J. Mycopathol, Res, **46(2)** : 289–295 (2008)

Influence of environmental factors on the uptake of chromium by *Pseudomonas stutzeri* TEM-317 isolated from tannery sludge

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Pseudomonas stutzeri TEM-317, the Cr-resistant bacterium isolated from tannery effluent could accumulate Cr from aqueous solutions. The process of accumulation of Cr increases with increase in cell age till it reaches the late exponential phase. Accumulation was maximum at 8 h of incubation at 37^{0}C. Irrespective of environmental conditions, the amount of Cr(III) accumulation was higher than that of Cr(VI). The optimum pH for biosorption of Cr(III) was 4.5, while that for Cr(VI) was 6.5. Uptake of Cr was proportional to biomass concentration and supplementation of glucose at low concentration enhanced the process. Drying of biomass at 80^{0}C prior to its utilization allowed maximum accumulation of the metal. It is proposed that the organism under optimized condition could serve as an effective tool in reducing the chromium load of polluted environment.

Key words: Bioaccumulation of chromium, bioremediation, chromium-resistant bacteria, chromium detoxification

INTRODUCTION

Chromium, a transition metal is of great environmental concern chiefly due to its variable oxidation states, which differ markedly in terms of toxicity and mobility. In the environment Cr occurs both in hexavalent and trivalent forms (Smith and Gadd, 2000). Trivalent Cr [Cr(III)] is extremely insoluble above pH 5.5, precipitating as $Cr(OH)_3$ or other oxides and hydroxides and are relatively immobile in soil systems. Hexavalent Cr [Cr(VI)], on the other hand occurs in soluble, pH dependent anionic forms with chromate $(CrO_4)^{2-}$ predominating above pH 6.0. Interconversion of these forms occurs in geologic and aquatic environments (Losi and Frankenberger, 1994). Hexavalent Cr, due to its ability to move across the biological membranes and to bind and interact with intracellular proteins and nucleic acids is considered to be toxic and mutagenic for most organisms, much more than Cr(III) (Komori *et al.*,1990).

Due to its widespread occurrence, high mobility in soil and water, chances of interaction of Cr(VI) with terrestrial and aquatic organisms, including man is very likely. However, Cr has been found to have little or almost no beneficial effect on different life forms. In fact, it exerts profound toxicity, which has evolved general awareness to develop ways and means for amelioration of Cr toxicity in the environment.

Microorganisms by virtue of their wide degree of adaptibility to various Cr-contaminated environments could serve as an effective tool for bioremediation or detoxification of chromium in comparison to the traditional chemical methods known. Mechanisms such as reduction of Cr(VI) to the less toxic Cr(III) and Cr accumulation, therefore, serve to detoxify the microbes immediate environment. These microbial processes are being utilized to reduce or eliminate the toxic effect of chromates on a larger scale (Komori *et al.*, 1990, Apel and Turick, 1991, Losi *et al.*, 1994).

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Bacterial chromate reduction has been studied quite extensively (Bopp and Ehrlich, 1988, Ishibashi *et al.*, 1990, Llovera et al., 1993, Campos *et al.*, 1995, Shen *et al.*, 1996). However, studies on detoxification of chromium by means of bioaccumulation and biosorption (Coleman and Param, 1983; Nair and Krishnamurthi, 1991, Garnham and Green, 1995) are few.

The present investigation was undertaken primarily to evaluate the influence of environmental factors on the uptake of chromium by the Cr-resistant *Pseudomonas stutzeri* TEM-317 under laboratory conditions.

MATERIALS AND METHODS

Bacterial culture and maintenance

The chromium-resistant bacterium *Pseudomonas stutzeri* TEM-317 (MTCC 2995) isolated from tannery sludge was used throughout the study. The isolate was grown and maintained on slopes of peptone yeast-extract (PYE) agar. The medium comprised of 1.0 g bacto-peptone, 1.0 g yeast extract, 0.5 g NaCl, 0.5 g $NH_3H_2PO_4$, 0.5 g KH_2PO_4, 0.2 g $MgSO_4$. $7H_2O$ in 1.0 L distilled water. The pH of the medium was adjusted to 7.2 (Luli et al., 1983).

Production of cell mass

The bacterial strain TEM-317 was grown in PYE medium (50 ml/250 ml flask) at 37°C on a rotary shaker (120 rpm) for 16 h. The flasks were inoculated with freshly grown culture at 1% level. Cell mass was harvested aseptically by centrifugation at 10,000 x g for 20 min and washed twice with sterile distilled water to remove the trace of medium constituents.

For chromium uptake studies freshly harvested cells were suspended aseptically in 5 ml of sterile aqueous solutions of chromium contained in 50 ml conical flask and incubated at 37°C under shaking condition (120 rpm). The cell suspension was prepared to provide about 0.4 mg (dry wt.) of cells/ml of solution. Cell-metal suspensions were withdrawn at regular intervals, the cell mass was pelleted by centrifugation at 10,000 x g for 5 min and washed twice with phosphate buffer (pH 7.2). The washed cells were then dried to constant weights at 80 ± 2°C and digested with 5 ml of HNO_3:HCl (1:3) mixture for 1h. The digested sample was then transferred to 100 ml volumetric flask and the volume was made to 100 ml using double distilled water. The total chromium content of the sample was determined using a Varian AA 20 Plus Atomic Absorption Spectrophotometer.

The influence of several factors like cell age, biomass concentration, incubation period, concentration of Cr, temperature, pH and metabolic state of cells on Cr uptake were determined.

Each experiment was performed in triplicates and the average results were presented in this work. All chemicals used were of analytical grade.

RESULTS

Accumulation of chromium by freshly grown cells of *Pseudomonas stutzeri* TEM-317 harvested at different stages of growth was found to show a gradual increase with increase in cell age up to 16 h of incubation (Fig. 1) which represented the late exponential phase of growth of the organism. At each stage of growth, the amount of trivalent chromium accumulation was higher than that of hexavalent form. Under optimum condition, the Cr(III) removal efficiency of cells was 33% more over that of Cr (VI).

Increase in incubation time produced a steady increase of chromium accumulation by the cell mass, attaining a maxima at 8 h of incubation. Prolongation of incubation period, however, failed to show further increase in accumulation, rather it maintained a static state with slightly reduced accumulation. In all stages of incubation, accumulation of hexavalent chromium was comparatively less than that of trivalent chromium (Fig. 2).

As shown in Fig. 3 the uptake of chromium increases with gradual increase in the initial metal concentration irrespective of the form of the metal. Maximum accumulation was recorded at 1000 µg/ml above which accumulation of Cr maintained more or less a constant state. Uptake of Cr(III)

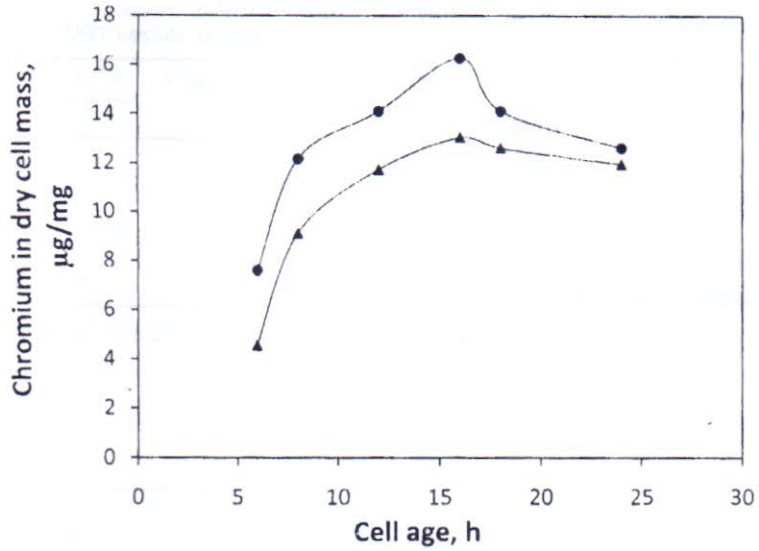

Fig 1. Effect of cell age on chromium [Cr(III)—●—; Cr(VI)—▲—] uptake by *Pseudomonas stutzeri* TEM-317.

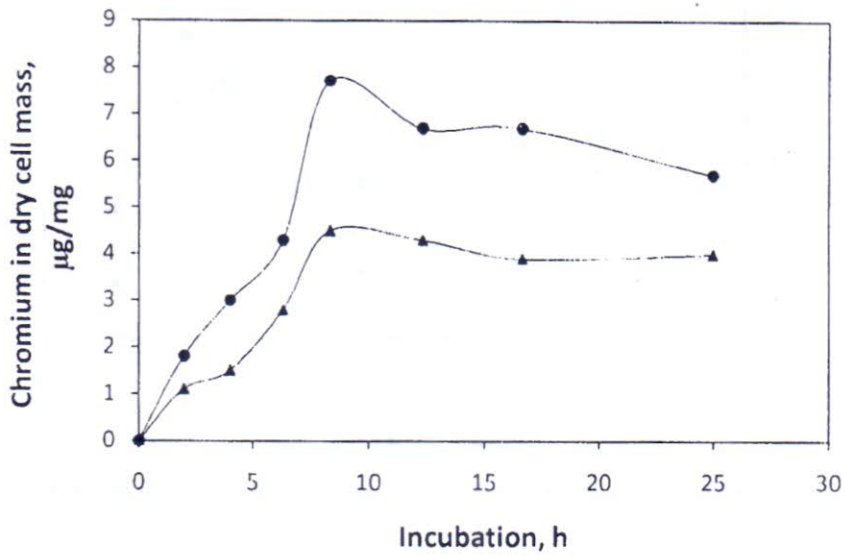

Fig 2. Effect of incubation period on chromium [Cr(III)—●—; Cr(VI)—▲—] uptake by *Pseudomonas stutzeri* TEM-317.

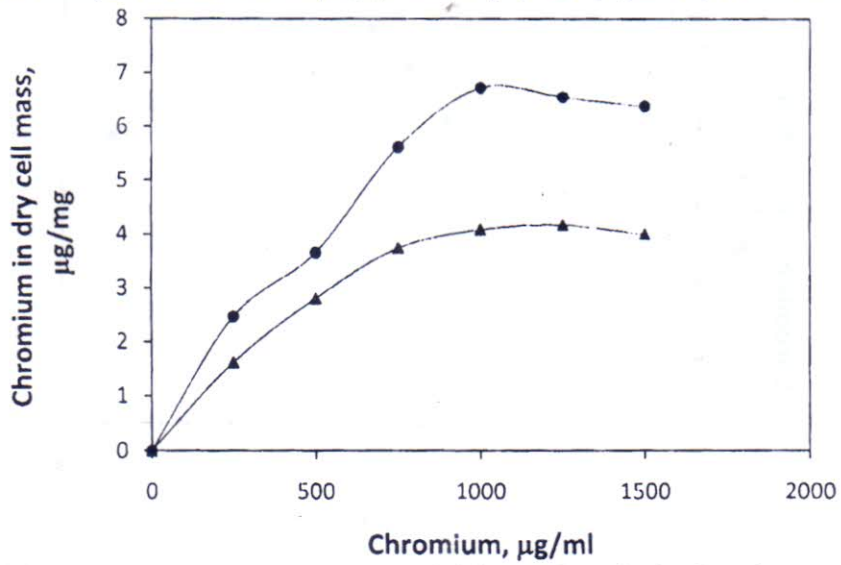

Fig 3. Effect of chromium concentration [Cr(III)—●—; Cr(VI)—▲—] uptake by *Pseudomonas stutzeri* TEM-317.

(as $CrCl_3$) was almost double compared to that of Cr(VI) as K_2Cr0_4 at identical concentration.

Variation of temperature resulted in remarkable difference in the accumulation of chromium per mg of cell mass (Figure 4). Uptake of chromium by bacterial cell mass was enhanced with increase in temperature, attaining a maximum value at 37°C. Further rise in temperature caused an inhibition of the accumulation process.

Table 1 : Effect of biomass concentration on chromium uptake by *Pseudomonas stutzeri* TEM-317.

Biomass concentration, mg/ml	Chromium uptake, µg/mga	
	Cr(III)	Cr(VI)
0.1	4.5	2.18
0.2	6.05	2.82
0.4	8.25	4.31
0.6	7.13	7.23
0.8	5.05	6.01

a Values represent average of triplicate readings.

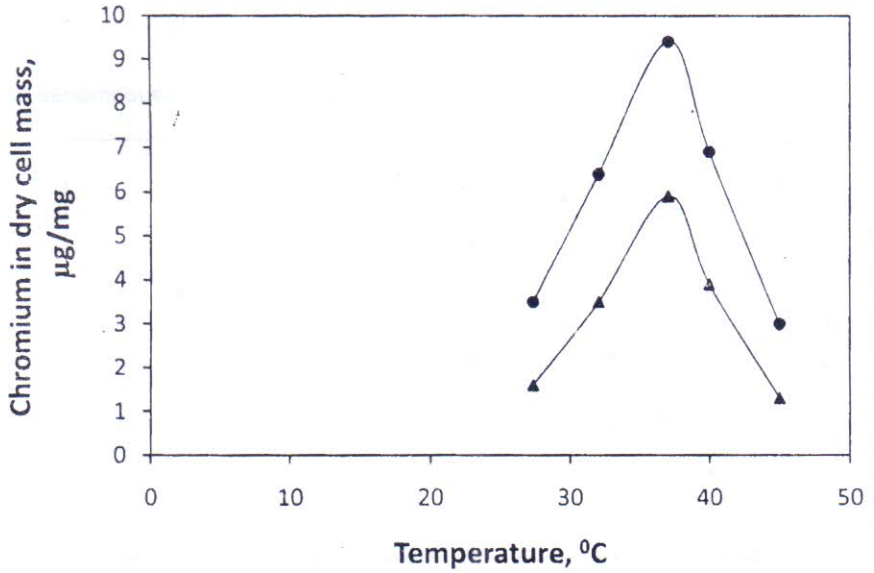

Fig 4. Effect of temperature on chromium [Cr(III)—●—; Cr(VI)—▲—] uptake by *Pseudomonas stutzeri* TEM-317.

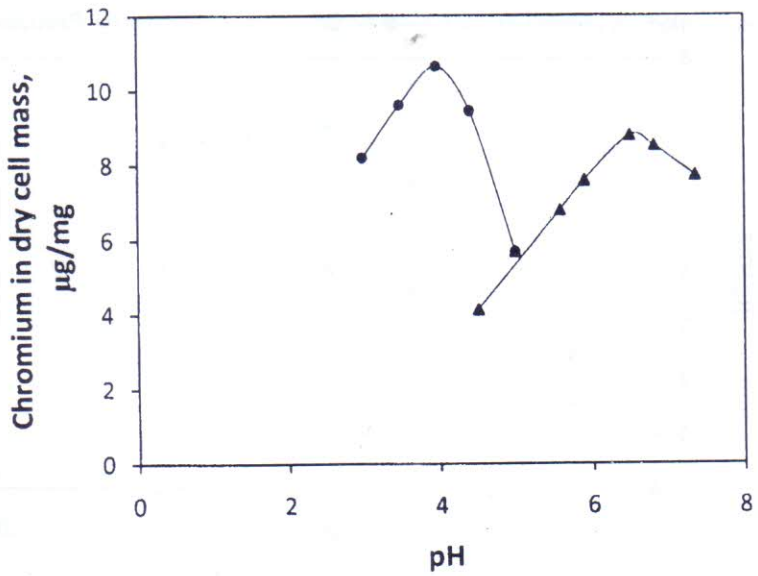

Fig 5. Effect of pH on chromium [Cr(III)—●—; Cr(VI)—▲—] uptake by *Pseudomonas stutzeri* TEM-317.

Since the solubility of the trivalent and hexavalent salts exists in different pH ranges, it was observed that accumulation of Cr(III) increased with increasing pH from 3 to 4, thereafter declined sharply at pH 5, while Cr(VI) uptake was found to increase with gradual increase of pH from 4.5 and attained maximum at pH 6.5. Further increase in pH resulted in significant decrease in the uptake (Figure 5).

Table 2 : Effect of glucose concentration on chromium uptake by *Pseudomonas stutzeri* TEM-317.

Glucose, % (w/v)	Chromium uptake, μg/mg[a]	
	Cr(IV)	Cr(IV)
Control (-glucose)	7.25	4.28
0.01	9.87	7.01
0.05	6.90	4.06
0.10	5.21	2.69

Values represent average of triplicates readings.

Irrespective of species, chromium accumulation increased with increasing concentration of biomass, uptake of trivalent form being more than that of the hexavalent form. Optimum biomass concentrations for the accumulation of Cr(III) and Cr(VI) were 0.4 and 0.6 mg/ml respectively (Table 1).

Variation of glucose concentration (0.01-0.1%, w/v) failed to produce any remarkable effect on biosorption of chromium by the bacterial isolate TEM-317. Only at lower concentration (0.01%) accumulation was increased to some extent but further increase in glucose concentration did not prove to be useful for the process of chromium uptake (Table 2).

Table 3 : Effect of metabolic state of *Pseudomonas stutzeri* TEM-317 cells on the uptake of chromium.

Metabolic state of cell	Chromium uptake, μg/mg[a]	
	Cr(III)	Cr(VI)
Control [a]	4.70	2.73
Metabolically inhibited [b]	4.21	1.86
Dead [c]	2.86	1.31
Dry [d]	4.80	3.49

Values represent average of triplicates readings.

a Cells from exponentially growing culture were harvested, washed and used as untreated control.

b Cell mass was exposed to 0.0001 M Na-azide for 20 min and washed thoroughly before use.

c Cells were treated with 1% w/v $HgCl_2$ for 10 min and washed thoroughly before use.

d Cell mass was dried at 80°C for 24 hours.

Comparison of various metabolic conditions of bacteria influencing the process of uptake of either forms of chromium revealed that dried cell mass bind higher amounts of chromium than viable cells. Compared to the living cells, accumulation was less in metabolically inhibited and dead cells (Table 3).

DISSCUSSION

The chromium-resistant bacterium *Pseudomonas stutzeri* TEM-317 under study was found to tolerate high concentrations of Cr chiefly by means of accumulation. The efficiency of chromium sorption has been found to increase with increasing cell age till late exponential phase of growth (Figure 1). Such observations could be attributed to the metabolic difference of the cells at different stages of growth. Culture age dependent metal uptake has been reported in *Zoogloea ramigera* (Norberg and Persson, 1984), *Pseudomonas aeruginosa* (Nair and Krishnamurthi, 1991) and *Thiothrix A1* (Shuttleworth and Unz, 1993). But the lowering in the uptake of chromium by the aged cultures of TEM-317 may be due to the loss of cell wall constituents during autolysis.

Prolongation of incubation period indicates a rapid physico-chemical interaction between the cell and metal ions up to a certain period of time (Figure 2). This result corroborates the findings of Aksu *et al.* (1991) with activated sludge bacteria. In short-term accumulation of chromate, the cyanobacteria attained the maximum value within 5 min with no further significant uptake over further 4 h incubation (Garham and Green, 1995).

The progressive increase in interaction with sites available on bacterial cell wall may probably account for the increase in Cr uptake with an increase in initial metal concentration (Figure 3). In *Pseudomonas aeruginosa,* similar increase of Cr. accumulation have been thought to be due to electrostatic attraction between oppositely charged sites available in cell wall (Nair and Krishnamurthi, 1991). In this context it might be mentioned that the chemistry of cell surface is complex and the

potential, therefore, exists for metals to sorb at a variety of sites (Beveridge, 1989; McLean and Beveridge, 1990).

The optimum temperature (370ºC) for biosorption of chromium by the isolate (Figure 4) might be indicative of the fact that the viable cells show equilibrium metal uptake capacity at this temperature. Aksu *et al.* (1991), however, have shown a much wider temperature range (25-45^{0}C) for maximum initial absorption rates for Cu, Ni, and Cr by activated sludge bacteria.

Several workers have indicated that pH exerts profound influence on binding of Cr by bacteria (Kuyucak and Volesky, 1988; Shuttleworth and Unz, 1993, Garnham and Green, 1995). In our study too we have observed that solution pH appears to be important for biosorption of both forms of chromium. The difference in optimum pH for biosorption of Cr(III) and Cr (VI) by the isolate (Figure 5) is chiefly due to the difference in solubility of the valence states of chromium. Li *et. al.*(2000) reported that pH 7.0 was most suitable for Cr(III) bioaccumulation by blue green algae *Spirulina platensis,* while it was pH 9.0 for bioaccumulation of Ni, Cd and Cr by *Pseudomonas aeruginosa* (Gupta *et al.*,2004).

The relationship of biomass concentration with the amount of chromium accumulated by the isolate (Table 1) could be explained by the fact that increase in biomass concentration increases the availablity of binding sites for the metal ions. Similar reports have been published by Al-Asheh and Duvnjak (1995) assuming that the metal uptake occurred at the cell surface only.

Glucose has been reported to enhance metal uptake by microbial cells (Norris and Kelly, 1977, Al -Asheh and Duvnjak, 1995). Increase in chromium uptake was observed with glucose as an additive, though only at a low concentration (Table 2). This could be explained as the result of increase of cellular activities, including the metal adsorption phenomenon. However, at higher concentrations, it may happen that glucose interferes with the cells metal binding sites thereby preventing their interactions with metal ions (Li *et al.*, 2000 Srinath *et al.*, 2002).

Metabolically inhibited cells as well as dead cells have failed to uptake equivalent amount of Cr. Dried cells, however, appeared to possess better binding capacities (Table 3). Such observations have been well documented by several workers (Kuyucak and Volesky, 1988; Gadd, 1990; Nair and Krishnamurthi, 1991; Smith and Gadd, 2000). It was apparent that chromium accumulation by the cells of TEM-317 was independent of cellular metabolism, which has received support from other metal removal studies (Lester and Strerritt, 1985).

It may be concluded that *Pseudomonas stutzeri* TEM-317, the chromate-resistant bacterium exhibits accumulation of Cr as an adaptive feature for resistance. The conditions for such accumulation have been optimized and it is evident that proper manipulation of such bioaccumulation properties of the organism could be an effective tool for remediation of chromium in polluted environment.

ACKNOWLEDGMENTS

Financial assistance from University Grants Commission, New Delhi to M. Basu is gratefully acknowledged. The authors also thank Dr. P. K. Mukherjee for helping in atomic absorption spectroscopic analysis.

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(Accepted for publication August 12, 2008)