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Studies on biological reduction of chromate by *Streptomyces griseus*

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ABSTRACT

Chromium is a toxic heavy metal used in various industries and leads to environmental pollution due to improper handling. The most toxic form of chromium Cr(VI) can be converted to less toxic Cr(III) by reduction. Among the actinomycetes tested for chromate reduction, thirteen strains reduced Cr(VI) to Cr(III), of which one strain of *Streptomyces griseus* (NCIM 2020) was most efficient showing complete reduction within 24 h. The organism was able to use a number of carbon sources as electron donors. Sulphate, nitrate, chloride and carbonate had no effect on chromate reduction during growth while cations such as Cd, Ni, Co and Cu were inhibitory to varying degrees. Chromate reduction was associated with the bacterial cells and sonication was the best method of cell breakage to release the enzyme. The enzyme was constitutive and did not require presence of chromate during growth for expression of activity. Chromate reduction with cell free extract (CFE) was observed without added NADH. However, addition of NAD(P)H resulted in 2–3-fold increase in activity. Chromate reductase showed optimum activity at 28 °C and pH 7.

1. Introduction

Industrialization is a hallmark of civilization, however, the fact remains that industrial emissions have been adversely affecting the environment leading to destruction of many agricultural lands and water bodies, thus becoming a matter of great concern. Hexavalent chromium (which exists as chromate) containing waste is generated from many industrial applications such as textile production, electroplating, metallurgy and petroleum refining. Chromium is an essential micronutrient required for the growth of many organisms. However, at high concentrations, Cr(VI) is toxic, mutagenic, carcinogenic and teratogenic [1]. According to the United States Environmental Protection Agency (USEPA), industries in the United States of America (USA) use more than 50,000 tonnes of Cr(VI) every year and release 5000 kg/d into the environment [2]. Cr(VI) undergoes reduction to less toxic Cr(III) in the presence of reducing agents and precipitates as hydroxides. Biological Cr(VI) reduction to Cr(III) has great potential for removing it from contaminated waters and waste streams.

Biological reduction of chromate has been studied mainly for the purpose of bioremediation, which is based on the concept that Cr(III) has negligible toxicity and minimal solubility compared with Cr(VI). Chromate reducing ability has been demonstrated in a wide range of microbes, including bacteria, actinomycetes and fungi [3–5]. A variety of Gram-negative [6–9] as well as Gram-positive bacteria [4,10–14] are known to reduce Cr(VI). Both aerobic [4,10,13,14] as well as anaerobic [1,15] chromate reduction are reported in the literature. Aerobic bacteria such as *Pseudomonas ambigua* G-1, *Pseudomonas putida* and *Streptomyces* sp. can reduce chromate by NAD(P)H-dependant chromate reductases [4,8,16]. In aerobic systems, most of the chromate reductases reported are soluble in the cytosol and reduce Cr(VI) to Cr(III) inside the cell or outside the plasma membrane [17,18].

Actinomycetes constitute a significant component of the microbial population in most soils and *Streptomyces* account for 90% of the total actinomycetes population. A few strains have also been reported from deep sea sediments. Being natural inhabitants of soil, water and manure, actinomycetes are continuously exposed to different metals present in these habitats. In order to survive in the polluted environment, they have to adapt or acquire tolerance or resistance to these metals. Their metabolic diversity and particular growth characteristics, mycelial form and relatively rapid colonization of selective substrates, make them the most suitable agents for bioremediation of metal and organic compounds. There are only few reports available in the literature on chromate reduction by actinomycetes. Das and Chandra [4] partially purified a membrane associated chromate reductase from *Streptomyces* sp. The crude cell extracts from this organism reduced Cr(VI) to Cr(III) in the presence of NAD(P)H. Amoroso et al. [19] reported chromium accumulation in two *Streptomyces* spp. isolated from river sediments. Polti et al. [20] reported qualitative and semi-quantitative screening of Cr(VI) resistance in actinomycete isolates. Chromate reduction during growth and by resting cells of *S. griseus* was reported by Laxman and More [13]. Chromate reduction by immobilized *S. griseus* in batch sytems and in a bioreactor has been reported [21,22]. Megharaj et al. [23] reported hexavalent chromium reduction by *Arthrobacter*

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sp. isolated from soil contaminated with tannery waste. *Arthrobacter crystallopoietes* produced a periplasmic chromate reductase that was stimulated by NADH [11]. Desjardin et al. [24] reported non-enzymatic chromate reduction by *Streptomyces thermocarboxydus* NH50. They isolated a small molecular weight (<1 kDa) reducing agent from culture filtrate but the identity of the reducing agent was not reported.

As evident from the literature, not much information pertaining to chromate reduction by actinomycetes is available in contrast to the extensive studies carried out in bacteria. Therefore, it is important to assess the predominance of chromate reductase activity among actinomycetes, knowledge regarding which will be useful in understanding its potential utility for detoxification of Cr(VI) contaminated industrial effluents.

The present paper deals with screening of actinomycete strains available in our culture collection for growth as well as chromate reduction and selection of a promising strain for detailed investigation on enzymatic reduction of Cr(VI) including the factors influencing chromate reduction.

2. Materials and methods

2.1. Microorganisms and culturing conditions

Actinomycete strains used in the present study were routinely sub-cultured once in two weeks on Broth-II agar slants as previously reported [13]. Growth and Cr(VI) reduction studies were carried out in submerged culture in sterile Broth-II liquid containing potassium dichromate as source of Cr(VI) as described earlier [13]. Ten percent (v/v) vegetative inoculum was used to inoculate the experimental flasks and incubated in a rotary incubator (200 rpm) at 28 °C for 2–5 days. Data presented are mean of two independent experiments conducted in duplicates. The error bars in figures indicate standard error of mean.

2.2. Screening of actinomycetes for chromium tolerance and reduction

For screening, nineteen actinomycete strains (16 strains from National Collection of Industrial Microorganisms, Pune, India and 3 new isolates) were grown in Broth-II medium containing 25 mg/L Cr(VI). Growth of the organism was determined as mycelial dry weight from 50 mL cultured medium. Mycelial biomass was collected by filtration, washed to remove media constituents and dried at 80 °C to constant weight. Relative growth was expressed as a percentage of those obtained in controls devoid of chromium, which was taken as 100%.

Samples were withdrawn at regular intervals and residual Cr(VI) in the culture supernatant was checked. The difference in initial and residual Cr(VI) was taken as Cr(VI) reduced and expressed as percentage of initial Cr(VI). Uninoculated controls were included for each experiment and incubated under identical conditions.

2.3. Effect of chromium concentration

Effect of chromium concentration on growth and reduction was studied by growing the organism in Broth-II medium containing 10–89 mg/L Cr(VI). Residual Cr(VI) concentration was monitored during growth and expressed as percentage of initial Cr(VI) values. Rate of Cr(VI) reduction (mg chromate reduced/L/h) was calculated from mg of chromate reduced during the incubation period.

2.4. Effect of electron donors

Effect of electron donors on chromate reduction during growth was investigated in Broth-II medium containing one of the following substrates (0.2%): glucose, sucrose, glycerol, ethanol, glycine, sodium salts of acetic acid, citric acid and tartaric acid.

2.5. Effect of anions and cations

Effect of anions and cations on chromate reduction during growth was investigated by growing the organism in Broth-II medium containing 25 mg/L Cr(VI) supplemented with anions such as carbonate, nitrate, chloride, sulphate and cations such as cadmium, cobalt, copper and nickel at equimolar concentrations.

2.6. Localization of reductase activity

Cells grown for 72 h in Broth-II medium with 25 mg/L Cr(VI) were used for localization studies. Cells were collected by centrifugation at 8000 rpm, 15 min and the culture supernatant was filter sterilized (on sterile 0.22 μm Millipore filters). Cells (2 g wet weight) and culture supernatant (50 mL) were separately incubated with fresh 25 mg/L Cr(VI) under sterile conditions at 28 °C, 180 rpm up to 24 h and residual Cr(VI) concentration was determined.

2.7. Methods of cell breakage for enzyme extraction

Cells grown in MGYP medium for 48 h were harvested by filtration followed by washing three times with 25 mM Tris HCl pH 7.0 buffer. Two grams wet cells suspended in 4 mL of 25 mM Tris HCl pH 7.0 were used for each method of cell breakage. The broken cell suspension was centrifuged at 4 °C, 12,000 × *g* for 10 min. The cell free extract (CFE) obtained was treated as source of enzyme and used for chromate reductase studies. Three different methods of cell breakage were employed viz. grinding with neutral alumina [4], crushing the frozen cells with liquid nitrogen and sonication [10]. Cells were frozen with liquid nitrogen and crushed on ice for 20 min, dispensed in buffer and processed further. Alternatively, cells were sonicated in an ice bath with an ultrasonic probe. Power was applied for 4 min with 10 s pulses at 60 W each.

2.8. Chromium (VI) analysis

Cr(VI) was determined spectrophotometrically using diphenylcarbazide (DPC) [25].

2.9. Conversion of Cr(VI) to Cr(III) by cell free extract (CFE)

Changes in Cr(VI) and Cr(III) during the conversion were monitored by DPC and absorbance at 595 nm respectively [4]. One millilitre of reaction mixture containing 25 mM Tris HCl buffer (pH 7.0), 64 mg/L Cr(VI) and CFE was incubated at 28 °C for 2 h (longer incubation period was required to detect noticeable changes as a result of Cr(VI) reduction). The CFE was heated at 100 °C for 5 min and assayed for residual chromate reductase activity.

2.10. Effect of dialysis and addition of NAD(P)H on chromate reduction

CFE obtained after sonication was dialyzed overnight against 25 mM Tris HCl pH 7.0 at 4 °C. One millilitre of assay mixture containing 25 mM Tris HCl buffer pH 7.0, 30 mg/L of Cr(VI) was incubated with dialyzed or undialyzed CFE and residual Cr(VI) was estimated by DPC.

The effect of NAD(P)H addition on chromate reduction by dialyzed CFE was studied using the following assay conditions: in one set, 1 mL reaction mixture contained 25 mM Tris HCl buffer pH 7.0, 0.4 mM Cr(VI) and CFE with or without 1.6 mM NAD(P)H. In another set, 1 mL reaction mixture contained 25 mM Tris HCl

Table 1
Cr(VI) reduction by actinomycetes during growth in Broth II + 25 mg/L Cr(VI).

Name of organism	Code	Residual Cr(VI) (%)		Rate of chromate reduction (mg Cr(VI)/L/h)	
		24 h	48 h	24 h	48 h
S. griseus	NCIM 2183	56.25	36.71	0.5157 ± 0.011	0.3731 ± 0.040
S. griseus	NCIM 2020	0	–	1.0420 ± 0.042	–
S. griseus	NCIM 2420	63.26	45.03	0.4335 ± 0.033	0.3248 ± 0.016
S. griseus	NCIM 2496	56.59	55.74	0.5122 ± 0.019	0.2612 ± 0.028
S. griseus	NCIM 2621	63.34	62.23	0.4325 ± 0.016	0.2228 ± 0.024
S. griseus	NCIM 2622	61.29	43.64	0.4560 ± 0.042	0.3320 ± 0.023
S. griseus	NCIM 2623	25.44	0.38	0.8781 ± 0.014	0.5866 ± 0.011
S. griseus	NCIM 2624	74.42	72.99	0.3013 ± 0.012	0.1591 ± 0.029
S. griseus	NCIM 2625	68.04	58.3	0.3772 ± 0.022	0.2461 ± 0.024
S. griseus	NCIM 2626	59.7	58.11	0.4746 ± 0.011	0.2466 ± 0.022
S. griseus	NCIM 2952	50.49	37.13	0.5842 ± 0.039	0.3706 ± 0.026
Isolate-2	GPR-2-8	55.85	33.48	0.5205 ± 0.018	0.3922 ± 0.033
Isolate-3	GPA	56.35	33.81	0.5145 ± 0.032	0.3900 ± 0.015
Uninoculated control	–	99.65	99.21	–	–

Rates of chromate reduction presented with standard error.

buffer pH 7.0, 0.4 mM Cr(VI) and 1.6 mM NAD(P)H with or without CFE and incubated at 28 °C for 30 min. The residual Cr(VI) in the reaction mixture was determined by DPC as mentioned earlier.

2.11. Chromate reductase assay

Chromate reductase assay was carried out by incubating 1 mL of assay mixture containing (to a final concentration) 0.4 mM Cr(VI), 1.6 mM NADH and CFE in 25 mM Tris HCl buffer, pH 7.0 at 28 °C, 30 min. One unit of reductase activity is defined as the amount of enzyme that reduced 1 nmol of Cr(VI) per min under the assay conditions.

2.12. Effect of addition of chromium as inducer and growth medium

To study the constitutive or inducible nature of chromate reductase, cells grown under identical conditions in Broth-II medium with and without 25 mg/L Cr(VI) were sonicated and reductase activity in CFE was determined. To study the effect of growth medium, cells grown for 48 h either in Broth-II or MGYP medium were sonicated and activity in CFE was estimated.

2.13. Fractional ammonium sulphate precipitation

The CFE obtained from cells grown on MGYP was used for fractional precipitation studies. The enzyme was fractionated with ammonium sulphate at following saturations (%): 0–20, 20–40, 40–60, 60–80. The supernatant obtained after centrifugation (12,000 × *g*, 15 min) was processed for next step of fractionation. The pellet was re-suspended in 25 mM Tris HCl, pH 7. 1 mM dithiothreitol (DTT) was added to prevent loss in activity.

2.14. Effect of pH and temperature on chromate reductase activity

Optimum pH and temperature for activity were determined for CFE and partially purified enzyme (40–60 ammonium sulphate precipitate) by incubating the reaction mixture at pH values ranging from 5 to 9 at 28 °C and at temperatures ranging from 20 to 60 °C at pH 7.0 respectively for 30 min. 25 mM sodium citrate (pH 5 and 6) and Tris HCl (pH 7–9) buffers were used. Relative activity was expressed as percentage of maximum activity taken as 100%.

2.15. Protein estimation

Protein content in the sample was determined according to Lowry et al. [26] using bovine serum albumin as standard.

3. Results and discussion

3.1. Screening of actinomycetes for chromium tolerance and reduction

Actinomycetes mainly belonging to genus *Streptomyces* were screened for Cr(VI) tolerance and reduction during growth on Broth-II medium containing 25 mg/L Cr(VI). Extent of growth in 48 h varied with strain and ranged from 13 to 90 mg (Fig. 1). Thirteen strains among the nineteen actinomycetes tested showed good growth while the remaining 6 strains did not grow in the presence of Cr(VI). These thirteen actinomycetes were investigated for Cr(VI) reduction. Cr(VI) concentration in the culture supernatant decreased from 25 mg/L to less than 18 mg/L in 48 h depending on the strain while it was undetectable within 24 h for *S. griseus* (NCIM 2020). Decrease in Cr(VI) was concomitant with increase in Cr(III) and was confirmed by estimating the chromium after oxidizing the

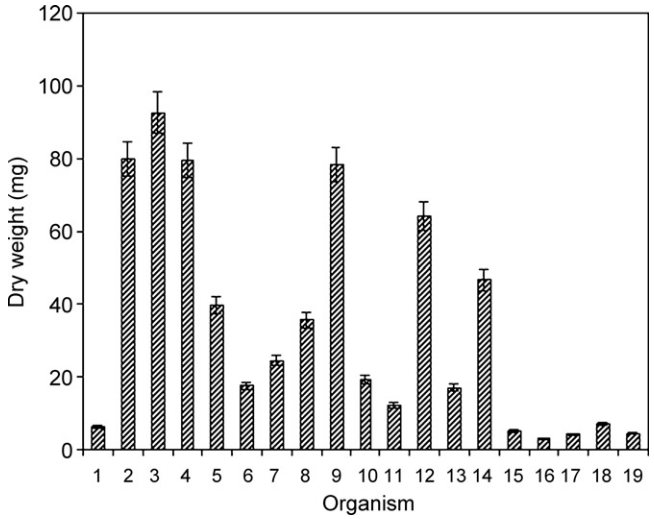

Fig. 1. Growth of actinomycetes in Broth-II + 25 mg/L Cr(VI).
1, GPR-2-1a; 2, GPR-2-8; 3, GPA; 4, NCIM 2183; 5, NCIM 2420; 6, NCIM 2496; 7, NCIM 2621; 8, NCIM 2622; 9, NCIM 2623; 10, NCIM 2624; 11, NCIM 2625; 12, NCIM 2952; 13, NCIM 2626; 14, NCIM 2020; 15, NCIM 2213; 16, NCIM 5008; 17, NCIM 2413; 18, NCIM 2086; 19, NCIM 2980.

Table 2
Rate of chromate reduction by actinomycetes.

Name of organism	Initial Cr(VI) (mg/L)	Growth medium	Incubation time	Rate of reduction (mg Cr(VI) reduced/L/h)	Refs.
Arthrobacter sp.	50	Minimal salts medium	30 mg in 46 h	0.652	[23]
Streptomyces sp.	≈36	Peptone yeast extract broth	35 mg in 72 h	0.494	[4]
Streptomyces M46	52	Minimal medium	26 mg in 72 h	0.361	[20]
Streptomyces griseus	15	Broth-II medium	15 mg[a] in 12 h	1.250	Present work
	30		30 mg[a] in 24 h	1.224	
	43		40 mg[a] in 24 h	1.697	
	64		54 mg[a] in 24 h	2.396	
	89		34.2 mg[a] in 12 h	2.850	

[a]mg Cr(VI) reduced.

trivalent chromium to hexavalent form in the supernatant. Concentration of Cr(VI) remained practically unchanged in an uninoculated control (Table 1). Eleven strains showing decrease in Cr(VI) concentration belonged to *S. griseus* while the other two are yet to be identified. Das and Chandra [4] reported a decrease of 25–30% of Cr(VI) in the culture filtrate in 72 h when *Streptomyces* sp. 3 M was grown in peptone yeast extract medium containing 100 mg/L $K_2Cr_2O_7$ [36 mg/L Cr(VI)] under aerobic conditions. Polti et al. [20] reported 40–50% removal from 1 mM (52 mg/L) Cr(VI) after 72 h of incubation in minimal medium by *Streptomyces* M46 and strains isolated from wastewaters of sugarcane plant. *Arthrobacter* sp. reduced 60% of 50 mg/L Cr(VI) in 46 h under aerobic conditions when grown in minimal salts medium [23]. Rate of chromate reduction among the actinomycetes tested in the present investigation varied for each strain and at the end of 24 h, it ranged between 0.3013 and 1.042 mg Cr(VI) reduced/L/h. *S. griseus* NCIM 2020 which showed highest rate of reduction was found to be most efficient and selected for further studies (Table 1).

3.2. Effect of chromium concentration

Good growth with no inhibition was seen up to 20–25 mg/L of Cr(VI). Increasing the chromium concentration increased inhibition of growth but visible growth was still observed in media containing 89 mg/L chromium. Reduction was nearly complete within 12 h when the initial Cr(VI) concentration ranged between 10 and 15 mg/L while it was slower and required longer incubation time of 36–48 h with higher initial Cr(VI) concentration (Fig. 2). Relative growths in the presence of 10, 15, 30, 43, 53, 64, 71, 89 mg/L Cr(VI) were 96.55, 89.66, 84.14, 76.55, 66.21, 55.17, 41.38 and 28% respectively clearly indicating that increased Cr(VI) concentration inhibited cell growth.

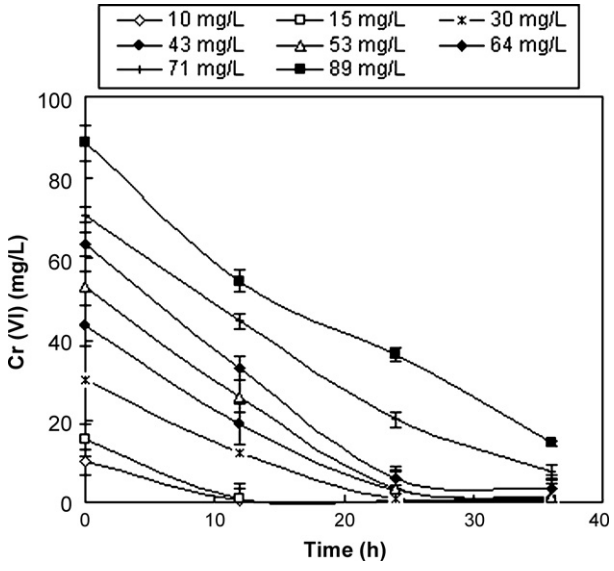

Fig. 2. Effect of initial Cr(VI) concentration on chromate reduction by NCIM 2020.

Rate of reduction (mg Cr(VI) reduced/L/h) by *S. griseus* (NCIM 2020) increased linearly with increasing Cr(VI) concentration but decreased with incubation time. Rate of reduction at 12 h increased from 0.788 mg/L/h for 10 mg/L to 2.85 mg/L/h for 89 mg/L. However at the fixed initial Cr(VI) of 30 mg/L, rate decreased from 1.487 mg/L/h in 12 h to 0.815 mg/L/h in 36 h. High initial rates of reduction are also reported for other bacterial cultures [14]. The highest rate of reduction with initial chromate concentration of 89 mg/L for *S. griseus* was 2.85 mg Cr(VI) reduced/L/h during the initial period of 12 h. To the best of our knowledge, this is the highest rate reported so far among actinomycetes (Table 2). Specific rate of chromate reduction (mg Cr(VI) reduced/g biomass/h) ranged between 0.188 and 1.491 for initial Cr(VI) concentrations of 10 and 53 mg/L respectively. Elangovan et al. [27] reported specific rate of Cr(VI) reduction for *Bacillus* sp. to be 0.794 for initial Cr(VI) of 20 mg/L.

3.3. Effect of electron donors

S. griseus NCIM 2020 was capable of utilizing a variety of substrates as sole sources of carbon and energy for chromate reduction. Growth as mycelial dry weight ranged between 40 and 60 mg in all the substrates tested except in two cases, one where there was no addition and other where glycine was used. Maximum Cr(VI) reduction was observed in the presence of glucose followed by glycerol and acetate. No reduction was observed in the absence of added electron donor or in the presence of glycine indicating the requirement for an electron donor for growth and reduction (Fig. 3).

These results are consistent with other reports indicating requirement of glucose or acetate. For example, Das and Chandra [4] reported 25–30% Cr(VI) reduction after 72 h of growth of *Streptomyces* sp. which increased to 97% in the presence of 0.5% glu-

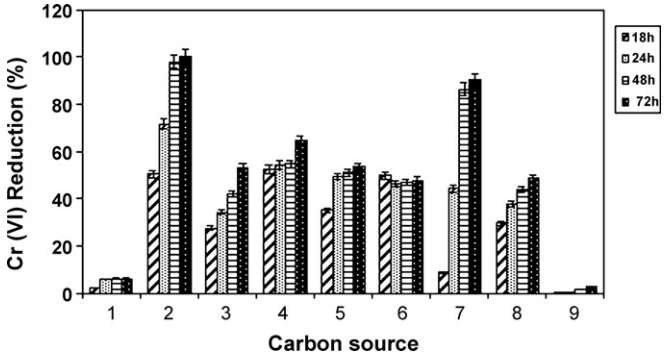

Fig. 3. Effect of carbon source on chromate reduction.
(1, none; 2, glucose; 3, sucrose; 4, acetate; 5, citrate; 6, tartarate; 7, glycerol; 8, ethanol; 9, glycine).

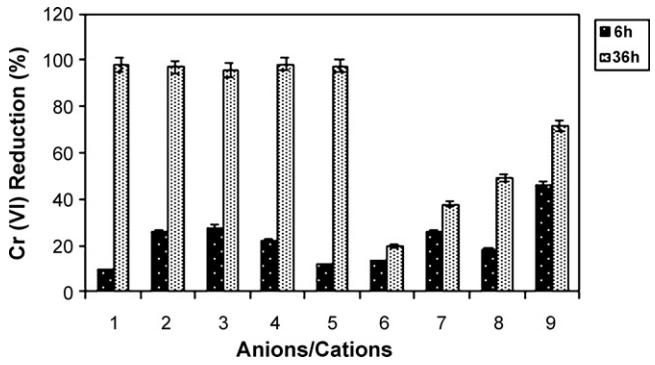

Fig. 4. Effect of anions/cations on chromate reduction.
(1, none; 2, carbonate; 3, nitrate; 4, chloride; 5, sulphate; 6, cadmium; 7, cobalt; 8, nickel; 9, copper).

cose. Though *B. sphaericus* used variety of organic compounds like amino acids and organic acids and nitrogenous substances as electron donors, pronounced reduction occurred only when glucose or yeast extract were used [14].

3.4. Effect of anions and cations

Cr(VI) removal from industrial effluents by reduction can be problematic as anions or cations or other heavy metals present in the wastewaters can influence chromate reduction. Effect of ions on Cr(VI) reduction during growth of *S. griseus* revealed that none of the anions tested had any significant effect while cations inhibited Cr(VI) reduction to varying degrees. Inhibition of growth was in the following order: $Cd^{2+} > Cu^{2+} > Co^{2+} > Ni^{2+}$. Inhibition of Cr(VI) reduction also followed similar trend with the exception of Cu^{2+} which showed least inhibition (Fig. 4). It is surprising to see that sulphate a known competitive inhibitor of chromate transport [18], did not inhibit Cr(VI) reduction in the present study. Chromate reduction by culture supernatants of *Streptomyces thermocarboxydus* NH50 was enhanced by 0.1 mM Cu^{2+} whereas Ni^{2+} and Cd^{2+} had no significant effect [24].

3.5. Localization of reductase activity

As chromate reduction was seen during growth of the organism, it was of interest to check if the reductase activity was extracellular or associated with cells. No chromate reduction was observed in culture supernatant even after prolonged incubation (96 h) while complete reduction within 24–48 h was noticed with cells indicating that the reductase activity was associated with the cells (data not shown).

3.6. Methods of cell breakage and conversion of Cr(VI) to Cr(III) by cell free extract (CFE)

Since the reductase activity was associated with the whole cells, it was necessary to break the cells to release the enzyme. 64 mg/L Cr(VI) was incubated with 0.5 mL of the CFE obtained from each method and residual concentration was monitored. Among the three methods tested for cell breakage, sonication was found to be the best which showed highest decrease in Cr(VI) concentration (35.31%) followed by liquid nitrogen (12.03%) while grinding with neutral alumina was poor (4.56%).

The CFE obtained after sonication was used for Cr(VI) reduction. The decrease in Cr(VI) concentration from 63.606 to 22.462 mg/L in the presence of CFE corresponded with increase in Cr(III) absorbance at 595 nm from 0.048 to 0.308 during the 2 h incubation. This clearly indicated that CFE was able to reduce Cr(VI) but was completely inactivated by heating at 100 °C for 5 min. Thus, Cr(VI) reduction by *S. griseus* appears to be enzymatic since it was catalyzed by CFE which was heat labile and proteinaceous in nature.

Table 3
Loss of chromate reduction activity on dialysis and restoration with NADH.

Component	Residual Cr(VI) (mg/L)	Cr(VI) reduction (%)
Undialyzed CFE	22.385	26.32
Dialyzed CFE	29.540	2.77
Dialyzed CFE + NADH	22.260	26.73

3.7. Effect of dialysis and addition of NAD(P)H on chromate reduction

As shown in Table 3, the Cr(VI) reducing activity was lost on dialysis. However, the activity was restored by the addition of NADH suggesting the loss of cofactor(s) required for reduction on dialysis. Similar observation was made by Horitsu et al. [6] in the case of *P. ambigua* G-1. Since NADH restored activity lost on dialysis, effect of addition of NADH and NADPH on chromate reduction was investigated. NADH and NADPH significantly increased the reduction and activities were nearly 3 times higher in the presence of added NAD(P)H (Table 4). There was no preference for electron donor and both NADH and NADPH acted equally well.

Our work is in concurrence with other studies where NAD(P)H dependant enzymatic reduction occurred under aerobic conditions [8,14–17]. NADH and NADPH also served as electron donors for reduction by *Streptomyces* sp. [4]. Addition of 1 mM NADH enhanced the Cr(VI) reductase activity in the cell free extracts of all the three isolates of *Bacillus* sp. G1DM20, G1DM22 and G1DM64 [28]. In contrast, chromate reductase from *P. ambigua* G-1 required NADH but not NADPH as electron donor for Cr(VI) reduction [6].

Unlike *P. putida* which required addition of NADH for activity [7], CFE from *S. griseus* NCIM 2020 was able to reduce chromate even in the absence of exogenous addition of NAD(P)H since the CFE itself appears to have the cofactors necessary for reduction (Table 4). Similar observations were also made by Horitsu et al. [6] with chromate-resistant *P. ambigua* G-1 strain which used internally stored reserves as electron donors. Addition of NAD(P)H significantly enhanced chromate reduction by 2–3-fold in *S. griseus*. Camargo et al. [10] reported similar results with *Bacillus* sp. ES 29 where addition of NADH as electron donor to the reaction mixture increased the reduction by 4.6 times after 30 min of incubation. The partial inhibition of chromate reductase by azide [13] and the ability of NADH/NADPH to act as electron donors suggests that an electron transport system is involved.

Table 4
Effect of NAD(P)H addition on chromate reduction.

	0 min	30 min		
	Initial Cr(VI) (mg/L)	Residual Cr(VI) (mg/L)	Residual Cr(VI) (%)	Reductase activity (U/mL)
NAD(P)H	25.01	23.50	94.03	–
CFE	21.18	16.57	78.92	8.53
CFE + NADH	21.23	5.77	27.49	27.94
CFE + NADPH	21.08	6.71	31.96	26.30

Table 5
Fractional ammonium sulphate precipitation.

Fraction	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg protein)
CFE	22.70	10.97	2.07
0–20	8.76	4.33	2.02
20–40	22.73	4.99	4.55
40–60	39.64	7.83	5.06
60–90	1.72	2.90	0.59

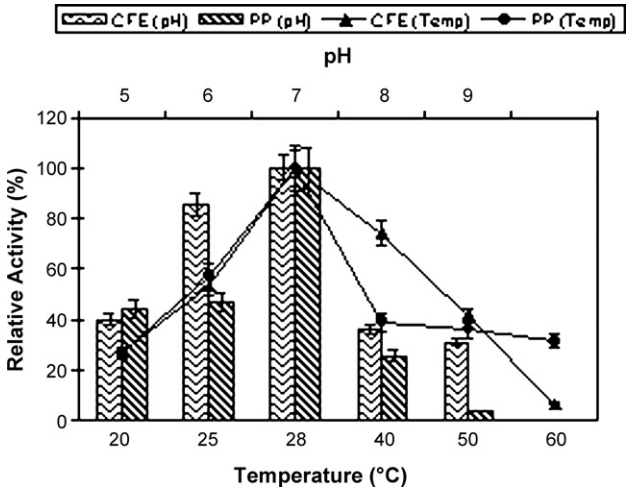

Fig. 5. Effect of pH and temperature.
(CFE—cell free extract; PP—partially purified enzyme).

3.8. Effect of growth medium and addition of chromium as inducer

The reductase activities in CFEs from cells grown in the absence and presence of Cr(VI) were 21.89 ± 2.32 and 25.47 ± 0.96 U/mL respectively. These results indicate chromate reductase to be constitutive in nature with slight induction in the presence of chromium. Therefore, it is apparent that addition of Cr(VI) during growth was not necessary for the expression of reductase activity. The activities in CFEs from cells grown in MGYP and Broth-II medium were 23.58 ± 1.07 and 22.32 ± 1.64 U/mL respectively. Growth medium and addition of Cr(VI) to growth medium had no significant effect on the reduction indicating that the activity was constitutive. Constitutive nature of the reductase was also reported in *Streptomyces* sp. [4].

3.9. Fractional ammonium sulphate precipitation

Major portion of chromate reductase activity was found in 40–60 ammonium sulphate precipitate fraction while part of it was also detected in 20–40 ammonium sulphate precipitate (Table 5).

3.10. Effect of pH and temperature on chromate reductase activity

Though both CFE as well as partially purified enzyme showed optimum activity at pH 7, CFE showed a broader peak compared to partially purified enzyme (Fig. 5). The CFE as well as partially purified enzyme showed maximum reductase activity at 28 °C however, they were active in the range of 25–40 °C but showed very low activity at the two temperature extremes of 20 and 60 °C (Fig. 5).

4. Conclusion

Our studies revealed that majority of the actinomycetes tested were tolerant and capable of reducing Cr(VI) to Cr(III) during growth. Among the *S. griseus* strains showing chromate reduction, *S. griseus* NCIM 2020 was most efficient with complete conversion of 25 mg/L Cr(VI) within 24 h. The organism was capable of chromate reduction even at higher concentrations and was able to utilize variety of carbon/energy sources/electron donors for reduction. The highest rate of reduction for *S. griseus* is 2.85 mg Cr(VI) reduced/L/h in the initial period of 12 h with 89 mg/L Cr(VI) concentration is the highest rate reported so far among actinomycetes. Reduction was unaffected by anions while cations inhibited reduction to varying degrees and was associated with cells. Reductase activity was heat labile and was lost on dialysis which was restored on addition of NADH. Addition of NADH/NADPH enhanced chromate reduction by 2–3-fold and the enzyme had no preference for NADH or NADPH. The enzyme was optimally active at 28 °C and pH 7.

The observation that reductase activity was found to be predominant in actinomycetes mainly belonging to *Streptomyces* is an important finding in the context that these organisms are widely distributed in nature. *S. griseus* can form dormant spores, which are resistant to heat and desiccation, can be easily stored, transported and readily germinated into vegetative cells by the addition of simple nutrients. These attributes make this organism a promising candidate for use in knowledge-based toxic metal remediation systems. An additional benefit of this organism is that it can effectively reduce Cr(VI) when grown on a simple mineral medium. The finding that the partially purified enzyme can function as a Cr(VI) reductase points out the possibility of developing cell free systems for treating Cr(VI) containing wastes.

Experiments are in progress to purify and characterize the enzyme and study the biochemical mechanisms responsible for Cr(VI) reduction.

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References

[1] K. Komori, A. Rivas, K. Toda, H. Ohtake, Biological removal of toxic chromium using an *Enterobacter cloacae* strain that reduces chromate under anaerobic conditions, Biotechnol. Bioeng. 35 (1990) 951–954.
[2] J.M. Chen, O.J. Hao, Biological removal of aqueous hexavalent chromium, J. Chem. Technol. Biotechnol. 69 (1997) 70–76.
[3] J.M. Chen, O.J. Hao, Microbial Cr(VI) reduction, Crit. Rev. Env. Sci. Technol. 28 (1998) 219–251.
[4] S. Das, A.L. Chandra, Chromate reduction in *Streptomyces*, Experientia 46 (1990) 731–733.
[5] K.M. Paknikar, J.V. Bhide, Aerobic reduction and biosorption of chromium by a chromate resistant *Aspergillus* sp., in: Biohydrometallurgical Technologies Proceedings of an International Biohydrometallurgy Symposium, Wyoming, USA, The Minerals, Metals & Materials Society, USA. vol. II, 1993, pp. 237–244.
[6] H.S. Horitsu, Y. Futo, S. Miyazawa, S. Ogai, K. Kanwal, Enzymatic reduction of hexavalent chromium by hexavalent chromium tolerant *Pseudomonas ambigua* G-1, Agric. Biol. Chem. 51 (1987) 2417–2420.
[7] Y. Ishibashi, C. Cervantes, S. Silver, Chromium reduction in *Pseudomonas putida*, Appl. Environ. Microbiol. 56 (1990) 2268–2270.
[8] C.H. Park, M. Keyhan, B. Wielinga, S. Fendorf, A. Matin, Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase, Appl. Environ. Microbiol. 66 (2000) 1788–1795.
[9] A.H. Caravelli, L. Giannuzzi, N. Zaritzky, Reduction of hexavalent chromium by *Sphaerotilus natans* a filamentous micro-organism present in activated sludges, J. Hazard. Mater. 156 (1–3) (2008) 214–222.
[10] F.A.O. Camargo, B.C. Okeke, F.M. Bento, W.T. Frankenberger, In vitro reduction of hexavalent chromium in a cell-free extract of *Bacillus* sp. ES 29 stimulated by Cu^{2+}, Appl. Microbiol. Biotechnol. 62 (2003) 569–573.
[11] F.A.O. Camargo, F.M. Bento, B.C. Okeke, W.T. Frankenberger, Hexavalent chromium reduction by an actinomycete, *Arthrobacter crystallopoietes* ES 32, Biol. Trace Elem. Res. 97 (2004) 183–194.
[12] R.N. Horton, W.A. Apel, V.S. Thompson, P.P. Sheridan, Low temperature reduction of hexavalent chromium by microbial enrichment consortium and a novel strain of *Arthrobacter aurescens*, BMC Microbiol. 6 (2006) 5.
[13] R.S. Laxman, S.V. More, Reduction of hexavalent chromium by *Streptomyces griseus*, Minerals Eng. 15 (2002) 831–837.

[14] A. Pal, A.K. Paul, Aerobic chromate reduction by chromium resistant bacteria isolated from serpentine soil, Microbiol. Res. 159 (2004) 347–354.
[15] J. McLean, T.J. Beveridge, Chromate reduction by a pseudomonad isolated from a site contaminated with chromated copper arsenate, Appl. Environ. Microbiol. 67 (2001) 1076–1084.
[16] T. Suzuki, N.H. Miyata, K. Horitsu, K. Kawai, K. Takamizawa, Y. Tai, M. Okazaki, NAD(P)H-dependent chromium(VI) reductase of *Pseudomonas ambigua G-1*: a Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III), J. Bacteriol. 174 (1992) 5340–5345.
[17] J. Campos, M. Martinez-Pacheco, C. Cervantes, Hexavalent-chromium reduction by a chromate-resistant *Bacillus* sp. strain, Antonie Van Leeuwenhoek 68 (1995) 203–208.
[18] C. Cervantes, J. Campos-Garcia, S. Devars, F. Gutierrez-Corona, H. Loza-Tavera, J.C. Torres-Guzman, R. Moreno-Sanchez, Interactions of chromium with microorganisms and plants, FEMS Microbiol. Rev. 25 (2001) 335–347.
[19] M.J. Amoroso, G.R. Castro, A. Duran, O. Peraud, G. Oliver, R.T. Hill, Chromium accumulation by two *Streptomyces* spp. isolated from river sediments, J. Ind. Microbiol. Biotechnol. 26 (2001) 210–215.
[20] M.A. Polti, M.J. Amoroso, C.M. Abate, Chromium(VI) resistance and removal by actinomycete strains isolated from sediments, Chemosphere 67 (2007) 660–667.
[21] A.C. Poopal, R.S. Laxman, Hexavalent Cr(VI) reduction by immobilized *S. griseus*, Biotechnol. Lett. 30 (2008) 1005–1010.
[22] A.C. Poopal, R.S. Laxman, Hexavalent Cr(VI) reduction by immobilized *S. griseus* in a bioreactor system, Biotechnol. Lett. 31 (2009) 71–76.
[23] M. Megharaj, S.A. Avudinaygan, R. Naidu, Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery wastes, Curr. Microbiol. 47 (2003) 51–54.
[24] V. Desjardin, R. Bayard, P. Lejeune, R. Gourdon, Utilization of supernatants of pure cultures of *Streptomyces thermocarboxydus* NH50 to reduce chromium toxicity and mobility in contaminated soils, Water, Air, Soil Pollut.: Focus 3 (2003) 153–160.
[25] P.F. Urone, H.K. Anders, Determination of small amounts of chromium in human blood, tissues and urine, colorimetric method, Anal. Chem. 22 (1950) 1317–1321.
[26] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with Folin reagent, J. Biol. Chem. 193 (1951) 265–273.
[27] R. Elangovan, S. Abhipsa, B. Rohit, P. Ligy, K. Chandraraj, Reduction of Cr(VI) by a *Bacillus* sp., Biotechnol. Lett. 28 (2006) 247–252.
[28] C. Desai, K. Jain, D. Madamwar, Evaluation of *in vitro* Cr(VI) reduction potential in cytosolic extracts of three indigenous *Bacillus* sp. isolated from Cr(VI) polluted industrial landfill, Bioresour. Technol. 99 (2008) 6059–6069.