

# Bioreduction of Cr(VI) by Viable Cells and Cell-Free Extract of a Moderate Halophile, *Halomonas Smyrnensis* KS802 and Evaluation of Their in Vitro Efficacy in Tannery Effluent

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## Research

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# Abstract

The present study was aimed to characterize the chromate reducing ability of cells and cell-free extract (CFE) of *Halomonas smyrnensis* KS802 (GenBank Accession No. KU982965) and evaluate their effectiveness in tannery effluents. Viable cells of the strain reduced 200  $\mu\text{M}$  Cr(VI) in basal medium for halophiles (MH) in 10 h and was inversely proportional to Cr(VI) concentrations. The rapid reduction by cells ( $10^8$  cells/mL) was achieved with 7.5% NaCl, at pH 7 and 37°C which increased with increasing cell density ( $10^{10}$  cells/mL). While acetate,  $\text{Cu}^{3+}$ ,  $\text{Fe}^{3+}$ ,  $\text{SO}_4^{2-}$ , and  $\text{CO}_3^{2-}$  were stimulating the reduction, the inhibitors retarded the process significantly. The NADH-dependent chromate reduction of the CFE was found to be constitutive with  $K_m$  and  $V_{max}$  values of 56.58  $\mu\text{M}$  and 3.37  $\mu\text{M}/\text{min}/\text{mg}$  protein respectively. The optimal reductase activity of the CFE was evident at 200  $\mu\text{M}$  Cr(VI), 10% NaCl, pH 8.0 and at 45°C. A higher concentration of CFE and electron donors increased the enzyme activity but was impacted negatively by toxic metals and anions. Both the cells and CFE were capable of reducing Cr(VI) remarkably from tannery effluent. FTIR and XRD spectra of chromate reducing cells confirmed possible complexation of reduced Cr-species with functional groups on cell surface.

## Introduction

Chromium (Cr) toxicity is one of the major causes of environmental pollution emanating from chrome tanning processes, electroplating, paint and pigment manufacturing industries (Shahid et al. 2017). Among the different valence states of Cr, trivalent [Cr(III)] and hexavalent [Cr(VI)] chromium are stable and have environmental significance. The hexavalent chromium is highly soluble, mobile, toxic, mutagenic and carcinogenic (Sultan and Hasnain 2007) compared to less soluble and mobile Cr(III). In addition to Cr(VI), most of the industrial effluents also contain other co-contaminating heavy metals, dyes, salts, ions etc. Therefore, treatment of hypersaline effluents polluted with Cr(VI) has become a challenging problem to the industries.

Due to high cost, low efficiency and generation of toxic wastes, the conventional physico-chemical methods of Cr(VI) removal from polluted effluents have been found unsuitable and as an alternate, microorganisms like bacteria, fungi and algae were implemented for bioremediation of toxic Cr(VI) in an eco-friendly way (Jeyasingh and Philip 2005). The chromium-resistant as well as tolerant bacteria inhabiting such polluted environments can survive and grow by adopting the mechanisms of biosorption, bioaccumulation, efflux, and most frequently by reduction of hexavalent chromium. Over the years, extensive work has been carried out on the removal of Cr(VI) by several species of bacteria isolated primarily from Cr-polluted environments (Thatoi et al. 2014). Extracellular, intracellular or membrane bound reductases of these bacteria have been reported to reduce Cr(VI) either aerobically or anaerobically, or by both (Losi et al. 1994).

Halophiles are functionally and taxonomically diverse communities found in hypersaline environments which exhibit optimum growth at a salinity of approximately 15–20% but are often able to grow in saturated brine. They are classified as: slight- (2–5% NaCl), moderate- (5–20% NaCl) and extreme

halophiles (20–30% NaCl) according their salt requirements (DasSarma and Aora 2002). In addition halophiles have the ability to adapt in high external osmotic pressure (NaCl) and maintain an osmotic balance between their cytoplasm and the hypersaline extracellular environment. This salt adaptation also makes the cellular structural components and both the intra- and extracellular enzymes to work under salt stress (Oren 2002). Moreover, environmental contamination due to industrial and municipal effluents discharged into saline and hypersaline entities make the halophiles tolerant to the toxic metals, dyes, hydrocarbons, pesticides etc (Gnanamani et al. 2010). Such adaptive features of halophilic and halotolerant microorganisms have directed the scientific and technical attention towards their increasing application in hypersaline wastewater treatments and removal of toxic chemicals.

Some of the halophilic microorganisms have already been reported for bioremediation of oil (Al-Maillem et al. 2008), dyes (Asad et al. 2007), oxyanion (Amoozegar et al. 2008), organic pollutants (Le-Borgne et al. 2008; Castillo-Carvajal et al. 2014) and polyaromatic hydrocarbons (Nanca et al. 2018). In the perspective of heavy metal tolerance of halophilic bacteria, very few studies on potential removal or detoxification of Cr(VI) (Amoozegar et al. 2007; Mishra et al. 2012; Subramanian et al. 2012; Focardi et al. 2012; Mabrouk et al. 2014; Biswas et al. 2018) were conducted till date. Moreover, metal removal by halophiles in the presence of salt has become crucial as waste water generated by most of the industries including the tannery waste contains higher concentration (1–10%, w/v) of salt as NaCl (Sivaprakasam et al. 2008).

A chromium tolerant moderately halophilic bacterium, *Halomonas smyrnensis* KS802 (GenBank Accession No. KU982965) isolated in this laboratory from a saline soil sediment collected from a multi-pond solar saltern of Gujarat, India has recently been established and documented as a potential candidate for reduction of Cr(VI) during growth in batch culture (Biswas et al. 2018). The present study was aimed to assess the efficacy of whole cells as well as cell-free extracts of *Halomonas smyrnensis* KS802 in reducing Cr(VI) and also to evaluate their potential for Cr(VI) reduction in tannery effluents under laboratory conditions.

## Materials And Methods

### Bacterial strain

*Halomonas smyrnensis* KS802 (GenBank Accession No. KU982965), a Cr(VI) reducing moderately halophilic bacterium, isolated from soil sediment of a multi-pond solar saltern located at Kandla port, Gujarat, India (Biswas et al. 2018) was used throughout the present study for comparative bio-reduction of Cr(VI) by whole cells and cell-free extract. The bacterial strain was maintained on MH agar medium (Ventosa et al. 1982) by repeated subculturing at an interval of 30 days. The medium contained (g/L): yeast extract 10; glucose, 10; protease peptone 5; NaCl, 25; MgCl<sub>2</sub>, 6H<sub>2</sub>O, 7; MgSO<sub>4</sub>, 7H<sub>2</sub>O, 9.6; CaCl<sub>2</sub>, 2H<sub>2</sub>O, 0.36; KCl, 2; NaHCO<sub>3</sub>, 0.06 and NaBr 0.026 (pH 7.2).

### Chromate reduction by whole cells

The isolate *H. smyrnensis* KS802 was grown in MH medium at 32 °C under continuous shaking for 24 h and cells were harvested by centrifugation (5,000 × g) for 10 min at 4 °C. The cell mass was washed thoroughly with sterile saline (0.9% NaCl) and re-suspended in phosphate buffer (pH 7.0). Chromate reduction by viable cells was conducted in Basal MH medium which contained (g/L) yeast extract 1.0; glucose, 1.0; NaCl, 25; MgCl<sub>2</sub>, 6H<sub>2</sub>O, 7; MgSO<sub>4</sub>, 7H<sub>2</sub>O, 9.6; CaCl<sub>2</sub>, 2H<sub>2</sub>O, 0.36; KCl, 2; NaHCO<sub>3</sub>, 0.06 and NaBr 0.026 (pH 7.2). Basal MH medium (20 mL/100 mL flask) supplemented with 200 µM Cr(VI) and 10<sup>9</sup> cells/mL was maintained for the reduction process. Similar set of experiment was performed with autoclaved (at 15 p.s.i. for 15 min) cell mass. A set without chromium was also maintained as negative control. The flasks were incubated at 32 °C under continuous shaking (120 rpm) and samples were withdrawn aseptically at regular interval and analyzed for residual Cr(VI) following standard S-diphenyl carbazide (DPC) method (Park et al. 2000). To one mL of cell-free culture supernatant, 3 mL of 0.166 M H<sub>2</sub>SO<sub>4</sub> and 1 mL of 0.05% DPC solution was added, mixed well and OD was read immediately at 540 nm. Residual Cr(VI) was estimated from the calibration curve prepared in the same way. The amount of Cr(VI) reduced was determined from the difference and expressed as % Cr(VI) reduced.

The effect of substrate [200 – 100 µM Cr(VI)], electron donors (0.1%), oxyanions (100 µM), metal ions (100 µM), metabolic inhibitors (200 µM), NaCl (5–20%), cell density (10<sup>8</sup> to 10<sup>11</sup> cells/mL), pH (5–9) and temperature (27–42 °C) on reduction process was performed in Basal MH medium under batch culture condition.

## Chromate reduction by cell-free extract

The cell-free extract (CFE) of *H. smyrnensis* KS802 was prepared according to Focardi et al. (2012) with minor modification. Cell mass from 36 h grown culture in MH medium supplemented with 200 µM Cr(VI) at 32 °C were harvested by centrifugation (5,000 × g), washed and sonicated in phosphate buffer (0.1 M; pH 7.0) at 5% of the original culture volume using an ultrasonic probe (Sonics, mod. 130) (5 cycles of 120 s on and 60 s off at 130 W). The sonicated cell suspension was centrifuged (12,000 × g, 4 °C, 20 min) and the supernatant was used as the cell-free extract. Total protein was estimated according to Lowry et al. (1951) using bovine serum albumin (Sigma, Milano) as standard.

Chromate reduction by the CFE was assayed using NADH (Sigma, Milano) as an electron donor. The reaction mixture (1 mL) contained 100 µM Cr(VI) and 5% NaCl in 0.8 mL of 25 mM potassium phosphate buffer (pH 7.0) and 100 µM NADH. An aliquot of 200 µL of CFE was added to the mixture to initiate Cr(VI) reduction. An assay mixture with similar composition but without CFE was used as the negative control.

Reduction of Cr(VI) was measured by estimating the decrease of Cr(VI) content in the reaction mixture after 30 min of incubation at 30 °C. Cr(VI) was quantified following standard S-diphenyl carbazide method (Park et al. 2000) as described above. One unit of chromate reductase was defined as the amount of enzyme per mg of protein which reduced 1 µM Cr(VI) per min at 30 °C. The effect of pH (3.0–11), temperature (20–80 °C), NaCl (0–25%), CFE (0.1–0.4 µL), electron donor (50–200 µM), anions (200 and 400 µM) and cations (200 and 400 µM) on reductase activity was assessed.

# Rate kinetics of Cr(VI) reduction

Reduction rates were described as the relationship between the decrease of chromium per unit time and the number of bacterial cells (Zhu et al. 2008) and expressed mathematically as:

$$V_R = \Delta C / N \cdot t$$

where  $V_R$  is the specific reduction rate in  $\mu\text{M}$  /number of cells/ h;  $\Delta C$ , the difference in the reduced substrate concentrations in micromole;  $N$ , the cell concentration and  $t$ , the time in h. However, relative reduction rate,  $V_{RR} = \Delta C / t$ , indicate the decrease in velocity of Cr(VI) ignoring the cell density in reduction system.

The kinetics of Cr(VI) reduction was calculated according to the following equation (Camargo et al. 2003):

$$y = a \cdot e^{-kt}$$

$$C / C_0 = a \cdot e^{-kt}$$

Linearized form becomes:  $\ln C / C_0 = \ln a - kt$

where  $a$  is constant,  $C / C_0$  is the fraction of Cr(VI) reduction at time  $t$ ,  $C$  is the concentration of Cr(VI) at time  $t$ ,  $C_0$  is the original Cr(VI) concentration, and  $k$  is the rate constant.

## Chromium reduction in tannery effluent

Untreated tannery effluent containing  $100 \mu\text{M}$  of Cr(VI) and 10% (w/v) NaCl collected from Kolkata based tanneries in Bantala, Kolkata, West Bengal, India was filter sterilized and used for the reduction studies. Both the raw effluent [ $100 \mu\text{M}$ , Cr(VI)], effluent supplemented with 500 and  $1000 \mu\text{M}$  Cr(VI) were used to examine the efficiency of viable whole cells ( $10^8$  and  $10^7$  cells/mL) and CFE (0.2 and 0.4 mg protein/mL). As usual, the hexavalent chromium reduction (%) was determined following the DPC method described above.

FTIR of used bacterial cell mass was recorded following the method of Chandra and Singh (2014). The cell mass harvested after treatment was washed thrice with 0.9% saline water and lyophilized at  $-56^\circ\text{C}$  in a lyophilizer (LSL Secroid). The dried cell mass and potassium bromide (1:24) were mixed, grinded and pressed to form pellet by using a hydraulic press. The FTIR spectrum ( $400-4000 \text{ cm}^{-1}$ ) of the pellet was recorded in a Jasco-6300, (USA) IR spectrometer.

Powder X-ray diffraction (p XRD) was performed according to the method of Dhal et al. (2010). In brief, p XRD patterns of the treated cell mass was recorded in a Rigaku Ultima Diffractometer 1840 using Ni-filtered  $\text{CuK}\alpha$  ( $\lambda = 1.315 \text{ \AA}$ ) radiation generated at 20–60 kV and 2–60 mA and the instrument was

calibrated with standard silicon using a scintillation scan detector at slow scan in  $2\theta = 3-80^\circ$ . The interplaner d-spacing at different  $\theta$  values were calculated.

A control set (cell mass without exposer to tannery effluent) was also analyzed using FTIR and p-XRD.

## Data analysis

All the experiments were done in triplicate sets and the results are represented as average of triplicate values  $\pm$  S.D.

## Results

### Chromate reduction by whole cells

Time course of Cr(VI) reduction by freshly grown viable whole cells ( $10^9$  cells/mL) of *H. smyrnensis* KS802 as conducted under batch culture in basal MH medium showed a gradual increase with time leading to complete reduction of 200  $\mu$ M Cr(VI) after 10 h of incubation under continuous shaking. Concomitant with chromate reduction, the total number of cells/mL of the medium also showed an increase (Fig. 1a) keeping parity with percent Cr(VI) reduced.

With increasing concentration of Cr(VI) in the reaction medium, a decreased bio-reduction of Cr(VI) by cells of *H. smyrnensis* KS802 was noted. It was evident that with a variation of concentration ranging from 200–1000  $\mu$ M of Cr(VI) under the experimental conditions, the reduction efficiency of the cell mass ranged from 100 – 23.5% in 10 h (Fig. 1b). Kinetics of Cr(VI) reduction at different substrate concentrations fitted well ( $R^2 \geq 0.99$ ) with the linearized form of exponential rate of equation. The highest ( $5 \times 10^2 \text{ h}^{-1}$ , 200  $\mu$ M/mL) and lowest ( $8 \times 10^3 \text{ h}^{-1}$ , 1000  $\mu$ M/mL) rates of bio-reduction was correlated with the lowest and highest concentrations of Cr(VI) respectively (Fig. 1c).

Among the different electron donors used, cells of *H. smyrnensis* KS802 preferred acetate, propionate as well as glucose as the most promising electron donors totally reducing 200  $\mu$ M Cr(VI) in 10 h. Other electron donors such as citrate, succinate and gluconate were also found effective for chromate reduction by *H. smyrnensis* cells (Table 1). Most of the low molecular weight metal oxyanions tested so far were found to be highly stimulatory to Cr(VI) reduction by cells of *H. smyrnensis* KS802 while,  $\text{TeO}_4^{2-}$  followed by  $\text{AsO}_4^{3-}$  were to some extent inhibitory to chromate reduction (Table 1). All the tested inhibitors lowered chromate reduction by whole cells. As against control, 2, 4-dinitrophenol (DNP) appeared to be stimulatory, whereas carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) was most inhibitory showing only 11.25% Cr(VI) reduction after 10 h (Table 1). Presence of some of the metal ions were detrimental to Cr(VI) reduction by viable cells of *H. smyrnensis* KS802. The order of such reduction was  $\text{Sn}^{2+}$  (81.25%) >  $\text{Ag}^+$  (61.25%) >  $\text{Hg}^{2+}$  (56%) >  $\text{Cd}^{2+}$  (52.25%) >  $\text{Zn}^{2+}$  (50%). However, presence of  $\text{Cu}^{3+}$  and  $\text{Fe}^{3+}$  was stimulatory to reduction of Cr(VI) while  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Pb}^{2+}$  mediated reduction of Cr(VI) was almost equivalent to the control (Table 1).

Table 1

Effect of electron donors, oxyanions, inhibitors and metal ions on Cr(VI) reduction by viable cells of *H. smyrnensis* KS802

Experimental condition		% Cr(VI) reduction				
		2 h	4 h	6 h	8 h	10 h
Electron donor (0.1%)	Glucose	13.35 ± 1.25	45.50 ± 2.36	61.25 ± 1.02	84.25 ± 2.84	100.0 ± 0.00
	Acetate	18.25 ± 2.52	51.25 ± 0.78	73.75 ± 1.50	88.75 ± 2.22	100.0 ± 0.00
	Citrate	10.25 ± 1.25	35.00 ± 2.48	53.25 ± 2.35	82.25 ± 1.25	95.00 ± 1.00
	Fumarate	12.50 ± 1.45	38.25 ± 1.85	51.25 ± 1.56	62.25 ± 1.25	66.25 ± 1.25
	Gluconate	11.50 ± 1.32	40.00 ± 2.00	57.50 ± 1.25	73.25 ± 1.75	86.25 ± 2.50
	Propionate	11.75 ± 2.35	42.50 ± 1.25	72.00 ± 1.35	87.25 ± 3.25	100.0 ± 0.00
	Succinate	16.50 ± 0.05	28.25 ± 0.95	56.25 ± 2.24	77.50 ± 2.76	91.25 ± 3.25
	Control	7.50 ± 1.25	15.25 ± 0.00	28.50 ± 2.25	45.00 ± 1.00	68.25 ± 2.50
Oxyanion (100 µM)	AsO <sub>4</sub> <sup>3-</sup>	15.75 ± 0.90	39.50 ± 1.25	58.50 ± 2.25	78.00 ± 2.50	82.25 ± 2.50
	CO <sub>3</sub> <sup>2-</sup>	27.25 ± 2.50	68.00 ± 2.50	92.00 ± 1.00	100.0 ± 1.65	100.0 ± 0.00
	IO <sub>3</sub> <sup>-</sup>	25.50 ± 2.50	61.25 ± 2.50	88.75 ± 2.00	100.0 ± 1.26	100.0 ± 0.00
	NO <sub>3</sub> <sup>-</sup>	18.00 ± 2.00	46.25 ± 3.25	73.00 ± 1.25	89.25 ± 1.35	100.0 ± 0.00
	PO <sub>4</sub> <sup>3-</sup>	14.50 ± 1.50	45.00 ± 1.50	67.00 ± 3.16	85.50 ± 1.22	100.0 ± 0.00
	SO <sub>4</sub> <sup>2-</sup>	31.50 ± 2.50	65.00 ± 1.56	95.50 ± 2.18	100.0 ± 1.25	100.0 ± 0.00
	TeO <sub>4</sub> <sup>2-</sup>	10.00 ± 0.05	37.00 ± 2.50	63.25 ± 3.56	72.50 ± 1.65	75.00 ± 3.75

CCCP, carbonyl cyanide-m-chlorophenyl hydrazone; DNP, 2, 4-dinitrophenol; DCC, *N,N*-di-cyclohexyl carboimide; NaN<sub>3</sub>, sodium azide; NaF, sodium fluoride.

Values of are presented as mean of three experiments ± SD

	Control	18.50 ± 0.75	42.50 ± 1.41	65.25 ± 1.25	82.50 ± 1.35	100.0 ± 0.00
Inhibitor (200 µM)	CCCP	2.50 ± 0.25	6.25 ± 1.25	8.75 ± 0.45	10.50 ± 0.85	11.25 ± 2.25
	DNP	14.75 ± 1.25	41.75 ± 1.75	65.75 ± 2.15	95.25 ± 2.00	100.0 ± 0.00
	DCC	11.75 ± 0.75	35.00 ± 1.00	46.25 ± 1.25	52.00 ± 1.25	56.25 ± 1.25
	NaN <sub>3</sub>	10.0 ± 1.00	29.25 ± 1.25	51.50 ± 1.25	66.25 ± 2.25	75.00 ± 0.00
	NaF	9.50 ± 1.25	29.50 ± 1.65	55.25 ± 2.25	72.25 ± 3.25	80.00 ± 2.00
	Control	11.75 ± 0.75	33.5 ± 1.85	58.50 ± 1.00	81.25 ± 2.00	100.0 ± 0.00
Metal (100 µM)	Ag (II)	7.50 ± 0.45	21.25 ± 1.22	40.25 ± 1.25	51.25 ± 1.56	61.25 ± 2.38
	Cd(II)	4.50 ± 0.56	18.75 ± 0.85	36.25 ± 1.25	45.00 ± 0.75	52.25 ± 1.25
	Co(II)	5.00 ± 0.25	17.50 ± 0.75	38.50 ± 1.50	48.00 ± 1.66	57.50 ± 2.25
	Cu (III)	18.25 ± 1.35	48.25 ± 1.55	85.00 ± 2.25	100.0 ± 0.00	100.0 ± 0.00
	Fe (III)	17.5 ± 0.85	44.25 ± 2.15	83.25 ± 0.88	100.0 ± 0.00	100.0 ± 0.00
	Hg (II)	6.25 ± 0.85	18.25 ± 1.05	33.25 ± 1.55	41.25 ± 2.11	56.00 ± 3.26
	Mn (II)	10.00 ± 0.75	38.50 ± 1.32	72.50 ± 0.75	89.75 ± 1.82	100.0 ± 0.00
	Ni (II)	5.50 ± 0.85	17.50 ± 1.35	53.75 ± 2.25	78.25 ± 1.34	100.0 ± 5.00
	Pb(II)	14.25 ± 0.75	34.50 ± 1.00	65.00 ± 1.00	81.75 ± 1.45	100.0 ± 0.00
	Sn (II)	12.25 ± 1.25	30.25 ± 1.25	68.00 ± 1.25	68.00 ± 1.32	81.25 ± 1.85
Zn (II)	2.25 ± 0.25	11.25 ± 0.56	29.75 ± 1.15	43.00 ± 0.85	51.25 ± 1.25	

CCCP, carbonyl cyanide-m-chlorophenyl hydrazone; DNP, 2, 4-dinitrophenol; DCC, *N,N*-di-cyclohexyl carboiimide; NaN<sub>3</sub>, sodium azide; NaF, sodium fluoride.

Values of are presented as mean of three experiments ± SD

Control	14.25 ± 1.25	34.50 ± 2.00	65.00 ± 1.35	83.75 ± 1.33	100.0 ± 0.00
CCCP, carbonyl cyanide-m-chlorophenyl hydrazone; DNP, 2, 4-dinitrophenol; DCC, <i>N,N</i> -di-cyclohexyl carboiimide; NaN <sub>3</sub> , sodium azide; NaF, sodium fluoride.					
Values of are presented as mean of three experiments ± SD					

Supplementation of NaCl (5–10%, w/v) in the reduction medium enhanced the chromate bioreduction and the fastest reduction rate (35.5  $\mu\text{M}/10^8$  cells/h) was recorded with 7.5% NaCl in 8 h of incubation (Fig. 2a). A remarkable inhibition of chromate reduction (41.25%), however was recorded at 20% NaCl. Reduction of Cr(VI) was significantly affected on increasing the cell density from  $10^8$  to  $10^{11}$  cells/mL (Fig. 2b) and the fastest complete reduction of 200  $\mu\text{M}$  Cr(VI) was achieved in 6 h with  $10^{11}$  cells/mL with maximum reduction rate of 53.5  $\mu\text{M}/10^8$  cells/mL/h. At low cell density ( $< 10^8$  cells/mL), only 18.5–56.75% reduction of 200  $\mu\text{M}$  Cr(VI) was recorded and was accompanied with reduced rate of bioreduction. So far the influence of pH and temperature is concerned, the complete Cr(VI) reduction was observed at pH 7.0 in 10 h (at the rate of 33.5  $\mu\text{M}/10^8$  cells /h) (Fig. 2c) and at 37 °C in 8 h (at a rate of 37.4  $\mu\text{M}/10^8$  cells/h) (Fig. 2d).

#### Chromate reductase activity of cell-free extract

Time course of chromate reductase activity of the cell-free extracts of induced and non-induced cells of *H. smyrnensis* KS802 followed an almost similar pattern although CFE of induced cells showed higher % of Cr(VI) reduction (Fig. 3a) compared to that of non-induced cells. An almost complete loss of enzyme activity of the CFE was detected when treated with trichloroacetic acid (TCA) and moist heat. While metabolic inhibitors like *N,N*-di-cyclohexyl carboiimide (DCC) and CCCP showed pronounced retardation of enzymatic activities (8.08 and 4.14 U/mg protein), EDTA, DNP and  $\beta$  marcapto-ethanol were found to stimulate the reductase activity with specific activity of 20, 22.5 and 24.25 U/ mg protein respectively (Fig. 3b).

Specific activity of the chromate reducing enzyme in CFE was proportional with Cr(VI) up to a concentration of 200  $\mu\text{M}$  Cr(VI) showing maximum activity of 24.95 U/mg protein (Fig. 4a). The linearized Lineweaver-Burk plot as drawn for saturation kinetics of the reductase activity was also effectively justified. The apparent Michelis-Menten constant ( $K_m$ ) and the maximum velocity ( $V_{max}$ ) were calculated as 56.58  $\mu\text{M}$  and 3.37 U/mg protein respectively (Fig. 4b).

The optimum pH, temperature and NaCl concentration for reductase activity of the CFE of the present strain was found to be pH 8.0, 45 °C and 10% NaCl with specific activity of 28.25, 29.5 and 29.42 U/ mg protein respectively (Fig. 5a, 5b and 5c) whereby the enzyme showed a broad range of tolerance of these factors.

An increased enzyme activity was found with increasing enzyme concentration (01-0.4 mg protein/ mL) and a saturation of the activity (33 U/mg protein) was noted when the reaction mixture was

supplemented with CFE equivalent to 0.4 mg protein/mL (Fig. 6a). Similarly, increase in concentration of acetate and NADH gradually increased the specific activity of the enzyme (Fig. 6b). Both the electron donor showed their efficiency towards Cr(VI) reduction but presence of NADH had a superior enzyme activity over acetate.

Considering the influence of oxyanions on Cr(VI) reduction by cells of *H. smyrnensis* KS802, effect of different concentrations of  $\text{CO}_3^{2-}$ ,  $\text{IO}_3^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{PO}_4^{3-}$ ,  $\text{NO}_3^-$  on reduction of Cr(VI) with CFE was tested. It was noted that at low concentration (200  $\mu\text{M}$ ), oxyanions like  $\text{CO}_3^{2-}$ ,  $\text{IO}_3^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{PO}_4^{3-}$ ,  $\text{NO}_3^-$  stimulated the enzymatic activity and the highest reductase activity was found in presence of  $\text{SO}_4^{2-}$  (33 U/mg protein) followed by  $\text{CO}_3^{2-}$  (31.25 U/mg protein) and  $\text{IO}_3^-$  (28.75 U/mg protein). However, at increased concentration (400  $\mu\text{M}$ ) of oxyanions, only  $\text{CO}_3^{2-}$ ,  $\text{IO}_3^-$  and  $\text{SO}_4^{2-}$  were still stimulatory while,  $\text{PO}_4^{3-}$  and  $\text{NO}_3^-$  at identical concentration was having negative effect on chromate reduction (Fig. 6c).

Toxic as well as heavy metals have versatile effect on enzyme activity. Stimulation of the reductase activity (33 U/ mg protein) was observed in presence of 200  $\mu\text{M}$   $\text{Fe}^{3+}$  and  $\text{Cu}^{3+}$  while at higher concentration (400  $\mu\text{M}$ ), the specific activity was lowered to 28.25 and 25 U/ mg protein. The highest inhibitory metal was  $\text{Zn}^{2+}$  followed by  $\text{Ni}^{2+}$  and  $\text{Pb}^{2+}$  (Fig. 6d).

#### Chromate reduction in tannery effluent

Raw tannery effluent containing 100  $\mu\text{M}$  Cr(VI) and tannery effluent supplemented with additional Cr(VI) (500 and 1000  $\mu\text{M}$ ) was treated with both the viable cells and CFE of *H. smyrnensis* KS802. An increase in % reduction of Cr(VI) in tannery effluents was found with increased number of viable whole cells as well as concentration of CFE. Raw effluent when treated with  $10^8$  and  $10^{10}$  cells/mL showed complete reduction of Cr(VI) after 10 h (Fig. 7a). Supplementation of Cr(VI) in the effluent, however has retarded the chromate reducing efficiency. Only 68.25% of 500  $\mu\text{M}$  and 35% of 1000  $\mu\text{M}$  Cr(VI) in tannery effluents was reduced by  $10^{10}$  cells/mL in 10 h. The CFE showed 100 and 68% reduction of Cr(VI) from tannery effluent with 0.2 and 0.4 mg protein/mL. Increased Cr(VI) in tannery effluent (500 and 1000  $\mu\text{M}$ ) have affected the reduction process strikingly. Only 48% reduction of 500  $\mu\text{M}$  Cr(VI), and 20% reduction of 1000  $\mu\text{M}$  Cr(VI) in tannery effluent was observed with highest concentration of CFE (0.4 mg protein/mL) (Fig. 7b).

Comparative FTIR analysis of bacterial biomass used for reduction of Cr(VI) from tannery effluents (Fig. 8b) with that of untreated cell mass (Fig. 8a) showed shifting of peaks as well as appearance of new peaks in the treated biomass. New peaks were visualized at 1744.45, 1240.76, 1179, 1103 and 976  $\text{cm}^{-1}$  while shifting of peaks at 3290.39, 3077.50, 1646.88, 1532.84, 1394.72, 1229 and 1660  $\text{cm}^{-1}$  were also evident (Fig. 8b).

As against the untreated cell mass of *H. smyrnensis* KS802 which showed no peaks in XRD analysis (Fig. 9a), the treated cell mass showed a number of sharp peaks of  $2\theta$  values at 27.5, 28.5, 31.8, 40.6, 45.4, 50.2, 54.0, 56.5, 58.84, 66.4, 73.25 and 75.39 with interplaner spacing (d-spacing) of 2.77, 2.65, 2.40, 1.90, 1.70, 1.55, 1.44, 1.39, 1.33, 1.20, 1.10 and 1.076 which indicated the crystallinity index of 0.83 (Fig. 9b).

## Discussion

Bioreduction of Cr(VI) in industrial wastes specially in tannery effluents which also contain toxic metals, oxyanions and high amount of NaCl could be achieved exploiting selective halophilic bacterial strains with unique adaptive features such as tolerance to high salinity and osmotic pressure, low oxygen and nutrient availability, and toxic substances including heavy metals (Oren, 2002). Therefore, studies on Cr(VI) reduction by halophiles and their enzymes could be of great importance in bioremediation of Cr(VI) contaminated industrial effluents. In this study, we have initially evaluated the chromate reduction potential of the previously reported moderately halophilic bacterium, *H. smyrnensis* KS802 (Biswas et al. 2018) using its viable whole cells and cell-free extracts.

Freshly grown cells of *H. smyrnensis* KS802 restored its chromate reducing activity and effectively reduced the Cr(VI) in the synthetic Basal MH medium with limited cellular growth possibly due to poor nutritional composition of the basal medium used (Fig. 1a). The anti-parallel effect of Cr(VI) concentration on reduction activity of cells of *H. smyrnensis* KS802 (Fig. 1b and Fig. 1c) reflected the interdependency of the enzyme and the substrate, Cr(VI) (McLean and Beveridge 2001).

Acetate a suitable electron donor for reduction of Cr(VI) by cells of *H. smyrnensis* (Table 1) might have favored the process by protecting the concerned enzyme from inactivation (Soni et al. 2012). The role of oxyanions as competing electron acceptors in Cr(VI) reduction process is well known and  $\text{SO}_4^{2-}$  in particular is likely to facilitate Cr(VI) uptake via the sulphate uptake pathway (Cervantes et al. 2001) and thereby promoting the process in *H. smyrnensis* KS802 cells (Table 1). The inhibitory effect of CCC,  $\text{NaN}_3$  and NaF at higher level has been explained due to retardation of cytochrome oxidase, enolase and disruption of chemiosmotic gradient (Opperman and Heerden 2007). The inhibitory activity of  $\text{Hg}^{2+}$  as in most cases appeared to be due to its binding to thiol (SH) group and inactivation of enzyme (Xu et al. 2012). But the  $\text{Cu}^{2+}$  and  $\text{Fe}^{2+}$  mediated improvement of Cr(VI) reduction by cells of *H. smyrnensis* KS802 could be attributed to their functions as cofactors for many enzymes in the cellular metabolism (Dogan et al. 2011).

It is a commanding feature of the KS802 cells to reduce Cr(VI) under high salt concentrations (Fig. 2a) compared to other *Halomonas* spp. so far reported (Focardi et al. 2012; Murugavelh and Mohanty 2012). The increase in Cr(VI) reduction with increased viable cells (Fig. 2b) is the general trend of bioreduction of Cr(VI) as reported previously (Dey and Paul 2014). Reduction of Cr(VI) at wide pH range indicated the conformational change of active site of enzyme with availability of  $\text{CrO}_4^{2-}$  (Karthik et al. 2017) (Fig. 2c) while, its thermotolerant feature (Fig. 2d) corroborated several previous studies (Amoozegar et al. 2007; Subramanian et al. 2012; Chandra and Singh 2014).

A higher specific activity of the CFE of induced cells compared to non-induced cells indicated the constitutive nature of the enzyme which was promoted to a higher degree under chromium stressed condition (Fig. 3a) (Mangaiyarkarasi et al. 2011). Loss of the chromate reduction ability of CFE with TCA and moist heat treatment established the involvement of proteinaceous enzymes in the process. The

enzyme mediated reduction by the CFE was further confirmed by the inhibition of the process following treatment with CCCP and DCC which functions as an uncoupler of proton gradient and ionophore in addition to inhibition of oxidative phosphorylation of ETS (Takamiya 1983). The stimulation of Cr(VI) reduction activity of CFE in presence of DNP suggested the involvement of other alternative intracellular Cr(VI) reduction mechanisms (Shen and Wang 1993).

Increase in Cr(VI) concentration have an inverse effect on reductase activity of CFE (Fig. 4a) and chemical kinetics of the reduction in relation with substrate concentration fitted well with linearized Lineweaver-Burk plot with low  $K_m$  value (56.58  $\mu\text{M}$ ) (Fig. 4b). This results indicated that the reduction of Cr(VI) was dependent on the substrate and low  $K_m$  value as found in Michelis-Menten equation suggested very high affinity of the substrate to the enzyme and therefore, at very low Cr(VI) concentration, the present enzyme could reach with maximal velocity ( $V_{max}$ ). Moreover, the variations of optimum pH (8.0), temperature (45 °C) and NaCl conc. of (10%) of the CFE (Fig. 5a, 5b and 5c) CFE of *H. smyrnensis* KS802 may offer suitability for bioremediation industrial effluents (Kiran et al. 2007).

With increasing concentrations of CFE, acetate and NADH, the reductase activity was increased gradually (Fig. 6a, 6b). It was pointed out that a Cr(V) intermediate is formed in presence of NADH which decreased reactive oxygen species (ROS) formation as well as chromium toxicity and thus highly stimulated the reductase activity (Bae et al. 2005). Reductase activity of CFE was positively influenced with tested oxyanions (Fig. 6c) which might be the resultant of non-competitive association of oxyanions and  $\text{CrO}_4^{2-}$  and facilitation of  $\text{CrO}_4^{2-}$  entry through respective oxyanions uptake pathways (Cervantes et al. 2001; Qian et al. 2016). Improvement of Cr(VI) reduction as noted with toxic metal ions (Fig. 6d) though contradicted the findings with non-halophilic strains (Sarangi and Krishnan 2008; Elangovan et al. 2010), promoting effect of  $\text{Fe}^{3+}$  on Cr(VI) reduction by *Halomonas* sp. has already been reported (Focardi et al. 2012).

Whole cells as well as CFE of *H. smyrnensis* KS802 was targeted for their application in bioreduction of Cr(VI) in tannery effluent which also contains a high concentration of NaCl, diverse toxic metals and oxyanions. The cells ( $10^8$ - $10^{11}$  cells/mL) (Fig. 7a) as well as crude enzyme (0.2–0.4 mg protein/mL) (Fig. 7b) exhibited remarkable efficacy in reducing Cr(VI) from tannery effluent indicating their potential for bioremediation.

Such potentials were reflected by possible interaction of chromium compounds with bacterial cell surface functional groups (Pradhan et al. 2007) as indicated by the shifting as well as appearance of new peaks in the FTIR analysis (Yee et al. 2004; Park et al. 2005) (Fig. 8a and 8b). Moreover, results of powder XRD analysis of the treated cells showed that a number of sharp peaks with a crystallinity index of 0.83 (Fig. 9a and 9b) indicated possible combination of crystalline reduced Cr-compounds on the cell surfaces during the reduction process. The nature of such complex and speciation of chromium, however, could not be determined during the present study.

## Conclusion

Characterization of the chromate reduction activities of viable whole cells and CFE from *H. smyrnensis* KS802 showed efficient bio-reduction of Cr(VI) under saline stressed environment co-contaminated with toxic metals and oxyanions. Efficacy of both cells and CFE in the anthropogenic toxic tannery effluents also proved promising at the laboratory level batch culture studies. An improvement of the Cr(VI) reductase activity under optimized conditions appeared to be fruitful for development of an well organized bioremediation process of Cr(VI) reduction for toxic effluents in large-scale bioreactor systems.

## Abbreviations

CFE, Cell free extract; Cr(VI), Hexavalent chromium;  $K_m$ , Michaelis-Menten constant;  $V_{max}$ , Maximal velocity; MH, Medium for Halophiles; Cr(III), trivalent chromium; DPC, S-diphenyl carbazide; OD, Optical density; NADH, Nicotinamide adenine dinucleotide (reduced); FTIR, Fourier transform infrared; XRD, X-ray diffraction; kV, kilo volt; mA, miliampere; Å, Angstrom, DNP, 2, 4-dinitrophenol; CCCP, carbonyl cyanide-m-chlorophenyl hydrazone; DCC, *N,N*-di-cyclohexyl carboiimide; TCA, trichloroacetic acid ; EDTA, Ethylenediamine tetraacetic acid; NaN<sub>3</sub>, Sodium azide, NaF, Sodium fluoride; ROS, Reactive oxygen species ETS, Electron transport system.

## Declarations

Not applicable.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

All data represented or analyzed during this study are included in this article.

### Competing interests

The authors declare no conflict of interest

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### Authors' contributions

Dr. Jhuma Biswas designed and performed the experiments and prepared the draft manuscript: Prof. A. K. Paul supervised the work and analysis of experimental data and finalized the manuscript.

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## Figures

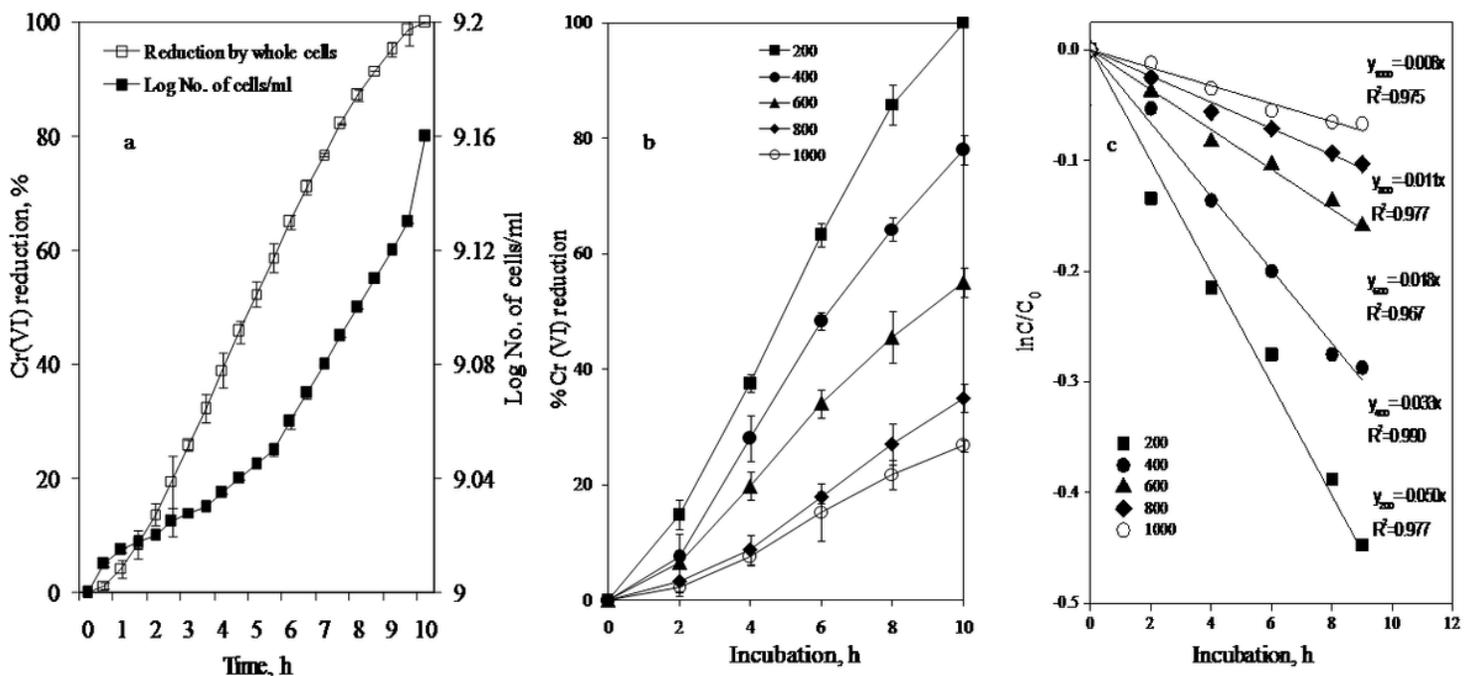


Figure 1

Time course of chromium (VI) reduction (□) by viable whole cells of *H. smyrnensis* KS802 and changes in log no. of cells/mL (■) during the process of reduction (a); Effect of Cr(VI) concentration (■, 200 μM; ●, 400 μM; ▲, 600 μM; ◆, 800 μM; ●, 1000 μM) on % Cr(VI) reduction (b) and rate of chromate reduction (c) by cells in basal MH medium.

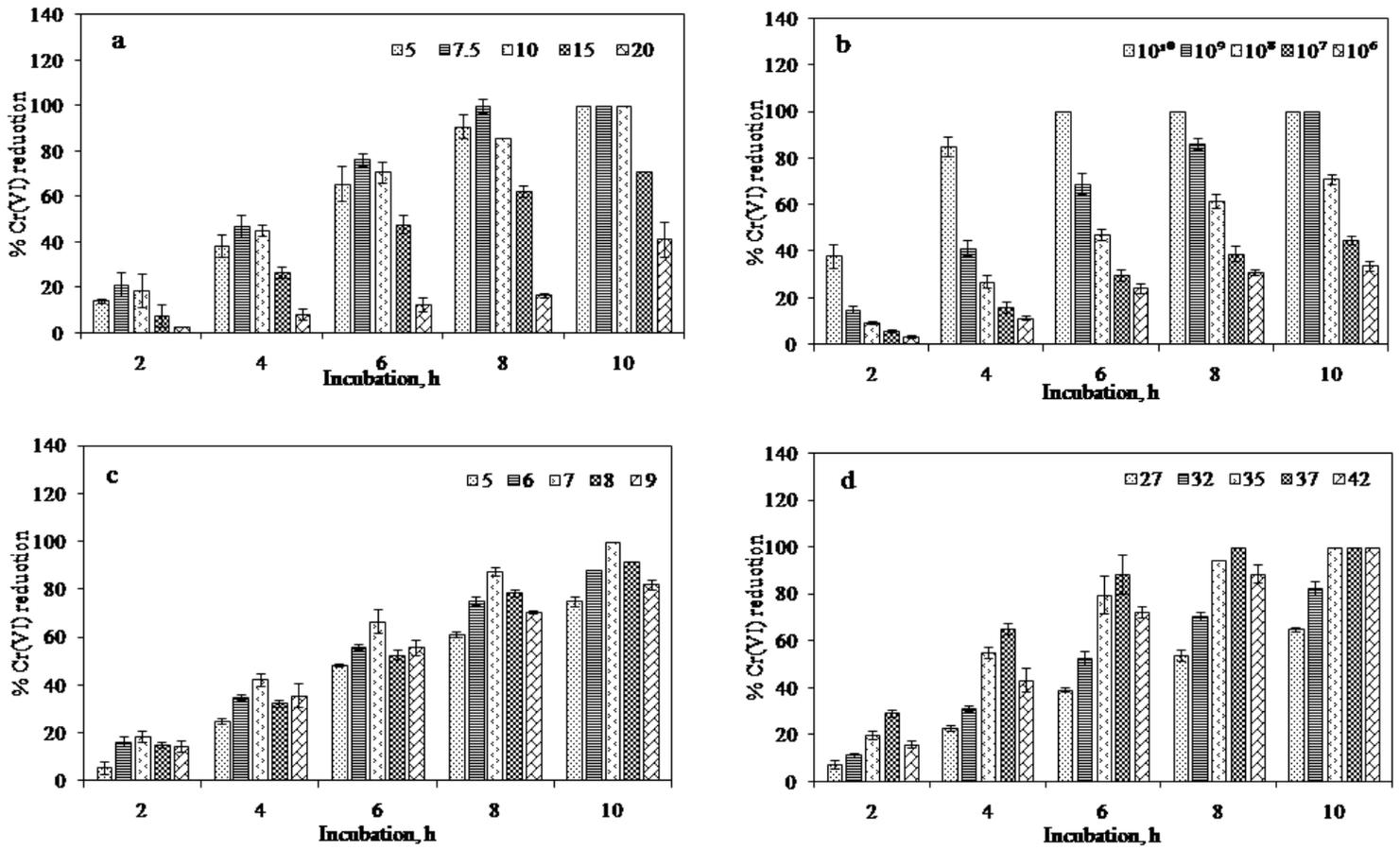


Figure 2

Effect of NaCl concentration (% w/v) (a), cell density (b), pH (c) and temperature (d) on % Cr(VI) reduction by free cells of *H. smyrnensis* KS802 in basal MH medium.

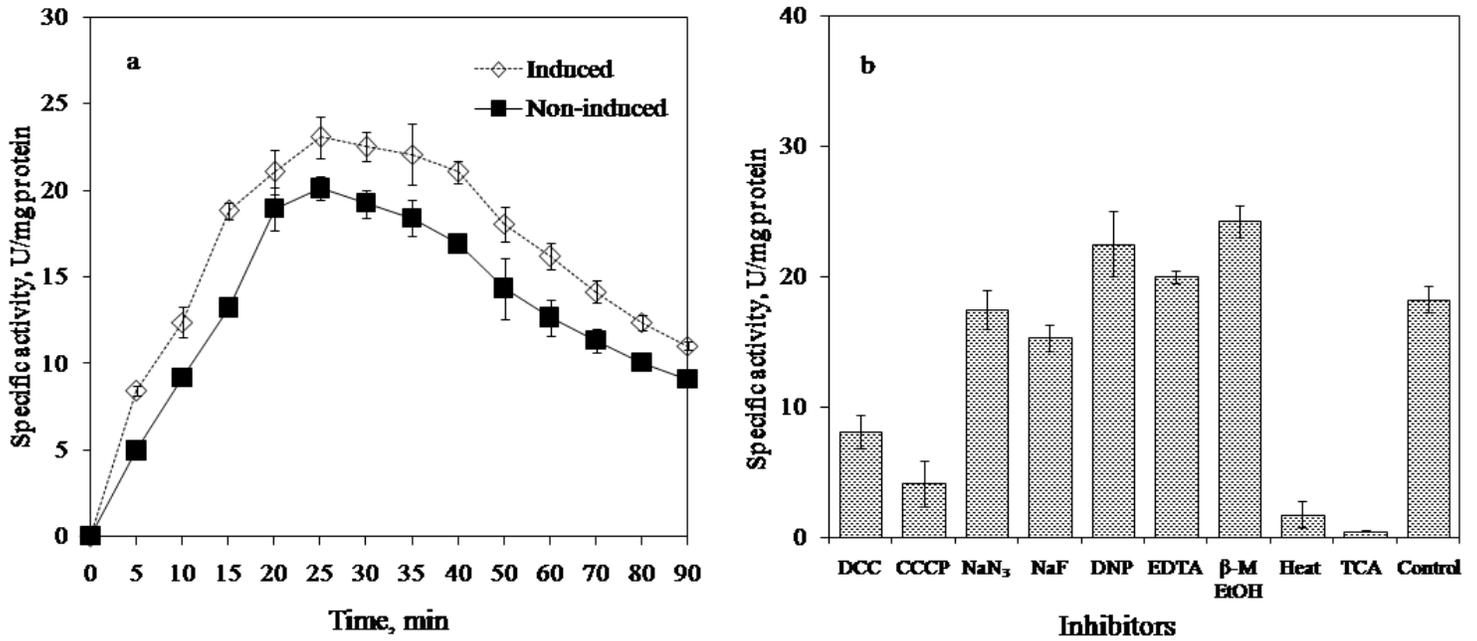


Figure 3

Cr(VI) reduction by cell free extract (CFE) prepared from induced [2 mM Cr(VI) in the medium for 72 h] and non-induced cells of *H. smyrnensis* KS802 (a) and effect of selected inhibitors on the enzyme activity of CFE.

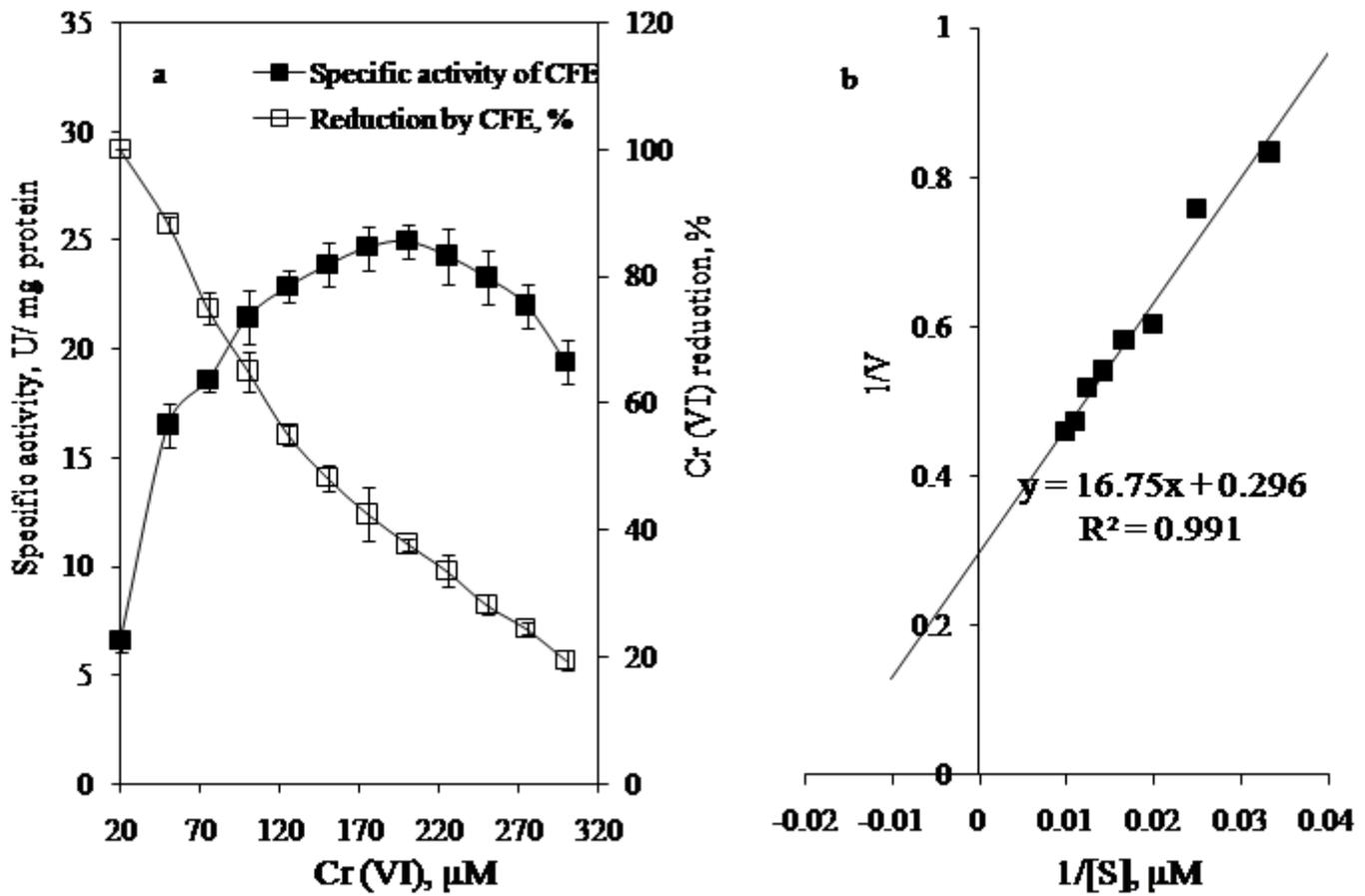
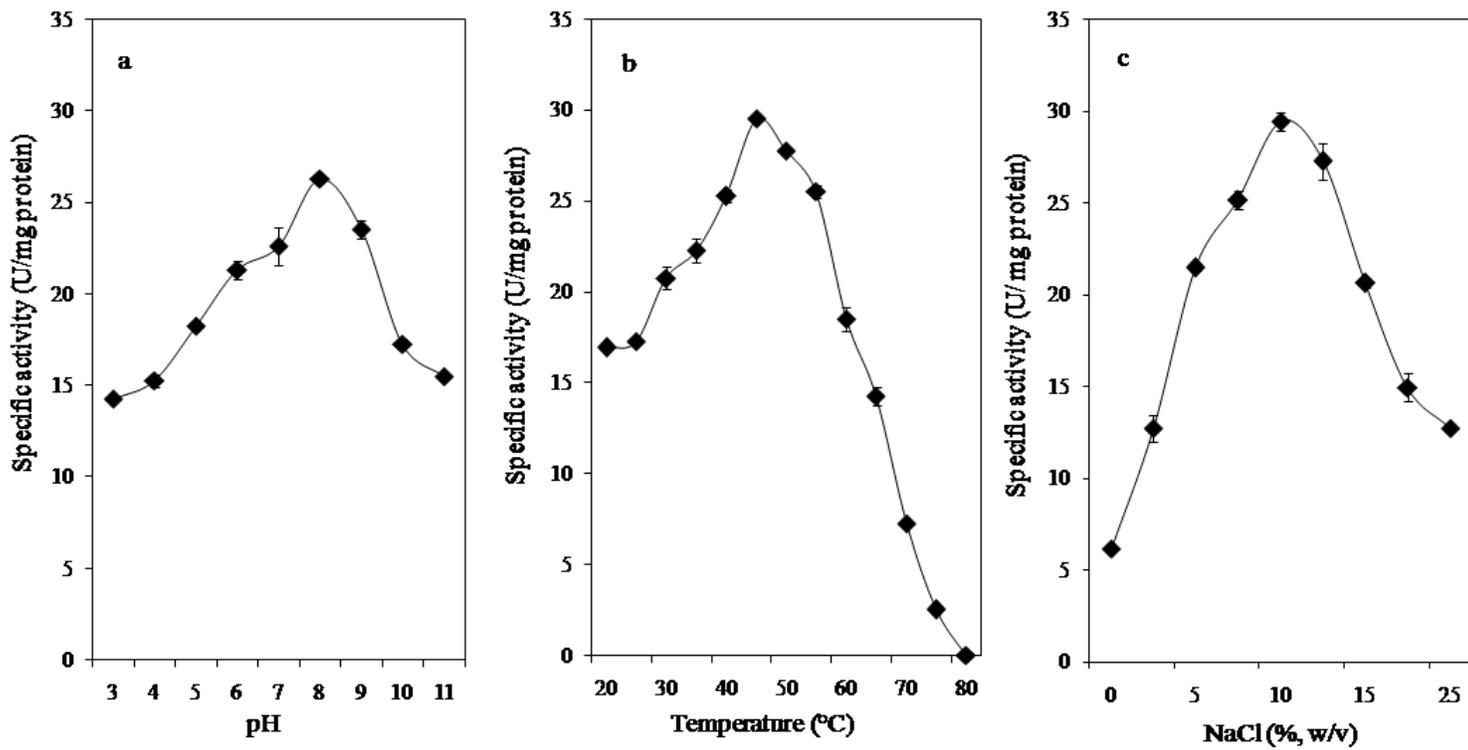


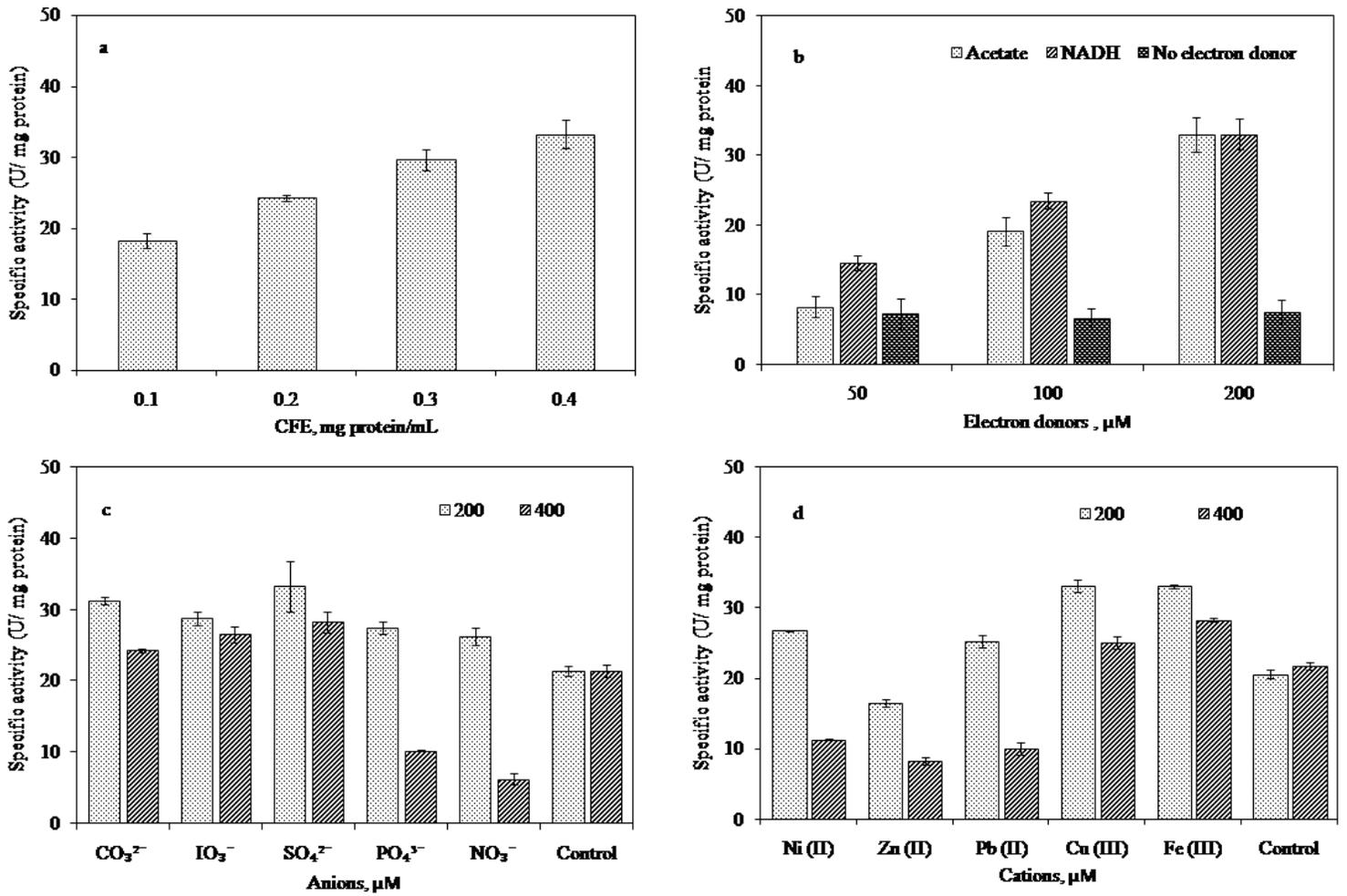
Figure 4

Kinetics of Cr(VI) reduction of cell free extract of *H. smyrnensis* KS802 at different concentrations of Cr(VI) for 30 min., at 30°C (a). Linearized Lineweaver-Burk plot for Cr(VI) reduction of CFE (b).



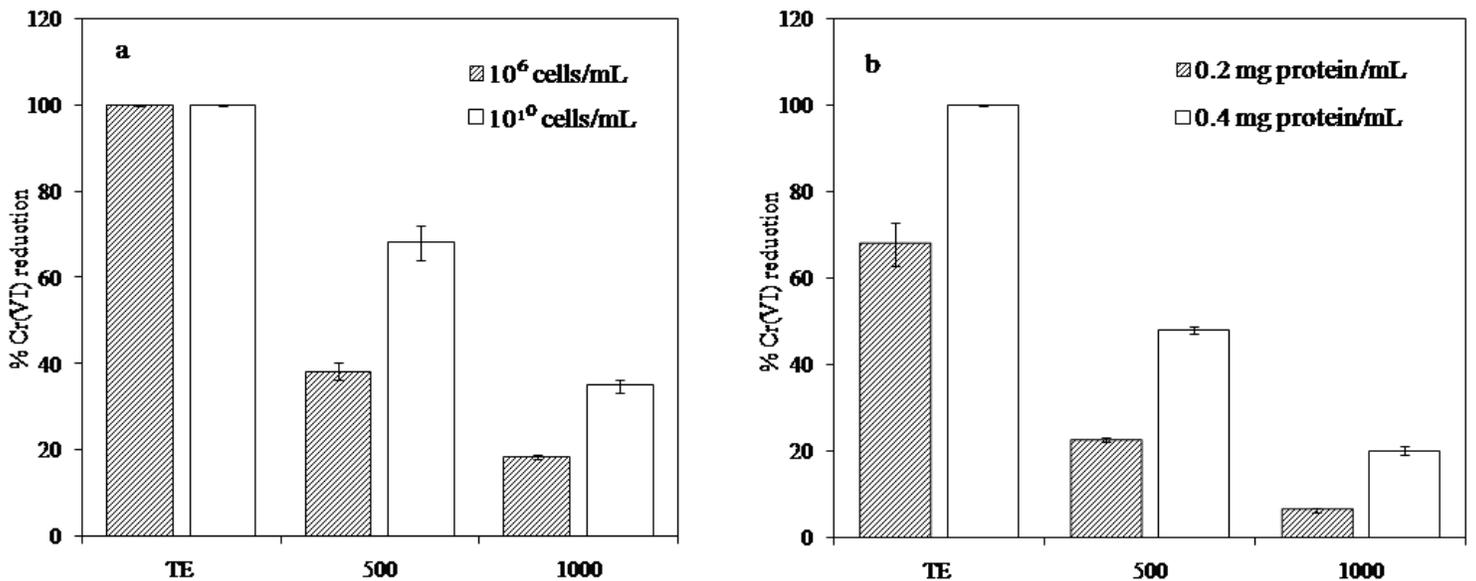
**Figure 5**

Effect of pH (a), temperature (b) and NaCl (c) on the chromate reduction activity of CFE of *H. smyrnensis* KS802.



**Figure 6**

Effect of different concentrations of CFE (a), electron donors (b), oxyanions (c) and metal ions (d) on the chromate reduction activity of CFE of *H. smyrnensis* KS802.



**Figure 7**

Chromate reduction in raw tannery effluent [TE = Cr(VI)100  $\mu$ M] and tannery effluent supplemented with additional Cr(VI) [ 500 = Final Cr(VI) con. 500  $\mu$ M; and 1000 = Final Cr(VI) con. 1000  $\mu$ M] using cells and CFE of *H. smyrnensis* KS802.

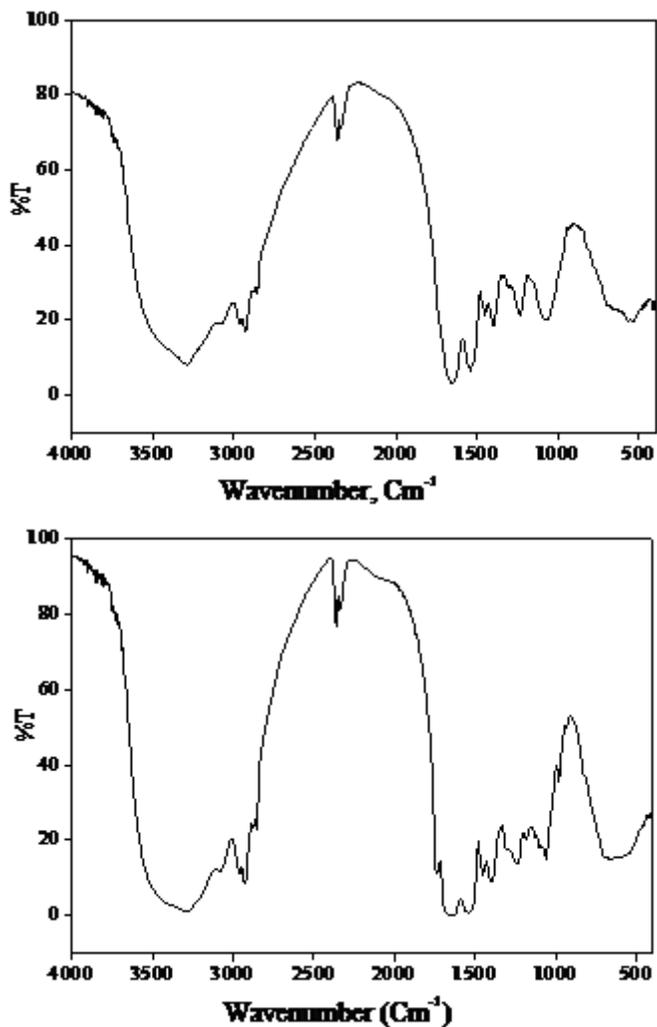


Figure 8

Comparative FTIR analysis of untreated cells of *H. smyrnensis* KS802 (a) and cells used for Cr(VI) reduction from tannery effluent (b).

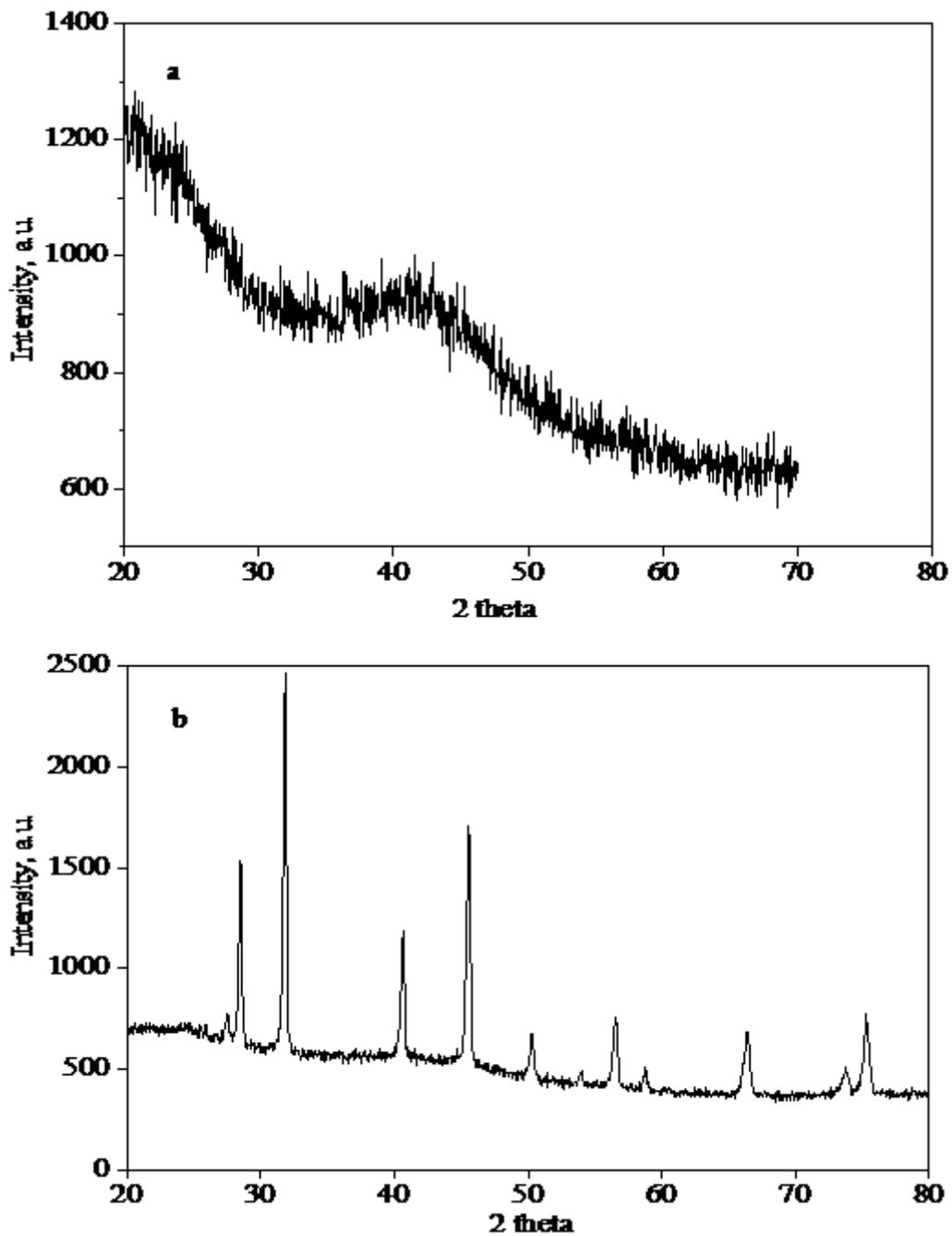


Figure 9

Powder XRD analysis of untreated cells of *H. smyrnensis* KS802 (a) and cells used for Cr(VI) reduction from tannery effluent (b).

## Supplementary Files

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