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RESEARCH PAPER

Investigation of the role of chromium reductase for Cr (VI) reduction by *Pseudomonas species* isolated from Cr (VI) contaminated effluent

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Abstract This study observed the role of pH, chromium (VI) concentrations, temperatures and chromium reductases for of Cr (VI) reduction. Bacteria isolated from effluent were identified as *Pseudomonas sp.* by molecular analysis. Bacterial strain MAI4 showed significant reduction at pH 7 (84%), 100 μg Cr (VI)/ml (86%) and 35 °C (86%). Increase in time of incubation increased Cr (VI) reduction by *P. entomophila* MAI4 significantly and 120 h of incubation showed maximum reduction of Cr (VI). *P. entomophila* MAI4 also showed significant reduction of Cr (VI) (80%) in industrial waste water. Bacterial strain MAI4 reduced Cr (VI) into Cr (III) after 120 h which was detected as 70 ± 3 μg/ml in cell pellet and 30 ± 2 μg/ml in supernatant, respectively. Chromium reductase found in cell free extracts (CFE) reduced almost all Cr (VI) to Cr (III) compared to cell debris. Based on reduction under *in vitro* and *in vivo* conditions, *Pseudomonas sp.* MAI4 could be used as a bioremediator of Cr (VI) in contaminated effluents.

Introduction

Chromium (VI) is the most important heavy metal pollutant among all the heavy metals and is released from industrial operations which pollutes agricultural soil as well as water bodies (Karthik, Elangovan, et al., 2017; Ortegel, Staren, Faber, Warren, & Braun, 2002; Oves, Khan, & Qari, 2017; Sultan & Hasnain, 2007). Accumulation of Cr (VI) in agronomic lands can limit soil productivity by decreasing the population of various soil microbes (Karthik, Oves, Sathya, Ramkumar, & Arulselvi, 2017; Wani et al., 2017). Cr (VI) is the most toxic form compared to Cr (III). High solubility and rapid permeability of Cr (VI) makes it more toxic than Cr (III) which can damage proteins and nucleic acids (Mishra et al., 2012). Toxicity of Cr (VI) compared to Cr (III) can modify gene expression which may be the cause

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of carcinogens; trivalent is an essential element for the growth of animals (Ackerley, Barak, Lynch, Curtin, & Matin, 2006; Krishna & Philip, 2005; Wang et al., 2013). Due to its carcinogenicity and mutagenicity, it has been designated as priority pollutant or class A pollutant by united states environmental protection agency (USEPA, 1996). Microbial reduction of hexavalent chromium to trivalent chromium is an important, cheap remediation technology (Jeyasingh & Philip, 2005; Wang et al., 2013), necessary for glucose metabolism (Vincent, 2000), enzymatic activation (Karuppanapandian, Sinha, Kamarul, & Manoharan, 2009) and DNA and RNA stabilization (Karuppanapandian et al., 2009). Chromium (VI) remediation has been reported in both soil and water contaminated with metal by bacterium *Pannonibacter phragmitetus* BB (Wang et al., 2014; Wani, Omozele, Wasiu, & Jamiu, 2015). *P. phragmitetus* BB has also been reported for reduction of Cr (VI) in chromite ore (Wang et al., 2013). Remediation of Cr (VI) has been reported under laboratory conditions for *Pseudomonas* sp. (Wani & Ayoola, 2015), *Brevibacillus brevis* (Wani et al., 2016), *Microbacterium* (Pattanapipitpaisal, Brown, & Macaskie, 2001), *Ochrobactrum intermedium* (Faisal & Hasnain, 2005) and *Micrococcus* (Sultan & Hasnain, 2005).

It has been reported that *chrA* gene is not the only mechanism which can contribute to Cr (VI) tolerance as *chrA* gene possesses changing resistance to Cr (VI) (0.35–200 mM) (Monsieurs et al., 2011; Viti, Marchi, Decorosi, & Giovannetti, 2014). It has also been studied that *chrA* genes can protect the cell from Cr (VI) toxicity only to certain extent. Stimulation of Chr efflux pump extrudes sulfate, favors sulfur starvation and thus is not good for growth (Branco et al., 2008; Viti et al., 2014).

Cr (VI) reduction (direct or indirect) and is affected by pH, temperature, different dose rates of chromium, incubation and microbial species (Soni, Singh, Awasthi, Singh, & Kalra, 2012). Intracellular, extracellular or membrane bound reductases produced by bacteria in direct process remediate Cr (VI) to Cr (III) (Gu et al., 2015; Joutey, Sayel, Bahafid, & Ghachtouli, 2015) whereas reductants or oxidants under indirect mechanism (DeFilippi & Lupton, 1992).

A number of reductases such as aldehyde oxidase, cytochrome P450, DT-diaphorase (Patra, Malik, Beer, Megharaj, & Naidu, 2010), nitroreductase (Kwak, Lee, & Kim, 2003), iron reductase (Gonzalez, Ackerley, Lynch, & Matin, 2005), flavin reductases (Ackerley et al., 2004), thioredoxin oxidoreductase (Tucker, Barton, & Thomson, 1998), etc. can detoxify Cr (VI) to Cr (III). Precipitation, coagulation, ion exchange, cementation, electro-dialysis, electro-coagulation, reverse osmosis causes secondary pollution, are costly conventional remediation processes of Cr (VI) (Ahluwalia & Goyal, 2007; Dhal, Thatoi, Das, & Pandey, 2013). Remediation of the Cr (VI) by tolerant microbes, a safe and cheap method, is the best technique (Joutey, Bahafid, Sayel, Ananou, & El Ghachtouli, 2014), thus its use is beneficial for contaminated biosphere (Wani & Khan, 2010).

Considering toxicity of Cr (VI) on one hand and the role of microbes in chromium detoxification on the other hand, the present study has been designed with the following objectives: (1) assessment of Cr (VI) removal by bacterial cells grown at varying pH, chromium concentrations and temperatures under *in vitro* conditions, (2) determination of Cr (VI) reduction in the industrial waste water (3) to find the location of Cr (VI) reduction using various factors (such as permeability, cell debris, cell free extract and cell denaturants).

Material and methods

Collection of contaminated sample

In this study, effluents (three replicates) contaminated with high concentrations of various metals were collected from alloy manufacturing industry of Abeokuta Ogun State Nigeria. Cr (VI) in effluents was detected by 1,5-diphenyl carbazide method (Eaton, Clesceri, & Greenberg, 1992). Other metals such as Cd, Ni, Pb, Cu, Hg, Mn, Fe, Al, Ca and Zn were analyzed after digesting effluent in nitric acid and hydrochloric acid (3:1) (McGrath & Cunliffe, 1985). Effluent was also assayed for organic carbon, nitrogen, phosphorus, sulphate, potassium, etc.

Isolation of chromium (VI) tolerant bacteria

100 μg/ml Cr (VI) nutrient agar plates were used to isolate resistant microbes. To obtain resistant microbes, waste water collected from alloy manufacturing industry of Abeokuta (7.15°N latitude, 3.35°E longitude and 67 m above sea level) was diluted and spreaded on Cr (VI) (100 μg/ml) amended nutrient agar plates which were incubated at 28 ± 2 °C for 24 h. Pure bacterial cultures were preserved on nutrient agar plates till they were used.

Morphological and biochemical identification of bacteria

Bacteria were identified using both morphological and biochemical tests. The biochemical tests used for the identification of the bacteria were catalase, oxidase, triple sugar iron agar, nitrate, citrate, VP, MR, lipase, indole, starch, mannitol, gelatin and fermentation of glucose, lactose, sucrose and arabinose (Holt, Krieg, Sneath, Staley, & Willams, 1994).

16S rRNA identification of bacteria

Molecular identification was performed by 16S rRNA gene sequence which was done commercially by Macrogen Inc., Amsterdam, Netherlands making use of universal primers 785F (5′GGATTAGATACCCTGGTA3′) and 907R (5′CCGTCAATTCMTTTRAGTTT3′). Identification was performed using nBLAST at NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

In vitro assay of tolerance to chromium (VI)

Tolerance of strain to Cr (VI) ($K_2Cr_2O_7$, Sigma, CAS Number 778509) (0–1000 μg/ml) was performed on nutrient agar plates as well as nutrient broth (Holt et al., 1994) at 28 ± 2 °C. Maximum dose rate of Cr (VI) which supported

growth was considered as maximum resistance level (MRL). Each experiment was repeated three times.

Bacterial growth

Bacterial growth in nutrient broth was measured up to a period of 120 h at pH values between 5 and 9 when incubated on shaker incubator. Experiments were replicated three times.

Effect of pH and initial chromium (VI) concentration on chromium (VI) reduction

Effect of different pH values (5–9) on Cr (VI) reduction was performed in nutrient broth (NB) amended with 100 μg/ml of $K_2Cr_2O_7$ at 28 ± 2 °C for 120 h. In second experiment, Cr (VI) reduction by strain MAI4 was performed in nutrient broth, pH 7 amended with Cr (VI) (0–150 μg/ml) at 28 ± 2 °C for 120 h. Aliquots (2 ml) were centrifuged at 6000 rpm for 10 min at 10 °C and left over Cr (VI) was done by 1,5-diphenyl carbazide method (Eaton et al., 1992). Acidified (pH 1–2) samples were added with 1,5-diphenyl carbazide (50 μg/ml) to observe for remaining Cr (VI) concentration at 540 nm. Nutrient broth without Cr (VI) and nutrient broth with Cr (VI) but not inoculated with MAI4 served as biotic and abiotic controls respectively.

Effect of temperature on Cr (VI) reduction

Effect of temperatures (25, 35 and 45 °C) on Cr (VI) removal by strain MAI4 was conducted in 100 μg Cr/ml, pH 7 amended nutrient broth. Bacterial culture was centrifuged at 6000 rpm for 10 min at 10 °C and left over Cr (VI) was detected by 1,5-diphenyl carbazide method (Eaton et al., 1992) after 120 h. Un-inoculated nutrient broth without Cr (VI) and with Cr (VI) served as biotic and abiotic control respectively.

Time course Cr (VI) reduction

Effect of time of incubation on Cr (VI) reduction by strain MAI4 in 100 μg Cr/ml, pH 7 amended nutrient was at 28 ± 2 °C was observed up to a period of 120 h.

Cr (VI) reduction in industrial effluent

100 ml of sterilized industrial waste water was inoculated with *Pseudomonas* MA4 which was incubated at a temperature of 28 ± 2 °C up to a period of 120 h. Cr (VI) reduction in industrial waste water was observed by the method of 1,5-diphenyl carbazide method (Eaton et al., 1992). Cr (VI) reduction in industrial waste water without inoculation was also observed which served as control.

Cr (VI) and Cr (III) analysis

Cr (VI) analysis in supernatant was determined by 1,5-diphenyl carbazide method (Eaton et al., 1992) and total chromium was assayed by AAS after digesting the sample (supernatant and pellet) in 1:1 HCl. The concentration of Cr (III) was calculated by deducting the remained Cr (VI) from the total chromium in the supernatant.

Detection of Cr (VI) reduction by resting and permeabilized cells

Full bacterial growth in nutrient was obtained after 18 h at 30 ± 2°. 0.1 M potassium phosphate buffer (pH 7.0) was used to wash cell debris (pellets) and was resuspended in same buffer to obtain resting cells. To obtain permeabilized cells, 0.2% (v/v) Tween-80 (Sigma, CAS 900656) was added to the resting bacteria cultures. Final volume of 100 μg Cr (VI)/ml amended resting and permeabilized cells was made up to 10 ml. The tubes were vortexed for 2 min and incubated at 30 ± 2 °C for 6 h. Aliquots (1 ml) obtained were assayed for Cr (VI) removal at regular time intervals. Permeabilized cells were heat killed at 100 °C which served as control.

Cell-free extract and detection of the expression of chromium reductase activity

Cell-free extracts of strain MAI4 were prepared using the method of Desai, Jain, and Madamwar (2008a, 2008b). Centrifuged bacterial growth (6000 rpm for 10 min at 4 °C) was washed and transferred to 20 ml of 0.1 M potassium phosphate buffer (pH 7.0). Obtained cells were put on an ice bath and subsequently broken into pieces by an ultrasonic Probe (Rivotek) (15 pulses of 15-s at 120W) for 30 min. Broken cells were ultracentrifuged at 175,000 × *g* for 90 min at 4 °C, filtered (0.22 μm filters) to get cell free extracts. Cell debris was also resuspended in the same volume of phosphate buffer. Cell free extracts and cell debris were observed for Cr (VI) reductase activity. In the next experiment we observed the role of chromium reductase for Cr (VI) removal. In this experiment, 10 mM silver nitrate (Sigma, CAS No. 7761888) at 30 ± 2 °C, 0.5% sodium dodecyl sulfate (SDS) (Merck, CAS No. 151213) at 30 ± 2 °C was added to Cr (VI) amended (100 μg/ml) cytoplasmic extract, heated to100 °C for 10 min to denature it. Experiments were performed three times. Reductase enzyme in cytoplasmic extract was calculated as a unit of chromate reductase/mg protein. Protein was estimated by Lowry, Roseberough, Lewis, and Randall (1951) method using BSA as standard.

Statistical analysis

The results of this study were analyzed by two way analysis of variance (ANOVA) and the calculation of LSD was performed at 5% level of probability.

Results and discussion

Detection of Cr (VI) and other metals in industrial effluent

150 μg/ml of Cr (VI) was found in the waste water whereas other metals such as Cd, Cu, Ni, Pb, Hg, Mn, Fe, Al, Ca and Zn were having concentration of 22, 36, 67,105, 25, 15, 18, 21, 14 and 220 μg/ml respectively. Organic carbon,

nitrogen, phosphorus and potassium was found to be 0.35, 0.79, 15.3 and 11.2 μg/ml respectively. Reports of higher contamination of metals in the industrial waste water has also been found (Wani & Khan, 2010). Zahoor and Rehman (2009) reported Cr (VI) in the waste water to be in the range 0.70–1.84 μg/ml. Wang et al. (2014) also found high concentration of metals including Cr (VI) in the industrial waste water which was found to be 534 mg/l.

Morphological, biochemical and molecular identification of bacteria

Bacterial isolate MAI4 was Gram negative, rod shaped, mucoid, aerobic, motile, produced serrate margin and cream colored colonies on nutrient agar medium. Strain MAI4 showed positive reaction to catalase, oxidase, triple sugar iron agar, nitrate, citrate, VP, mannitol. MAI4 could utilize glucose but could not ferment lactose, sucrose, indole, MR, arabinose, starch and lipase. On the basis of morphological and biochemical properties shown above, strain MAI4 was identified as *Pseudomonas* species and 16S rRNA gene sequence confirmed that the strain MAI4 belongs to *Pseudomonas sp*. exhibiting 100% similarity with *Pseudomonas sp*. strain 160711-042 _013 (MH458856.1). 16S rRNA gene sequence of strain MAI4 was deposited in NCBI and was given the accession number as MH458856.

Bacterial assay for Cr (VI) tolerance

Tolerance to Cr (VI) was assessed on nutrient agar as well as in nutrient broth. MAI4 showed higher tolerance of 1000 μg/ml on nutrient gar plates and 450 μg/ml in nutrient broth. Significant difference in tolerance of strain MAI4 on nutrient agar and broth is due to the formation of complex between nutrient agar and Cr (VI) which will not expose bacteria to maximum concentrations of metal while as in broth bacteria are directly exposed to the metal. Maximum tolerance of bacterial isolate MAI4 on nutrient agar plates as well as in nutrient broth was the reason for selecting this isolate for further studies. Many studies reported maximum tolerance of bacteria to Cr (VI) (Karthik, Oves, et al., 2017; Wani et al., 2016; Wani & Omozele, 2015) which is influenced by various growth factors (Rajkumar, Nagendran, Kui, & Wang, 2005). For example, *Intrasporangium* sp. Q5-1 tolerated Cr (VI) up to a concentration of 17 mM (Yang, He, & Wang, 2009) while *Bacillus* spp. PZ3 and *Streptococcus* spp. PZ4 showed a tolerance of 700 μg/ml (Wani, Omozele, et al., 2015; Wani, Zainab, Wasiu, & Jamiu, 2015). Changing tolerance to Cr (VI) was observed in *chrA* gene (0.35–200 mM) (Monsieurs et al., 2011; Viti et al., 2014; Wani, Hussaini, et al., 2018), which confirmed that bacteria has also other mechanisms of resistance.

Bacterial growth and pH

Bacterial strain MAI4 was assessed in the presence of varying pH. Maximum growth of the bacterial strain MAI4 was recorded at pH 7, which was followed by 6, 8, 5 and 9. At pH 7 growth of bacterial strain MAI4 was found to be 38.5 ± 4.2 cfu/ml after 24 h of incubation. Maximum growth was found after 48 h of incubation (120.4 ± 9.6 cfu/ml). As the time of incubation increased from 48 to 120 h, there was decreasing trend in the bacterial growth. Least bacterial growth was found at 120 h of incubation which was found to be 6.4 cfu/ml. Similar trend in the bacterial growth was found at pH 6, 8, 5 and 9. There was less growth at pH value of 5 and 9 compared to other pH values. Similarly Soni et al. (2012) also observed bacterial growth over a period of 120 h. Maximum growth of bacterial cells occurred up to 48 h of incubation at pH 7. After 48 h there was no increase in growth of the bacterial cells and the population of cells started decreasing from 48 h and there less population of bacteria at 120 h of incubation.

Effect of pH, Cr (VI) concentration, temperature and time of incubation on Cr (VI) reduction

Selection of appropriate pH can greatly affect bioreduction of Cr (VI) (Karthik, Oves, et al., 2017; Oves et al., 2017; Wani, Hussaini, et al., 2018; Wani et al., 2017). Variations in pH and change in ionic form of active site of chromium reductase enzyme affect activity of chromium reductase enzyme (Karthik, Elangovan, et al., 2017). Cr (VI) reduction by *Pseudomonas sp*. MAI4 at different pH values is shown in (Fig. 1). pH 7 was found to be most suitable pH for bioreduction of Cr (VI) to Cr (III) and maximum and significant amount 84% of Cr (VI) was reduced by strain MAI4 at pH 7. Even at acidic and alkaline pH, there was significant reduction of Cr (VI) to Cr (III) which was 52 and 48% at pH 6 and 8 respectively in nutrient broth amended with 100 μg Cr/ml at 120 h of incubation. There was very less reduction at pH 5 and 9 thus confirmed that pH 7 was the best pH for reduction of Cr (VI) to Cr (III). Biotic and abiotic controls do not show any Cr (VI) reduction and 100% Cr (VI) was recovered from the medium (data not shown). Change in reduction of chromium (VI) by microorganisms is due to variation in the release of chromate reductase enzyme (Gu et al., 2015; Wani, Wani, & Wahid, 2018). Since Cr (VI) reduction is an enzyme mediated process, enzyme ionization and protein confirmation may be affected due to change in pH of the medium (Wani, Hussaini, et al., 2018; Wani, Wani, et al., 2018). For this purpose, further experiments of reduction were conducted by keeping pH of media constant at 7.0.

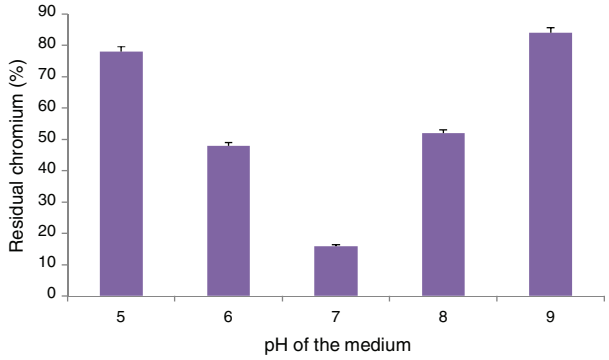

Figure 1 Effect of pH on Cr (VI) reduction by *Pseudomonas sp*. MAI4 at 120 h of growth in nutrient broth having a concentration of 100 μg/ml of Cr (VI) at 28 ± 2 °C.

Impact of changing doses of chromium on Cr (VI) reduction by strain MAI4 was variable (Fig. 2). Significant reduction of Cr (VI) by MAI4 was recorded at 100 and 200 μg Cr (VI)/ml (Fig. 2) at 120 h. Non-significant reduction was observed at 300 μg/ml of Cr (VI). Strain MAI4 reduced 86 μg Cr/ml at a concentration of 100 μg Cr/ml, 168 μg Cr/ml at 200 μg/ml Cr (VI) and 78 μg Cr/ml at 300 μg Cr/ml respectively. Reduction of Cr (VI) by the bacterial strain MAI4 is greatly influenced by temperature. In this study, effects of various temperatures on Cr (VI) removal by MAI4 were investigated under *in vitro* conditions (Fig. 3). 35 °C was found best temperature for the reduction of Cr (VI) which was followed by 25 °C. Both temperatures showed significant reduction of Cr (VI), whereas 45 °C showed less reduction by strain but it was still able to reduce Cr (VI) at extremely high temperature and showed that bacteria can reduce Cr (VI) at thermophilic range of temperature. Strains MAI4 showed a reduction of 84% at 35 °C whereas reduction of 82% was observed at 30 °C. The effect of time of incubation on Cr (VI) reduction was observed up to 120 h of growth of bacterial strain MAI4 in nutrient broth amended with 100 mg/l at pH 7.0 (Fig. 4). As the time of incubation increased, Cr (VI) reduction by the strain MAI4 also increased. Bacterial strain reduced 86% of Cr (VI) within a period of 120 h of incubation.

Similarly, Das et al. (2014) also reported pH 7 as an effective pH for reduction of Cr (VI). Varied reduction due to

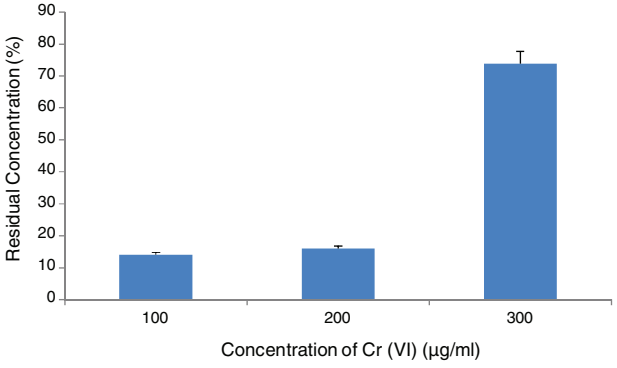

Figure 2 Effect of Cr (VI) concentrations on Cr (VI) reduction by *Pseudomonas sp.* MAI4 in nutrient broth having pH of 7, 28 ± 2 °C after 120 h of growth.

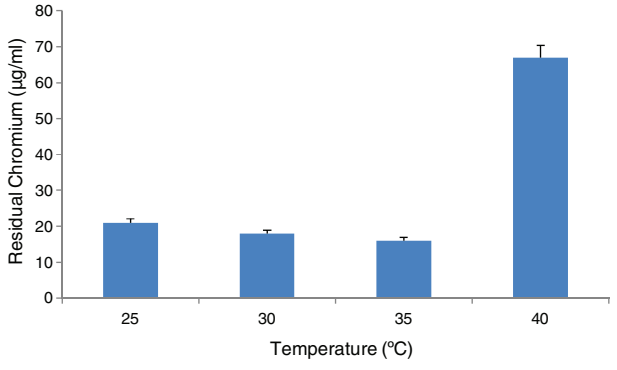

Figure 3 Effect of temperature on reduction of hexavalent chromium by *Pseudomonas sp.* MAI4 after 120 h of growth in nutrient broth at pH of 7.

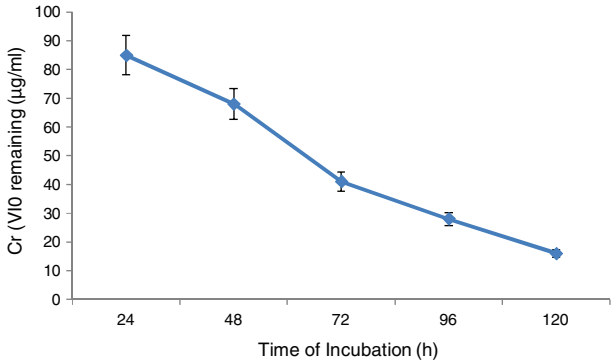

Figure 4 Time course chromium (VI) reduction by *Pseudomonas sp.* MAI4 in nutrient broth (pH 7.0) amended with 100 μg/ml Cr (VI) at 35 °C.

varied pH is due to secretion of chromate reductase which occurs between pH 6.5 and 9 (McLean, Beveridge, & Phipps, 2000; Wani, Hussaini, et al., 2018; Wani, Wani, et al., 2018). Significant removal of Cr (VI) at lower concentrations of Cr (VI) has also been reported by Yang et al. (2009) and Karthik, Oves, et al. (2017). Similarly Wani, Omozele, et al. (2015), Wani, Zainab, et al. (2015) and Wani et al. (2017) also reported significant removal of Cr^{6+} (pH 6.0, 7.0 and 50 and 100 μg Cr/ml).

Soni et al. (2012) also studied the effect of pH, chromium concentration, temperature and time of incubation on Cr (VI) reduction by the bacterial strains. Das et al. (2014) found 100 mg/l, pH 7 and 35 °C to be the most suitable concentration, pH and temperature for Cr (VI) reduction. There was complete reduction of Cr (VI) after 144 h of incubation by *B. amyloliquefaciens*. Temperature of 35 °C was found to be the most suitable for the reduction of Cr (VI) by *Bacillus* species (Dhal et al., 2010). Growth and reduction of Cr (VI) are negatively affected by extreme temperature which arises from reduction in viability or due to the arrest of physiological activity of the cell. Higher temperature results in protein denaturation and DNA damage as well as change in the structure of the membrane. In a study Soni et al. (2012) found that increase in time of incubation resulted in increase in Cr (VI) reduction.

Studies indicated varied tolerance to Cr (VI) by *chrA* gene (0.35–200 mM) (Viti et al., 2014; Wani, Hussaini, et al., 2018).

Cr (VI) reduction in industrial waste water

Chromium (VI) reduction by *Pseudomonas sp.* MAI4 in the industrial waste water contaminated with chromium Cr (VI) (150 μg/ml Cr (VI)), pH 6.8 is shown in Fig. 5. *P. entomophila* MAI4 reduced 80% of Cr (VI) in the industrial waste water after 120 h. Cr (VI) reduction was not observed in unsterilized and sterilized industrial waste water without bacterial inoculation (Controls) and 100% of Cr (VI) was recovered after 120 h of incubation. Bacteria significantly reduced Cr (VI) in natural environment such as industrial waste water which was found to be contaminated with a number of metals, thus confirming its potential as a bioremediator under natural environment. Results of Cr (VI) reduction in industrial waste water inoculated with bacterial isolates were

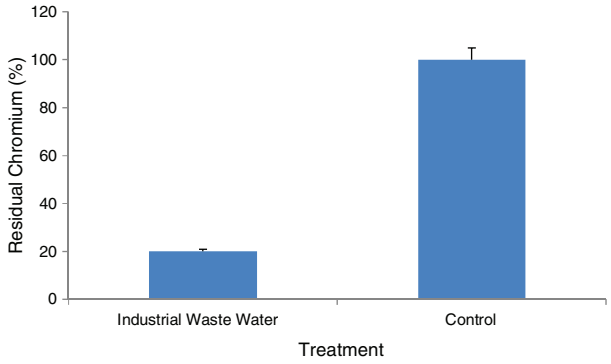

Figure 5 Cr (VI) reduction by *Pseudomonas sp.* MAI4 in industrial waste water having initial Cr (VI) concentration of 150 μg/ml after 120 h of growth.

observed by Zahoor and Rehman (2009). They observed that *Bacillus* sp. JDM-2-1 and *S. capitis* reduced 86% and 89% of Cr (VI) in the industrial waste water after 144 h of incubation respectively. Reduction of Cr (VI) into Cr (III) in contaminated effluents of the metal finishing industry has been reported (Ganguli & Tripathi, 2002; Hardoyo & Ohtake, 1991).

Fate of Cr (VI) in supernatant

Bacterial strain MAI4 reduced 86% of Cr (VI) to Cr (III) at pH 7 at 120 h. 25.8 ± 2.0 μg/ml Cr (III) was found in supernatant and 60.2 ± 3.0 μg/ml in pellet after its reduction by MAI4 after 120 h which confirmed maximum accumulation of Cr (III) in pellet. These results are in agreement to those reported by other researchers (Gu et al., 2015; Karthik, Elangovan, et al., 2017; Oves et al., 2017; Wani, Hussaini, et al., 2018). Pan et al. (2014) also reported maximum accumulation of Cr (III) on the cell debris than supernatant and cytoplasm after reduction of Cr (VI) to Cr (III) by planktonic cells. Cr (III) was found to be 37.5% (supernatant), 44% (cell debris) and 18.5% (cytoplasm). On the other hand, Megharaj, Avudainayagam, and Naidu (2003) reported maximum amount of Cr (III) in the supernatant as soluble end product after the reduction of Cr (VI) by *Arthrobacteria* and *Bacillus*.

Chromium (VI) reduction by chromium reductase enzyme

Cr (VI) reduction by the bacterial strain MAI4 was studied in both resting and permeabilized cells (Fig. 5) and Tween-80 was used to achieve cell permeabilization. Above activities were observed in 100 μg/ml Cr (VI) amended bacterial cells up to 4 h at 28 °C (Fig. 6). Reduction was more in permeabilized cells (84%) than resting (75%) and control (10%) cells after 4 h of incubation. Bacterial cells were broken into cell free extract (CFE) and cell debris and were observed for Cr (VI) reduction (Table 1). CFE showed significant reduction of Cr (VI) (0.54 μmol min^{-1} mg^{-1} protein) when amended with 100 μg/ml of Cr (VI) only within 4 h of incubation, whereas in cell debris, no Cr (VI) reduction was found. These results showed that Cr (VI) reduction was mainly dependent on

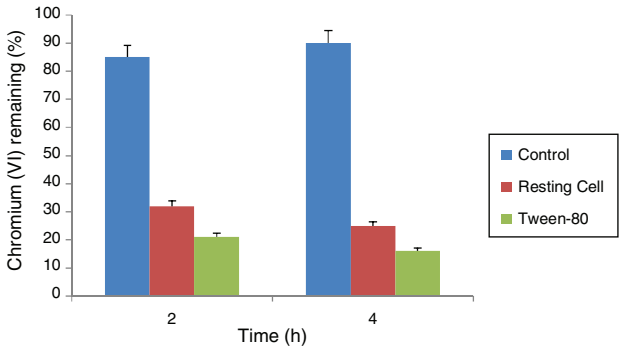

Figure 6 Cr (VI) reduction by resting and permeabilized cells of *Pseudomonas sp.* MAI4 up to 6 h of incubation supplemented with 100 μg/ml of Cr (VI).

Table 1 Subcellular localization of chromate reduction ability in *Pseudomonas sp.* MAI4 at 100 μg/ml Cr (VI) concentration.

Cell components	Chromate reduction ability (μmol min^{-1} mg^{-1} protein)
Cell free extract	0.54 ± 0.175
Cell debris	0.02 ± 0.003
Cell free extract control	0.01 ± 0.005

CFE. Furthermore, to see the role of reductase for Cr (VI) reduction, we assayed the effect of 100 °C heat killed cells (NC) and protein denaturants (silver nitrate and SDS) on chromium (VI) reduction in CFE. Fig. 7 showed that there was complete halt in chromium (VI) reduction by denaturants which concluded reduction was due to CFE. Untreated cell free extract did not show chromium (VI) reduction which also concluded reduction to be through chromium reductases.

Cr (VI) reduction by resting, permeabilized cells and CFE confirmed role of cytosolic fraction in the Cr (VI) detoxification (Gu et al., 2015; Wani, Hussaini, et al., 2018; Wani, Wahid, Singh, & Kehinde, 2018; Wani, Wani, et al., 2018). Tween-80 released proteins (reductase) from the cytoplasm which reduced Cr (VI). There was no reduction

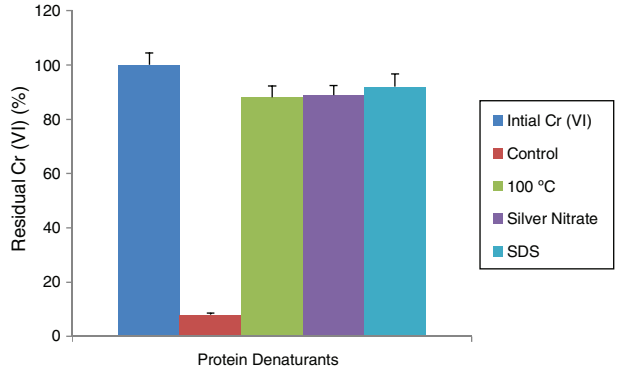

Figure 7 Effect of heat and protein denaturants on Cr (VI) reduction by cell free extract of *Pseudomonas sp.* MAI4 supplemented with 100 μg/ml of Cr (VI).

of Cr (VI) in heat treated and protein denatured ($AgNO_3$ and SDS) extracts, which showed that the reduction of Cr (VI) was indeed enzymatic and not by adsorption. Similar results of Cr (VI) reduction by chromium reductase enzyme (soluble) associated with cytoplasmic membrane were also observed by Pal, Dutta, and Paul (2005) and Elangovan, Abhipsa, Rohit, Ligy, and Chandraraj (2006). Desai et al. (2008a, 2008b) reported role of soluble chromate reductase in *Pseudomonas* sp. G1DM21 for Cr (VI) reduction. In another study Priester et al. (2006) reported the role of chromium reductase for Cr (VI) reduction after its release from cytoplasm after cell lysis. Many studies confirmed the role of chromium reductase for reduction of Cr (VI) to Cr (III) (Ravindranath, Henne, Dorothea, Thompson, & Irudayaraj, 2011); Wani, Wahid, et al., 2018). Furthermore cell debris did not show Cr (VI) reduction which concluded chromium reductase to be responsible for Cr (VI) reduction. This was also confirmed by Soni et al. (2012) and Gu et al. (2015) who did not found the role of cell debris for Cr (VI) reduction.

Conclusion

Viability of microbes are lost under the influence of increasing doses of Cr (VI), therefore Cr (VI) resistant and reducing microbes are significantly necessary to be isolated from metal polluted habitat to establish efficient bioremediation strategies. *Pseudomons sp*. MAI4 showed significant Cr (VI) reduction at pH 7 (84%), 100 μg/ml of Cr (VI) (86%), 35 °C (86%) as well in industrial waste water (80%). Cell debris accumulated more amount of Cr (III) (60.2 μg/ml) whereas cell free extract accumulated less amount of Cr (III) (25.8 μg/ml) after reduction of Cr (VI) by *Pseudomonas* MAI4. Cr (VI) reduction by MAI4 increased as time of incubation increased. Results of this study confirmed chromium reduction due to cell free extract of *Pseudomonas sp*. MAI4 compared to cell debris. No Cr (VI) reduction was observed in cells treated with heat and protein denaturants, thus confirmed role of chromium enzyme for reduction. Based on above properties, MAI4 can be used a potential bio-inoculant for detoxification of Cr (VI) under *in vitro* and *in vivo* conditions. Further investigations are exploited to address issues involving Cr (VI) uptake kinetics, interaction of bacteria with the metal, and the characterization of the gene and enzyme involved in detoxification. Investigation is also needed to address role of symbiotic association of cowpea and bio-inoculant for metal detoxification in natural environment.

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Conflicts of interest

The authors declare no conflicts of interest.

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