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Effect of Environmental Factors on Biological Reduction of Hexavalent Chromium by *Pseudomonas mendocina*†

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In this study, the effects of pH, initial chromium concentrations, organic acids (alginic acid, galacturonic acid, glucuronic acid and citric acid) and their binary combinations on the bacterial chromium reduction were investigated. The results revealed that the Cr(VI) reduction for *Pseudomonas mendocina* was high at optimum pH value (6). The Cr(VI) reduction rate of *P. mendocina* decreased with the increase in initial chromium concentration. The Cr(VI) reduction ability of the bacterium increased in the presence of organic acids especially galactronic acid and glucuronic acid. Binary combinations of galactronic acid and glucuronic acid caused a dramatic increase in the rate of chromate reduction. Experiments with heat-inactivated cells indicated that biosorption onto cell material had a negligible impact for the loss of Cr(VI) from the solution. As a result of SDS-PAGE analysis, it was observed a protein band approximately 31 kDa in periplasmic extracts of *P. mendocina* cells.

Keywords: Biosorption, Biodegradation, Bioremediation, Chromate reduction, Electrophoresis, *Pseudomonas*.

INTRODUCTION

Hexavalent chromium has been commonly used in the steel, refractory, leather tanning, wood treatment, pigment and chemical industries[1]. During the last few decades the uncontrolled release of industrial wastes has contaminated the soil and water with Cr(VI) throughout the world[2-5].

Hexavalent chromium is a strong oxidizing species with toxic and carcinogenic influences on all living organisms, including human. Contact with Cr(VI) compounds over a prolonged period of time is a risk factor for developing lung cancer[6]. Trivalent chromium Cr(III) is a common representative of chromium compounds and less harmful compared to Cr(VI)[7]. Moreover, Cr(VI) penetrates into the cells *via* the sulfate transporters due to its structural and electrical similarity with sulfate ions, whereas, intracellular penetration of Cr(III) is rather not possible[8].

Generally Cr(VI) is much more mobile in soil than Cr(III). The major Cr(VI) species include; chromate, bichromate and dichromate are thermodynamically stable over a large pH range in the environment and hardly absorbed onto soil colloids under alkaline to sub-neutral conditions. Therefore, Cr(VI) posing a threat to surface and groundwater quality[9,10]. Batch sorption studies, primarily performed with pure mineral phases, suggest that Cr(III) is highly reactive and may strongly sorb to the mineral phases[11]. Chromium(VI) exhibits weak to medium binding affinity for metal oxides such as Fe- and Al-oxides depending on the environmental conditions[12-14]. One of the most important factors affecting chromium mobility in underground systems is natural organic substances that are abundant in soil and water. These organic substances act as electron donors and convert Cr(VI) compounds to Cr(III) compounds.

Conventional methods like chemical procedures, used for the removal of hexavalent Cr are expensive and lack specificity[15]. As an alternative, biological approaches by utilizing microorganisms have the potential to remove toxic metals selectively and with considerable operational flexibility. Hence they can be used in a range of bioreactor configurations both *in situ* and *ex situ*[16,17]. Therefore, bacterial bioremediation is of considerable interest as an environment friendly and affordable solution to chromate pollution[4]. Chromium remediation studies have been carried out with several bacteria, such as *Pseudomonas*[18], *Bacillus*[19], *Providencia*[20] and *Achromobacter*[21].

In this background the present study was designed to understand the effect of environmental factors such as different organic acids and their binary combinations, pH level and

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Cr(VI) concentration on the microbial Cr(VI) reduction by *Pseudomonas mendocina* bacterium. Furthermore, the biosorption of chromium was determined by dead *P. mendocina* cells and chromate reductase enzyme induction of periplasmic extracts of *P. mendocina* bacterium.

EXPERIMENTAL

Unless otherwise stated, all chemicals used in the experiments were reagent grade. Water for all experiments was supplied from a Human Power-Pure water system (Zeener Power, Korea). The stock solution of Cr(VI) was prepared by dissolving 2.829 g $K_2Cr_2O_7$ (294.19 g/mol) (Merck) in 1 L UV-water, which was autoclaved separately and added to the media before the start of experiments. The D(+)-glucuronic acid sodium salt monohydrate ($C_6H_9NaO_7$ H_2O) (Merck), D(+)-galacturonic acid monohydrate ($C_6H_{10}O_7$ H_2O) (Sigma-Aldrich), alginic acid sodium salt monohydrate (Sigma-Aldrich) and citric acid monohydrate ($C_6H_8O_7H_2O$) (Merck) were used as organic acids (ligands) in all the experiments. In addition, diphenylcarbazide (Merck) reagent was prepared in acetone. All stock solutions were stored in amber glass bottles in darkness at 4 °C.

Culture conditions of microorganism: The waste water isolates of *Pseudomonas mendocina* used in this study were obtained from Bacteriology Labrotory Culture Collection, Department of Biology, Pamukkale University. To determine the best chromium reduction medium, three different medium were used such as, Nutrient Broth (NB; g/L: peptone 5, yeast extract 2, meat extract 1, sodium chloride 5), triptic soy broth (TSB; g/L: pepton from casein, 17, pepton from soy meal 3, D(+)-glucose 2.5, sodium chloride 5, di-potassium hydrogen phosphate 2.5) and Lauri Bertani-Miller (LB-Miller; g/L: tripton 10, yeast extract 5, sodium chloride 10). All media were autoclaved at 121 ºC for 15 min and stored at 4 ºC until use.

Chromium-resistance evaluation of bacterial strain: The minimum inhibitory concentration (MIC) of Cr(VI) resistant strain was determined by broth dilution method[22] in triptic soy broth medium with Cr(VI) concentrations ranging from 10 to 500 mg/L. The bacterium samples (2 %) were inoculated to this series and incubated at 37 ºC. After 24 h, tube series were checked. The minimum concentration of chromium in the medium inhibiting complete growth was taken as the minimal inhibitory concentration (MIC). minimal inhibitory concentration experiments were performed triplicate.

Reduction of Cr(VI): The 250 mL flasks containing 100 mL of triptic soy broth with a desired concentration of Cr(VI) were inoculated with 2 mL cultures of *Pseudomonas mendocina* at logarithmic phase. The initial pH of the media was adjusted to 7 (± 0.2) using an appropriate amount of $NaHCO_3$ (0.11 mM). All media were autoclaved at 121 ºC for 15 min before use in microbial Cr(VI) reduction experiments. Cultures were then incubated at 37 ºC with constant shaking at 125 rpm. Immediately after inoculation with bacteria, samples were drawn at regular time intervals (every 12 h) and centrifuged at 6000 rpm for 20 min. The concentration of Cr(VI) in the supernatant was determined colorimetrically using diphenylcarbazide reagent at 540 nm by UV spectrophotometer[23]. The growth of cells was also routinely monitored by measuring optical density (OD) at 600 nm. The experiments were carried out in duplicate.

Effects of different pH levels on bacterial chromium reduction: In order to specify effects of pH to bacterial Cr(VI) reduction, triptic soy broth mediums containing 25 mg/L Cr(VI) were prepared at different pH series such as, 6, 7, 7.5, 8, 9. To stabilize the pH, $NaHCO_3$ buffer was used for pH 6, 7, 7.5 and 8 and NH_3-NH_4Cl buffer was used for 9. Then mediums were autoclaved at 121 ºC for 15 min. The bacterium samples (2 %) were inoculated to this series media.

Effects of initial chromium concentration on bacterial chromium reduction: To identify effects of different chromium concentrations, triptic soy broth mediums of different initial chromium concentrations such as, 10, 15, 20 and 25 mg/L for the bacterium were prepared. The media were autoclaved at 121 ºC for 15 min. The bacterium samples (2 %) were then inoculated to this series.

Effects of organic acids and their binary interactions to bacterial chromium reduction: The effect of organic acids and their binary interactions on the bacterial chromium reduction were investigated by using alginic acid, galacturonic acid, glucuronic acid and citric acid as electron donors. Organic molecules were added for each of them as 1 g/L.

Biosorption: *P. mendocina* bacteria, after being incubated for 24 h in 1000 mL triptic soy broth medium, were centrifuged at 6000 rpm at +4 ºC for 20 min. The obtained pellets were dried under aseptic conditions for 12-16 h at 80 ºC. After that, heat killed cells were used as a biosorbent in the biosorption experiments. The biosorbents with the final concentration of 1 g cell/L were suspended in 100 mL of the chromium solution (10, 15, 20 and 25 mg/L). The samples were incubated at 37 ºC and their chromium concentration was periodically determined according to diphenylcarbazide method.

Chromate reductase enzyme induction: For analysis of chromate reductase enzyme induction of *P. mendocina* bacterium, it was grown in media containing 10 and 15 mg/L Cr(VI) and without Cr(VI) (control) at 37 °C for 24 h with constant shaking at 125 rpm. Then, to determine the existence of induced chromium reductase, osmotic shock method was used by obtaining periplasmic fractions from overnight grown bacteria for all of chromium concentrations[24,25]. In order to compare the protein profiles of the periplasmic fractions of the cells grown with and without chromate, the concentrated periplasmic fractions were suspended in 100 µL of 1x cracking buffer (0,0625 M *tris*-HCl, pH 6.8; 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol and 0,001 % bromophenol blue) and mixed thoroughly by vortexing for 1-2 min. The lyzed suspensions were then boiled and loaded on 10 % SDS-PAGE [26]. Proteins were resolved by electrophoresis at 100 V for 2 h (stacking) and 200 V for 4 h (resolving). Gels were stained with coomassie brilliant blue R-250.

RESULTS AND DISCUSSION

It is well known that hexavalent chromium is toxic to all forms of life including humans and exhibits mutagenic, teratogenic and carcinogenic effects on biological systems due to its strong oxidizing nature[27]. Therefore, the concentration of Cr(VI) in the effluents before discharging in environment needs to be reduced to the permissible limit (*e.g.*, < 0.05 mg/L as per US-EPA)[28] by using appropriate technology. The ability

of some bacteria to reduce Cr(VI) has raised the possibility of using these microorganisms as a biotechnological tool for bioremediation of chromium-polluted zones[29,30]. Bioremediation of Cr(VI) using Cr-resistant bacteria provides a safe, effective and alternative viable process[31]. Bacterial chromium reduction is affected by multiple factors such as pH, chromium concentration, carbon sources, natural organic acids, metal ions and temperature.

Chromium resistance evaluation of bacterial strain: The study made to determine the medium showed that the best medium for the *Pseudomonas mendocina* growth and rapid Cr(VI) reduction was triptic soy broth medium. All the experiments were then carried out at 37 °C, in triptic soy broth medium. The chromium-resistance of the bacterium was determined by identifying the minimum inhibition concentration using the broth dilution method. While applying the broth dilution method, chromium concentration was kept at 10-500 mg/L and the overall volume was 5 mL. The minimum concentration of the dilution tube where there was not an observable growth was taken as MIC. Moreover, the results were verified by inoculating petri dish and we found 25 mg/L MIC value for the bacterium.

Effects of pH: The initial pH of the culture plays a crucial role in chromium reduction. Many researchers have investigated the optimum pH values for chromium reduction through bacteria. Wang and Xiao[32] reported that, the optimum pH value for reduction of chromium by *Bacillus* sp. and *Pseudomonas fluorescens* is 7 and chromium reduction is inhibited at pH 6. On the other hand, it was found that optimum initial pH was 9 for another *Bacillus* sp. XW4 isolate and one Gram-positive isolate[33]. The variation observed in optimal pH indicates that it is important to determine the optimum pH value in different cultures and to modify the pH in order to achieve maximum Cr(VI) reduction of chromium detoxification. Therefore, the effect of pH variation on Cr(VI) at pH levels of 6, 7, 7.5, 8 and 9 was assessed in the present study. It was found that the optimum pH value was 6 and all 25 mg/L Cr(VI) was reduced at 36th h. Therefore, other chromium reduction experiments were performed at pH 6. At pH 7, 7.5 and 8 the chromium reduction was very similar and it completely reduced at 48th h of incubation at pH 7 and 7.5 whereas, at pH 8, reduction was slightly slow and completed at the 60th h. However, at pH 9, there was very limited Cr(VI) reduction. The effect of pH on chromium reduction in growth media is shown in Fig. 1. The general trend with reference to the influence of pH on reduction was pH 6 > pH 7 = pH 7.5 > pH 8 > pH 9 (Fig. 1).

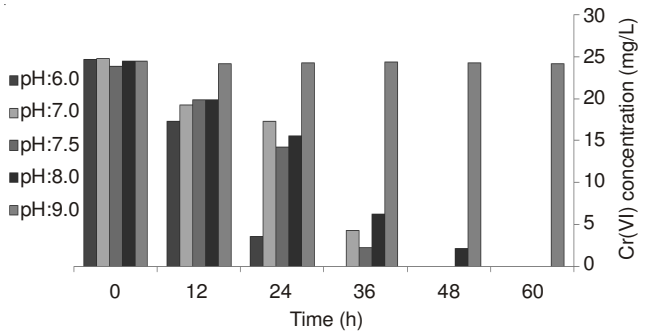

Fig. 1. Effect of different pH levels on Cr(VI) reduction by *P. mendocina* with 25 mg/L Cr(VI) in triptic soy broth media

Effects of initial chromium concentration: The effect of initial Cr(VI) concentration on Cr(VI) reduction by using four different Cr(VI) concentrations (10, 15, 20, 25 mg/L) has been shown in Fig. 2. The complete Cr(VI) reduction was observed for all concentration at 36 h. At the first 24 h of incubation, Cr(VI) reduction rates of *P. mendocina* bacterium in media containing 10, 15, 20 and 25 mg/L Cr(VI) were 76.29, 66.45, 51,19 and 44.13 %, respectively. A similar trend was also observed in *Pannonibacter phragmitetus* LSSE-09[34] and *Ochrobactrum* sp.[35]. The decrease in chromate reduction rate with the increase of initial Cr(VI) concentration might be due to chromate toxicity.

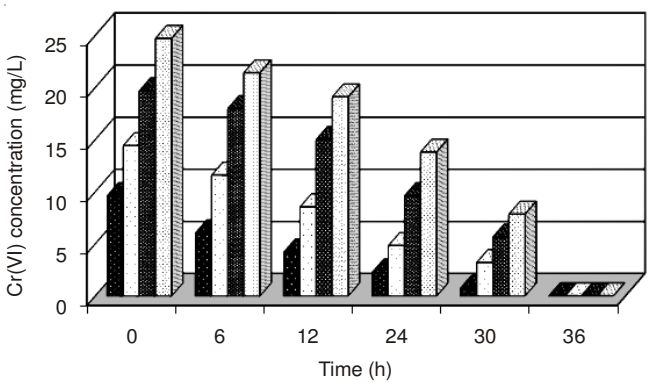

Fig. 2. Effect of initial Cr(VI) concentrations on chromate reduction in triptic soy broth media

Effects of organic acids and their binary interactions: The effects of organic acids such as galacturonic acid, glucuronic acid, alginic acid and citric acid on Cr(VI) reduction by *Pseudomonas mendocina* was tested at 10, 15, 20 and 25 mg/L Cr(VI) containing medium (Fig. 3a-d). Among the organic acids used in the study, galactronic acid and/or glucuronic acid was the most effective on Cr(VI) reduction. Other organic acids (alginic acid and citric acid) were not affected the chromium reduction of *P. mendocina* bacterium considerably. At 10, 15, 20 and 25 mg/L Cr(VI) concentration, complete Cr(VI) reduction was observed in the control (without organic acids) at 36 h. However, when galactronic acid or glucuronic acid was added in growth medium, Cr(VI) reduction completed at 30 h for 20 and 25 mg/L Cr(VI) and 12 h for 10 and 15 mg/L Cr(VI). This might be due to actual organic acids which serve as an electron donor. Previous studies on Cr(VI) reduction by *Pseudomonas putida* P18 and *Pseudomonas aeruginosa* P16 showed that the glucuronic acid, galactronic acid, alginic acid and bacterial EPS were used as organic ligand and reduction was faster in the medium containing these organic acids. Furthermore, the influence trend of these organic acids was alginic acid > glucuronic acid > EPS > galactronic acid[18]. Desai *et al.*[36] observed an enhanced chromate reductase activity by *Pseudomonas* sp. G1DM21 in the presence of electron donors such as citrate, acetate and succinate.

In natural media such as soil and water, these kinds of organic compounds undoubtedly exist together and interact with each other. The presents study therefore used the binary combinations of natural organic acids and determined the relationship between these combinations and Cr(VI) reduction. The results showed that there are synergistic effect between

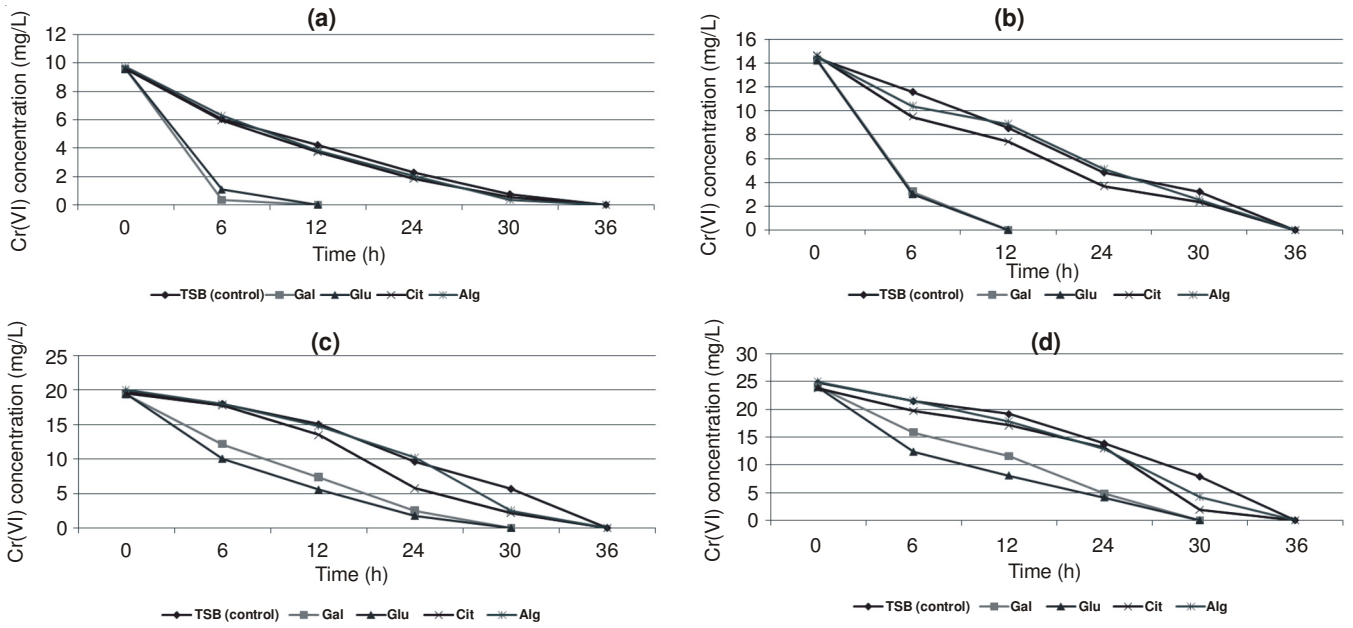

Fig. 3. Effect of different organic acids (1 g/L) on Cr(VI) reduction by *P. mendocina* Gal: galactronic acid, glu: glucuronic acid, cit: citric acid, alg: alginic acid, (a) 10 mg/L Cr(VI) containing media, (b) 15 mg/L Cr(VI) containing media, (c) 20 mg/L Cr(VI) containing media, (d) 25 mg/L Cr(VI) containing media

galactronic acid and glucuronic acid, galactronic acid and citric acid, glucuronic acid and citric acid, glucuronic acid and alginic acid combinations (Fig. 4). Using this combinations, the Cr(VI) reduction time was decreased and it was approximately half, compared to separate use of organic acids. The reduction time for 25 mg/L Cr(VI) is 12 hr in binary combinations of galactronic acid and glucuronic acid (30 h for one by one) (Fig. 4). On the other hand, binary combination of galactronic acid and alginic acid did not affect the Cr(VI) reduction time significantly. Interestingly, alginic acid and citric acid combination extended the Cr(VI) reduction time from 36 to 54 h. There is very scanty information published regarding the binary combinations of organic acids for Cr(VI) reduction[37,38].

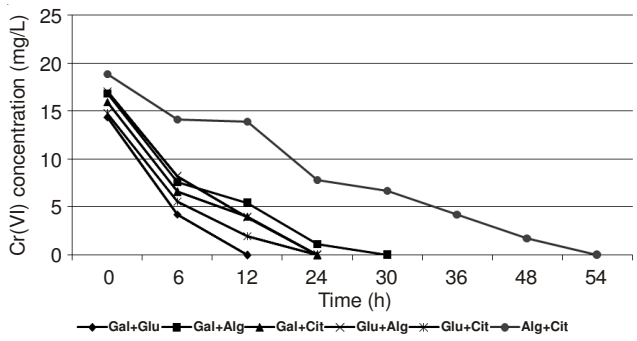

Fig. 4. Effect of binary combinations of organic acids with 25 mg/L Cr(VI) on chromium reduction by *P. mendocina* Gal: galactronic acid, glu: glucuronic acid, cit: citric acid, alg: alginic acid

Biosorption: Experiments with heat-inactivated cells indicated that biosorption of cell material had a negligible impact for the loss of Cr(VI) from solution (Fig. 5). In the heat killed cells of *P. mendocina* bacterium biosorped only 1.33, 1.84, 4.82 and 0.95 % in 25, 20, 15 and 10 mg/L Cr(VI) solution, respectively at 60th h of incubation. After the 60 h of incubation biosorption stopped and chromium concentration was stabilized.

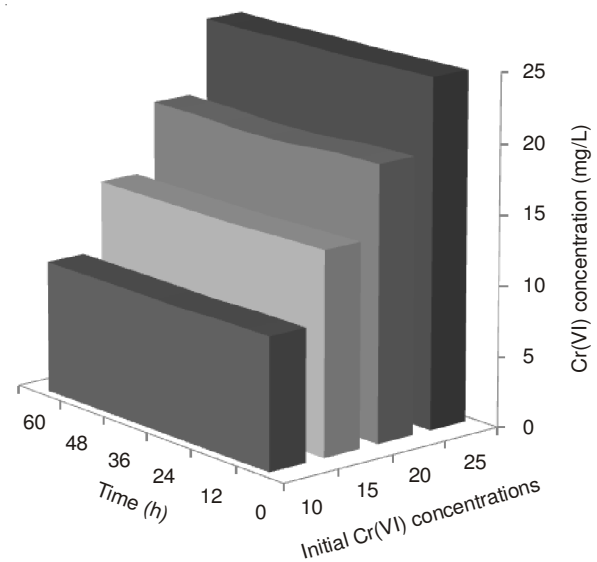

Fig. 5. Biosorption of different initial Cr(VI) concentrations by heat-inactivated cells of *P. mendocina*

Chromate reductase enzyme induction: Chromate reduction by bacteria occurs under aerobic or anaerobic conditions and it has been associated with soluble or membrane-associated enzyme activities[27,39]. It was determined by various methods such as SDS-PAGE, NMR and MS that induced of chromate reductase at the presence of chromium in growth medium of bacteria. In the present investigation a protein that has a molecular weight around 31 kDa was induced in the presence of chromium (15 mg/L) (Fig. 6). Similar types of protein induction studies have been done and chromate reductase has been purified from *P. putida* MK1 which has a molecular weight of 20 kDa on SDS-PAGE[40]. Similarly, in *P. aeruginosa*[24] and *Ochrobactrum* sp.[34] chromate reductase was shown to have a molecular weight of 30 kDa. In another study, it was reported that the presence of an induced protein having

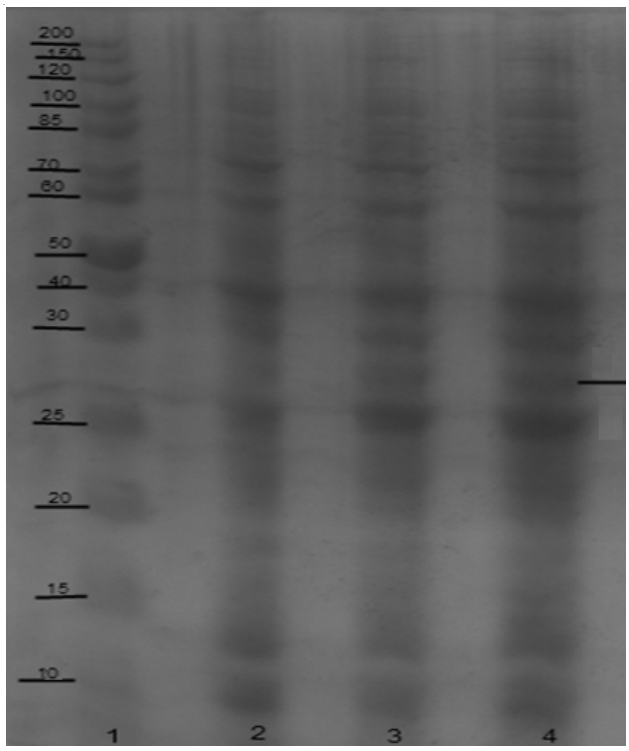

Fig. 6. Comparison of periplasmic protein profiles of *P. mendocina* 1: Marker (SM-0661), 2: without chromium, 3: with 10 mg/L Cr (VI), 4: with 15 mg/L Cr (VI)

molecular weight around 25 kDa in the presence of chromium in *Bacillus* sp. JDM-2-1 and *Staphylococcus capitis* bacteria[41].

Conclusion

The use of microorganisms for detoxification of environment contaminated with Cr(VI) has received considerable attention in recent years, because of their ability to tolerate and reduce Cr(VI). The effects of environmental factors (pH, organic compounds and their combinations, Cr concentration) on Cr(VI) reduction to achieve maximum reduction by the *Pseudomonas mendocina* strain has been reported. It has been found that galactronic acid and/or glucuronic acid are the most effective organic acids which can be used for Cr(VI) reduction. The optimum pH was 6 and initial chromium concentration has negative effect on bacterial chromium reduction. Also we determined the Cr(VI) was not biosorped onto cell surface by *P. mendocina* bacterium. Additionally, we detected a protein that has a molecular weight around 31 kDa by SDS-PAGE. The presence of this protein in the periplasmic extracts of cells grown in the presence of chromate, but its absence of the protein in cells grown without chromate, points out a possible role of this protein in chromate reduction.

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