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REDUCTION OF TOXIC HEXAVALENT CHROMIUM BY BACTERIAL STRAINS ISOLATED FROM THE EFFLUENTS OF TANNERIES

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Abstract

Four chromium resistant bacterial strains CrT-4, CrT-5, CrT-6, and CrT-7 that reduce hexavalent chromium Cr(VI) to trivalent chromium were isolated from the effluents of East-Pak Chrome tannery situated near Lahore-Sheikhupura road. These chromium resistant bacterial strains were able to grow both in liquid as well as in solid media in the presence of very high concentration of chromate i.e. up to 25mg ml^{-1} in nutrient broth and 40 mg ml^{-1} in nutrient agar. Strains CrT-4, CrT-6, and CrT-7 were gram-negative motile rods. They have circular convex colonies with entire margins whereas strain CrT-5 was gram-positive cocci with irregular flat colonies. All the strains were facultatively anerobes. CrT-4, CrT-6, and CrT-7 showed affinities with Pseudomonadaceae while CrT-5 shared character with Micrococcaceae. In majority of strains optimum pH was 8 in chromate-supplemented media but they preferred pH 7 in Chromate free media. All of them give better growth at 37° C. They were resistant to different antibiotics and metallic salts and reduce hexavalent chromium aerobically. The rate of hexavalent chromium reduction is more with low initial chromate concentration (100 μg ml^{-1}) and high inoculum size. For optimum chromate reduction all of them preferred pH 7 at 37° C. Four strains could also caused reduction of hexavalent chromium in industrial effluents and CrT-4 exhibited the highest reduction potential.

Introduction

Industrial wastewater and mining byproducts poses a serious pollution threat worldwide. Different methods of degradation are being investigated and applied in reducing or eliminating the many toxic compounds found in chemical and industrial effluents.

Chromium is discharged along with heavy metals in industrial processes such as leather tanning, chromeplating, paint formation etc. Although trace amount of chromium is necessary for the normal carbohydrate and lipid metabolism in humans being. Above certain level it is toxic, (Turick and Apel, 1997; Wang and Shen, 1997; Vankowa *et al.,* 1999), mutagenic (Liu and Dixon, 1996; Wang and Shen, 1997; Cheng and Dixon 1998), carcinogenic (Liu and Dixon, 1996; Hamilton *et al.,* 1998; Singh *et al.*, 1998; Shimada *et al.*, 1998; Shumilla *et al.*, 1999) and teratogenic (Snow, 1994; Junaid *et al.*, 1995; Asmatullah *et al.*, 1998).

Chromium occurs in oxidation state ranging Cr (II) to Cr (VI), but trivalent chromium, Cr(III), and hexavalent chromium, Cr(VI) are more stable forms. Trivalent chromium compounds are less soluble and readily precipitate by forming insoluble hydroxide at neutral pH. To minimize/remediate the environmental contamination much efforts has been made to treat hexavalent chromium. Common methods used for the treatment of chromate include chemical reduction by using reducing agents, which consumes high energy and large quantity of chemicals.

Studied have shown that highly toxic and highly soluble hexavalent chromium can be reduce to less toxic and less soluble hexavalent chromium by the action of chromium resistant bacteria (DeLeo and Ehrlih, 1994; Shen and Wang , 1995; Peitzsch *et al.*, 1998; Fredrickson *et al.*, 2000). Both aerobic and anaerobic

chromate reduction with different bacterial genera are now known (Campos *et al.*, 1995; Turick *et al.*, 1997; Peitzsch *et al.*, 1998; Fredrickson *et al.*, 2000). Hence biological detoxification of hexavalent chromium by the bacterial strains offers reliable and inexpensive alternative.

The present work is focused on evaluation of hexavalent reduction potential of bacterial strains isolated from the effluents of tanneries. These strains were further assessed for extraction/removal of hexavalent chromium from the effluents of electroplating industry.

Materials and methods

From the effluent samples of tanneries, chromium resistant bacteria were isolated. Bacterial growth was obtained on nutrient agar plates supplemented with 1000 μg ml^{-1} of potassium chromate. Apparently different colonies were picked purified which were taken to higher level of potassium chromate. Isolated strains were characterized following Gerhardt *et al* (1994). Growth responses of these isolates in the presence of different concentration of potassium chromate (.5, 1, 2, 4, 8, 15, and 25 mg ml^{-1}) were determined on nutrient agar and in nutrient broth at 37°C after 24 hours of incubation. Optical density of culture in nutrient broth was measured at 600nm.

The growth behavior of bacterial strains at different temperature (20, 24, 28, 32, 37 and 42° C) and pHs (5, 6, 7, 8, and 9) was exhibited in chromate supplemented, (1 mg ml^{-1}) and chromate free nutrient broth. The resistance profile of these strains against different metallic salts $NiSO_4$, $ZnSO_4$, 700 μg ml^{-1}., $CuSO_4$, $Pb(NO_3)_2$, 1000 μg ml^{-1}, $MnSO_4$, 1500 μg ml^{-1}, $CoCl_2$, 500 μg ml^{-1} and $HgCl_2$, 50 μg ml^{-1}), and antibiotics (streptomycin, 500 μg ml^{-1}, ampicillin, 300 μg ml^{-1}, tetracycline, 30 μg ml^{-1}., kanamycin, 40 μg ml^{-1}, chloramphenicol, 5 μg ml^{-1}, cefradine, 100 μg ml^{-1} and erythromycin, 50 μg ml^{-1}) were accomplished in nutrient agar supplemented with these salts or antibiotics after incubation of 24 hours at 37°C. For chromate reduction DeLeo and Ehrlich (1994) medium was used. Three chromate concentrations (100, 500, and 1000 μg ml^{-1} of K_2CrO_4) and two different inoculum size i.e 2.4^7 and 9.6^7 cells ml^{-1} were applied. After every twenty-four hours, samples were taken aseptically and were analyzed following DeLeo and Ehrlich (1994). The impact of some metallic salts (Zn, Ni, Mn, Cu, 100 μg ml^{-1}, Co and Hg, 50 μg ml^{-1} on the reduction potential of these isolates was also analyzed.

To check the Cr(VI) reduction ability of these isolates in the effluents, sample were collected from an electroplating industry in sterilized bottles. The physio-chemical parameters of the samples were as follows: pH 5-6, temperature 27-29°C, Cr (VI) 300 μg ml^{-1}, Fe 21 μg ml^{-1}, Cu 10 μg ml^{-1}, Zn 4 μg ml^{-1}, Ni 11 μg ml^{-1}, Co 2 μg ml^{-1}, Pb 1 μg ml^{-1}, Mn 1 μg ml^{-1}. Following nutritional requirements for bacterial cultures were also added in the effluent samples (in g/L^{-1}): tryptone 10; yeast extract 5; NaH_2PO_4 6.9; $C_2H_8O_7$, 1(DeLeo and Ehrlich, 1994). All these experiments were performed at pH7 and 37°C.

Results

Four chromium resistant bacterial strains CrT-4, CrT-5, CrT-6 and CrT-7 were isolated from the effluents of tanneries. All of them could resist up to 40 mg ml^{-1} and 25 mg ml^{-1} of potassium chromate in nutrient agar and broth, respectively.

Strains CrT-4, CrT-6 and CrT-7 were aerobic, gram-negative motile rods whereas CrT-5 was facultative anaerobic, gram-positive cocci. They were non-spore former. They gave positive results for oxidase (except CrT-5), catalase but for voges-proskauer, urease, methyl red (except CrT-5), sodium malonate (except CrT-5), H_2S production tests were negative. All could reduce nitrate but were unable to denitrify and hydrolyse starch and arginine. All strains could utilize sucrose and maltose as sole carbon source but for glucose (except CrT-5), manitol (except CrT-5) and lactose (except CrT-5) utilization they gave negative results. CrT-4 and CrT-6 exibited yellow- brown pigments. Strains CrT-4, CrT-6 and CrT-7 could be affiliated with family Pseudomonadaceae and strain CrT-5 shared most of its characteristics with family Micrococcaceae. Fig 1(a) reveals the effect of different concentrations of potassium chromate on the growth of bacterial strains in nutrient broth. Nevertheless the growth of all the strains decreased with increasing concentration of chromate salt, but all of them could grow in the presence of 25 mg ml^{-1} of K_2CrO_4. CrT-6 exhibited relatively better growth at all the concentration and CrT-5 showed poor growth at these concentrations. In the presence of chromate (1 mg ml^{-1}) the lag phase of these strains prolonged (Fig-1b). Initially growth was better in Cr-free medium but later on it excelled in chromium-supplemented medium.

The temperature preference remains the same i.e. 37°C, both in the presence and absence of chromate salt. At low temperature i.e. 20°C the growth was drastically reduced (fig.2a). These strains preferred pH 7 in simple nutrient broth but preferences changes to alkalinity (pH 8) in the presence of 1 mg ml^{-1} of potassium chromate. In the presence of chromate, the growth was very poor at pH5 as compared to chromate free nutrient broth (Fig.2b).

All the strains exhibited sensitive behavior to $HgCl_2$, but could resist at different concentration of Ni, Zn, Pb, Mn, Co and Cu (Table.1). These strains show sensitivity against 500 μg ml^{-1} of streptomycin, 40 μg ml^{-1} of kanamycin., 50 μg ml^{-1} of erythromycin and 100 μg ml^{-1} of cefradine. All the strains confer resistance to 300 μg ml^{-1} of ampicillin. Excluding CrT-5, these strains also confer resistance to 30 μg ml^{-1} of tetracycline and 5 μg ml^{-1} of chloroamphenicol. In addition to conferring resistance to chromate, these strains could reduced toxic hexavalent chromium into trivalent chromium aerobically (Fig.3). Initially reduction potential of these strains was evaluated using three concentration of potassium chromate i.e. 100, 500 and 1000 μg ml^{-1} and two cell concentrations i.e. 2.4^7 and 9.6^7 cells ml^{-1}. With an initial chromate concentration of 100 μg ml^{-1}, all the strains could reduce almost all the chromate present in the culture within 96 hours (Fig.3). Strain CrT-5 reduced more chromate at all the concentrations as compared to the others strains with an inoculum size of 2.4 cells ml^{-1}. But with high inoculum size strain CrT-4 reduced more chromate at low concentrations i.e.100 and 500 μg ml^{-1}. When cell concentration was increased four-folds, strain CrT-5 reduced more chromate at high chromate concentration i.e. 1000 μg ml^{-1}. With the passage of time the reduction potential of the strains became lowered. At high concentration i.e. 500 μg ml^{-1}, strains could not completely reduce all the chromate present in the medium. The reduction was more with high inoculum size (9.6^7 cells ml^{-1}). With an increase in chromate concentration i.e. at 1000 μg ml^{-1}, the percentage reduction was less but overall all the strains reduce more chromate as compared to the previous two cases. Addition of metallic salts of Cu, Ni, Mn, Pb, Zn and Co did not affect significantly

the reduction ability of these strains. Nevertheless some increase in reduction ability was evident in CrT-4 (Mn), CrT-5 (Co), CrT-6 (Cu), CrT-7 (Ni, Cu, Co) with the addition of some metallic salts (Table-2). Reduction potential of these strains was also estimated in effluents samples supplemented with basic nutritional requirements of bacterial strains. After determining the concentration of hexavalent chromium in the effluents, effluent sample having 150 μg ml^{-1} (sample 1) and 300 μg ml^{-1} (sample 2) were used for estimating the reduction ability of these strains. In sample 1, all the strains could reduce about 80-90% of hexavalent chromium within 36 hours. In the sample 2, strains CrT-4, CrT-5, CrT-6 and CrT-7 reduce almost 73%, 64.66%, 65% and 67% of hexavalent chromium, respectively (Table-2). Hence CrT-4 exhibited maximum reduction in both industrial effluents.

Discussion

Metals, beings elements, can neither be degraded nor metabolized; they are an example of ultimate persistence. In spite of crucial requirements of many metals for biological life, above critical level they exhibit toxic effects. Chromium is one of such metal, which is frequently used, in many industrial processes and its continuous discharge in the effluents, hence elevating its concentration in the environment. It is imperative to keep its concentration low and bacterial processes offer a strategy to extract and reuse of spent chromium. Hence four chromium resistant bacterial strains CrT-4, CrT-5, CrT-6 and CrT-7 were isolated. Three of them CrT-4, CrT-6, CrT-7 belongs to the family Pseudomonadaceae and one CrT-5 to Micrococcaceae. These strains could grow in a very high concentration of potassium chromate, both in nutrient agar and broth. Chromium resistant strains reported by other workers were also resistant to chromate, but their tolerance was not as high as in these strains. They could resist against potassium chromate at a high concentration in nutrient agar medium as compared to broth. This may be due to the binding of metals ions by various components of cultures media. Growth was much better in chromate free nutrient broth as compared to chromate supplemented nutrient broth. Itoh *et.al* (1994) reported that the viability of *E.coli* cells shows decreased in the presence of chromate. In the presence of chromate lag phase was prolonged in all the strains. This may be due to the inhibition of cell division due to chromate salt. Ogawa *et al* (1989) reported that chromate salt, increased the generation time and decrease cell division. Growth of all the strains was much reduced at pH5 in the presence of chromate, which may be due to the solubility of metals ions at acidic pH.

All these strains were able to reduced toxic hexavalent chromium, aerobically. Several bacterial genera are now known including *Pseudomonas ambigua* (Suzuki *et al.,* 1992), *Desulfovibrio vulgaris* (Lovely and Philips, 1994), *Enterobacter cloacae* HO-1(Fujie *et al.*, 1996), *Alcaligenes eutrophus* (Peitzsch *et al.*, 1998) and *Dinococcus radiodurans* R1 (Fredrickson *et al.*, 2000) that are successfully used as chromate reducing agents. The rate of hexavalent chromium was more with an initial Cr (VI) concentration of 100 μg ml^{-1} as compared to the higher concentration i.e. 500 and 1000 μg ml^{-1}. Bopp (1980) demonstrated that at higher Cr (VI) concentration product inhibition of Cr (VI) reduction by Cr (III) occurred. On further incubation Cr (VI) reduction would have been ceased. Almost all the strains could reduce 100 μg ml^{-1} of Cr (VI) within 72 hours at both inoculum size i.e. 2.4^7 and 9.6^7 cells ml^{-1}. *Pseudomonas fluorescence* LB 300 reduced 69% of

Cr (VI) with an initial chromate concentration of 200 mg l^{-1} in 289 hours (DeLeo and Ehrlich, 1994). In the first 30 hours the rate of reduction was much faster, but with passage of time it dropped slowly (Fig.3) which might be the result of the formation of Cr (III) and toxic substance in growth medium or other factors might be involved. Cell density has impact on chromium reduction of these strains. High inoculum size cause relatively more reduction. As the amount of Cr (VI) increased, the rate of %age chromate reduction decrease but overall the more amount of Cr (VI) was reduced with same number of cells at same time.

DeLeo and Ehrlich (1994) demonstrated that the rate of reduction of Cr (VI) increased with increasing initial Cr (VI) concentration. At higher concentration of chromate i.e. 500 and 1000 μg ml^{-1}, complete reduction did not occur within 96 hours. At lower concentration different heavy metals did not inhibit the Cr (VI) reducing capability of these strains. But at higher concentration they might be problematic. Some increase in reduction ability of CrT-4 (Mn), CrT-5 (Co), CrT-6 (Cu) and CrT-7 (Ni, Cu, Co) was observed with some metallic salts. In *Pseudomonas putida*, Ag and Hg strongly inhibit the rate of chromate reduction at a concentration of 30-60 mM (Ishibashi *et al.*, 1990).

Hexavalent chromium reduction potential of these strains exhibits that the strains could be useful for the treatment of industrial wastewater. Strain CrT-4 reduced 93.33% and 73% of Cr (VI) in effluent sample 1 and 2 respectively, while all other strains reduced about 86-90% in sample 1 and 64-67% in sample 2 (Table-2). Such studies with wastewater are valuable for examining the feasibility of these strains for the biological treatment of contaminated Cr (VI). An example of the application of bacterial detoxification of Cr (VI)- contaminated industrial effluent had been reported previously (Ohtake and Silver, 1992). Hence bacterial strains (CrT-4, CrT-5, CrT-6 and CrT-7) reported here exhibiting multiple metal and antibiotics resistances and strong hexavalent chromium, reducing ability, might be the good candidates for the bioremediation of Cr (VI) from waste water contaminated with hexavalent chromium.

Table 1. Heavy metals resistance profile of chromium resistant bacterial isolates.

STRAINS	HEAVY METALS (μg ml^{-1})						
	$NiSO_4$	$ZnSO_4$	$Pb(NO_3)_2$	$CuSO_4$	$CoCl_2$	$HgCl_2$	$MnSO_4$
CrT-4	400	700	1000	700	400	-	1500
CrT-5	400	400	600	1000	200	-	1500
CrT-6	500	700	1000	700	500	-	1500
CrT-7	700	700	1000	1000	300	-	1500

Table. 2 A. Effects of different heavy metals on the reduction potential of the strains. B. Chromate reduction in industrial effluents. a*. Initial chromate concentration 150 μg ml^{-1}. b. Initial chromate concentration 300 μg ml^{-1}.**

SOURCE	STRAINS			
	CrT-4	CrT-5	CrT-6	CrT-7
	A. Metals			
Control	34%	38%	31.5%	28.6%
Ni (200)	34.6%	38.7%	31.8%	30.6%
Mn (200)	36.2%	36.6%	30.6%	29.6%
Zn (200)	32.6%	37.8%	29.4%	28.2%
Cu (200)	35.8%	38.2%	34.2%	31.6%
Co (50)	34.4%	41%	32.2%	30.8%
	B. Industrial effluents			
a*	93.33%	86.66%	88.86%	90.66%
b**	73%	64.66%	65%	67%

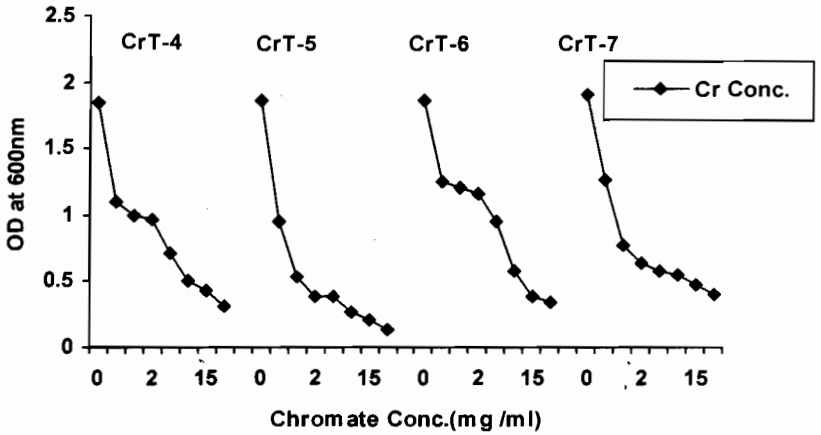
Legend to figures

Fig.1 Growth responses of bacterial strains a) at different concentration of potasssium chromate, b) after different time of incubation in the presence and absence of chromate (1 mg ml^{-1}).

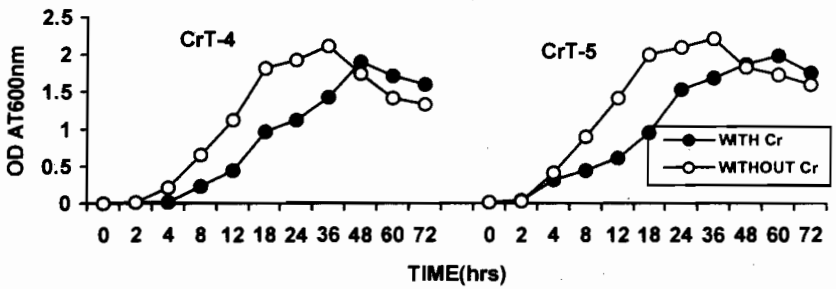
Fig.2 Growth responses of bacterial strains in chromate supplemented (1 mg ml^{-1}) and chromate free l-broth at different a) temperatures (20-42°c) and b). growth pHs (5-9).

Fig.3 Reduction of toxic hexavalent chromium at different time intervals with two inoculum size i.e. 2.6^7 and 9.6^7 cells ml^{-1} .Initial chromate concentrations 100 µg ml^{-1} (a), 500 µg ml^{-1} (b), 1000 µg ml^{-1}(c).

a.



b.



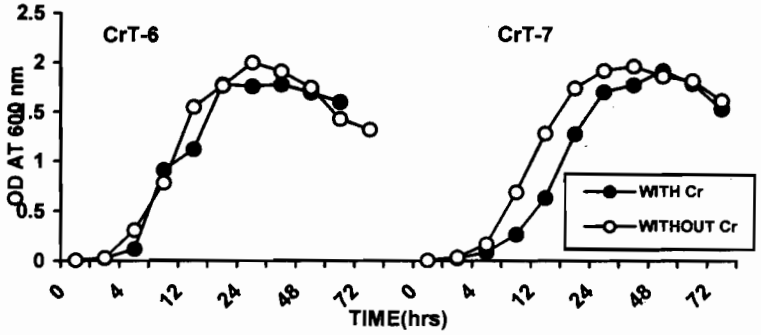
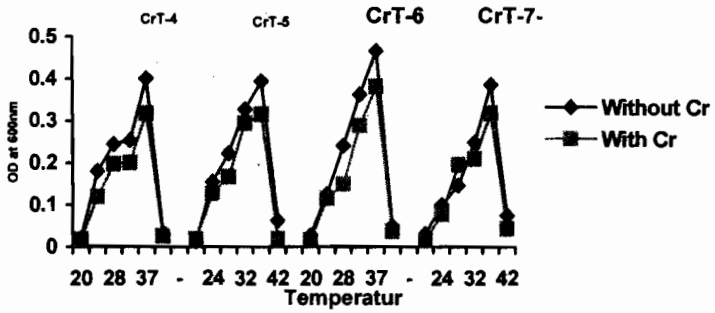

CrT-6
CrT-7
OD AT 600 nm
2.5
2
1.5
1
0.5
0
0 4 12 24 48 72 0 4 12 24 48 72
TIME(hrs)
WITH Cr
WITHOUT Cr

Fig.1

a.


CrT-4
CrT-5
CrT-6
CrT-7-
OD at 600nm
0.5
0.4
0.3
0.2
0.1
0
20 28 37 - 24 32 42 20 28 37 - 24 32 42
Temperatur
Without Cr
With Cr

b.

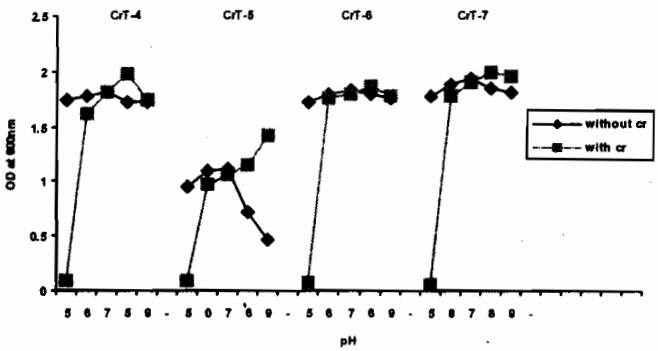

CrT-4
CrT-5
CrT-6
CrT-7
OD at 600nm
2.5
2
1.5
1
0.5
0
pH
without cr
with cr

Fig.2

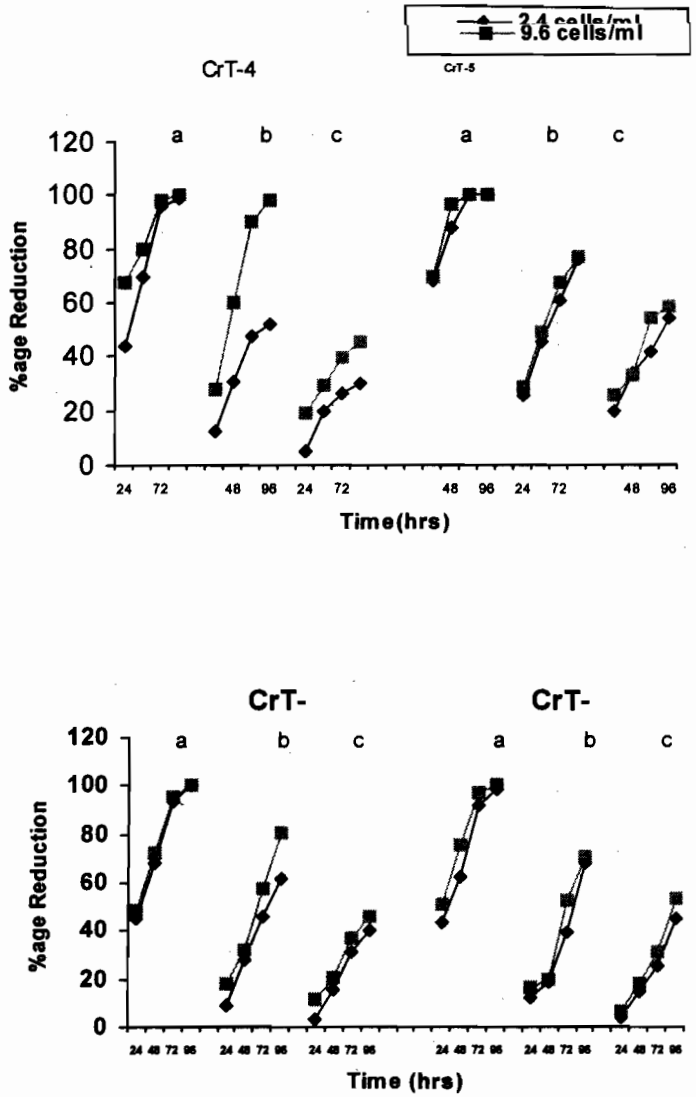


Fig.3

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