml of each medium were inoculated with 10 µl of culture containing 40 to 50 cells and incubated at 28 ± 2°C. The growth of the ciliates was observed by using a haemocytometer every day for 5 days in the case of RT-1, and 8 days in the case of RE-1 and RE-2. Readings in triplicate were taken and their means were calculated (Haq et al., 2000) for preparation of growth curves.

Copper resistance and determination of LC_{50}

To study the effect of copper on growth of RT-1, RE-1 and RE-2, two sets of culture were prepared. Each of the three sterilized 250 ml flasks containing 50 ml of Bold-basal salt medium (pH 7.5) was supplemented with 5 to 7 wheat grains, inoculated with culture containing 40 to 50 cells and incubated at 28±2°C. In the control set of cultures, no metal ions were added to the medium. In the treated set, copper ions at 10 µg/ml concentration were added from a stock solution of $CuSO_4.5H_2O$ (50 mg/ml of $Cu^{+2)}$ for up to eight days in treated set of cultures. Organisms were counted every 24 h by haemocytometer, and growth curves were prepared. LC_{50} was determined for each ciliate. The concentration of copper at which cell population was reduced to 50% compared to that of untreated (control) culture, was considered as LC_{50}.

Determination of copper uptake ability

Copper uptake ability was determined in three sets of 500 ml flasks; positive control (with metal ions but without ciliates), negative controls (without metal ions but with ciliates) and treated cultures (with metal ions and ciliates). These experiments were performed in triplicate, and each of the three flasks contained 100 ml Bold-basal salt medium (pH 7.5) supplemented with 8 to 9 wheat grains. Ciliate cultures were grown in the negative controls and treated flasks by inoculating log phase growing ciliates (40 to 60 organisms) at 28±2°C. Copper was added to the positive controls and treated sets of flasks by adding a stock solution of $CuSO_4.5H_2O$ according to the LC_{50} calculated for each ciliate. Samples were removed from each flask of RT-1 after 24, 48, 96, 120 and 168 h and from each flask of RE-1 and RE-2 after 48, 72, 96, 120, 168 and 192 h of incubation. Samples were centrifuged at 9803 x *g* (Beckman Coulter Allegra™ 25R Centrifuge) for 10 min and the supernatants collected in separate tubes. Pellets were washed with 0.8 to 0.9% NaCl and digested with concentrated HNO_3. Copper concentrations in the cell free extracts and in the digested pellets were determined by atomic absorption spectrophotometry (Thermo Unicam-969, Solaar, LabX, ON, Canada) using an air-acetylene flame. Reduction in the amount of copper ions in the medium containing ciliates, as well as increases in copper concentrations in the pellet, was interpreted as

copper processing abilities of the organisms. The percent reduction in the amount of copper ions in the supernatant was also calculated and compared with the percentage increase in copper in the pellet.

Purification of *Tetrahymena* RT-1

Among the three locally isolated ciliates, *Tetrahymena* RT-1 showed the most significant tolerance of copper. This culture contained both bacteria and seasonal contamination of algae, yeasts and fungi. In order to obtain an axenic culture, algae were excluded by keeping the cultures in darkness. Yeasts were excluded by removing organic substances from the culture medium. The culture was plated on potato dextrose agar (PDA) to check for fungal growth. Three antibiotics *viz*., ampicillin (25 µg/ml), chloramphenicol (35 µg/ml), and gentamycin (25 µg/ml) were added to the medium to prevent the growth of bacteria.

Optimization of growth conditions

Optimum temperature

To ascertain the optimum growth temperatures for RT-1, sterilized Bold-basal salt medium (50 ml) supplemented with 0.1% glucose, was inoculated with 40 to 50 RT-1 cells and incubated at different temperatures *viz*., 15, 20, 25, 30, 37 and 42°C for 5 days. Growth was assessed by counting the number of organisms at each temperature, every day, under light a microscope.

Optimum pH

For the determination of optimum pH for RT-1, Bold-basal salt medium (50 ml) containing 0.1 % glucose, at different pH *viz*., 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 was inoculated with 40 to 50 log phase growing RT-1 cells and incubated at 25±2°C for 5 days. Growth was determined by counting the number of organisms daily at each pH, under a light microscope.

Growth conditions were also optimized in ATCC medium 357. The experiments were conducted at 15, 20, 25, 30 and 37°C to optimize the temperature and at pH 6.5, 7.0, 7.5 and 8.0 to determine optimum pH.

RESULTS AND DISCUSSION

Growth of ciliates in different media

Ciliate RT-1 exhibited maximum growth on day 2 (Figure 1A) in all five media tested. The number of cells increased from 4 x 10^4 cells/ml at the time of inoculation to 3.6 x 10^6 cells/ml (a 90-fold increase) in Bold-basal salt medium; from 3.8 x 10^4 to 2.2 x 10^6 cells/ml (a 57.8-fold increase) in LB medium; from 4 x 10^4 to 2.0 x 10^6 cells/ml (a 50-fold increase) in PY medium, and from 4.2 x 10^4 to 3.8 x 10^6 cells/ml (a 90.4-fold inrease) in ATCC medium 357, after 48 h of incubation. The RT-1 culture showed least growth in wheat and rice grain medium (that is, from 4.1 x 10^4 cells/ml to 9.44 x 10^5 cells/ml (a 22.8-fold increase). Thus, RT-1 can be grown most successfully in either Bold-basal salt medium or ATCC medium 357.

The lag phases of RE-1 and RE-2 (Figure 1B, C) were longer than that of RT-1. Both ciliates showed maximum growth in Bold-basal salt medium. RE-1 attained maximum growth on day 4 in Bold-basal salt, LB and PY media in which the cell number increased from 4 x 10^2 cells/ml at the time of inoculation to 3.2 x 10^4 cells/ml (a 80 –fold increase); from 3.8 x 10^2 to 2.0 x 10^4 cells/ml (a 52-fold increase); and from 3 x 10^2 to 1.8 x 10^4 cells/ml (a 60-fold increase) respectively, after 4 days of incubation. In ATCC medium 357 (which is *Tetrahymena* specific medium), RE-1 exhibited the fastest growth rate (a 125-fold increase in cell numbers; from 2 x 10^2 to 2.5 x 10^4 cells/ml) after five days of incubation. Similarly, in wheat and rice grain medium, the culture attained maximum growth on day 5, although, the increase in cell counts was only about 30-fold (from 3.1 x 10^2 to 8.86 x 10^3 cells/ml).

In contrast to RE-1, the ciliate RE-2 attained maximum growth on day 4 in all five media. Again, Bold-basal salt medium resulted in the best growth that is, an 89.47-fold increase (from 3.8 x 10^2 cells/ml at the time of inoculation to 3.4 x 10^4 cells/ml on day 4). The order of preference in the remaining four media was ATCC medium 357 > PY medium > LB medium > wheat and rice grain medium. Thus, Bold-basal salt medium was shown to be the most preferred, while wheat and rice grain medium, the least favorable for maintenance, growth and metal uptake experiments on RE-1 and RE-2.

Generally, protozoan cultures grow in mineral salts medium supplemented with organic compounds (Holz, 1964; Weekers and Vogels, 1994). All three ciliates examined in this study, RT-1, RE-1 and RE-2, exhibited maximum growth on Bold-basal salt medium which is consistent with the ciliates ability to synthesize their bio-molecules utilizing these metal ions as essential requirement or by utilizing scant organic molecules released by other organisms in the culture medium (Rehman et al., 2005). Many protozoa can switch from one source of nutrition to another if circumstances change, indicating their adaptability which is perhaps why RT-1 showed good growth on ATCC Medium 357.

Effect of copper on ciliate growth

Figure 2 shows growth curves of axenic cultures of the ciliates RT-1, RE-1, and RE-2 in Bold-basal salt media with no added copper and in copper-containing media at optimum conditions (pH 7.5, 28±°C). A gradual increase in the number of cells, both in the control and treated culture was obvious. However, the lag phases were prolonged in all the three copper-containing ciliate cultures. RT-1 reached maximum growth on day 4 in the copper containing medium as against day 2 in the control culture medium. After 7 days of incubatuion in the copper-amended cultures, the cell population of RT-1 was reduced by 34% compared to the control (Figure 2A). Thus, in the presence of copper, the growth of RT-1 was

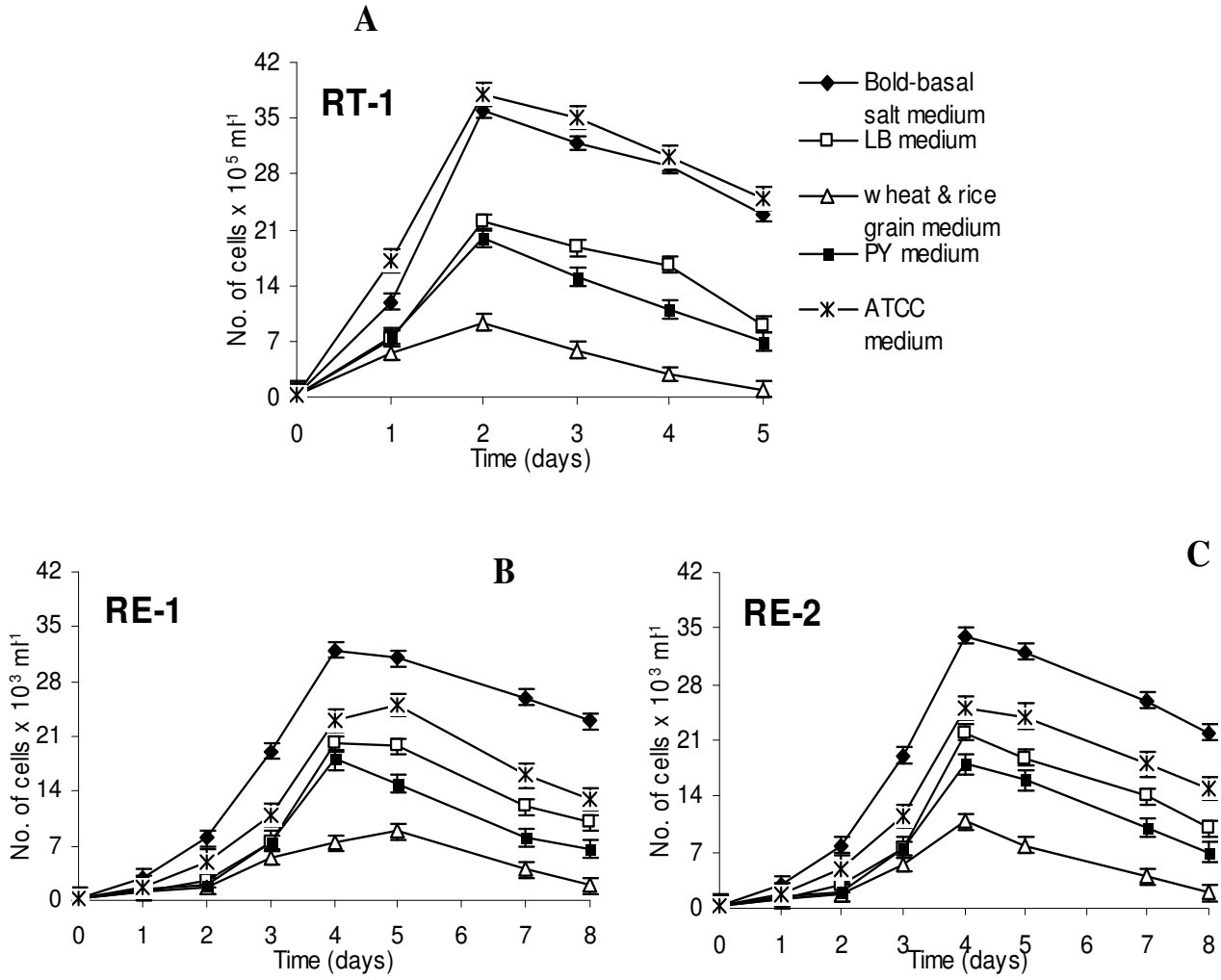

Figure 1. Growth curves of ciliates *Tetrahymena* RT-1 (A), *Euplotes* RE-1 (B), and *Euplotes* RE-2 (C) in different media at pH 7.2-7.5 and 28 ± 2°C. ♦, Bold-basal salt medium; □, LB medium; ∆, wheat and rice grain medium; ■, PY medium; and -x-, ATCC medium 357.

affected to such an extent that growth rate remained half of that of the copper-free cultures. The maximum cell count achieved during the growth cycle in cultures with 60 ppm of copper was $16x10^5$ cells/ml, as against $32x10^5$ cells/ml, in the control culture (without copper).

The LC_{50} of copper was found to be 48 ppm against RE1 and 49 ppm for RE2. The maximum growth for RE-1 was attained on day 7 in the copper-treated cultures as opposed to day 5 for the control cultures (Figure 2B). In RE-2, maximum growth in the presence of copper was realized on day 5 while it was obtained on day 4 in control (Figure 2C). The overall decrease in the maximum cell counts of RE-1 and RE-2 in copper-containing media (48 and 49 ppm, respectively) was, 2.4- and 2.15-fold, respectively, on days 7 and 5.

Metal tolerance is the maximum metal concentration, at which organisms survived and multiply. It is very difficult to establish comparisons of the toxic effects of heavy metals among the reported studies using ciliates due to the diversity of the experimental conditions (Nilsson, 1989; Martin-Gonzalez et al., 1999; Gutierrez et al., 2003). This study reports a general trend in decreased cell growth with increasing copper concentration in the medium. It has previously been reported from other laboratories (Sudo and Aiba, 1973; Brady et al., 1994) that a concentration of 25 to 27 ppm copper reduced the growth of *V. microstoma* and *Opercularia* sp. from activated sludge by 50%. Yamaguchi et al. (1973) reported complete inhibition of *T. pyriformis* growth at 1 ppm copper. Salvado et al. (2001) reported that the population of ciliate *Euplotes affinis* did not decrease after 24 h exposure to 5 to 10 ppm copper and over a period of 36 to 48 h, it increased its population, which is considered as evidence of gradual succession of species.

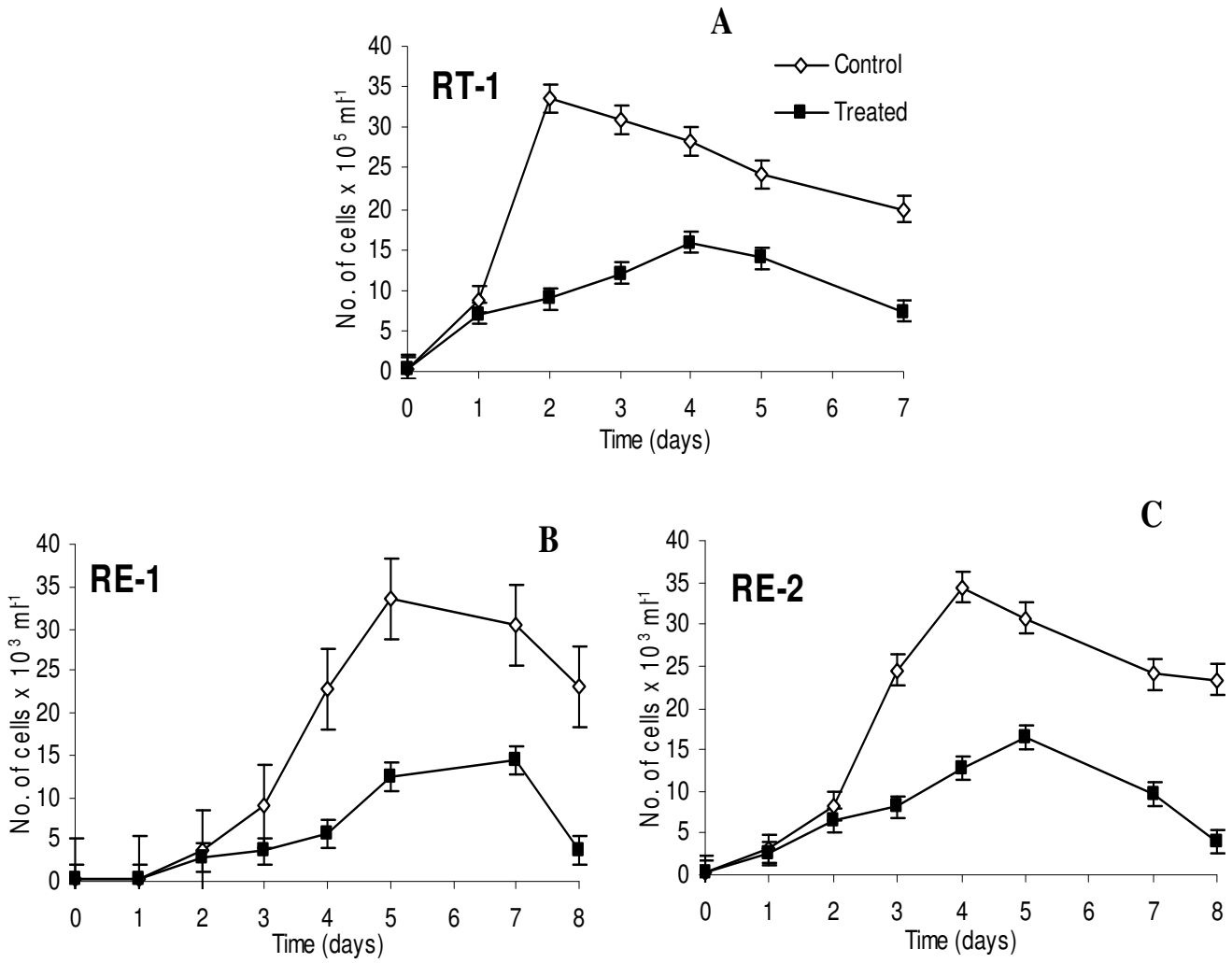

Figure 2. Effect of copper on the growth of ciliates *Tetrahymena* RT-1 (A), *Euplotes* RE-1 (B) and *Euplotes* RE-2 (C) in Bold-basal salt medium at pH 7.5 and 28 ± 2°C (◊, Control; ■, Cu^{2+} treated).

Table 1. Half lethal concentration (LC_{50}) values of copper against Tetrahymena RT-1 and Euplotes RE-1 and Euplotes RE-2 in Bold-basal medium supplemented with wheat grains at pH 7.5 and 28±2°C.

Ciliate	LC_{50}
RT-1	942 µM (= 60 ppm)
RE-1	758 µM (= 48 ppm)
RE-2	765 µM (= 49 ppm)

Repetitious growth in the medium containing the same copper concentration resulted in improved growth, arguing for a mechanism of tolerance by adaptation (Brady et al., 1994).

LC_{50} values of copper against the three ciliates are summarized in Table 1. The ciliates used in this study can tolerate rather high copper concentrations compared to previous reports from other laboratories (Nilsson, 1989; Madoni et al., 1992; Gilron and Lynn, 1998). *T. pyriformis* has been reported to be the ciliate, most resistant to copper (Nilsson, 1989; Schaefer et al., 1994; Nicolau et al., 2001). Madoni et al. (1992) has reported that copper is generally more toxic to ciliate populations than Cd, Hg or Zn. According to them, the LC_{50} after 24 h ranged between 0.01 and 0.02 ppm in the tested species, while *Euplotes mutabilis* showed maximum resistance against copper at a concentration of 22 µg/l (0.022 ppm).

Copper uptake

Copper uptake by RT-1 was determined by inoculating 100 ml Bold-basal salt medium (pH 7.5) containing 8 to 9

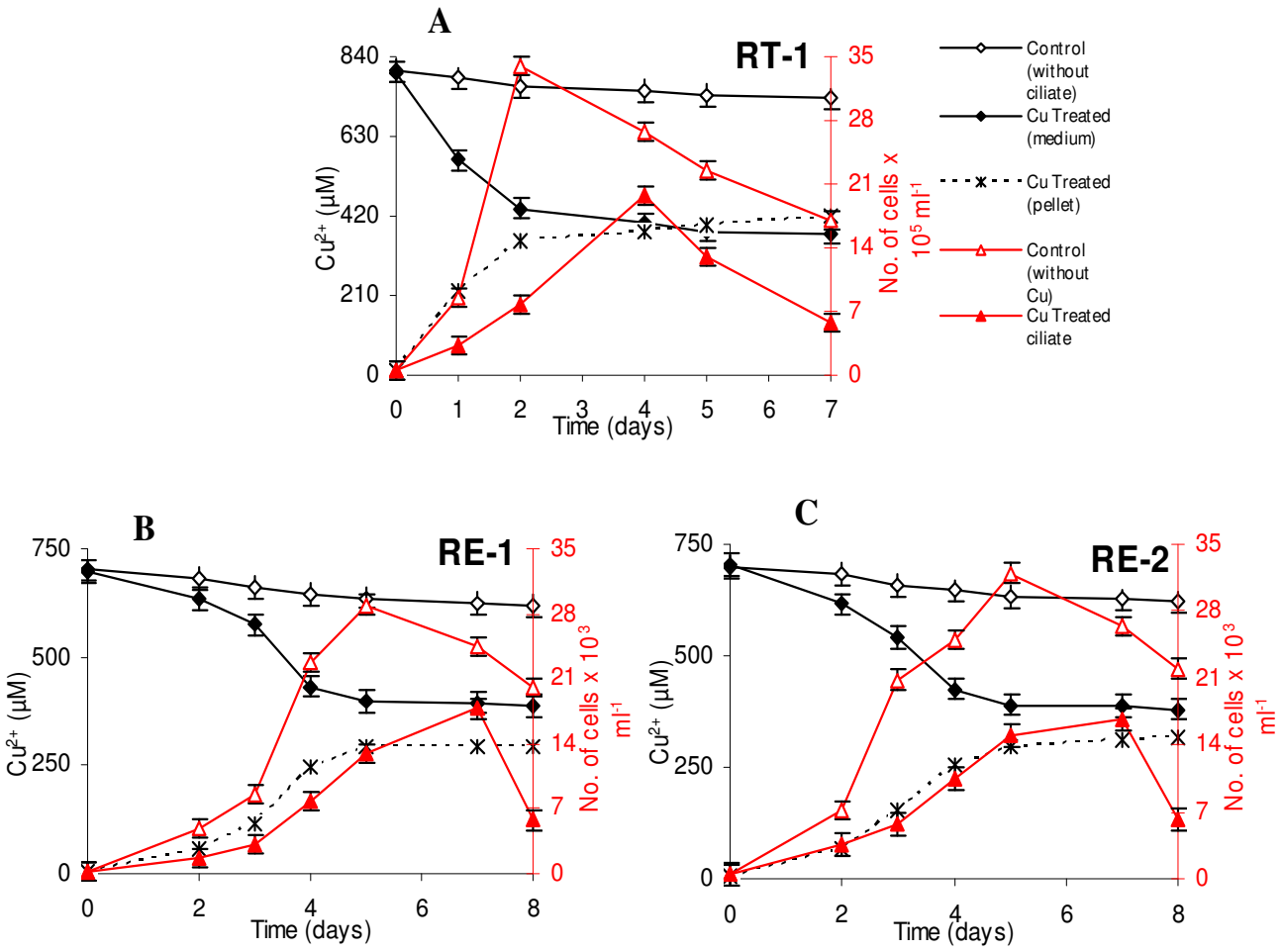

Figure 3. Removal of copper ions by *Tetrahymena* RT-1 (A), *Euplotes* RE-1 (B), and *Euplotes* RE-2 (C) from Bold-basal salt medium containing copper (800 µM for RT-1 and 700 µM for RE-1 and RE-2) and 8-9 wheat grains per 100 ml (pH 7.5 and 28 ± 2°C). [◊, level of Cu^{2+} in positive control (without ciliate); ♦, copper ions in treated (with ciliates) medium; and -x-, copper ions in treated ciliate cells (pellet)]. Effect of copper on their growth is also shown in red (Δ, negative control (without copper) and ▲, copper treated).

wheat grains and 800 µM (51 µg ml^{-1}) Cu^{2+} with 10 µl of log phase ciliates at 28 ± 2°C. The control RT-1 culture had 4.8 x 10^4 cells/ml on day 1 and reached its maximum growth on day 2 (Figure 3), however, when Cu^{2+} was added, the maximum number was reached on day 4. After seven days of growth, RT-1 population was 33.88% smaller than the control, and the concentration of copper in the medium containing RT-1 was reduced by 63% (Figure 3A), which is the amount of copper absorbed by the ciliate from the culture medium in 7 days .

The control RE-1 culture contained 2.8 x 10^2 cells/ml on day 1, which increased to 2.9 x 10^4 cells/ml on day 5 of incubation. However, in the presence of 700 µM (46 ppm) Cu^{2+}, the number increased from 3.4 x 10^2 cells/ml on day 1 to 17.76 x 10^3 cells/ml on day 8 (Figure 3B). Thus, in the presence of 700 µM copper, RE-1 cell count was 71% lower compared to the control cell population, after eight days of growth. The ciliate removed about 56% Cu^{2+} from the medium in eight days.

Likewise in the RE-2 control culture, the number of cells increased from, 3.7 x 10^2 cells/ml on day 1, to 3.2 x 10^4 cells/ml on day 4. In the presence of 700 µM of copper ions (46 ppm), the cell count increased from, 3.8 x 10^2 cells/ml on day 1, to 6.3 x 10^3 cells/ml on day 8 of incubation. Thus, in copper treated culture, the cell count was about 71% lower than the control culture. RE-2 removed about 57% in 8 days.

Figure 4 shows the comparison of copper ion concentration in supernatant *vs.* pellet in ciliate RT-1, RE-1 and RE-2. A gradual decrease in copper ion concentration in supernatant and increase in pellet, with time, indicated metal uptake by the ciliate. Out of the three ciliate cultures, only RT-1 removed copper ions more efficiently during the log phase of growth. Metal uptake was more

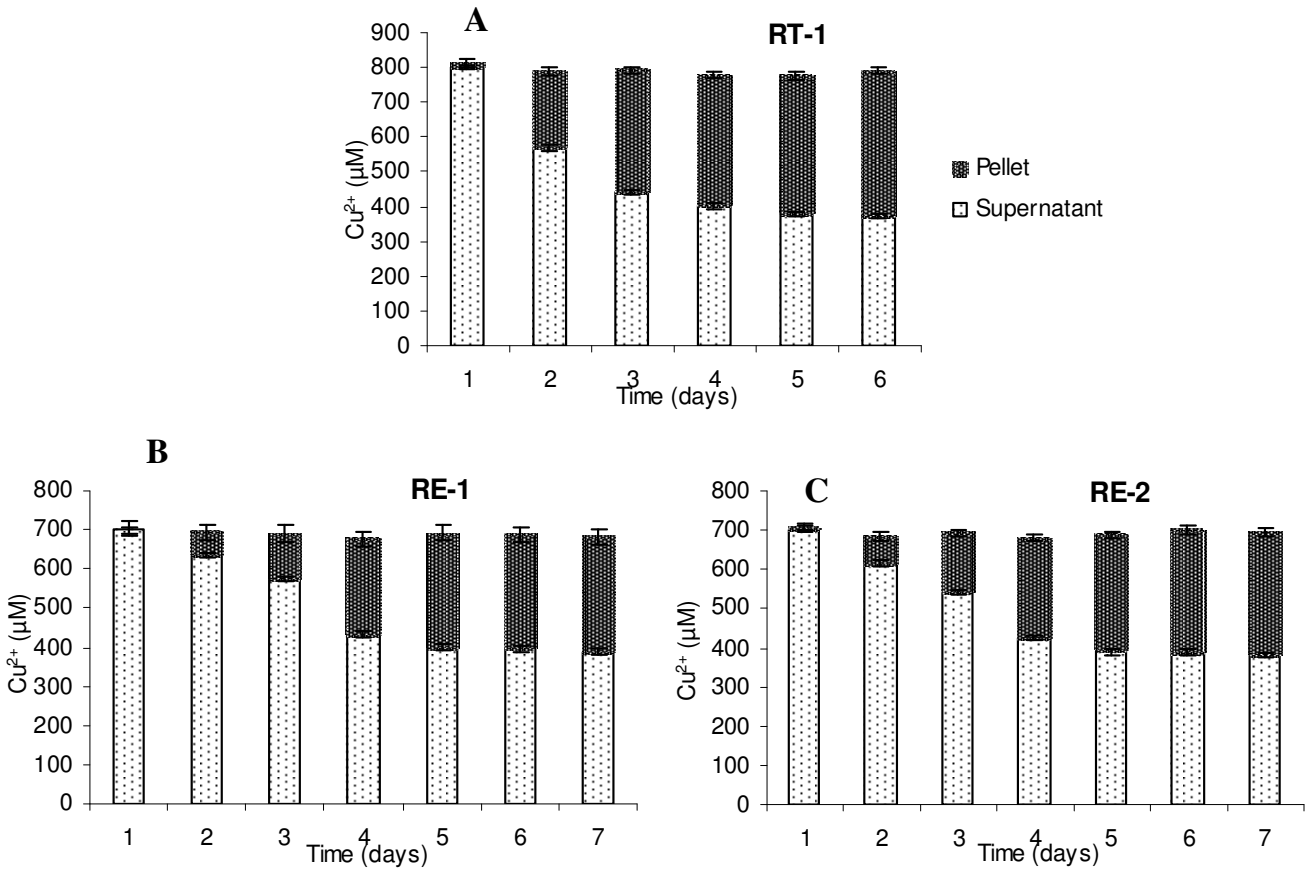

Figure 4. Uptake of copper ions by *Tetrahymena* RT-1 (A), *Euplotes* RE-1 (B), and *Euplotes* RE-2 (C) from the Bold-basal salt medium (pH 7.2 and 28 ± 2°C) containing copper (800 µM for RT-1 and 700 µM for RE-1 and RE-2) and 8-9 wheat grains per 100 ml. There is gradual decrease of copper ion concentration in the supernatant with time with concomitant increase in the pellet.

efficient until day 3 in the case of RT-1, when 63% copper was removed from the medium. In the case of RE-1, the copper uptake was more efficient till day 5 when 56% copper ions were removed from the medium, whereas, RE-2 removed 57% copper ions from the medium. Both the species of *Euplotes* showed maximum cell count on day 7 of incubation, whereas RT-1 showed maximum cell count on day 4 in the presence of 60 and 48 ppm copper ions in the medium, respectively.

Metal uptake by microorganisms is a complex process that depends on the chemistry and concentration of the metal ions, the specific surface properties of the organisms, cell physiology and physicochemical influences of the environment (for example, pH, and temperature). The metal uptake process usually involves adsorption of metal ions at the cell wall or cell membrane via interactions with functional groups and/or transport into the cell with subsequent chemical changes, like reduction, oxidation etc. Microbial removal of heavy metals invoves biosorption, which is a metabolism-independent binding of heavy metals to anything, whereas bioaccumulation is an active process requiring metabolic activity of living organism. The activation energy for bioaccumulation (≈ 63 kJ/mol, Davis et al., 2003) corresponds to a biochemical process. This process involves transport of metal ions across the cell membrane and its subsequent transformation.

All the three ciliates checked for accumulation of copper from the aqueous medium, showed good copper accumulation capabilities. RT-1 was found to take up 44% copper from the medium after two, and 53% after seven days. RE-1 and RE-2 accumulated 42.3 and 45% copper in 8 days, respectively (Table 2).

Growth and characterization of *Tetrahymena* RT-1

RT-1 could be maintained easily under laboratory conditions. It showed high tolerance to copper ions (up to 800 µM). An axenic culture was prepared and growth conditions were optimized. For comparison, copper uptake by two ATCC cultures of this genus, *T.*

Table 2. Removal of copper ions by the ciliates (*Tetrahymena* RT-1, *Euplotes* RE-1 and *Euplotes* RE-2) from Bold-basal salt medium containing copper (800 µM for *Tetrahymena* RT-1 and 700 µM for *Euplotes* spp.) and 8-9 wheat grains per 100 ml (pH 7.5 and 28 ± 2°C). The table indicates the copper concentration removed from the medium and that taken up by the ciliates.

Organism	Day	Amount of copper (µM)		
		In the medium	Removed from the medium	Detected in the organism
T-1	0	800.8	13.7	12.67
	2	440.4	360.4	351.7
	7	370.8	430.0	419.4
RE-1	0	698.2	11.8	4.0
	4	432.6	265.6	245.2
	8	388.0	310.2	295.3
RE-2	0	700.4	11.0	6.3
	4	425.7	274.6	255.2
	8	380.0	320.4	315.3

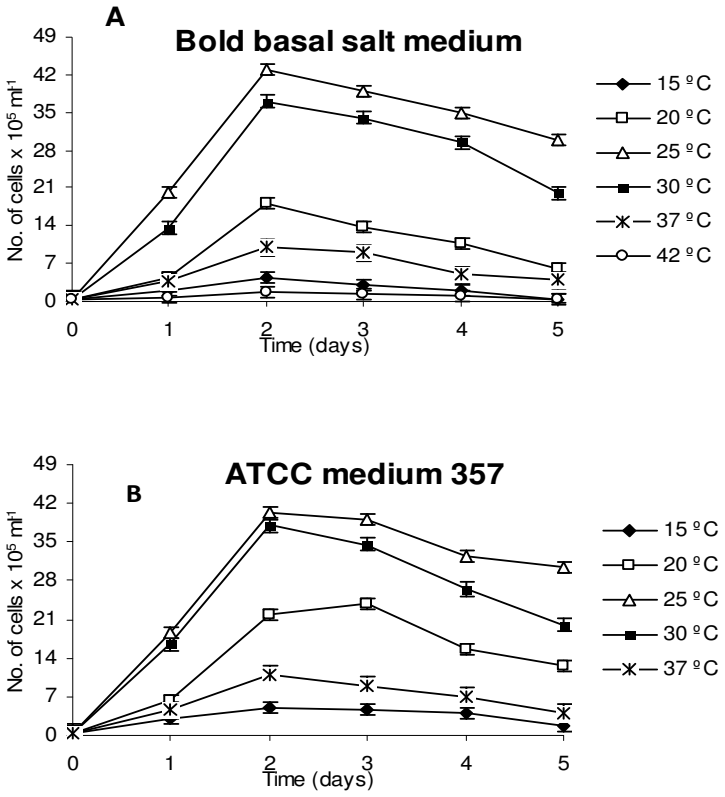

Figure 5. Growth curves of *Tetrahymena* RT-1 in Bold-basal salt medium (A), and ATCC medium 357 (B) at different temperature, *viz.*, 15°C (♦), 20°C (□), 25°C (Δ), 30 ^{0}C (■), 37°C (-x-), and 42°C (○).

thermophila and *T. pyriformis,* was also studied. RT-1 showed maximum growth at 25°C in both Bold-basal salt medium as well as ATCC 357 medium (Figure 5). The growth was retarded at temperatures above 30°C and below 20°C.

The optimum pH for the growth of RT-1 was found to

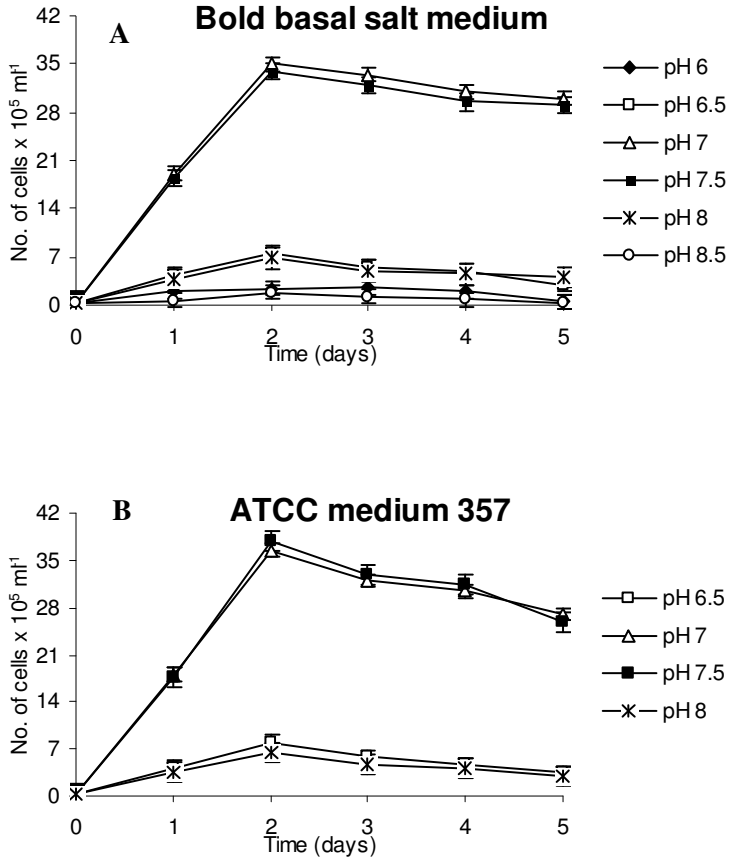

Figure 6. Growth curves of *Tetrahymena* RT-1 in Bold-basal salt medium (A), and ATCC medium 357 (B) at different pH, *viz.*, 6 (♦), 6.5 (□), 7 (Δ), 7.5 (■), 8 (-x-), and 8.5 (○).

be pH 7 in Bold-basal salt medium and pH 7.5 in ATCC 357 medium (Figure 6). In contrast to temperature, RT-1 was found highly sensitive to even a slight change in the pH. Maximum cell counts were obtained at pH 7 and 7.5. At pH 6.5 and 8, the number of ciliates decreased eight fold in Bold-basal salt medium and six fold in ATCC medium 357. RT-1 was found to be highly sensitive to even a slight change in the pH. Maximum cell count was obtained at pH 7 and 7.5. Several authors reported the optimum growth of protozoa at pH 7.5±0.2 (Rehman et al., 2005; Haq et al., 2000; Madoni et al., 1996). Nilsson (2003) reported optimum growth of *T. pyriformis* at pH 7. The culture began to deplete at pH ≤ 6.5 and ≥ 8 in both Bold-basal salt medium and ATCC medium 357.

RT-1 showed significantly high tolerance to copper ions (upto 800 µM, that is, 52 ppm) and LC_{50} 66 ppm, and thus, could uptake copper ions most efficiently. The results showed that it grew equally well in Bold-basal salt medium supplemented with wheat grains as well as in *Tetrahymena* specific ATCC 357 medium. RT-1 showed maximum growth at 25°C in both media. Several authors have reported the growth of *T. pyriformis* at 28°C (Nilsson, 2003; Piccinni et al., 1994). It was stated that the multiplication rate was slightly lower at low temperatures (≈20°C) although at this temperature, the culture can be kept a bit longer as compared to higher temperatures (≈37ºC). Twagilimana et al. (1998) reported that the ciliate cultures incubated at high and low temperatures deteriorated slowly, the survivors became rounded and the divisions rare, though some were able to remain alive for several days.

Growth of *Tetrahymena* spp. in the presence of copper

The effect of copper on the growth of RT-1, *T. thermophila* and *T. pyriformis* was studied in triplicate. The treated sets of culture were inoculated with log phase growing culture and were given stress of 500 µM copper. In control set of flasks having RT-1 inocula, no copper stress was given. Figure 7 shows growth curves of the ciliates in control and copper containing Bold-basal salt medium supplemented with 0.1% glucose at pH 7.5

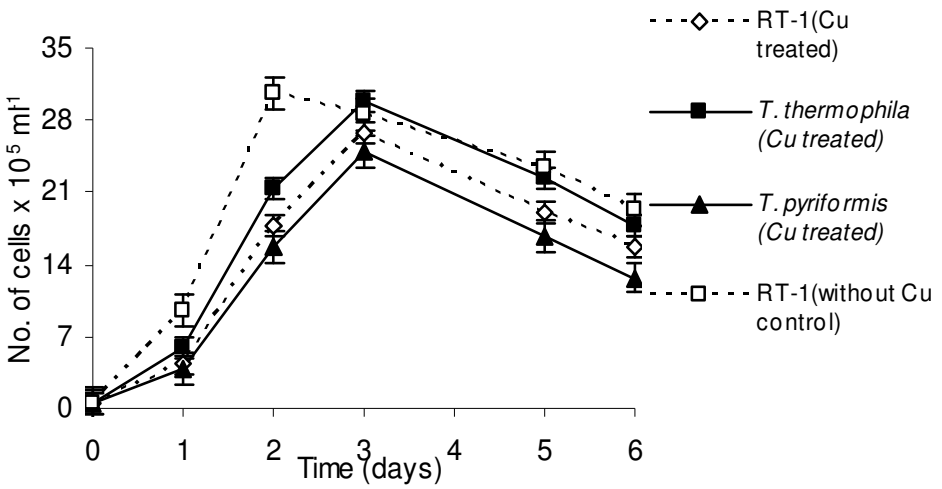

Figure 7. Effect of copper (500 µM) on the growth of ciliates, *Tetrahymena* RT-1 (◊), *Tetrahymena thermophila* (■), and *Tetrahymena pyriformis* (▲) in Bold-basal salt medium supplemented with 0.1% glucose at pH 7.2 and 28 ± 2°C. □ represent *Tetrahymena* RT-1 control without copper.

and 28 ± 2°C.

In the presence of copper, all the three ciliates attained maximum growth on day 3, whereas the control RT-1 culture attained maximum cell count on day 2. Since the dose at which the three ciliates were exposed to, was non toxic, their growth pattern was not signigficantly different from that of RT-1 grown in the absence of copper.

Exposure to copper ions induces efficient copper resistance possibly as a result of adaptation or other genetic means. In contrast to zinc (which is required as a structural or catalytic co-factor in hundreds of proteins), copper is part of a considerably lower number of proteins. This fact, combined with the extreme toxicity of free intra-cellular copper, is probably the reason why organisms from prokaryotes to mammals are in possession of specific copper chaperones that bind copper with high affinity and deliver it to specific target proteins. They are probably important also to compete with the non-specific copper binding sites of many cellular proteins in various subcellular compartments (Balamurugan and Schaffner, 2006).

Copper uptake by *Tetrahymena* spp.

Figure 8 shows uptake of copper ions by RT-1, *T. thermophila* and *T. pyriformis* from the medium containing 500 µM copper ions. RT-1 removed 47% copper from the medium after two days and 61% after six days of incubation. The amount of copper ions taken up by the cells (pellet) was 219.7 µM (44 %) in 2 days and 269.4 µM (54%) in 6 days.

The ATCC culture *T. thermophila* removed 68% copper from the medium after six days of incubation. The other ATCC culture, *T. pyriformis* was comparatively less efficient as the amount of copper removed by it from the medium was only 59% after six days of incubation. The *T. thermophila* cells (pellet) absorbed 62%, while *T. pyriformis* cells (pellet) took up 43% of copper from the medium. Thus, it could be concluded that RT-1 is more efficient than *T. pyriformis* and less efficient than *T. thermophila* in the removal of copper from the medium.

Figure 9 shows that the rate of metal uptake/absorption was more efficient up till day 3 in the case of RT-1 as copper removal from the medium is 61% (306.7 µM) after 6 days of incubation. Copper uptake ability of *T. thermophila* was 62% after 6 days of incubation. In *T. pyriformis,* copper uptake ability was found to be only 43% after 6 days of incubation (Table 3).

T. thermophila is well-established as a model eukaryote, elaborating typical eukaryotic components (for example, microtubules, membrane systems) into a highly organized cell whose structural and functional complexity is comparable to or exceeds that of human and other metazoan cells (Gorovsky, 1973; Brownell et al., 1996; Orias, 2000; Mochizuki et al., 2002; Yao and Chao, 2005; Miao et al., 2009). It has been acknowledged as an efficient test organism for the detection of pollutant in aquatic ecosystems (Cronin et al., 1991). Several test methods for xenobiotic toxicity using this ciliate have been proposed (Roberts and Berk, 1990; Noever et al., 1994; Sauvant et al., 1995; Larsen et al., 1997). Among the five ciliates tested in this study for copper uptake from the medium, RT-1 and *T. thermophila* showed greater efficiency than the others. The very fact that these ciliates can uptake 54 and 61% copper, respectively, from the medium, make them suitable candidates for use in the

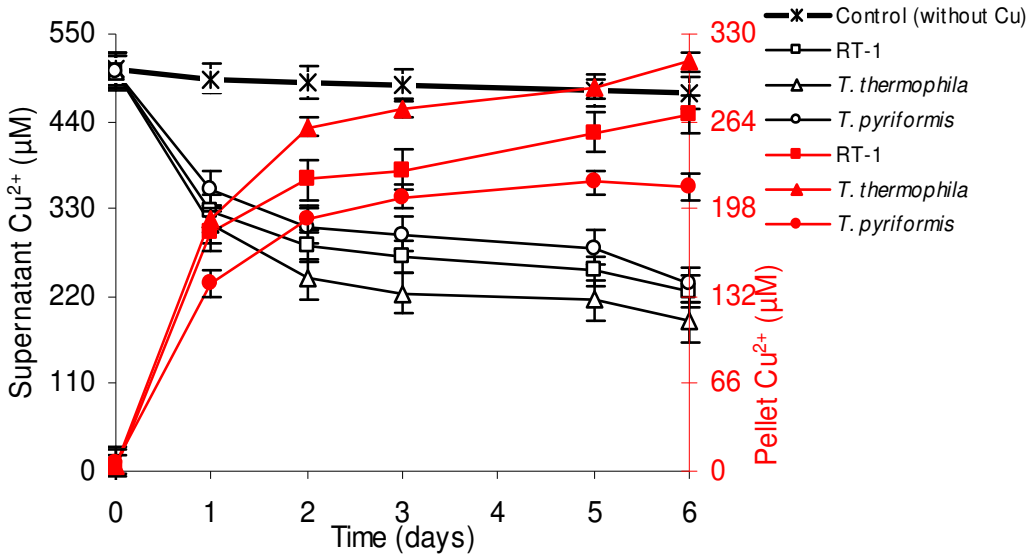

Figure 8. Removal of copper ions from Bold-basal salt medium containing 500 µM copper (pH 7.2 and 28 ± 2°C) by *Tetrahymena* spp. The lines with open symbols show removal of copper ions from the culture medium by RT-1 (□), *T. thermophila* (Δ), and *T. pyriformis* (◊). The lines in red with solid symbols show uptake of copper ions from the medium containing 500 µM copper by ciliate cells (pellets) of RT-1 (■), *T. thermophila* (▲) and *T. pyriformis* (♦) over a period of six days. The bold line (-x-) shows the concentration of copper ions in the control culture medium without ciliates.

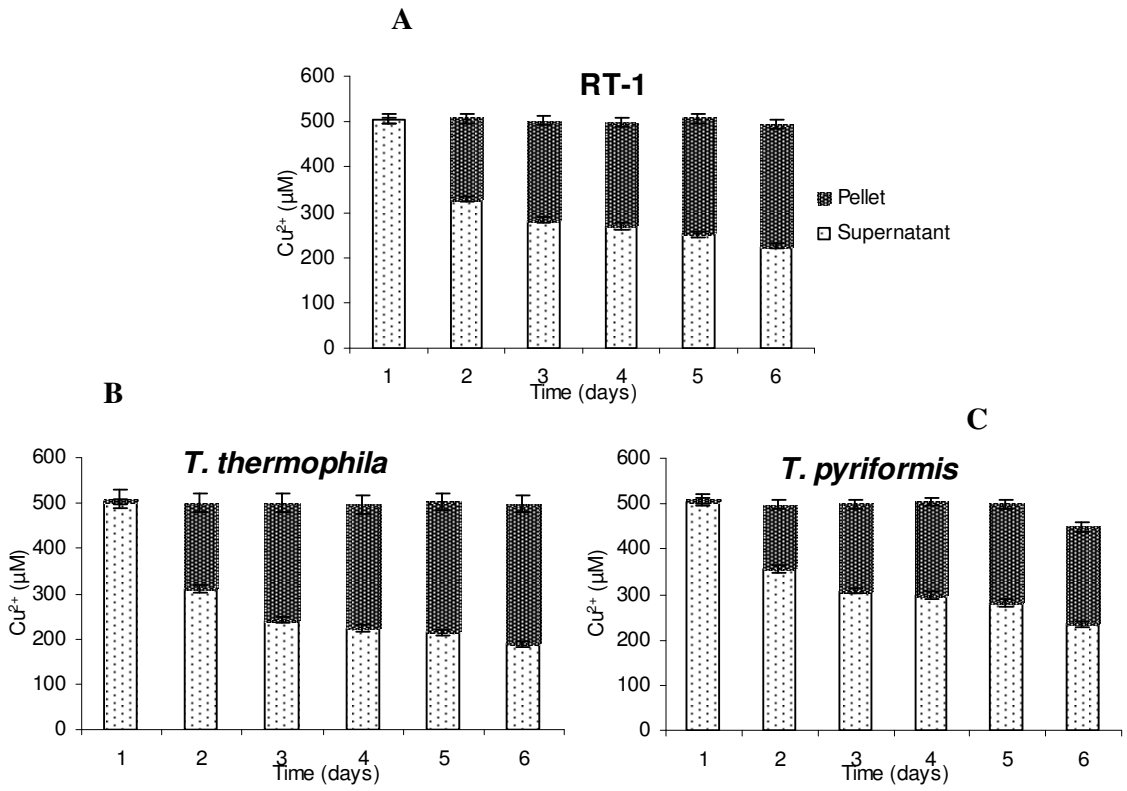

Figure 9. Uptake of copper ions by *Tetrahymena* RT-1 (A), *T. thermophila* (B) and *T. pyriformis* (C) from the Bold-basal salt medium containing 500 µM copper supplemented with 0.1% glucose at 28 ± 2°C and pH 7.2. There is gradual decrease in concentration of copper ions in the medium with concomitant increase in the pellet, over a period of six days.

Table 3. Removal of copper ions by the RT-1 and standard cultures (T*etrahymena thermophila* and *Tetrahymena pyriformis*) from Bold-basal salt medium containing 500 µM copper at pH 7.5 and 28 ± 2°C. The table indicates the copper concentration removed from the medium and that taken up by the ciliates.

Organism	Day	Amount of copper (µM)		
		In the medium	Removed from the medium	Detected in the organism
RT-1	0	502.8	11.7	2.6
	2	282.4	220.4	219.7
	6	224.8	278	269.4
T. thermophila	0	503	11.5	4.7
	2	241.6	261.4	259.7
	6	188.6	314.4	309.4
T. pyriformis	0	504.2	10.3	5.4
	2	308	196.2	190.7
	6	234.8	269.4	214.4

consortia of organisms for remediation of wastewater contaminated with heavy metals, particularly copper.

Conclusions

The three ciliates RT1, RE-1 and RE-2, isolated from the industrial wastewater showed high tolerance to copper ions. The LC_{50} of copper against the three isolates were found to be 60, 48 and 49 ppm, respectively. Moreover, RT-1 showed significantly high tolerance to copper ions and could absorb 53% of available copper ions. The copper uptake ability of the axenic culture (61.20%) was compared with that of ATCC cultures - *T. thermophila* (68%) and *T. pyriformis* (59%). The copper uptake ability of the local isolates, particularly RT1, makes them highly suitable candidates from bioremediation of industrial wastewater contaminated with copper ions.

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