World Journal of Microbiology and Biotechnology (2018) 34:126
https://doi.org/10.1007/s11274-018-2510-z

ORIGINAL PAPER

Isolation and characterization of chromium(VI)-reducing bacteria from tannery effluents and solid wastes

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Received: 22 March 2018 / Accepted: 28 July 2018 / Published online: 6 August 2018
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Abstract

In the present investigation, five novel Cr(VI) reducing bacteria were isolated from tannery effluents and solid wastes and identified as *Kosakonia cowanii* MKPF2, *Klebsiella pneumonia* MKPF5, *Acinetobacter gerneri* MKPF7, *Klebsiella variicola* MKPF8 and *Serratia marcescens* MKPF12 by 16S rDNA gene sequence analysis. The maximum tolerance concentration of Cr(VI) as $K_2Cr_2O_7$ of the bacterial isolates was varying up to 2000 mg/L. Among the investigated bacterial isolates, *A. gerneri* MKPF7 was best in terms of reduction rate. The optimum temperatures for growth and Cr(VI) reduction by the bacterial isolates were 35 and 40 °C, respectively except *A. gerneri* MKPF7 which grew and reduced Cr(VI) optimally at 40 °C. The optimum pH for growth and Cr(VI) reduction by *K. cowanii* MKPF2, *A. gerneri* MKPF7 and *S. marcescens* MKPF12 was 7.0 whereas the optimum pH for growth and Cr(VI) reduction by *K. pneumoniae* MKPF5 and *K. variicola* MKPF8 were 7.0, 8.0 and 6.0, 7.0, respectively. All the bacterial isolates showed maximum tolerance against Ni^{2+} and Zn^{2+} whereas minimum tolerance was observed against Hg^{2+} and Cd^{2+}. The bacteria isolated in the present study thus can be used as eco-friendly biological expedients for the remediation and detoxification of Cr(VI) from the contaminated environments.

Keywords Cr(VI) detoxification · Tannery effluents · Tannery solid wastes · *Kosakonia cowanii* MKPF2 · *Acinetobacter gerneri* MKPF7 · *Klebsiella variicola* MKPF8 · *Serratia marcescens* MKPF12

Introduction

Leather tanning and its related industrial pollution is one of the major industrial as well as environmental pollution problems in Bangladesh. There are about 250 tanneries in Bangladesh and 95% of which are established in Hazaribagh, a heavily populated suburban area of the Dhaka city. This area is well known for its tannery industry and has also been listed as the fifth, among the list of top ten polluted places of the world (Bernhardt and Gysi 2013). Almost 90% of tanneries placed in Hazaribagh area in Bangladesh are involved in chrome tanning process and discharge liquid effluents and solid wastes that contain 180 mg/L of chromium (Shams et al. 2009). Leather tanning industries use chromium compounds like chromium(III) salts extensively in the tanning process and release the untreated effluents and solid wastes containing higher level of chromium in the natural environments which results severe anthropogenic chromium pollution (Cheung and Gu 2007; Garg et al. 2012).

Chromium is considered as one of the most regulated toxic substances, sustaining in the environment in various forms

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(Hora and Shetty 2014). It has several oxidation states ranging from Cr(II) to Cr(VI) but only Cr(III) and Cr(VI) are stable in the natural environments (Kotas and Stasicka 2000). Chromium(III) and chromium(VI) have their individual physiochemical and toxicological properties (Ishibashi et al. 1990), for example, Cr(VI) has severe toxic, mutagenic and carcinogenic characteristics to living systems (Nishioka 1975; Wang et al. 1990; Costa 1997). This is because of its high solubility, bioavailability and oxidizing nature that accelerate rapid active transport of Cr(VI) through biological membranes (Singh et al. 2005) and subsequently cause oxidative damage to DNA, proteins and fats (Cervantes et al. 2001). Chromium(III) is a crucial micronutrient for higher organisms which is 100 times less toxic, relatively insoluble in water, more stable and some 1000-fold less mutagenic than Cr(VI) (DeFlora et al. 1990; Dinakarpandian et al. 2004; Pal et al. 2005).

Conventional treatment technologies for detoxification of Cr(VI) compounds are ion exchange (Maksin et al. 2012), precipitation, ultrafiltration, reverse osmosis and electrodialysis (Rengaraj et al. 2007). All these methods experienced a lot of disadvantages like less removal efficacy, the production of huge quantities of chemical sludge, and high price of chemicals used for Cr(VI) reduction, particularly for the removal of comparatively low concentrations of Cr(VI) (Kratochvil and Volesky 1998). The bacterial reduction of more toxic Cr(VI) to less toxic Cr(III) is considered as an essential stage in the detoxification process of Cr(VI) polluted environments (Megharaj et al. 2003). Several bacteria have chromate reductase capability that can transform Cr(VI) to Cr(III), which is much less toxic and less soluble. Thus, biological reduction gives a sustainable alternative for Cr(VI) bioremediation (Ackerley et al. 2004). The major advantages of biological Cr(VI) detoxification are the lower costs and the significant smaller quantities of the produced sludge (Liu et al. 2006).

In the present study, five novel chromium(VI) resistant bacteria from chromium contaminated tannery effluents and solid wastes have been isolated and identified by molecular techniques based on 16S rDNA gene sequence analysis. Moreover, determination of maximum tolerance concentration (MTC) of Cr(VI), reduction potentials, tolerance of other heavy metals, effects of temperatures and pH on growth and Cr(VI) reduction of the isolated Cr(VI) reducing bacteria have been investigated in order to use them in bioremediation based detoxification of Cr(VI) contaminated sites.

Materials and methods

Isolation of Cr(VI) resistant bacteria from tannery effluent and solid wastes

Tannery effluents and solid wastes samples were collected from Hazaribagh tannery industrial area, Dhaka, Bangladesh. For isolation of Cr(VI) resistant bacteria M9 minimal salts medium (Eisenstadt et al. 1994; Megharaj et al. 2003) was used which contained (g/L): Na_2HPO_4, 6; KH_2PO_4, 3; NH_4Cl, 1; NaCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.246; $CaCl_2$, 0.01. The medium was modified with 0.5% (w/v) glucose as additional carbon source and 100 mg/L potassium dichromate ($K_2Cr_2O_7$) as a source of Cr(VI) and pH of the medium was adjusted to 6.9 by using 1 N NaOH. Cr(VI) resistant bacteria were spreaded on solid agar plates and incubated at 37 °C for 24–72 h. The total number of bacteria per gram solid wastes and per ml of effluents was then counted by viable plate count method. Suitable single colonies were selected and purified through repeated subculture onto nutrient agar medium, labeled and kept at 4 °C in a refrigerator or in 20% glycerol stock for further investigations.

Determination of the MTC of Cr(VI) as $K_2Cr_2O_7$ of the bacterial isolates

The MTC of Cr(VI) as $K_2Cr_2O_7$ to the bacterial isolates was investigated in M9 minimal salts broth by broth dilution method (Calomoris et al. 1984). 500 mL of M9 minimal salts broth was prepared in a 1000 mL conical flask and was then transferred to 12 100 mL conical flasks containing 0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and 2000 mg/L of $K_2Cr_2O_7$ as Cr(VI). The conical flasks containing different concentrations (0–2000 mg/L) of $K_2Cr_2O_7$ as Cr(VI) were then autoclaved. After cooling at room temperature, the sterilized medium containing varying concentrations (0–2000 mg/L) of $K_2Cr_2O_7$ as Cr(VI) were inoculated aseptically with 100 µL of the 24 h bacterial culture (OD, 0.25) previously grown on nutrient broth. Another conical flask containing only medium served as the control. All the conical flasks including control were then incubated at 37 °C in a shaking incubator for 24–48 h. After incubation of 24 and 48 h, the growth of the bacterial isolate was monitored by measuring optical density (OD) at 600 nm using a spectrophotometer. The maximum concentration of $K_2Cr_2O_7$ as Cr(VI) which supports the bacterial growth in the medium after 48 h of incubation was considered as MTC of the bacterial isolates.

Reduction ability of Cr(VI) to Cr(III) by the bacterial isolates

In order to measure the ability of the bacterial isolates to reduce Cr(VI) to Cr(III), the 1,5-diphenylcarbazide method (Fulladosa et al. 2006) was used. A total 15 centrifuge tubes containing sterilized M9 minimal salts medium and two initial Cr(VI) concentration (10 and 20 mg/L) were inoculated aseptically with 24 h enriched cultures (OD, 1.60) of the bacterial isolates. Five centrifuge tubes; each contained

individually 35 mL medium, 1 mL cell suspension of each bacterial isolates and 4 mL Cr(VI) solution. Five centrifuge tubes; each contained individually 31 mL of medium, 1 mL cell suspension of each bacterial isolates and 8 mL Cr(VI) solution. Five centrifuge tubes; each contained individually 39 mL of medium and 1 mL cell suspension of each bacterial isolates served as control. All the centrifuge tubes including control were then vortexed and incubated at 37 °C and 180 rpm in a shaking incubator for 0–36 h. The growth and Cr(VI) reduction of the bacterial isolates were monitored at a specific time interval by measuring optical density with a spectrophotometer at 600 and 540 nm, respectively.

Determination of hexavalent chromium Cr(VI)

The hexavalent chromium was determined colorimetrically with an UV–Vis spectrophotometer (U-2910, HITACHI, USA) by following the method of Ilias et al. (2011). The samples (6 mL) from the cultures containing Cr(VI) and controls (containing medium and inoculum only) were harvested in centrifuge tubes after 0, 3, 6, 12, 18, 24 and 30 h intervals, centrifuged at 6000 rpm for 10 min and supernatants were used to estimate the Cr(VI) remained in the culture medium. The supernatants (3 mL) was transferred in the test tubes and 375 µL diphenylcarbazide (DPC) reagent was added to the test tubes, mixed gently and kept at room temperature for 20 min in order to develop the red–violet color of DPC–Cr(VI) complex. A control was run identical manner. The concentration of Cr(VI) in the samples was then estimated by the prepared standard curve of $K_2Cr_2O_7$.

Determination of total chromium in the culture supernatants

In order to cross-check the Cr(VI) reduction by the bacterial isolates, the total chromium in the culture supernatants was determined by using a flame atomic absorption spectrophotometer (FAAS-6300, SHIMADZU, Japan). The previously preserved culture supernatants in refrigerator which contains chromium were transferred to the plastic sample pots (6 mL) and aspirated into the air acetylene flame of the atomic absorption spectrophotometer. The concentration of total chromium of the samples was then determined by using the calibration curve of standard chromium (Cr) solution.

Identification of the bacterial isolates by 16S rDNA gene sequence techniques

The genomic DNA of the bacterial isolates was isolated by Maxwell 16 Cell DNA Purification Kit. The gene fragments specific for the extremely variable V3 region of the bacterial 16S rDNA gene was amplified by polymerase chain reaction (PCR) as described by Ovreas et al. (1997), using two universal bacterial primers 8F (Eden et al. 1991) and 534R (Bruet al. 2008) in a thermal cycler (GeneAtlas G, Astec, Japan). The sequence of 8F primer was 5′-AGA GTTTGATCCTGGCTCAG-3′ and the sequence of 534R primer was 5′-ATTACCGCGGCTGCTGGCA-3′. The PCR reactions were carried out in thin wall tubes using hot start PCR protocol as described by Aquilla et al. (1991). The PCR reaction mixtures contained 12.5 µL of Go Taq hot start master mix (Cat: M7432, Origin: Promega, USA) included deoxyribonucleotide triphosphates (dNTPs), buffer ($MgCl_2$) and Taq polymerase, 1 µL 8F primer, 1 µL 534R primer, 9.5 µL double de-ionized water and 1 µL extracted bacterial DNA in a total volume of 25 µL.

The PCR amplification protocol used with the 8F and 534R primers was: initial denaturation at 95 °C for 3 min, melting at 95 °C for 30 s, annealing at 50 °C for 30 s, elongation at 72 °C for 35 s; a total of 34 cycles, followed by a final extension step at 72 °C for 5 min and hold the PCR products at 4 °C overnight. The PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide, ETBr (Origin: Promega, USA) and visualized with an Alpha imager (Model: mini, Origin: Protein Simple, USA) for the presence of minimum 250 bp PCR products. The amplified 16S rDNA gene PCR products were purified using Wizard SV Gel and PCR purification kit (A9281, Promega, USA) according to the manufacturer instructions. Nucleotide sequences were determined by the dideoxynucleotide method. An ABI-Prism big dye terminator cycle sequencing kit was used in combination with an ABI-prism 877 integrated thermal cycler and the PCR products were purified by a standard protocol. The sequences were analyzed by an ABI-Prism 3700 genetic analyzer (Perkin Elmer Applied Biosystems, Warrington, UK).

The chromatogram sequencing files were edited using Chromas 2.5.1 software. Multiple sequence alignment of all the sequences obtained and the sequences of the present study were carried out using the Bio-Edit software. The identity of the 16S rDNA gene sequences was checked with the 16S rDNA gene sequences of other organisms that had already been submitted to Gene Bank database using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) algorithm. The phylogenetic trees were created based on the sequences of 16S rDNA genes of the bacterial isolates in the present study and those sequences downloaded after BLAST analysis by maximum likelihood method using MEGA 6 software (Tamura et al. 2013). An additional single tree with all the sequences together in comparison to multiple external groups have also been constructed using the same procedure. The 16S rDNA sequences of the bacterial isolates were submitted to the National Centre for Biotechnology and Information (NCBI) gene bank database with the help of "Bankit" to get accession numbers.

Effects of temperature on growth and Cr(VI) reduction of the bacterial isolates

The effects of temperature on growth and Cr(VI) reduction were studied in M9 minimal salts medium. In order to determine the effects of temperature, 35 mL of sterilized M9 minimal salts medium was added in six sets; each sets consisted of seven centrifuge tubes from which five centrifuge tubes contained 10 mg/L of Cr(VI) each [35 mL medium + 4 mL sterilized stock solution of Cr(VI)]. Two centrifuge tubes contained only medium (35 mL). The six sets of centrifuge tubes were then inoculated aseptically with 1 mL of enriched bacterial cultures (OD, 1.6) of each bacterial isolates. From each sets, one centrifuge tube containing only medium did not inoculate with bacterial cultures which served as control. The centrifuge tubes were then vortexed and each sets of centrifuge tubes including controls were then incubated at 25, 30, 35, 40, 45, and 50 °C in six shaking incubator with a shaking of 180 rpm for 18 h. The growth was measured after 18 h by taking absorbance at 600 nm with a spectrophotometer. The culture was centrifuged and the supernatant was used to determine the residual Cr(VI) concentration by 1,5-diphneylcarbazide method.

Effects of pH on growth and Cr(VI) reduction of the bacterial isolates

To determine the effects of pH on growth and Cr(VI) reduction by the bacterial isolates, centrifuge tubes having 35 mL of sterilized M9 minimal salts medium were prepared in five sets, each sets consisted of eight centrifuge tubes, from which six centrifuge tubes contained 10 mg/L of Cr(VI) [35 mL medium + 4 mL sterilized stock solution of Cr(VI)]. The remaining two centrifuge tubes of each sets contained only medium which served as controls. The centrifuge tubes containing medium and Cr(VI) (10 mg/L) were then adjusted to pH 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 with predetermined amounts of sterilized 1 N HCl and 1 N NaOH, then inoculated with 1 mL of enriched cultures of each bacterial isolates separately, except one centrifuge tubes of each sets which served as control for the determination of growth of the bacterial isolates. The five sets of centrifuge tubes including controls were then incubated at 37 °C and 180 rpm for 18 h. Growth was measured after 18 h by taking absorbance at 600 nm with a spectrophotometer. The culture was centrifuged and the supernatant was used to determine the residual Cr(VI) concentration by 1,5 diphenylcarbazide method as described above.

Tolerance of the bacterial isolates to other heavy metals (Cu^{2+}, Zn^{2+}, Ni^{+}, Pb^{2+}, Cd^{2+} and Hg^{2+})

In order to investigate the other heavy metals tolerance of the bacterial isolates, the procedures described by Zahoor and Rehman (2009) was followed where M9 minimal salts was used as medium. For determining the metals tolerance of the bacterial isolates, 2000 mg/L stock solutions of different metallic salts ($CuCl_2$, $NiCl_2$, $ZnCl_2$, $Pb(NO_3)_2$, $CdSO_4$ and $HgCl_2$) was prepared. The metal resistance was assayed by increasing the concentration of individual metals gradually with 25 mg/L in M9 minimal salts medium. Culture centrifuge tubes contained 100 mL of medium and different metallic ions were inoculated aseptically with 20 µL of enriched bacterial cultures. The centrifuge tubes were then incubated at 37 °C and 180 rpm in a shaking incubator for 24 h. After 24 h the growth of the bacterial isolates was monitored by using a spectrophotometer at 600 nm.

Statistical analysis

Each set of experiments were carried out in triplicate. Experiments were repeated separately to ensure reproducibility. All the results are expressed by mean ± SD (standard deviation) values. All statistical analysis was performed by Graph Pad Prism version 7 software.

Results

Isolation of Cr(VI) resistant bacteria

The total number of Cr(VI) resistant bacteria in the tannery effluent and solid waste samples are presented in the Tables 1 and 2, respectively. The average number of Cr(VI) resistant bacteria in the effluent samples were 1.0×10^2, 1.756×10^2 and 1.89×10^2 CFU/mL at 24, 48 and 72 h, respectively. The average number of Cr(VI) resistant bacteria in the tannery solid wastes samples were 2.515×10^3, 2.915×10^3 and 3.469×10^3 CFU/g at 24, 48 and 72 h of incubation, respectively. Twenty-five (ten from effluents and fifteen from solid wastes) morphologically distinct bacterial isolates were purified and preserved for further investigation.

Identification of Cr(VI) resistant bacteria

The Cr(VI)-resistant bacterial isolates were identified by both morphological and molecular techniques based on 16S rDNA gene sequence analyses. BLASTN analysis of the bacterial isolates MKPF2, MKPF5, MKPF7, MKPF8 and MKPF12 showed close association with *Kosakonia cowanii* 0122 (KP236256.1), *Klebsiella pneumoniae* KP-1/yak-2014 (KP866814.1), *Acinetobacter gerneri* MTCC (AB860302.1), *Klebsiella variicola* JM16 (KC853296.1) and *Serratia marcescens* A-a (KJ787109.1), respectively with 99% identity (Table 3). The partial 16S rDNA gene sequences of the bacterial isolates were deposited to the GeneBanK of NCBI (National Centre for Biotechnology and Information)

Table 1 Enumeration of Cr(VI) resistant bacteria in the tannery effluents

Sample ID	Number of bacteria at 24 h (CFU/mL)	Number of bacteria at 48 h (CFU/mL)	Number of bacteria at 72 h (CFU/mL)
H. T. E-1	1.1×10^2	2.0×10^2	2.0×10^2
H. T. E-2	1.0×10^2	2.3×10^2	2.32×10^2
H. T. E-3	1.5×10^2	2.6×10^2	2.70×10^2
H. T. E-4	0.5×10^2	1.2×10^2	1.3×10^2
H. T. E-5	1.3×10^2	2.4×10^2	2.45×10^2
H. T. E-6	0.3×10^2	1.2×10^2	1.5×10^2
H. T. E-7	1.3×10^2	1.8×10^2	2.0×10^2
H. T. E-8	0.10×10^2	0.26×10^2	0.51×10^2
H. T. E-9	1.4×10^2	1.6×10^2	1.65×10^2
H. T. E-10	1.5×10^2	2.2×10^2	2.5×10^2
Max	1.5×10^2	2.6×10^2	2.70×10^2
Min	0.10×10^2	0.26×10^2	0.51×10^2
Mean	1.0×10^2	1.756×10^2	1.89×10^2
SD	0.498×10^2	0.675×10^2	0.633×10^2

Max maximum, *Min* minimum, *SD* standard deviation, *CFU* colony forming unit

Table 2 Enumeration of Cr(VI) resistant bacteria in the tannery solid wastes

Sample ID	Number of bacteria at 24 h (CFU/g)	Number of bacteria at 48 h (CFU/g)	Number of bacteria at 72 h (CFU/g)
H. T. S-1	2.50×10^3	3.10×10^3	3.40×10^3
H. T. S-2	2.70×10^3	3.30×10^3	3.56×10^3
H. T. S-3	3.10×10^3	3.20×10^3	3.45×10^3
H. T. S-4	2.80×10^3	3.50×10^3	3.75×10^3
H. T. S-5	2.20×10^3	3.25×10^3	3.60×10^3
H. T. S-6	2.30×10^3	2.90×10^3	3.21×10^3
H. T. S-7	1.80×10^3	2.70×10^3	2.90×10^3
H. T. S-8	2.45×10^3	3.26×10^3	3.52×10^3
H. T. S-9	2.71×10^3	3.50×10^3	3.80×10^3
H. T. S-10	2.61×10^3	3.40×10^3	3.50×10^3
Max	3.10×10^3	3.50×10^3	3.80×10^3
Min	1.80×10^3	2.70×10^3	2.90×10^3
Mean	2.515×10^3	2.915×10^3	3.469×10^3
SD	3.407×10^2	8.963×10^2	2.476×10^2

Max maximum, *Min* minimum, *SD* standard deviation, *CFU* colony forming unit

under Accession Numbers of KX299066.1, KX299069.1, KX299068.1, KX299070.1 and KX299067.1, respectively. The phylogenetic trees (Fig. 1) showed that the bacterial isolates MKPF2, MKPF5, MKPF7, MKPF8 and MKPF12 strongly clustered with *K. cowanii, K. pneumoniae, A. gerneri, K. variicola and S. marcescens*, respectively. An additional single tree with all the sequences together in the present study comparing with multiple external groups (all are Cr(VI) reducing bacteria) has also been constructed and illustrated in Fig. 2, which also indicates that all the isolated bacteria have significant association to other Cr(VI) reducing bacterial strains. Subsequently the bacterial strains were identified as *K. cowanii* MKPF2, *K. pneumonia* MKPF5, *A. gerneri* MKPF7, *K. variicola* MKPF8 and *S. marcescens* MKPF12, respectively.

Maximum tolerance concentration (MTC) of Cr(VI) as $K_2Cr_2O_7$ of the bacterial isolates

In the present study, out of 25 bacterial isolates from tannery effluents and solid wastes only 5 bacterial isolates showed higher resistant to Cr(VI) as $K_2Cr_2O_7$ up to 2000 mg/L. Others bacterial isolates did not show Cr(VI) resistant at a concentration above 500 mg/L. So, only five bacterial isolates were selected for further investigations based on their MTC of Cr(VI) as $K_2Cr_2O_7$. The MTC of Cr(VI) as $K_2Cr_2O_7$ of the bacterial isolates is presented in Fig. 3. The bacterial isolates *K. cowanii* MKPF2, *K. pneumonia* MKPF5, *A. gerneri* MKPF7, *K. variicola* MKPF8 and *S. marcescens* MKPF12 were able to tolerate 2000, 1800, 1800, 1400 and 2000 mg/L of $K_2Cr_2O_7$ as Cr(VI), respectively in M9 minimal salts medium.

Reduction of Cr(VI) by the bacterial isolates

Chromium(VI) reducing capability of the bacterial isolates was determined by adding 10 and 20 mg/L of Cr(VI) as two initial concentrations and 2% inoculum in M9 minimal salts medium. Cr(VI) reduction potentialities with correspondence growth of the bacterial isolates are shown in Fig. 4. Both initial concentrations of Cr(VI) were completely reduced at 24 and 30 h of incubation by the bacterial isolates *K. cowanii* MKPF2, *K. variicola* MKPF8 and *S. marcescens* MKPF12 whereas *A. gerneri* MKPF7 and *K. pneumonia*

Table 3 Molecular identification of Cr(VI)-resistant bacteria by 16S rDNA gene sequence analysis

Isolates	Organisms	Accession no.	Max. score	Total score	E. value	Identity (%)
MKPF2	*Kosakonia cowanii*	KX299066.1	874	874	0	99
MKPF5	*Klebsiella pneumoniae*	KX299069.1	885	885	0	99
MKPF7	*Acinetobacter gerneri*	KX299068.1	872	872	0	99
MKPF8	*Klebsiella variicola*	KX299070.1	891	891	0	99
MKPF12	*Serratia marcescens*	KX299067.1	889	889	0	99

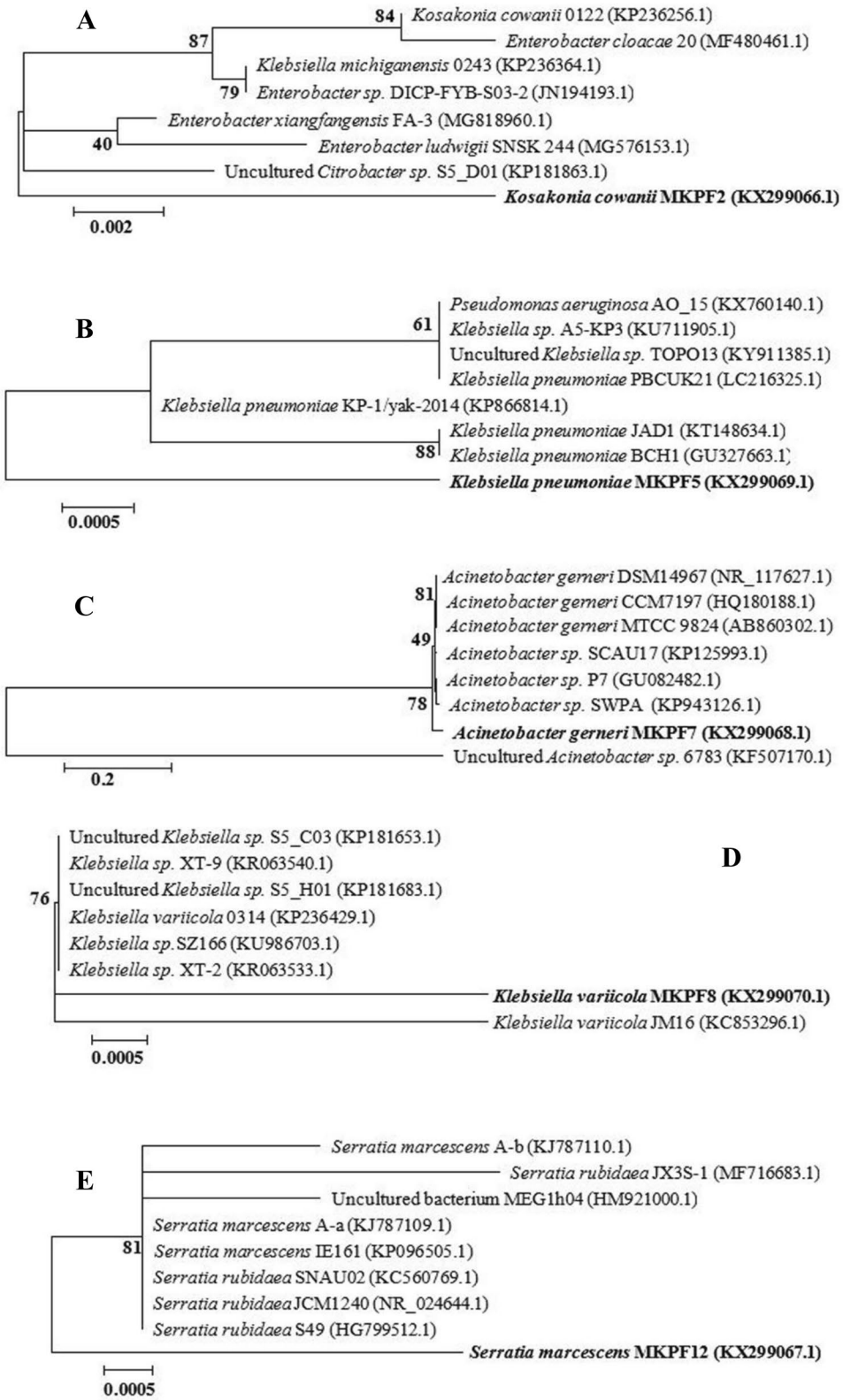

Fig. 1 The phylogenetic trees of the bacterial isolates; **a** *K. cowanii* MKPF2, **b** *K. pneumoniae* MKPF5, **c** *A. gerneri* MKPF7, **d** *K. variicola* MKPF8 and and **e** *S. marcescens* MKPF12 resulting from the comparison of 16s rDNA gene sequences of the closest phylogenetic neighbors obtained by BLAST (n) analysis using maximum likelihood method based on the Jukes–Cantor model of MEGA 6 package version. The trees with the highest log likelihood are shown here. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved eight nucleotide sequences. All positions containing gaps and missing data were eliminated. The values at nod represent the percentage of 1000 bootstrap interactions. Scale bars are the base substitution per position. GeneBank accession numbers are given in brackets. Bolded strains are the isolated Cr(VI) resistant bacteria in the present study

MKPF5 reduced the same concentrations at 18 and 24 h, 30 and 36 h, respectively from the culture medium. The concentration of total chromium in the culture supernatants of ultimate samples of all the bacterial isolates is presented in Table 4. In the culture supernatants, supplemented Cr(VI) of the culture medium (10 and 20 mg/L) was not noticed

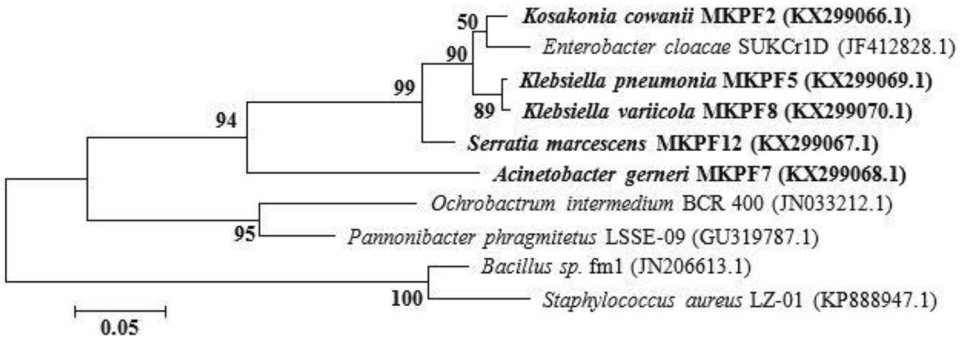

Fig. 2 The phylogenetic tree of the bacterial isolates; **a** *K. cowanii* MKPF2, **b** *K. pneumonia* MKPF5, **c** *A. gerneri* MKPF7, **d** *K. variicola* MKPF8 and and **e** *S. marcescens* MKPF12 resulting from comparison of 16s rDNA gene sequences of the multiple external groups [Cr(VI) reducing bacteria] by BLAST (n) analysis using maximum likelihood method based on the Jukes–Cantor model of MEGA 6 package version. The tree with the highest log likelihood is shown here. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved ten nucleotide sequences. All positions containing gaps and missing data were eliminated. The values at nod represent the percentage of 1000 bootstrap interactions. Scale bars are the base substitution per position. GeneBank accession numbers are given in brackets. Bolded strains are the isolated Cr(VI) resistant bacteria in the present study

at all. Almost all the reduced Cr [Cr(III)] was traced in the culture supernatants.

Effects of temperature on growth and Cr(VI) reduction by the bacterial isolates

Cr(VI) reduction (10 mg/L) by the bacterial isolates in the present study was evaluated in six different temperatures, namely 25, 30, 35, 40, 45 and 50 °C in M9 minimal salts medium at 180 rpm for 18 h of incubation. The effects of temperature on growth and Cr(VI) reduction by the bacterial isolates are illustrated in Fig. 5. All the bacterial isolates grew and reduced Cr(VI) at a wide range of temperatures ranging from 25 to 45 °C. The growth and Cr(VI) reduction by all the bacterial isolates increased with increasing temperatures up to 40 °C and decrease above 40 °C. The optimum temperatures for growth and Cr(VI) reduction by the bacterial isolates were 35 and 40 °C, respectively except *A. gerneri* MKPF7 which grew and reduced Cr(VI) optimally at 40 °C.

Effects of pH on growth and Cr(VI) reduction by the bacterial isolates

To optimize the pH on growth and Cr(VI) reduction by the bacterial isolates, chromium reduction experiments were performed at different initial pH values (5.0, 6.0, 7.0, 8.0, 9.0 and 10) which are presented in Fig. 6. All the bacterial isolates reduced Cr(VI) in a wide range of pH (5–10). However, no significant difference was observed in the presence or absence of Cr(VI) on the growth of all the bacterial isolates at different pH values.The optimum pH for growth and Cr(VI) reduction by *K. cowanii* MKPF2, *A. gerneri* MKPF7 and *S. marcescens* MKPF12 was 7.0 whereas the optimum pH for growth and Cr(VI) reduction by *K. pneumonia* MKPF5 and *K. variicola* MKPF8 were 7.0, 8.0 and 6.0, 7.0, respectively.

Tolerance of other heavy metals by the bacterial isolates

The resistance to heavy metals other than chromium by the bacterial isolates is presented in Fig. 7. All the bacterial isolates showed maximum tolerance against Ni^{2+} and Zn^{2+} whereas minimum tolerance was observed against Hg^{2+} and Cd^{2+}. The order of resistance against different metallic salts by the bacterial isolates *K. cowanii* MKPF2, *K. pneumonia* MKPF5, *A. gerneri* MKPF7, *K. variicola* MKPF8 and *S. marcescens* MKPF12 was $Ni^{2+} > Zn^{2+} > Cu^{2+} > Pb^{2+} > Cd^{2+} > Hg^{2+}$, $Zn^{2+} > Ni^{2+} > Pb^{2+} > Cu^{2+} \gg Cd^{2+} > Hg^{2+}$, $Zn^{2+} > Ni^{2+} > Cu^{2+} > Cd^{2+} > Pb^{2+} > Hg^{2+}$, $Ni^{2+} > Cu^{2+} > Zn^{2+} > Pb^{2+} > Cd^{2+} > Hg^{2+}$ and $Ni^{2+} > Zn^{2+} > Pb^{2+} > Hg^{2+} > Cu^{2+} > Cd^{2+}$, respectively. Data indicated that the isolated bacteria have a great potential in heavy metals tolerance.

Discussion

The total number of Cr(VI) resistant bacteria in tannery effluents of the present study is comparatively lower than that of the study of Shakoori et al. (2000) which may be due to the use of higher concentration of $K_2Cr_2O_7$ (100 mg/L) as Cr(VI) in the culture medium, and the use of less nutrient enriched medium (M9 minimal salts medium)

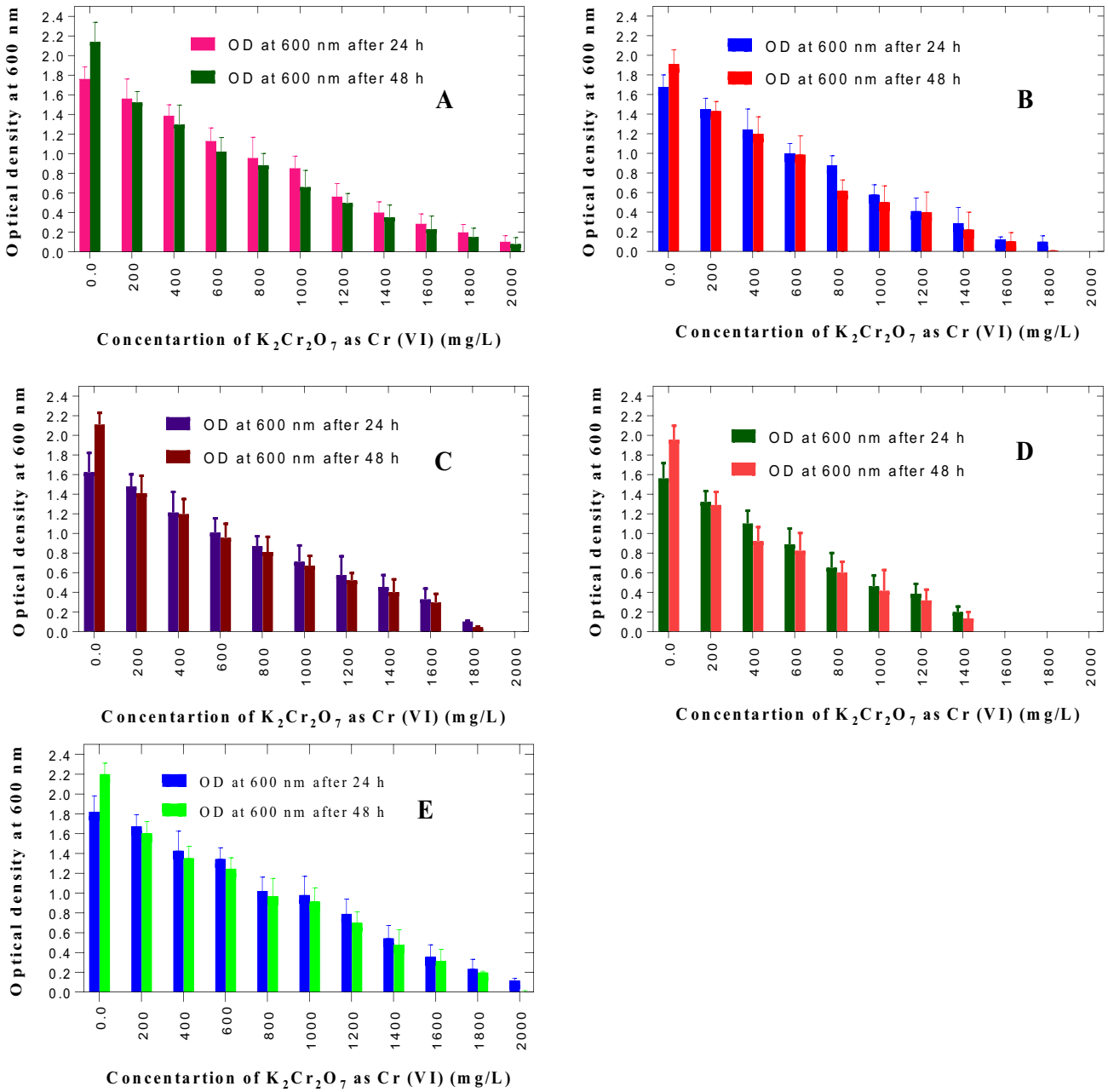

Fig. 3 The MTC of Cr(VI) as $K_2Cr_2O_7$ of the bacterial isolates; **a** *K. cowanii* MKPF2, **b** *K. pneumonia* MKPF5, **c** *A. gerneri* MKPF7, **d** *K. varicola* MKPF8 and **e** *S. marcescens* MKPF12. Values are mean numbers ± standard deviations generated from three independent experiments

as culture medium. Shakoori et al. (2000) isolated Cr(VI) resistant bacteria from tannery effluents by supplementing 1 mg/L Cr(VI) as $K_2Cr_2O_7$ in the Luria Bertani agar medium and counted the total number of bacteria ranged from 48×10^6 to 128×10^6 CFU/mL. The average number of Cr(VI) resistant bacteria in the tannery solid wastes were 2.515×10^3, 2.915×10^3 and 3.469×10^3 CFU/g after 24, 48 and 72 h of incubation, respectively. The average number of bacteria both in the tannery effluents and solid wastes were higher at 72 h when compared to 24 and 48 h which may be due to the slow growing nature of the Cr(VI) resistant bacteria. Between solid wastes and effluents, the number of Cr(VI) resistant bacteria was comparatively greater in solid wastes samples than in effluents samples. This greater number of Cr(VI) resistant bacteria in the solid waste samples may be due to the continuous deposition of Cr from Cr enriched tannery's leather-cut wastes in the solid wastes that provides a natural environment for enrichment of chromium resistant bacteria. On the other hand, the effluent samples were collected from drain

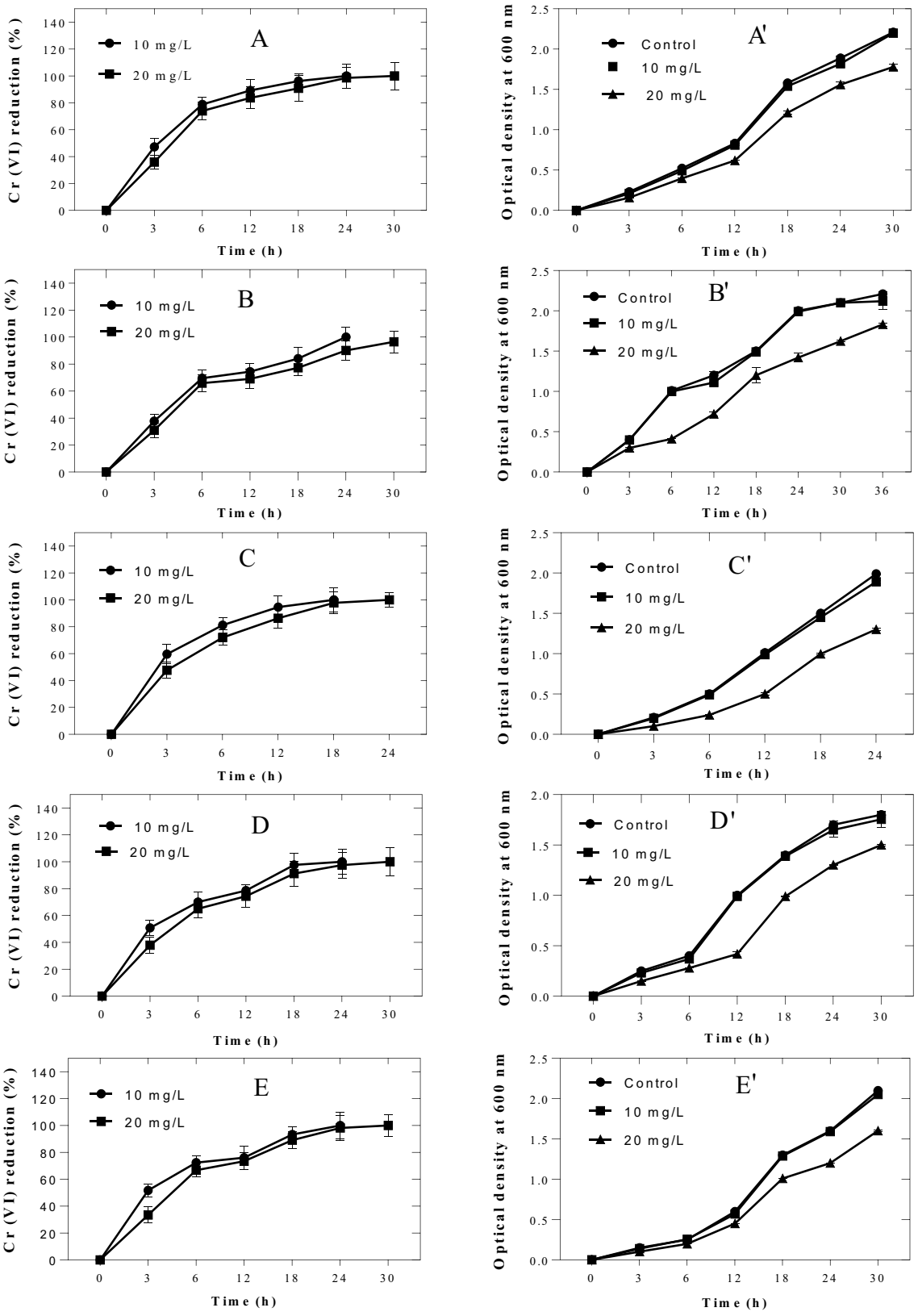

Fig. 4 Percentages (%) of Cr(VI) reduction and growth of the bacterial isolates; **a** *K. cowanii* MKPF2, **b** *K. pneumonia* MKPF5, **c** *A. gerneri* MKPF7, **d** *K. variicola* MKPF8 and **e** *S. marcescens* MKPF12. Values are mean numbers ± standard deviations generated from three independent experiments

Table 4 Determination of total chromium in the culture supernatants by atomic absorption spectrophotometer (AAS)

Bacterial isolates	Concentration of total chromium (mg/L) in the culture supernatants	
	10 mg/L	20 mg/L
Kosakonia cowanii MKPF2	9.61 ± 0.34	19.55 ± 0.42
Klebsiella pneumoniae MKPF5	9.66 ± 0.31	19. 63 ± 0.61
Acinetobacter gerneri MKPF7	9.85 ± 0.32	19.81 ± 0.36
Klebsiella variicola MKPF8	9.27 ± 0.37	19.55 ± 0.45
Serratia marcescens MKPF12	9.78 ± 0.33	19.55 ± 0.51

water and contained excess amount of salts and other toxic chemicals which may not support suitable conditions and enough time to adapt and colonize the bacteria themselves with such environment.

BLASTN analysis of the bacterial isolates MKPF2, MKPF5, MKPF7, MKPF8 and MKPF12 showed close association with *K. cowanii* 0122 (KP236256.1), *K. pneumonia* KP-1/yak-2014 (KP866814.1), *A. gerneri* MTCC (AB860302.1), *K. variicola* JM16 (KC853296.1) and *S. marcescens* A-a (KJ787109.1), respectively with 99% identity and the bacterial strains were identified as *K. cowanii* MKPF2, *K. pneumonia* MKPF5, *A. gerneri* MKPF7, *K. varicola* MKPF8 and *S. marcescens* MKPF12, respectively.

Chromium tolerant bacteria which are able to detoxify Cr(VI) have been recorded worldwide from chromium contaminated sites (Srinath et al. 2001).Several authors recorded *Acinetobacter* sp. as Cr(VI) resistant genus which can also efficiently detoxify Cr(VI) under laboratory conditions (Srivastava and Thakur 2007; Essahale et al. 2012; Bhattacharya and Gupta 2013; Hora and Shetty 2014).Gonzalez et al. (2014) isolated a native strain *Serratia* sp. C8 from chromium contaminated environments which was able to reduce Cr(VI) at varying concentrations. Mondaca et al. (2002) and Campos et al. (2005) also noticed Cr(V)-resistant and reduction potentials of *S. marcescens*.

The contamination of heavy metals result in alteration of microbial population which ultimately facilitates few bacterial species to resist high concentration of metal incorporation (Stepanauskas et al. 2005; Ansari et al. 2008). Chromium pollution causes a discriminatory burden on microbial flora of tannery effluents and soils (Viti et al. 2003). Although, there is no correlation between Cr(VI)-resistant and reduction by the bacterial isolates (Ohtake et al. 1990). The growth of all the bacterial isolates decreased with increasing concentration of $K_2Cr_2O_7$ that may be due to the antibacterial activity of $K_2Cr_2O_7$ as reported by Degiam et al. (2011) and Ramesh et al. (2012). Chromium(VI)-resistant ability of bacteria has been investigated worldwide. In the previous research, two Cr(VI) reducing bacteria were isolated from tannery effluents that can tolerate up to 2000 mg/L of $K_2Cr_2O_7$ as Cr(VI) (Ilias et al. 2011).

Some chromium resistant bacteria which can resist or reduce Cr(VI) at concentrations of 1500–2500 mg/L $K_2Cr_2O_7$ as Cr(VI) had been reported by Camargo et al. (2003). However, the real tolerance of bacteria to environmental pollutants must be determined in liquid media after their isolation from solid agar media (Megharaj et al. 2003). In the present investigation, M9 minimal salts medium has been used to determine MTC of Cr(VI) of the bacterial isolates which reduced the problem of "toxicity musking" of nutrient enriched media to the isolated Cr(VI)-resistant bacteria. Usually, the determination of Cr(VI) toxicity and its reduction by bacteria had been investigated in highly nutrient-rich media containing east extract and tryptone (Basu et al. 1997; Shakoori et al. 2000). In such media, the actual toxicity of Cr(VI) could be concealed or underestimated due to the complexation of Cr(VI) with organic compounds present in such media.

In the previous study, it had been reported that *Acinetobacter* sp. B9 was capable of reducing 67% of initial Cr(VI) within 24 h of incubation (Bhattacharya and Gupta 2013). Ilias et al. (2011) isolated two Cr(VI) resistant bacteria *Staphylococcus aureus* and *Pediococcus pentosaceus* which were able to reduce 20 mg/L of Cr(VI) completely from the culture medium at 6 and 24 h, respectively. It has been reported that *Bacillus* sp. ES29 reduced 90% of 2 mg/L of Cr(VI) from medium in < 6 h (Camargo et al. 2003). All the bacterial isolates in the present study showed almost similar growth pattern during reduction of Cr(VI). No significance growth difference was found between control and 10 mg/L Cr(VI) incorporation but growth was decreased substantially in 20 mg/L of Cr(VI).

The reduction of Cr(VI) in the present study is growth concomitant of the bacterial isolates and time dependent, the total time required for complete reduction of Cr(VI) increased with increasing concentration of Cr(VI) (Megharaj et al. 2003; Ilias et al. 2011). The comparison of reported Cr(VI) reducing bacteria with the ones in the present investigation in terms of their Cr(VI) resistant levels, reduction capability and optimum temperatures and pH for growth and Cr(VI) reduction has been illustrated in the Table 5. In the culture supernatants, supplemented Cr(VI) of the culture medium (10 and 20 mg/L) was not noticed at all. Almost all the reduced Cr [Cr(III)] was traced in the culture supernatants. Thus, it can be concluded that Cr(VI) was completely reduced by all the bacterial isolates in the extracellular environment as very trace quantities of total Cr were determined in the culture supernatants of all the bacterial isolates which may bind within the cell pellets of the bacterial isolates (Ilias et al. 2011).

Temperature is considered as a critical factor influencing bacterial Cr(VI) reduction process (Essahale et al.

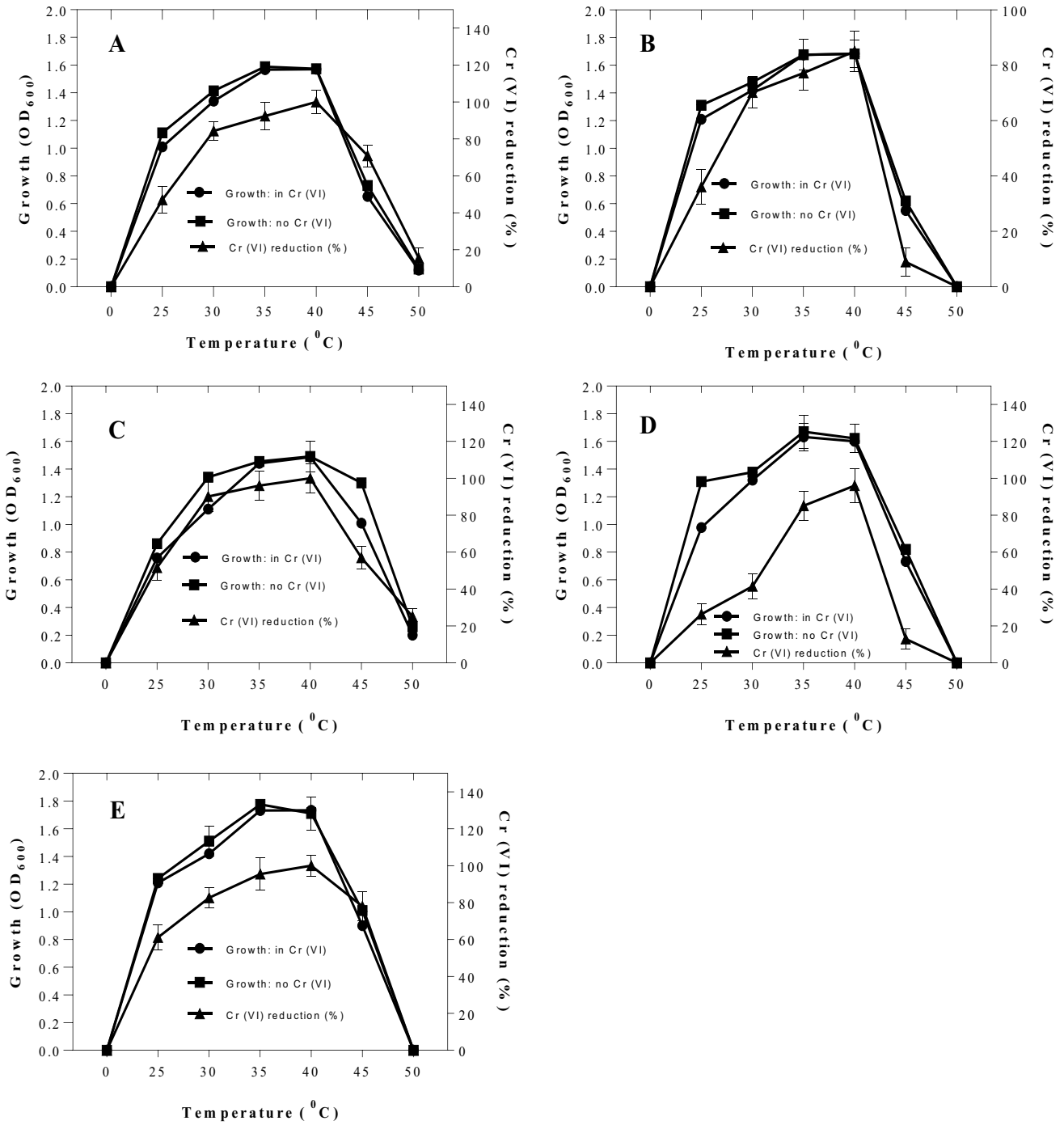

Fig. 5 Effects of temperature on growth and Cr(VI) reduction by the bacterial isolates; **a** *K. cowanii* MKPF2, **b** *K. pneumonia* MKPF5, **c** *A. gerneri* MKPF7, **d** *K. variicola* MKPF8 and **e** *S. marcescens* MKPF12. Values are mean numbers ± standard deviations generated from three independent experiments

2012; Shi et al. 2012; Ge et al. 2013). In the present study, the reduction of Cr(VI) was satisfactory (25–100%) from 25 to 45 °C by all the bacterial isolates. The optimum temperatures for growth and Cr(VI) reduction by the bacterial isolates *K. cowanii* MKPF2, *K. pneumonia* MKPF5, *K. variicola* MKPF8 and *S. marcescens* MKPF12 were 35 and 40 °C, respectively whereas the optimum temperature for growth and Cr(VI) reduction by *A. gerneri* MKPF7 was 40 °C. The effects of temperature in the present study is congruent with the results from previous studies such as: Ilias et al. (2011) isolated two Cr(VI) reducing bacteria IFR-2 and IFR-3 from tannery effluents and the optimum

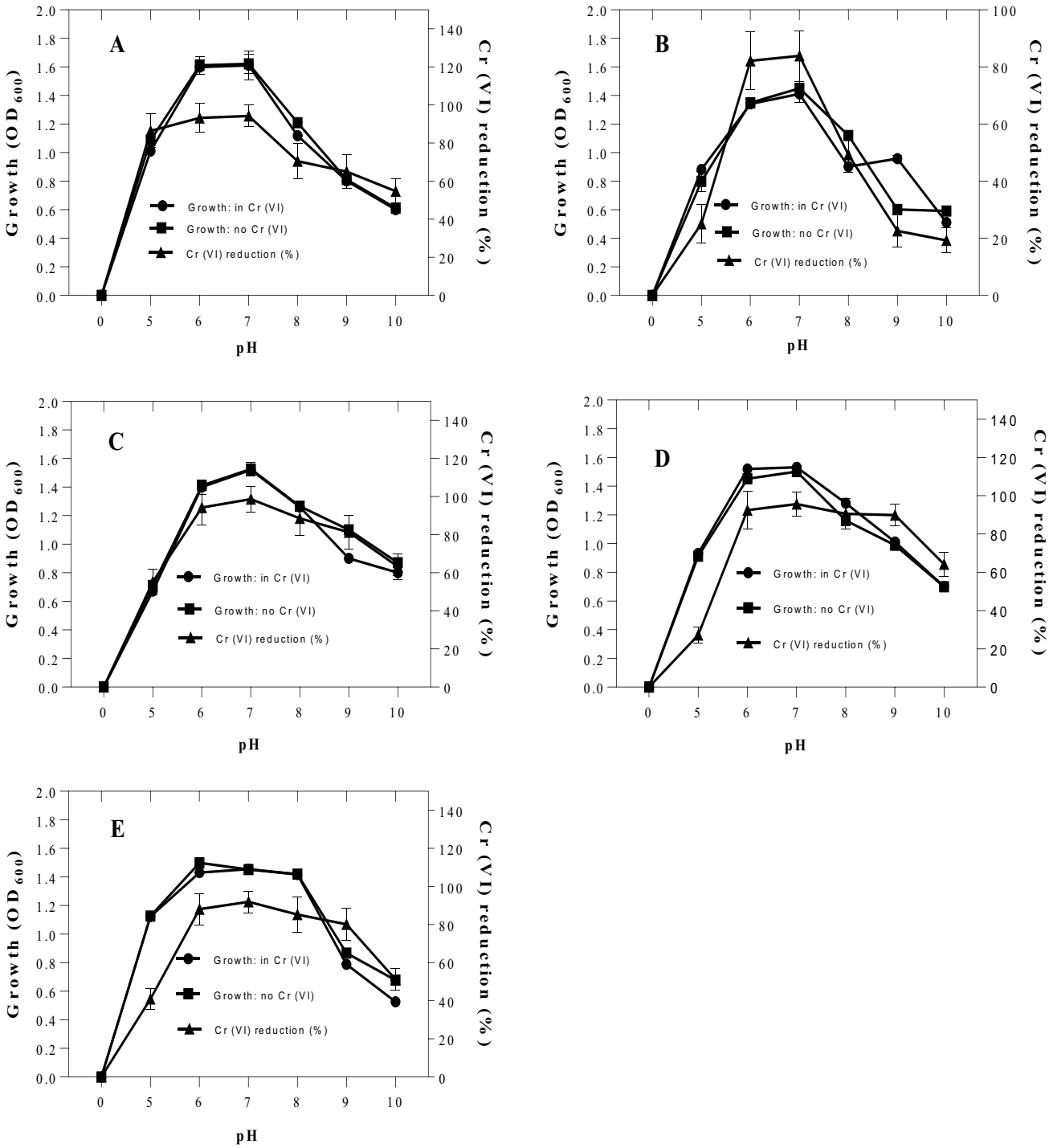

Fig. 6 Effects of pH on growth and Cr(VI) reduction by the bacteria isolates; **a** *K. cowanii* MKPF2, **b** *K. pneumonia* MKPF5, **c** *A. gerneri* MKPF7, **d** *K. variicola* MKPF8 and **e** *S. marcescens* MKPF12. Values are mean numbers ± standard deviations generated from three independent experiments

temperatures for growth and Cr(VI) reduction for both of the isolates were 35–40 °C. Cheung and Gu (2007) reported optimum temperature for bacterial growth and Cr(VI) reduction ranged from 35 to 37 °C.

Thacker and Madamwar (2005) reported a maximum growth and reduction of Cr(VI) at 35 °C by the bacterial isolate DM1. Ibrahim et al. (2011) studied growth and Cr(VI) reduction by *Amphibacillus* sp. KSUCr3 at various

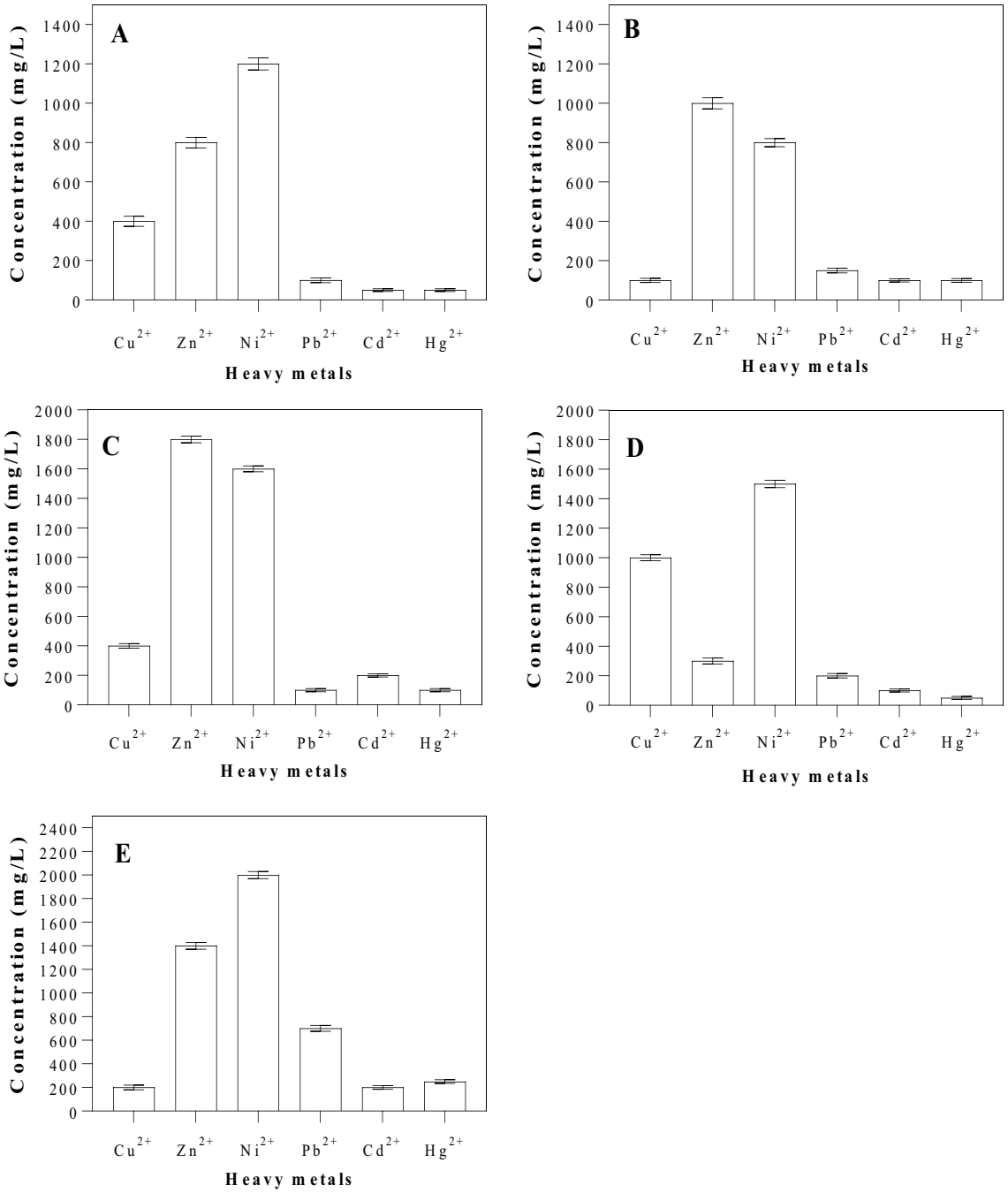

Fig. 7 Tolerance of other metallic ions (Ni^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+} and Hg^{2+}) by the bacterial isolates; **a** *K. cowanii* MKPF2, **b** *K. pneumonia* MKPF5, **c** *A. gerneri* MKPF7, **d** *K. variicola* MKPF8 and **e** *S. marcescens* MKPF12. Values are mean numbers ± standard deviations generated from three independent experiments

temperatures (25–45 °C). The growth and Cr(VI) reduction increased with increasing temperature up to 40 °C and decreased above 40 °C for all the bacterial isolates in the present study. Higher temperature (50 °C) ceased the growth and Cr(VI) reduction of all the bacterial isolates except *K. cowanii* MKPF2 and *A. gerneri* MKPF7. It was interesting that these bacterial isolates showed their Cr(VI) reduction capability at 50 °C temperature. *Pseudomonas ambigua* displayed Cr(VI) reduction activity over a wide range of temperatures from 40 to 70 °C (Suzuki et al. 1992). A chromate reductase was purified from thermophillic *Thermuss cotoductus* SA-01 which showed Cr(VI) reduction activity at 60 °C (Opperman et al. 2008).

Table 5 Comparison of reported Cr(VI) reducing bacteria with the ones in the present study in terms of Cr(VI) resistant levels, reduction capability and optimum temperatures and pH for growth and Cr(VI) reduction

Cr(VI) resistant bacteria	Cr(VI) resistant levels	Cr(VI) reduction capability	Optimum temperatures for growth and Cr(VI) reduction		Optimum pH for growth and Cr(VI) reduction		References
			Growth (°C)	Cr(VI) reduction (°C)	Growth	Cr(VI) reduction	
Kosakonia cowanii MKPF2	2000 mg/L $K_2Cr_2O_7$ as Cr(VI)	20 mg/L Cr(VI)	35	40	7.0	7.0	Present study
Klebsiella pneumoniae MKPF5	1800 mg/L $K_2Cr_2O_7$ as Cr(VI)	20 mg/L Cr(VI)	35	40	7.0	8.0	Present study
Acinetobacter gerneri MKPF7	1800 mg/L $K_2Cr_2O_7$ as Cr(VI)	20 mg/L Cr(VI)	40	40	7.0	7.0	Present study
Klebsiella variicola MKPF8	1400 mg/L $K_2Cr_2O_7$ as Cr(VI)	20 mg/L Cr(VI)	35	40	6.0	7.0	Present study
Serratia marcescens MKPF12	2000 mg/L $K_2Cr_2O_7$ as Cr(VI)	20 mg/L Cr(VI)	35	40	7.0	7.0	Present study
Staphylococcus aureus IFR-2	2000 mg/L $K_2Cr_2O_7$ as Cr(VI)	20 mg/L Cr(VI)	35–40		7.0	8.0	Ilias et al. (2011)
Pediococcus pentosaceus IFR-3	2000 mg/L $K_2Cr_2O_7$ as Cr(VI)	20 mg/L Cr(VI)	35–40		7.0	8.0	Ilias et al. (2011)
Arthrobacter sp.	100 mg/mL Cr(VI)	50 µg/mL Cr(VI)	–	–	–	–	Megharaj et al. (2003)
Bacillus sp.	100 mg/mL Cr(VI)	20 µg/mL Cr(VI)	–	–	–	–	Megharaj et al. (2003)
Bacillus sp. FM1	1000 mg/L Cr(VI)	100 mg/L Cr(VI)	–	37	–	8.0	Masood and Malik (2011)
Leucobacter sp. G161	1000 mg/L Cr(VI)	400 mg/L Cr(VI)	–	35	–	8.0	Ge et al. (2013)
Pannonibacter phragmitetus	–	2000 mg/L Cr(VI)	–	30	–	9.0	Shi et al. (2012)
Acinetobacter AB1	400 mg/L of Cr(VI)	50 mg/L Cr(VI)		30	–	10.0	Essahale et al. (2012)
Bacillus pumilis	–	200 µg/mL K_2Cr_2O7 as Cr(VI)	–	37	–	7.0	Rehman and Faisal (2015)
Cellulosimicrobium cellulans	–	400 µg/mL $K_2Cr_2O_7$ as Cr(VI)	–	37	–	7.0	Rehman and Faisal (2015)
Exiguobacterium	–	200 µg/mL $K_2Cr_2O_7$ as Cr(VI)	–	37	–	7.0	Rehman and Faisal (2015)
Ochrobactrum intermedium	–	100 mg/L Cr(VI)	–	30	–	7.0	Kavita and Keharia (2012)
Bacillus sp. JDM-2-1	4800 µg /mL	100 µg/mL	37	37	6.0	6.0	Zahoor and Rehman (2009)
Staphylococcus capitis	2800 µg/mL	100 µg/mL	37	37	7.0	7.0	Zahoor and Rehman (2009)

The bacterial Cr(VI) reduction is an enzymatic process. Fluctuations in pH values affect the ionization frequency of enzyme, the structures of proteins and subsequently the activity of enzyme (Camargo et al. 2003). The optimum pH for growth and Cr(VI) reduction for *K. cowanii* MKPF2 was 7.0. The growth and Cr(VI) reduction was decreased with increasing pH above 7.0 for *K. cowanii* MKPF2. Interestingly, significant Cr(VI) reduction was occurred at acidic pH 5.0 (86.40%) and alkaline pH 9.0 (65.058%), 10 (54.761%) by this bacterial isolate, although significant growth was not observed in these pH values. *Pannonibacter phragmitetus* LSSE-09 reduced Cr(VI) in a wide range of pH under aerobic condition with an optimum at 7.0 (Xu et al. 2012). Cr(VI) resistant bacteria control a cytoplasmic pH lower than the ambient pH under alkaline environments which may results alkaline pH homeostasis to lower the cytoplasmic pH in alkaline conditions (Padan et al. 2005). The stabilization of Cr(III) in acid medium preferring Cr(VI) reduction due to the positive value of the reduction potential standard of the Cr(VI)/Cr(III) redox couple (Nragu and Nieboer 1988), which may be the reason for significant Cr(VI) reduction at acidic pH 5.0 by *K. cowanii* MKPF2. The bacterial isolate *K. pneumonia* MKPF5 showed optimum growth and Cr(VI) reduction at pH 7.0 and 8.0, respectively. Chromate

reduction by *Klebsiella* sp. was reported by Wani and Omozele (2015), and the optimum pH for Cr(VI) reduction was within 6–7. Ilias et al. (2011), reported optimum pH for bacterial Cr(VI) reduction within the range of 7–8. Optimal reduction of Cr(VI) was directly related to the optimum pH (8.0) for the *Bacillus* isolates (Camargo et al. 2003).

The optimum pH for growth and Cr(VI) reduction of *A. gerneri* MKPF7 was 7.0. The pH values ranged from 6.0 to 8.0 for optimal reduction of Cr(VI) by *Acinetobacter* sp. (Bhattacharya and Gupta 2013). However, Panda and Sarkar (2012) informed pH 7.0 to be optimum for reduction of Cr(VI) in case of *Acinetobacter* sp. PCP3 and *Acinetobacter* sp. PD 12 S2, respectively. On the contrary, optimal pH for Cr(VI) reduction was 10.0 for *Acinetobacter* sp. AB 1 (Essahale et al. 2012). The effects of different initial pH of media on Cr(VI) reduction showed maximum Cr(VI) reduction by the bacterial isolate *K. variicola* MKPF8 in the pH range of 6.0–9.0. Growth and Cr(VI) reduction increased with increasing pH up to 9.0. However the optimum pH for growth and Cr(VI) reduction for this bacterial isolate were 6.0 and 7.0, respectively. McLean et al. (2000) stated a wide range of pH (6.0–9.0) for Cr(VI) reduction by *P. synxantha*. The bacterial isolate *S. marcescens* MKPF12 showed maximum chromate reduction at pH 6.0, 7.0 and 8.0 and the reduction percentages were 88.12, 92.01 and 85.321% respectively. However, the optimum pH for growth of *S. marcescens* MKPF12 was 7.0 (Fig. 5). At acidic pH 5.0, the bacterial isolate *S. marcescens* MKPF12 reduced 41.012% Cr(VI), whereas at alkaline pH 9.0 and 10.0 the reduction percentages were 63.121 and 51.233%, respectively. The optimum pH for Cr(VI) reduction and removal by *Serratia* sp. C8 from industrial effluents was within 6–7 (Gonzalez et al. 2014).

Cr(VI) containing waste water from industrial processes usually contains other heavy metals, therefore, the resistance of the bacterial isolates to different heavy metallic salts was also investigated in minimal salts medium. The resistance of Cr(VI) reducing bacterial isolates to different metallic ions has obvious advantages for their application in bioremediation (Masood and Malik 2011). All the bacterial isolates showed maximum tolerance against Ni^{2+} and Zn^{2+} whereas minimum tolerance was observed against Hg^{2+} and Cd^{2+} that may be due to the toxic and growth inhibitory effects of Hg^{2+} and Cd^{2+} to the bacterial isolates (Basu et al. 1997).

Conclusions

The average number of Cr(VI) resistant bacteria in the tannery effluents and solid wastes was 1.0×10^2 CFU/mL and 2.51×10^3 CFU/g after 24 h of incubation, respectively. Isolated bacteria have great potential of resistance against different heavy metals. All the bacterial isolates of present study detoxified Cr(VI) at a wide range of pH (5–10) and temperatures (25–45 °C). Thus, it can be concluded that Chromium(VI) detoxifying bacteria isolated from the natural environments can be used to reduce environmental as well as industrial pollutants Cr(VI) problems in Bangladesh, in a cost-effective and environmental friendly manner.

Compliance with ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human and animal participants The manuscript does not contain clinical studies or patient data.

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