



Identification of bacterial strains from tannery effluent and reduction of hexavalent chromium

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Abstract: Four chromium-resistant bacteria were isolated from tannery effluent collected from Burgelarab, Alexandria, Egypt. These isolates displayed different degrees of chromate reduction under aerobic conditions. Based on 16S rDNA gene sequence analysis, two of them (S3 and S4) were identified as *Acinetobacter*, and *Pseudomonas* sp., respectively. The minimum inhibitory concentration for *Acinetobacter* sp. strain S3 was 160 mg l⁻¹, while it was 200 mg l⁻¹ for *Pseudomonas* sp. strain S4. However, strain S4 was able to reduce a wide range of Cr (VI) concentrations from 20 to 200 mg l⁻¹; while, it was reducing 64.4% of Cr (VI) at 160 mg l⁻¹ within 72 hr. Immobilization experiments demonstrated that strain S4 in calcium alginate gel matrix was more effective than the using of free cells in chromium reduction.

Key words: Chromate-resistant bacteria, Cr (VI) reduction, Minimal inhibitory concentration, Immobilization
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Introduction

Chromium is one of the most toxic heavy metals discharged into the environment through various industrial wastewaters, such as leather tanning, electroplating, paints, pigment production, steel manufacture. Others industrial processes using catalysts discharge worldwide huge amounts of chromium every year and it has become a serious health problem. The effluents of these industries contain chromium at concentrations ranging from tenths to hundreds of milligrams per liter (Dermou *et al.*, 2005). Safe value in water for drinking purposes is 0.05 mg l⁻¹ and recommended value for discharge is less than 5 mg l⁻¹ (Directive EPA, USA, 2003; Debabrata *et al.*, 2006).

Chromium (Cr) exists in several oxidation states, but the most stable are trivalent Cr (III) and hexavalent Cr (VI) species, with different chemical characteristics and biological effects (Cervantes *et al.*, 2001; Nath *et al.*, 2009). It is an essential trace metal, but overexposure to Cr (VI) produces allergic dermatitis; ulceration in the skin, mucous membranes and nasal septum; renal tubular necrosis and increases risks of respiratory-tract cancer and cytotoxic and genotoxic effects (cell death, cell transformation and gene mutation) (Lu and Yang, 1995; Flavio *et al.*, 2004).

The conventional methods to detoxify and remove Cr (VI) from the environment involve chemical reduction followed by precipitation, ion exchange and absorption on coal, activated carbon, alum, kaolinite, and flyash (Ohtake and Silver, 1994; Arundhati and Paul, 2005). However, biological treatments arouse great interest because of their lower impact on the environment. The processes by which microorganisms interact with toxic metals enabling their removal/and recovery are bioaccumulation, biosorption and

enzymatic reduction (Srinath *et al.*, 2002). Recent studies have shown that certain species of bacteria are capable of transforming Cr (VI), into the much less toxic and less mobile Cr (III) (Dermou *et al.*, 2005; Camargo *et al.*, 2005; Pal and Paul, 2005).

Chromate-reducing bacteria have been isolated and characterized mostly from chromium-contaminated soil (Mclean and Beveridge, 2001; Viti *et al.*, 2003), wastewater and industrial effluents (Ganguli and Tripathi, 2001; Pattanapitpaisal *et al.*, 2001; Srinath *et al.*, 2001). Most of the previous studies on biological reduction of Cr (VI) were conducted in batch reactors (flasks) using mainly pure cultures. For instance, Wang and Xiao (1995) studied several factors affecting hexavalent chromium reduction in pure cultures of bacteria in flasks. Shakoory *et al.* (2000) isolated a dichromate-resistant Gram-positive bacterium from effluent of tanneries and used flasks as batch reactors.

Immobilized microbial cells are used in organic synthesis clinical and chemical analysis, food industries, medicine, and environmental applications as well (Chibata and Tosa, 1981). The expansion of biotechnology and the expected developments has encouraged effects to immobilize enzymes and cells for applied purpose (Bickerstaff, 1987). For a particular application it is necessary to find an immobilization procedure that would be simple and inexpensive. Immobilization of the biomass within a suitable matrix provide a physical support for cells, ideal size, mechanical strength, rigidity and porous characteristics to the biological material (Bucke, 1983; Trujillo *et al.*, 1995). The aim of this research was to isolate the microbial isolates from tannery effluent and to access the Cr reduction capacity at different concentration of Cr (VI). In addition to this, studying the Cr (VI) reduction capacity of immobilized bacterial isolate for removing of Cr (VI) from aqueous solution.

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Materials and Methods

Isolation and Identification of chromate-resistant bacteria:

Chromate-resistant bacteria were isolated from tannery effluent obtained from a company in Borg Al-Arab, Alex. Egypt. For the isolation and enumeration of bacteria, samples were serially diluted and plated on Luria–Bertani (LB) agar (tryptone: 10 g l⁻¹; yeast extract: 5 g l⁻¹; NaCl: 10 g l⁻¹; glucose: 0.1 g l⁻¹) adjusted at normal pH value (7.0). The molten medium was amended with Cr (VI) as K₂Cr₂O₇ to final concentration 40 mg l⁻¹ using sterile filtered Cr (VI) stock solutions. Plates were incubated at 30°C in the dark and read after 2 days. Subsequently, four isolates were selected according to their morphological shapes for further studies.

The minimum inhibitory concentration (MIC) of four Cr (VI)-resistant isolates were determined by broth dilution method (Calomiris *et al.*, 1984) in LB medium with Cr (VI) concentrations ranging from 20 to 500 mg l⁻¹. The minimum concentration of metal in the medium inhibiting complete growth was taken as the (MIC). From these results two isolates (S3 and S4) were selected for further analysis.

Characterization of growth and chromium reduction by the isolates:

Chromate-resistant bacterial isolates (S3 and S4) were inoculated into LB broth (pH 7.0) containing different concentration of Cr (VI) (from 20 to 200 mg l⁻¹) and incubated for 72 hr at 30°C with orbital shaking (200 rpm). The inoculum was 2% of the total volume of medium. Bacterial cell density (diluted 10-fold with water) of the liquid cultures was determined by measuring optical density at 600 nm by use of UV/Vis. spectrophotometer (DU.530 Beckman). Hexavalent chromium reduction was determined from the difference between total chromium and Cr (VI) concentration and Cr (III) concentration.

Reduction of chromium was determined from extracted solution by using UV spectrophotometers at 540 nm with 1,5-diphenylcarbazide as a pink colored complex agent (Snell and Snell, 1959; APHA, 1992; Park *et al.*, 2005). Total chromium [Cr (VI) + Cr (III)] was measured using a Perkin- Elmer Analyst 300 atomic absorption spectrophotometer (AAS).

Molecular identification: Amplification of 16S rDNA with eubacterial universal primers 27F and 1492R was done (Lane, 1991). Genomic DNAs and/or PCRs were performed using EZ-10 Spin Column DNA purification kit according to the manufacturer's instructions (BIO BASIC INC). Sequencing was performed using ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase and an Applied Biosystems 373 DNA sequencer (Perkin-Elmer, Foster City, Calif.).

The sequences were analyzed using the CHECK CHIMERA and the SIMILARITY RANK programs of the Ribosomal Database Project (Altschul *et al.*, 1990) also analyzed using the BLAST program (National Centre for Biotechnology Information) to determine the closest available database sequences. Selected rDNA sequences were aligned using the Clustal W program (Shingler,

1996). Published sequences were obtained from GenBank. A phylogenetic tree was constructed using Clustal W by distance matrix analysis and the neighbour-joining method (Saitou and Nei 1987). Phylogenetic trees were displayed using TREEVIEW (Page, 1996).

Immobilization and Chromium reduction: Selected isolate S4 was processed for immobilization as for the method of Paul *et al.*, 2005 and Srinath *et al.*, 2003. The batch adsorption experiments were carried out to determine the reduction of Cr (VI) by immobilized *pseudomonas* sp. strain S4 and its free cells. Using a 250 ml Erlenmeyer flask containing 50 ml of LB broth (pH 7.0), Cr (VI) at concentration 120 mg l⁻¹, and 2 mg cell dry weight were added. Incubated at 30°C with orbital shaking (200 rpm) and the samples were taken from each flask every day for 7 days. The inoculum was 5% of the total volume of medium. Bacterial cell density of the liquid cultures was determined by measuring optical density at 600 nm. Hexavalent chromium reduction was determined from the difference between total chromium and Cr (VI) concentration using atomic absorption spectrophotometer (AAS) and 1, 5-diphenylcarbazide method.

After the first cycle (3 days), the alginate beads containing encapsulated cells were filtered, washed and used in a second cycle for reduction of Cr (VI) at concentration 120 mg l⁻¹. Three repeated batch cycles were performed.

Results and Discussion

Isolation, evaluation and identification: A total of nine Cr-resistant bacteria were isolated from tannery effluent in the present study. Four selected isolates according to their morphological shape were plated in media amended with 40 mg l⁻¹ Cr (VI). Similarly Srinath *et al.* (2002) isolated 71 strains that are capable of bioaccumulating Cr(VI) from tannery effluent. Two strains, identified as *Bacillus circulans* and *Bacillus megaterium*, showed excellent bioaccumulation ability (34.5 and 32.0 mg g⁻¹ dry weight, respectively). Also Xuejun Quan *et al.* (2006) isolated three isolates from chromium slag samples which collected from Chemical group Company, China.

The effect of Cr (VI) concentrations ranging from 20 to 500 mg l⁻¹ on the growth of the isolates was evaluated (Fig. 1). Increasing the Cr (VI) concentration to a certain limit is affected negatively on the growth of four isolates. The most significant growth decreased after addition of 40 mg l⁻¹ of Cr (VI) was observed with isolate S1. However, isolate S2 was affected by the chromium concentration added (60 mg l⁻¹). Isolates S3 and S4 grow well when exposed to the highest chromium concentration, up to till 100 mg l⁻¹. The detoxification efficiency of the four respective isolates follows the sequence: S1> S2> S3> S4. The two isolates (S3 and S4) were selected for further experiments (Flavio *et al.*, 2004) he found that isolates showed different abilities to resist Cr (VI) in the medium, which was directly related to varying Cr (VI) concentrations.

The 16S rDNA were partially sequenced following PCR amplification and compared with sequences deposited in databases.

Table - 1: Effect of various concentrations of Cr (VI) (mg l^{-1}) on the cell growth and chromate reduction using strains S3 and S4.

Cr (VI) concentration (mg l^{-1})	S3		S4	
	OD	% of Cr (VI) reduction	OD	% of Cr (VI) reduction
20	1.722	100	1.844	100
40	1.656	100	1.895	100
80	1.534	83.1	1.555	92.5
120	1.513	69.2	1.524	72.5
160	0.111	55	1.356	64.4
200	0.095	53.5	0.133	62

Table - 2: Cr (VI) reduction (%) and the cell density by free and immobilized *Pseudomonas* strain S4 at initial Cr (VI) concentration 120 mg l^{-1}

Days	Strain S4 free		Strain S4 immobilized	
	OD	% of Cr(VI) reduction	OD	% of Cr (VI) reduction
0	0	0	0	0
1	0.62	60	1.65	63.32
2	1.587	64	1.746	66.66
3	1.758	72.5	1.902	80
4	1.802	81.4	2.182	83.32
5	1.725	82	2.194	85
6	1.768	82.1	2.32	85.1
7	1.763	82.12	2.269	85.11

Totally ~700 bp of the 16S rDNA of isolates S3 and S4 was determined. The phylogenetic tree (Fig. 2) showed that isolate S3 is closely related to the genus *Acinetobacter* exhibiting similarity values greater than 90%. However, isolate S4 was belonging to the genus *Pseudomonas* with similarity value > 99%. The nucleotide sequence data reported in this study have been deposited in the NCBI nucleotide sequence database (GenBank) under the accession number of FJ827752 and FJ827753, respectively. It was emphasized that *Acinetobacter* sp. strain and *Pseudomonas* sp. strain have been described for their ability to reduce hexavalent chromium into insoluble low valence form Cr (III) aerobically (McLean and Beveridge, 2001; McLean *et al.*, 2000; DeLeo and Ehrlich, 1994).

Efficiency of the selected strains: As shown in Table 1, significant differences in both growth rate and Cr (VI) reduction potentials were observed. 100% reduction of Cr (VI) was reported for both strains S3 and S4 at concentrations from 20 and 40 mg l^{-1} . However, by increasing the concentrations of chromium ion the reduction ratio decreased significantly to reach 53.5% of S3 and 62% of S4 at Cr (VI) concentration 200 mg l^{-1} . In addition, the growth rate of both isolates takes the same direction of the chromium reduction results.

As shown in Fig. 3 a progressive decrease in growth with increasing Cr(VI) concentrations was observed. In addition, results obtained pointed out that, the exact MIC for S3 is 160 mg l^{-1} but for S4 is 200 mg l^{-1} . From these results the isolate S4 is the most potent one which was selected for immobilization test.

This MIC is very similar to that of *B. circulans* and *B. megaterium* reported by Srinath *et al.* (2002), which MIC of Cr

(VI) is reached as high as 130 and 170 mg l^{-1} Cr (VI), respectively. While other work done by Pal and Paul (2004) 34 Cr-resistant bacteria were isolated from serpentine soil. The majority (about 62%) of these isolates showed an MIC value of $>600 \text{ mg l}^{-1}$ Cr (VI), but only about 9% of isolates tolerated $>800 \text{ mg l}^{-1}$ Cr (VI).

The effect of initial Cr(VI) concentration on reduction was investigated over a range of $20\text{--}400 \text{ mg l}^{-1}$ Cr (VI) (Fig. 4). The % of reduction efficiency decreased by increasing the Cr (VI) concentration and reached to 53.5 and 62% for S3 and S4 respectively at 200 mg l^{-1} Cr.

Out of these results, the rate of chromate reduction is greatly influenced by the initial Cr (VI) concentration (Pattanapitpaisal *et al.*, 2001; Wang and Xiao, 1995; Shen and Wang, 1994); however, complete reduction is done at the lowest concentration of metal. In contrast with Arundhati *et al.* (2004) their isolate AND303, likewise failed to cause complete reduction even at initial concentration of 20 mg l^{-1} Cr (VI). Other results have been reported by Sikander *et al.* (2007) for *Ochrobactrum* intermedium strain SDCr-5 was probed over a Cr (VI) concentration range of $100\text{--}1500 \mu\text{g ml}^{-1}$. The rate of Cr (VI) reduction by strain SDCr-5 increased by increasing Cr (VI) concentrations up to $1500 \mu\text{g ml}^{-1}$.

Chromium reduction measurement using free and immobilized cells: The ability of chromate-resistant bacterial *Pseudomonas* S4 for reduction of Cr (VI) at 120 mg l^{-1} using free and immobilized cell using calcium alginate were estimated as described before in the materials and methods section. The reaction occurred in 250 ml Erlenmeyer flask containing 50 ml of LB broth (pH 7.0) and incubated at 30°C with orbital shaking (200 rpm) and the samples were taken daily for 7 times.

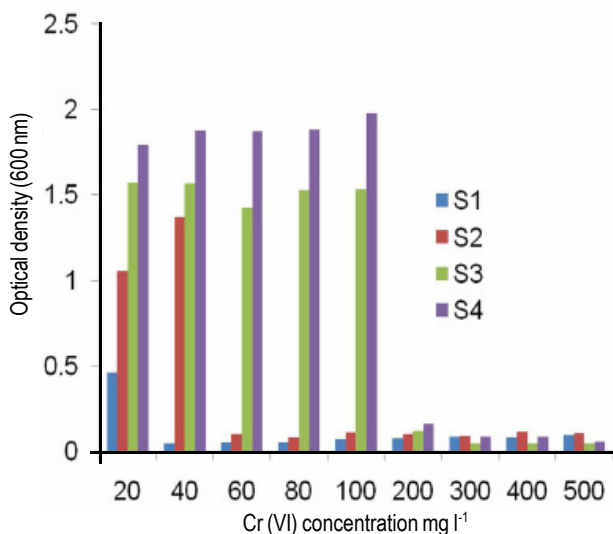


Fig. 1: Effect of different Cr (VI) concentrations on the growth of four selected isolates (S1, S2, S3 and S4)

From Fig. 5 and Table 2, it was found that cellular growth was increased with increasing the time until the fourth day, after that there was no significant different in the growth. The cell density of media containing immobilized cell is greater than the free cells. The removal percentage of Cr (VI) ion increased with increasing the time which reach to the maximum reduction after 4 days, the reduction is 72.5 and 81.4% for third and fourth days respectively using free cell and still constant until the seventh day as shown in Fig. 6.

Similarly McLean *et al.* (2000) with pseudomonad strain CRB5 reported the reduction rate decreased during the first 24 hr at Cr (VI) concentrations of 30 and 40 $\mu\text{g ml}^{-1}$. Also DeLeo *et al.*, (1994) reported 99.7% reduction of 112.5 $\mu\text{g ml}^{-1}$ Cr (VI) by *P. fluorescence* LB300 within a period of 289 hr. The pseudomonad strain CRB5, however, showed complete reduction of 20 $\mu\text{g ml}^{-1}$ of chromate after 120 hr Mclean *et al.* (2001). However Sikander, *et al.* (2007) show that the rate of Cr (VI) reduction by *Ochrobactrum* intermedium strain SDCr-5 decrease with time irrespective to initial Cr (VI) concentration used.

The Cr(VI) reduction was observed with immobilized cell suspensions of *Pseudomonas* S4 strain which reach to 80 and 83.3% at the third and fourth days respectively and still constant until the seventh day as shown in Fig. 6. The reduction of Cr (VI) was high with immobilized cell comparing with the free cell, also using the immobilized cell is preferable due to its capability for using several times with the same efficiency, which make it more economically. Similar work is done by Sikander *et al.* (2007) showing the higher reduction with premeabilized cell of *Ochrobactrum* intermedium strain SDCr-5.

Ca-alginate immobilized S4 was tested in several consecutive chromium reduction experiments to investigate the possible deactivation of cells with repeated use. It was observed that immobilized cells of *Pseudomonas* S4 could be reused three times without losing their chromium reduction activity each for 3 days with 80% of reduction efficiency of Cr (VI).

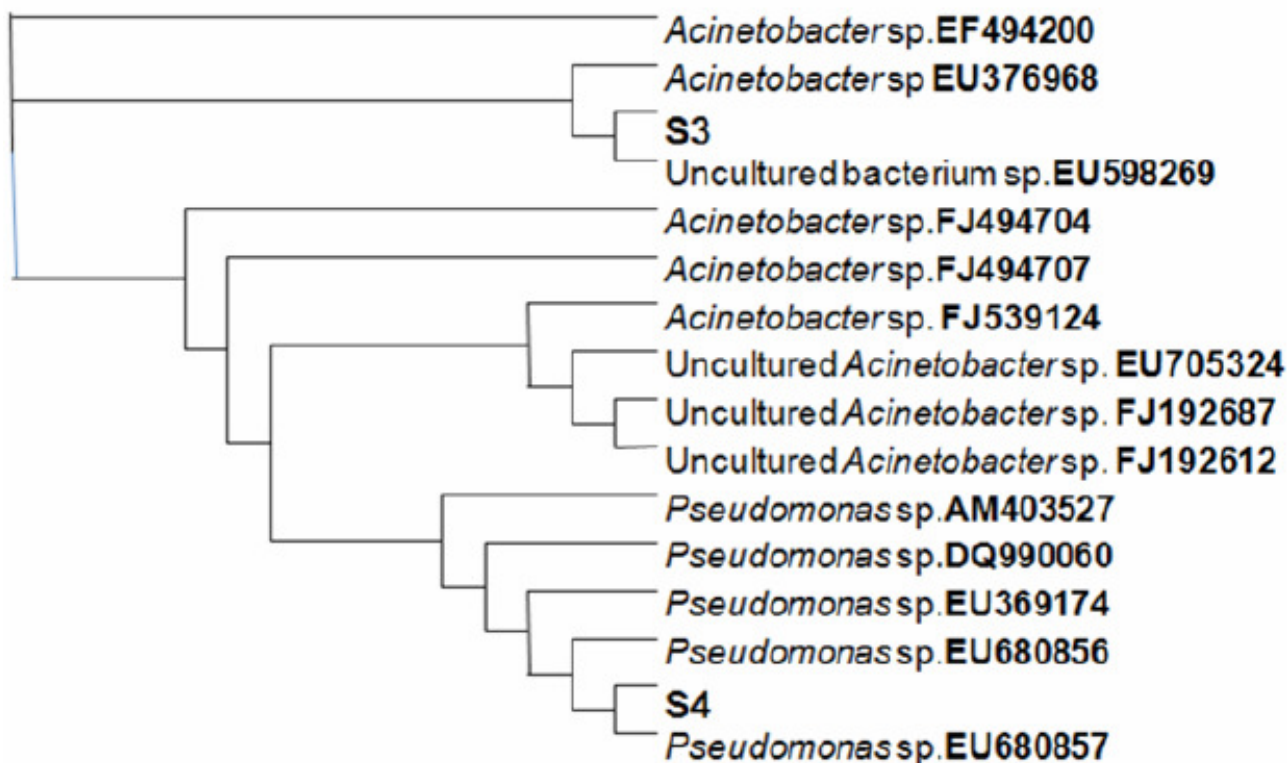


Fig. 2: Phylogenetic tree showing the relationships among selected isolates

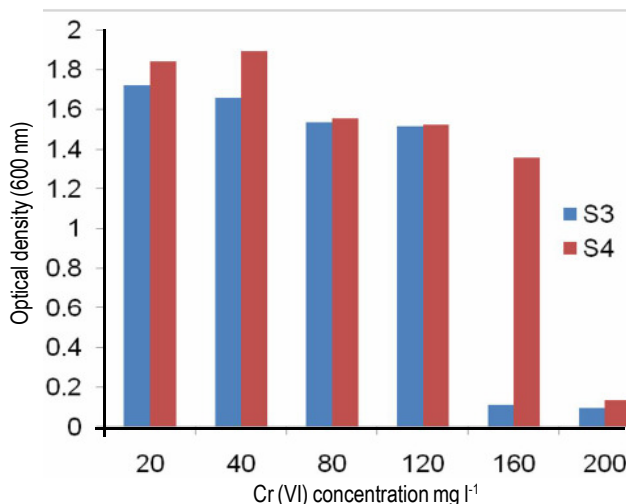


Fig. 3: Effect of various concentrations of Cr (VI) (mg l⁻¹) on the growth of strains S3 and S4

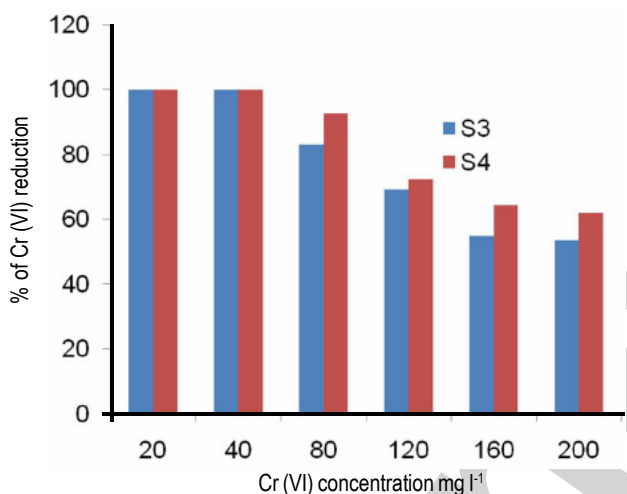


Fig. 4: Influence of initial Cr (VI) concentrations on the rate of chromate reduction by S3 and S4

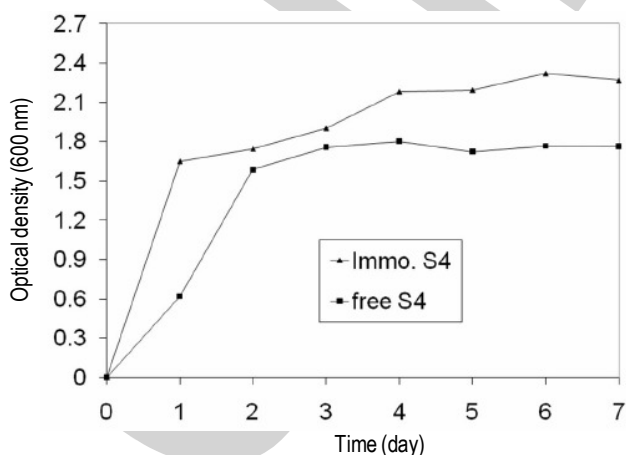


Fig. 5: The comparison of the growth rate of immobilized S4 and free S4 at constant Cr concentration 120 mg l⁻¹ within different time intervals

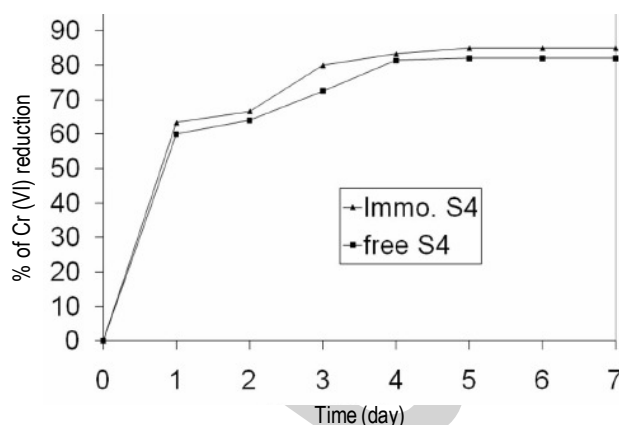


Fig. 6: The comparison of Cr (VI) reduction efficiency using immobilized S4 and free S4 at constant Cr concentration 120 mg l⁻¹ within different time intervals

The results revealed the isolation and identification of isolates with potency for reduction of Cr (VI) to Cr (III). Further the two isolates was identified as *Acinetobacter*, and *Pseudomonas*. Results indicated that immobilized *Pseudomonas* sp. strain S4 could be efficiently used for reduction of Cr (VI).

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