Published in the Russian Federation
Russian Journal of Biological Research
Has been issued since 2014.
ISSN: 2409-4536
E-ISSN: 2413-7413
Vol. 8, Is. 2, pp. 69-79, 2016

DOI: 10.13187/ejbr.2016.8.69
www.ejournal23.com

UDC 579.6; 582.28(063)

Optimization of Process Parameters for Effective Bioremediation of Chromium Contaminated Soil by *Trichoderma Pseudokoningii*

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Abstract

Release of hexavalent chromium in environment is usually the outcome of anthropogenic activities. Apart from its carcinogenicity, hexavalent chromium also contributes damage to the gastrointestinal, respiratory, reproductive and immunological systems. Hence removal of this toxic metal is very essential for the safety of both humans and animals. Microbial bioreduction of Cr (VI) to less toxic Cr (III) has proved to be an ecological and economical option for chromate detoxification. In this paper, we report the conversion of hexavalent chromium by the chromium reducing fungal strain of *Trichoderma pseudokoningii*, isolated from tannery effluent enriched soil near Kolkata. The study reveals that the isolated strain could grow well at a concentration of 1000 mg/L chromium, but spore formation became scanty as the concentration increased. Removal of hexavalent chromium was found to be accomplished through bio reduction rather than biosorption or bioaccumulation since no membrane bound and intracellular fraction bound Cr (VI) could be traced. Extra cellular chromium reduction was found to be highest when the culture medium (pH = 7) was supplemented with 0.5 % (w/v) pure dextrose and 0.09 % peptone as sole carbon and nitrogen source respectively. Reduction of potassium dichromate Cr (VI) at a concentration of 220 mg/L was highest after 144 hours of inoculation, i.e. at the stationary phase of growth of the strain. The reduction rate was enhanced in presence of cystine and DTT which might be due to the increased rate of activity of chromium reductase enzyme having thiol groups at its active site. Addition of acid treated human hair and feather in the culture medium boosted the power of chromate reduction by the strain. The gradual chromate reduction by the strain in soil kept in near natural conditions was confirmed by the data of atomic absorption spectroscopy which indicated the prospective role of the strain in successful bioremediation.

Keywords: hexavalent chromium, chromate reduction, bioremediation, *Trichoderma pseudokoningii*.

Introduction

Chromium (Cr) can exist in nine valence states (Smith *et al*., 2002), of which only trivalent chromium Cr (III) and hexavalent chromium Cr (VI) are stable in natural environment and hence are ecologically important. Studies have revealed that Cr (VI) is approximately 100 times more toxic (Beszedits, 1988) and 1000 times more mutagenic than Cr (III) (Lofroth *et al*., 1978).

Hexavalent chromium polluted soils and sediments are usually the result of sewage sludge disposal or dumping of chromate wastes from industrial and manufacturing activities (McGrath and Smith, 1990). Chromium contamination of environment is of concern because of the mobility and toxicity of Cr (VI) and is recognized to be highly toxic, carcinogenic, mutagenic and teratogenic for mammals including humans (Flores *et al.,* 1999). Cr (VI) exposure in humans can induce allergies, irritations, eczema, ulceration, nasal and skin irritations, perforation of eardrum, respiratory track disorders and lung carcinoma (Poopal *et al*, 2009; Gibb *et al*, 2000a, Gibb *et al*, 2000b). Moreover, Cr (VI) shows the capability to accumulate in the placenta, damaging fatal development (Saxena *et al,* 1990).

Cr (VI) in soil and water also alters the structure of soil microbial communities leading to the reduction of microbial growth and related enzymatic activities, with a consequent persistence of organic matter in soils and accumulation of Cr (VI) (Shi *et al*, 2002).

In various states of India, mainly in Tamilnadu, Uttar Pradesh and West Bengal (Vijayanand and Hemapriya**,** 2014), tanneries are still employing the chrome tanning processes and release effluents into the environment without proper pre-treatment. It causes uncontrolled enrichment of Cr (VI) in nature. As "the maximum acceptable concentration of 0.05 mg/L for chromium in drinking water has been established on the basis of health considerations" (Sterrett, 1978), removal of excess Cr (VI) becomes the need of the hour.

Since the removal of hexavalent chromium by conventional processes is expensive and lacks specificity (Katiyar and Katiyar, 1997), bioremediation with the help of suitable bacteria and fungi is the best option left. A number of microbes are reported to remove hexavalent chromium (Ahluwalia, 2014), of which most were able to adsorb the toxic metal. Fungi, in general, are well known for their ability to biosorb and bioaccumulate metals but few reports about biotransformation, especially on chromium reduction (Das and Santra, 2012) are available.

The present research deals with the optimization of the parameters for bioreduction of hexavalent chromium by a chromate tolerant fungal strain and elucidation of its prospective application in bioremediation of chromium contaminated soil.

Materials and methods

Microorganism and its cultivation

A chromium tolerant fungal strain, *Trichoderma pseudokoningii,* isolated from tannery effluent rich soil in West Bengal, India (Ray *et al*, 2013) was used throughout this study. The fungal strain was cultivated in 500 mL Erlenmeyer flasks each containing 100 mL of Basal Medium (BM) composed of ($g \cdot l^{-1}$): peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1; $MgSO_4 \cdot 7H_2O$ 0.1, and glucose 0.5 (pH: 7) for 48 hours.

Measurement of growth

Growth of the mycelial type culture was measured by weighing the dried (at 80°C for 120 min) mycelial mat on dried pre weighed filter paper (Whatman No 1).

Tolerance to Cr (VI)

The efficacy of the strain to tolerate hexavalent chromium was tested by supplementing the medium with different concentrations of Cr (VI), namely, 0-1000 $mg \cdot l^{-1}$. The flasks were incubated for 0-12 days followed by an assessment of their growth by measuring the mycelial dry weight.

Studies on Chromium removal by the selected strain

The fungal strain was grown overnight in 500 ml flasks each containing 100 mL of chromium enriched medium. For determination of chromium concentration of the extracellular fraction, the fungal culture was centrifuged at 10.000 RPM for 5 minutes and the supernatant was used as the sample. To determine the intracellular chromium concentration, the harvested mycelium was washed twice with 0.1 M phosphate buffer (pH = 7), mechanically disrupted in a Sonicator (Rivotek, India) and the cell mass was extracted with 20 ml phosphate buffer (pH = 7). The supernatant obtained after removing the cell debris by centrifugation at 10.000 RPM for 20 min was used as the intracellular content. The separated pellet, after washing with 0.1 M phosphate buffer (pH = 7) was used as membrane fraction.

The chromium reducing activity of the three fractions thus obtained (the extracellular, the intracellular or cytoplasmic and the membrane fraction) were estimated by the decrease in chromium concentration in the sample with time using Cr (VI) specific colorimetric reagent S-diphenyl carbazide (DPC) 0.25% (w/v) prepared in acetone (AR). The reaction mixture containing 200 µl sample and 330 µL of 6 M H_2SO_4 was mixed with 400 µL of freshly prepared diphenylcarbazide solution and final volume was made to 10 ml using glass distilled water (Thacker *et al*., 2006). The residual chromium was measured at 540 nm by a spectrophotometer (Shimadzu, Japan) and the Cr (VI) concentration was calculated from the standard curve of $K_2Cr_2O_7$.

Measurement of total Chromium

The total Cr content was determined by AAS (Varian spectra AA 220) after acid digestion with concentrated HNO_3, with the addition of 33% H_2O_2 (Page *et al*., 1982).

Optimization of parameters for chromate reduction by the strain

The strain was grown in different flasks containing Cr (VI) supplemented media with a range of initial pH (4-9) at a fixed temperature and with a fixed pH at various temperatures (7˚-37˚C) to determine the most suitable pH and temperature respectively for achieving the maximum efficacy of the strain for chromate reduction.

Effect of exogenous additives on chromate removal

The Cr (VI) enriched growth medium was supplemented with untreated and acid treated indigenous sources of cysteine namely feathers, human hair, dried onion scales, Indian gooseberry fruit (*Phyllanthus emblica*) followed by a measurement of the residual chromium in the culture medium. For acid treatments, feathers and hairs were incubated with 50 mL of 1.0 N analytical-grade HCl for 24 h at 37˚C followed by thorough rinsing with distilled water and total drying.

Assessment of chromate reduction efficacy of the strain from chromium rich soil samples

Soil samples (20 g), were sterilised and mixed with various concentrations (160-40mg·l^{-1}) of Cr (VI) and sprinkled with the culture of the working strain (10 ml) and kept at a near natural environment in earthen pots for 10 days with proper control of non-inoculated soil for each set. After acid digestion (USEPA, 1996), total chromium of all the soil samples was measured by atomic absorption spectroscopy.

Chemicals

All chemicals used were of analytical grade purchased from Sigma chemicals Co. (St. Louis, USA), Merck, Germany and Himedia, India.

Each experiment was done in triplicate and their values were averaged.

Results and discussion

The chromate tolerant fungal strain *Trichoderma pseudokoningii* (Ray *et al*, 2013) was found to grow both on solid state fermentation (PDA plate) and in submerged culture supplemented with up to 1000 mg·l^{-1} of Cr (VI). In submerged culture, around 40% growth was observed up to a concentration of 720 mg·l^{-1}, above which, the growth of the strain was severely hindered. (Fig. 1). Hence, for better expression of its chromate removal efficacy, further experiments were designed with the cultures supplemented with a sub lethal concentration of hexavalent chromium (220 mg·l^{-1}) allowing 80% growth of the strain. Similar level of tolerance to Cr (VI) was reported from a yeast strain DBVPG 6502 (Baldi *et al*, 1990), whereas two strains namely *Aspergillus parasiticus* and *Aspergillus niger* were cultivated in a medium supplemented with only 20 mg·l^{-1} of Cr (VI) (Shugaba *et al*, 2012).

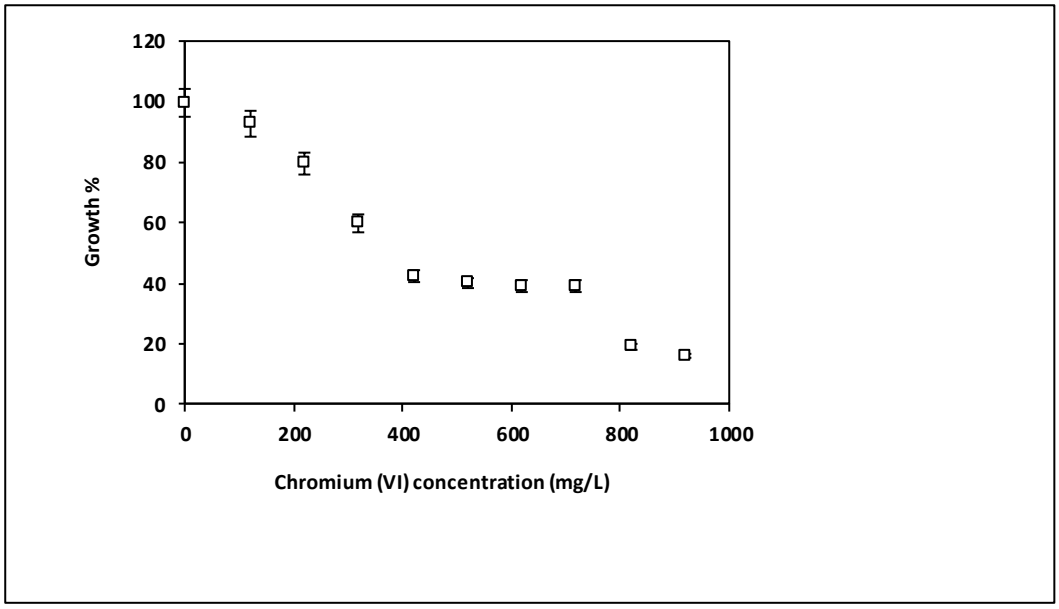

Fig. 1. Growth (dry weight) of *Trichoderma pseudokoningii* with different concentrations of Cr (VI)

Estimation of both the extra and intercellular chromate content indicated that about 68% of extra cellular hexavalent chromium was reduced within 4 days of incubation. Although a little amount of hexavalent chromium could be detected in the intracellular fluid only after 2 days of cultivation, the total hexavalent chromium was disappeared within 8 days of cultivation (Fig. 2). The existence of a small amount of intercellular chromate and absence of detectable Cr (VI) in the membrane fraction indicated the chromate removal process involved in the present strain was not through biosorption, as found in other fungal strains (Pal and Vimala, 2011; Khambhaty *et al*, 2009, Kumar *et al*, 2008; Mungasavalli *et al*, 2007). (Table 1). The total disappearance of hexavalent chromium from the medium and cellular environment indicated the chromate removal was not accomplished by bioaccumulation; instead a bio reduction method was adopted by the strain to convert the hexavalent into trivalent form.

Table 1. Comparative account of hexavalent chromium removing fungal strains

Fungi	Initial concentration of Cr(VI) (mgL^{-1})	Mechanism involved	References
Aspergillus niger	400	Biosorption	Khambhaty *et al*, 2009
Aspergillus niger	30	Biosorption	Kumar *et al*, 2008
Aspergillus niger	10	Biosorption	Mungasavalli *et al*.2007
Aspergillus niger	125	Bioaccumulation	Holda *et al,* 2013
Aspergillus parasiticus	20		Shugaba *et al*, 2012
Aspergillus niger			Shugaba *et al*, 2012
Aspergillus sp. N3 and N5	60		Fukuda *et al*, 2008
Aspergillus foetidus	5		Prasenjit and Sumathi 2005
Aspergillus oryzae	5	Biosorption	Reya Issac *et al*, 2012
Aspergillus flavus, *Aspergillus sp,A. niger*	600	Reduction-coupled biotransformation	Bennett *et al*, 2013
Aspergillus sojae	5	Biosorption	Reya Issac *et al*, 2012

Aspergillus flavus Aspergillus clavatus	300	Biosorption and biotransformation	Das, 2014
Candida sp	500	Biosorption	Baldi *et al*, 1990
Mucor hiemalis	50	Biosorption	Pillicsanmer *et al*, 1995.
Rhizopus oryzae	400	Reduction	Sukumar, 2010
Rhizopus nigricans	100	Biosorption	Sudha and Abraham 2001
Rhizopus arrhizus	200	Biosorption	Preetha and Viruthagiri 2007.
Rhizopus arrhizus	50	Biosorption	Shroff and Vaidya, 2012
Rhizopus arrhizus	100	Biosorption	Prakasham *et al*, 1999.
Phanerochaete chrysosporium	100		Pal and Vimala .2011.
Penicillium sp	60		Kumar *et al*, 2008
Penicillium janthinellum	30		Kumar *et al*, 2008
Penicillium citrinum	125	Bioaccumulation	Holda *et al,* 2013
Halo tolerant *Penicillium*	400	Biosorption	Patel and Power, 2014
Paecilomyces sp,	1000	Biosorption	Juan Fernando Cárdenas and Ismael Acosta ,2011.
Fusarium solani	500	Biosorption	Sen and Ghosh Dastidar,2011
Fusarium solani	500	Bioreduction	Sen *et al,* 2013
Gloeophyllum sepoarium			Achal *et al,* 2011
Ganoderma lucidum and *Mucor hiemalis*	20	Biosorption	Verma *et al*, 2014
Trichoderma viride	125	Bioaccumulation	Holda *et al*, 2013
Trichoderma harzianum	40	reduction	Sarkar *et al*, 2013
Trichoderma harzianum	30	Biosorption	Sarkar *et al*, 2010
Trichoderma pseudokoningii	220	Reduction	Present work

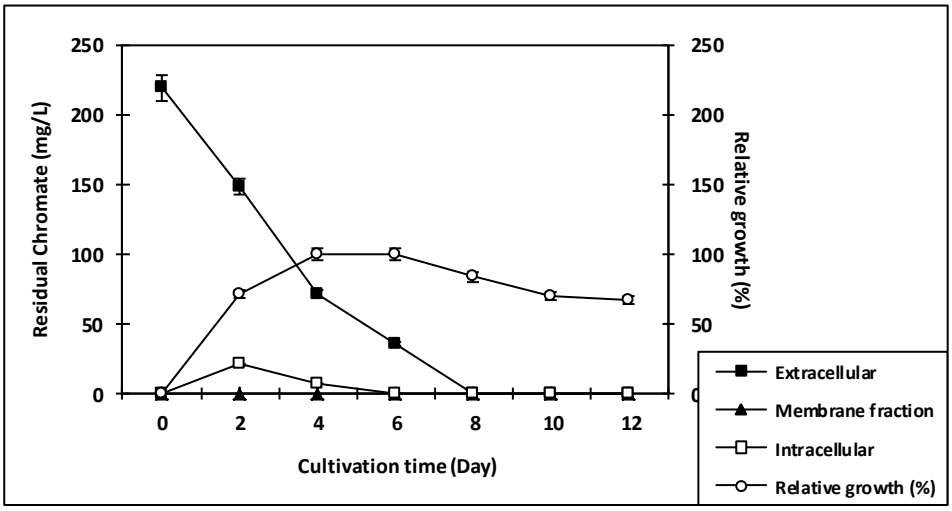

Fig. 2. Kinetics of extra and intra cellular chromate reduction by *Trichoderma pseudokoningii*

Initial Cr (VI) concentration: 220 ml/L, 100% growth = 150 mg

The Cr (VI) reduction was found to achieve its highest level at a neutral pH (Fig. 3) and the efficacy of chromate reduction was positively correlated with growth pattern of the strain. The preference of neutral pH for chromate removal was also found in *Aspergillus* sp N2 and *Penicillium* sp N3 (Fukuda *et al*, 2008) and in *Gloeophyllum sepoarium* (Achal *et al*, 2011).

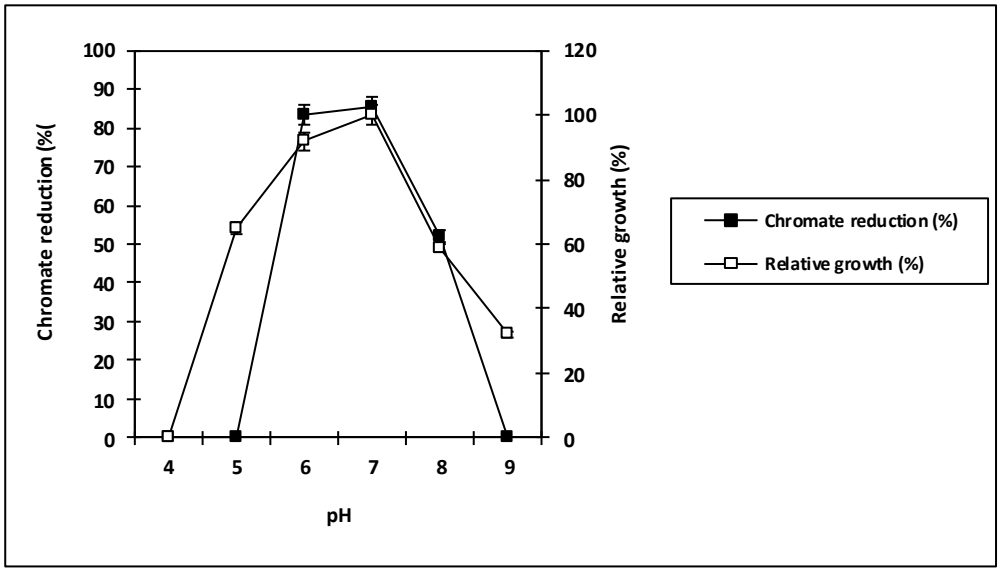

Fig. 3. Effect of pH on growth and chromate reduction by *Trichoderma pseudokoningii*

The growth pattern of the strain was closely related to chromium reduction and as the strain showed highest growth at 27°C (Fig. 4), maximum amount of hexavalent chromium was found to be reduced at 27°C, above which the reduction rate declined. Similar temperature preference was reported from *Gloeophyllum sepoarium* (Achal *et al*, 2011) and *Paecilomyces sp* (Cárdenas-González and Acosta-Rodríguez, 2011) and *Trichoderma harzianum* (Sarkar *et al*, 2013).

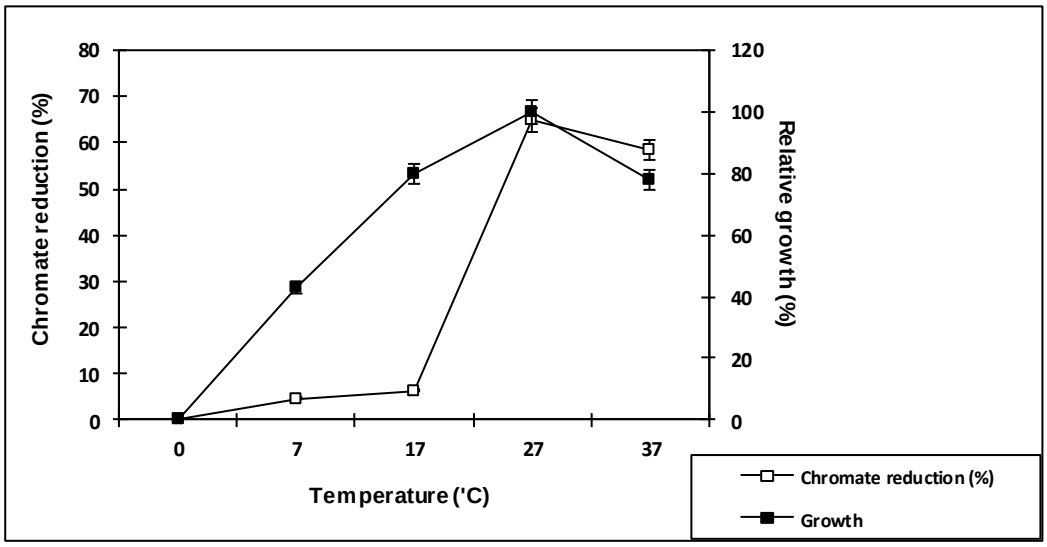

Fig. 4. Effect of temperature on growth and chromate reduction by *Trichoderma pseudokoningii*

The growth and bio reduction efficacy of the working strain was increased in presence of glucose Similar report was obtained from Cr (VI) removal by *Serratia* sp (González *et al,* 2014). The most preferred concentration of glucose was found to be 0.5 % (w/v) (Fig. 5).

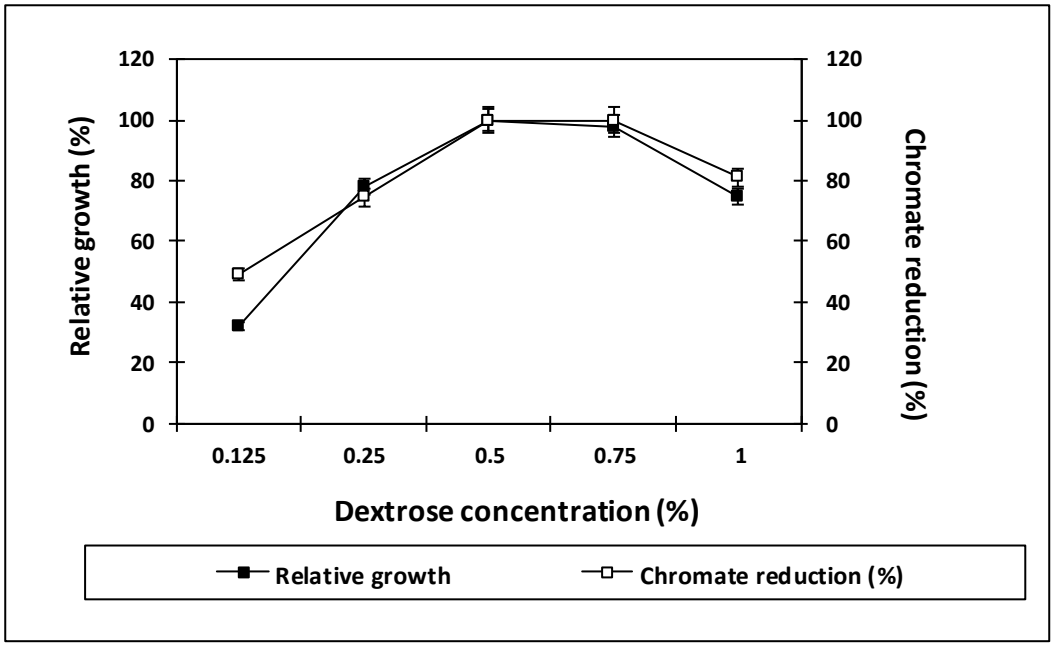

Fig. 5. Effect of dextrose concentration on growth and chromate reduction by *Trichoderma pseudokoningii*

Amongst the nitrogen sources tested peptone was found to promote growth as well as chromate reduction efficiency of the strain (Fig. 6).

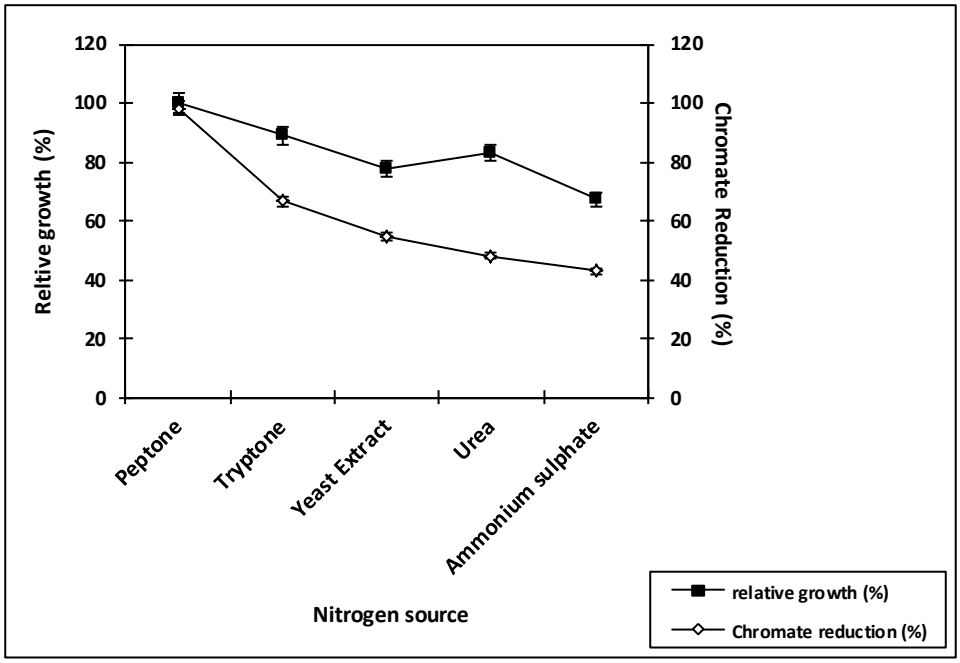

Fig. 6. Effect of nitrogen source on growth and chromate reduction by *Trichodermapseudokoningii*

Chromate reduction after 5 days of cultivation (with 80% chromate reduction without additive) in presence of various additives indicated that exogenous thiols like cysteine and DTT could enhance the chromate reducing activity of the strain (Fig. 7). This could be due to the increased activity of the chromate reductase through the activation of the catalytic site by the exogenous thiols. On the other hand the surfactants and chelators remarkably affected the efficacy.

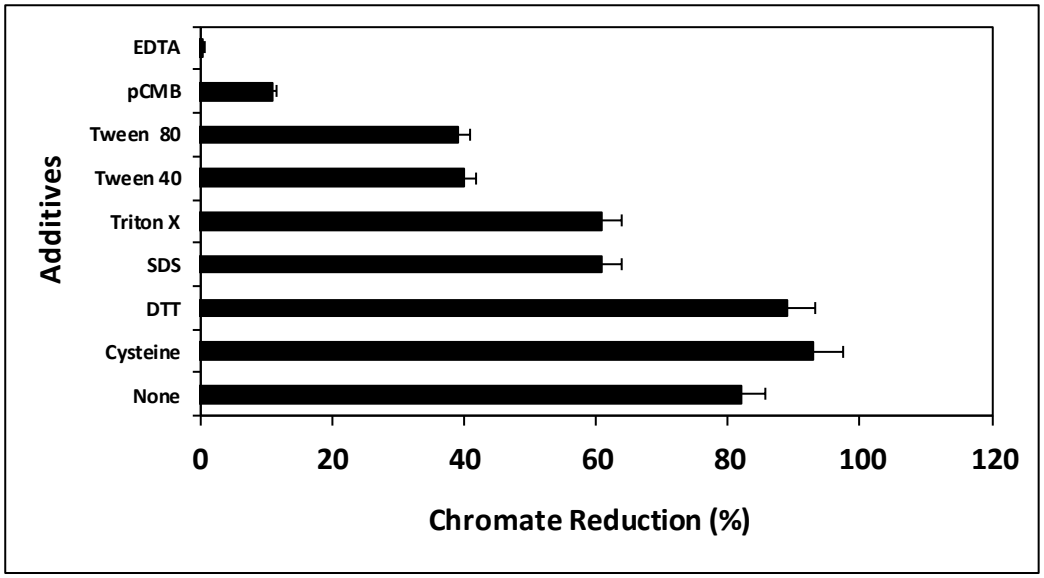

Fig. 7. Effect of additives on growth and chromate reduction by *Trichoderma pseudokoningii*

Since exogenous cysteine enhanced chromate reduction, some natural sources of cysteine were tried (Table 2), of which human hair and feather itself could reduce the chromate content even in the absence of the fungal spores. Acid treatment of human hair and feather gave remarkable results in chromate reduction and could be effectively used for bioremediation of polluted soil along with the fungal spores.

Table 2. Effect of natural additives on chromate reduction

Medium supplement	**Residual concentration of Cr (VI) (mg/L)***	
	Non inoculated	**Inoculated with fungal spores**
Medium supplemented with Cr (VI)	212	130
Medium supplemented with Cr (VI) and untreated human hair	200	125
Medium supplemented with Cr (VI) and acid treated human hair	126	42.6
Medium supplemented with Cr (VI) and untreated feather	210	129
Medium supplemented with Cr (VI) and acid treated feather	170	119
Medium supplemented with Cr (VI) and dried onion scales	200	112
Medium supplemented with Cr (VI) and vitamin C.	200	110
Medium supplemented with Cr (VI) and dried Indian gooseberry fruit.	200	110

*Initial Cr (VI) concentration: 212 mg/L, cultivation time: 72 hrs.

Removal of Cr (VI) from soil

The soil samples treated with increasing concentration of hexavalent chromium and kept in near natural conditions for 10 days showed almost no change in total chromium content, whereas chromium infested soil samples with fungal spores showed remarkable reduction in chromate content (Table 3).

Although the chromate removal by the fungal strain was more effective in batch culture than that of the soil, which might be due to the presence of some natural constraints existing in the

microenvironment of soil. However 97-99% chromate removal within 10 days confirmed the fact that the strain could be used as successful bioremediation of hexavalent chromium pollution.

Table 3: Change in chromium content in the soil (as estimated by Atomic absorption spectrometry)*

Cr (VI) concentration (mg/L)	Total Chromium content (mg/L)	
	Non inoculated soil	**Soil inoculated with fungal culture**
400	397± 0.26	10.58±0.31
320	318.8± 0.21	5.23±0.30
240	237.2 ± 0.36	5.02±0.23
160	157.6± 0.18	2.36±0.26

*Time of exposure: 10 days.

Conclusion

The present study concludes that the presently described fungal strain of *Trichoderma pseudokoningii,* isolated from tannery effluent showed the ability to tolerate remarkably high concentrations of hexavalent chromium which was probably acquired as an adaptive feature for surviving in chromate infested environment. Further the increased rate of chromate bioreduction in presence of natural ingredients like human hair, feather, onion scales, and gooseberry fruits opens up a promising way of chromate detoxification of the polluted soil near industries of tanning, plating and pigment manufacture.

Acknowledgments

The authors wish to thank RSIC, Bose Institute, Kolkata for providing the facility of Atomic Absorption Spectrophotometer and DST-FIST, Government of India for providing infrastructural facility at Bethune College, Kolkata, India.

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