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Reduction and bioaccumulation of hexavalent chromium by *bacillus megaterium* from a tannery's activated sludge

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ABSTRACT/RESUME

Abstract:*Hexavalent Cr(VI) is used in leather industry and when left untreated, it exhibits its known carcinogenic and mutagenic effect toward many organisms including humans. Several bacteria possess the capability to reduce Cr(VI) to a less dangerous form: the trivalent Cr(III), others are able to accumulate it or to adsorb it, so microbial bioremediation gives immense opportunities for the development of technologies to detoxify Cr(VI)-contaminated soils or water as an alternative to the existing physicochemical technologies.*
This work deals with the evaluation of the ability of Bacillus megateriumA3-1,isolated from local tannery activated sludge, to resist high concentrations of Cr(VI), by determining the MIC, on the one hand, and secondly byinvestigating the involved Cr(VI) removal mechanisms, namely enzymatic reduction and bioaccumulation.Our results indicated that, B.megaterium A3-1 exhibited an MIC for Cr(VI) of 80 mg/L, and could reduce chromate to 100 % when present at a concentration of 30 mg/L after 72 h at 37 °C, furthermore, the rate of Cr(VI) accumulation was 27.50 mg/g biomass dry weight at the same initial concentration.

I. Introduction

Heavy metals are the major toxic pollutants of waste water from various industrial sectors, including tanneries. This wastewater represents a major risk to the environment if it is not appropriately treated before being discharged into the environment. Among the various heavy metals that are involved in environmental pollution, chromium (VI) is considered one of the most dangerous due to its oxidant, mutagenic, and carcinogenic properties. Generally, all statutory bodies worldwide cite the Cr(VI) as a toxic chemical to be controlled in priority [1].
Contaminated wastewater contains significant amounts of Cr (VI), when released into the environment; they represent a toxicological risk to humans, animals and plants [2]. The Cr (VI) is mainly introduced into the environment by industrial use, while chromium in trivalent form Cr (III) naturally predominates in the environment [3]. Cr(VI) is highly diffused in sediment and surface waters; it is characterized by a higher solubility, mobility and bioavailability than the Cr(III) and all other forms of chromium [4].

Despite its toxicity, the resistance of microorganisms to high concentrations of Cr(VI) is reported; it offers a significant opportunity to reduce the Cr(VI) to Cr(III) and great promise for application in bioremediation. The biological reduction of Cr(VI) to Cr(III) is a potentially useful process for the recovery of Cr(VI)-contaminated sites [5,6]. Bioremediation technology also includes phycoremediation that uses photosynthetic microorganisms such as microalgae and cyanobacteria in the removal of metal contaminants [7]. In this aspect, yeasts and fungi in particular have been the most extensively studied microorganisms [8].

Chromium resistant bacteria display various mechanisms; the three main strategies involved are the prevention of Cr(VI) entry to the cell (mutation of the sulphate transport system), the Cr expulsion outside the cell (ChrA efflux pumps) or the enzymatic or non enzymatic reduction to Cr(III) [9-11].

In the current context, the present work aims to evaluate the ability of *B.megaterium*A3-1 isolated from the activated sludge of the waste water treatment plant of the tannery of Jijel to remove chromium (VI) and to study the prospective involved mechanisms.

II. Materials and methods

II.1. Bacterial strain and culture media

The bacterial strain *Bacillus megaterium*A3-1 used in this study was previously isolated from the activated sludge of the wastewater treatment plant of the tannery of Jijel. It was identified based on 16S DNA sequencing and assigned the accession number KP163918. The strain was grown at 37°C in nutrient broth (Difco).

II.2. Minimum inhibitory concentration (MIC) determination

The method used to determine the minimum inhibitory concentration of chromium is the liquid medium dilution method described [12], corresponding to a modified protocol of [13]. Following an increasing concentration gradient of Cr (ranging from 0 mg/L to 200 mg/L), a first series of test tubes with a total volume of 10 ml, containing nutrient broth is prepared to which is added 1 ml of *B.megaterium* inoculum ($OD_{600\ nm}$1.0) previously activated. On the other hand, a second set of representative blank tubes for each concentration was also prepared. The tubes are then incubated with shaking at 37°C for 24h. Bacterial growth is monitored by measuring the OD at 600 nm at t0 and t24.The minimum inhibitory concentration is the lowest concentration (expressed in mg/L) capable of inhibiting the growth of the bacterium.

II.3. Effect of Cr VI on bacterial growth

In sterile 250 ml Erlenmeyer flasks, the bacterial strain A3-1 previously activated wasadded to 10% (OD_{600nm} 1.8) in 50 ml of nutrient broth in the presence of a concentration of 10, 20, 30 and 40 mg / l Cr(VI). Meanwhile, a control containing only nutrient broth and culture was also prepared. After incubation at 37°C with stirring, the OD at 600 nm was measured for each flask every 60 minutes during the first 4 h, then at the times 24, 48 and 72 h, experiment was carried out in duplicate.

II.4. Hexavalent chromium removal test

Sterile250 ml Erlenmeyer flask containing a final volume of 100 ml of nutrient broth with a concentration of 30 mg/L of Cr(VI), was inoculated with 10% of the bacterial culture (OD_{600nm} 1.8) previously activated [14]. Two control flasks were also prepared: the first isexempt of bacterial culture, to assess the impact of abiotic factors on the fate of chromium. The second is devoid of Cr, to estimate the amount of initial intracellular Cr (which indicates the level of Cr bioaccumulation). The Erlenmeyer flasks were incubated at 37°C with shaking for a period of 72 h.

To monitor the concentration of chromium in time, samples of 10 ml are collected at times t0, t24, t48 and t72. The samples are centrifuged for 20 min at 6000 rpm to obtaincell-free supernatants[15]. In parallel, other samples are taken every 24 h to monitor the growth of *B.megaterium*, as mentioned above. Determination ofCr(VI) concentration was carried out according to the standard colorimetric method of Greenberg et al.(1985). The Cr(VI) of the test sample reacts with a complexing agent, 1,5-diphenycarbazide (DPC) in acid pH. Thus, after 10 to 15 minutes, the complex formed gives the purplish pink color whose intensity is proportional to the hexavalent chromium concentration and is measured spectrophotometrically at 540 nm [16].The rate of chromium reduction is calculated using the following formula [10,12,17].

$R\% = [(C_0-C_t) / C_0] \times 100$
C_0: initial concentration of Cr(VI)
C_t: concentration of Cr(VI) in time t
t: time of incubation.
Experiment was carried out in duplicate.

II.5. Bioaccumulation assessment

At the end of the incubation period (72h) of the previous test, two samples of 5 ml were collected and centrifuged for 10 min at 6000 rpm. A first test tube was used for measuring the dry weight of biomass, after removing the supernatant, and the second tube was used for cell lysis. The collected pellet was washed three times with deionized water and cell lysiswas induced by the addition of 5 ml of nitric acid 1% during 24 h at 4 °C. Thereafter, the bacterial lysate was centrifuged for 10 min at 6000 rpm, and the supernatant obtained was filtered and used for assessing the concentration of the intracellular Cr using the DPC assay. The amount of accumulated chromium is calculated and expressed as mg Cr/g dry weight biomass [18].

II.6. CrVI-reductaseassay

The reaction mixture for the enzyme assay contains Cr(VI) at a concentration of 1 mg/L in 0.8 ml of phosphate buffer (0.1 M, pH 7). After 5 minutes pre-incubation at 30°C, the enzymatic reaction is initiated by adding 0.2 ml of the enzyme

(supernatant collected from samples at t0, t24, and t48 from the previous test) and the reduction of Cr(VI) was measured after 30 min incubation. The residual Cr(VI) was determined by the colorimetric method using DPC.One unit of enzyme activity is defined as the amount of enzyme that reduces 1µmol/min of Cr(VI) at 30°C [19]. A blank is prepared with the same components it was however heated at 100°C for 30 min to remove the reducing activity of the enzyme.The total proteins concentration was determined according to the method of Bradford [20].

III. Results and discussion

This work concerned the study of Cr(VI) removal by *B.megaterium*strain A3-1, through the evaluation of the resistance level to the metal and subsequently the ability of the bacterium to reduce it to Cr(III) or to accumulate it.

III.1. Determination of the minimum inhibitory concentration (MIC)

The MICof Cr(VI) is needed to be knownbefore analyzing the microbial growth in the presence of the metal as the bacteria will not grow when concentrations equal to or greater than the MIC are present in culture medium. The results showed that the MIC of chromium for *B.megaterium*A3-1 is 80 mg/L, the bacteria is resistant to metal concentrations below this value, but grow hardly beyond it and starts to decline at a concentration of Cr (VI) of 90 mg/L.

Similar results were also reported by other researchers, likeSrinath et al.[18], who reported that among 71 isolated microbial strains of tannery effluents, including *B.megaterium* and *B.circulans*, 55 strains had an MIC ranging from 51 to 100 mg/L, five strains only had an MIC of less than 100 mg/L, and 11 were able to tolerate concentrations above 100 mg/L of Cr(VI). Farag and Zaki[21], have also noted that among four strains of the genus *Bacillus*, the bacterial growth of two of them, was affected after addition of a concentration of 40 mg/L and 60 mg/L of chromium in the medium, respectively, while the two others highly resisted and displayed an MIC of 160 mg/L and 200 mg/L for each strain.

Several studies have shown the high Cr resistance exhibited by microorganisms isolated from contaminated sites. This is the case of *Corynebacteriumhoagii* which is resistant to aCr concentration of 22 mM [22], and *Bacillus* sp. JDM-2-1 and *Staphylococcuscapitis*which tolerate up to 4800 µg/ml and 2800 µg/ml of Cr, respectively [23].*B. subtilis*SS-1 showing an MIC of 600 mg/L [12], and *Providencia*sp exhibiting an MIC of 1000 mg/L [24] were also reported.

This variation of the critical value of chromiumMIC depends on thevariable experimental conditions, like the choice of the method for determining the MIC (liquid medium, solid, or with discs), the composition of the culture medium (poor or rich), the nature of the metal salt used, seeded inoculum and finally the bacterial species studied and the environmental habitat from which it was isolated.

III.2. Hexavalent chromium effect on bacterial growth

This test aims to monitor the bacterial growth in the absence and presence of different concentrations of Cr(VI) (10, 20, 30, 40 and 50 mg/L) by measuring the OD at 600 nm (Figure 1). The growth rate after 72 h of incubation was also calculated (Table 1).

In general, it appears clearly that in the absence of Cr, cell growth increased rapidly during the early h of incubation, the growth rate is higher than that showed in the presence of metal. It was also noticed that in the presence of Cr at concentrations up to 30 mg/L, the strain still grows and the growth curves have almost the same profile; whereas, microbial growth becomes weaker and very disturbed at concentrations of 40 mg/L and 50 mg/L. The development of bacterial culture occurs in several phases which are clearly visible in Figure 1. After the initial growth phase (the first three hours of incubation), comes the exponential growth phase during which the cell division proceeds more rapidly, followed by the stationary phase. Therefore, the higher theCr concentrations are, the longer are the lag phases, probably corresponding to a period of adaptation of the strain with the metal present at high quantities where the preparation of the necessary equipment to tolerate and / or resist Cr takes place.

Accordingly, Singh et al. [25], studied *Bacillus cereus* growth curve and showed that the lag phase duration depended strongly on the concentration of Cr(VI) in the medium. They found that the Cr(VI) did not significantly delays the bacterial growth until 400 µg/ml. Cell growth was strongly influenced by the Cr(VI) at a concentration of 1200 µg/ml, while at 600, 800 and 1000 µg/ml,a weaker influence on growth was noted. The lag phase was 12 h long for all the tested concentrations below 800 µg/ml. In the presence of 1000 µg/ml, a 24 h lag phase and a stationary phase during the period of 96-108 h were shown.

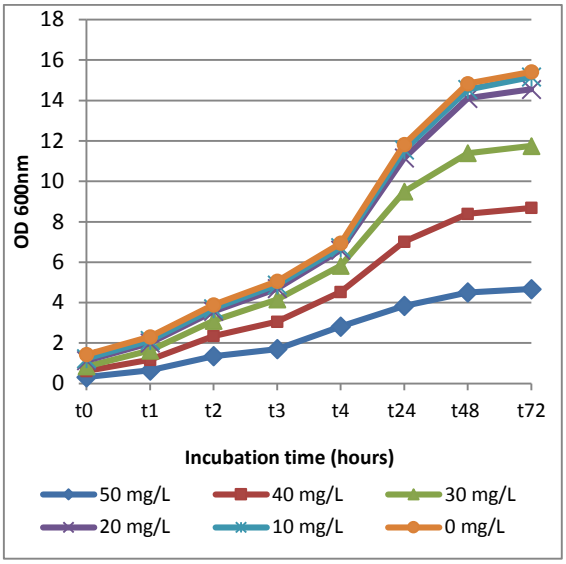

Figure 1. *B. megaterium A3-1 growth pattern in nutrient broth in presence of different concentrations of Cr at 37 °C*

Tableau 1.*B. megateriumA3-1 growth rate in presence of different concentrations of Cr after 72h of incubation at 37 °C.*

Cr(VI) concentration (mg/L)	Growth rate (h^{-1})
0	0.1932
10	0.1686
20	0.1784
30	0.1210
40	0.0662
50	0.0048

It can be concluded from the calculated growth rates, that the bacterial growth is higher (μ = 0.1932 h^{-1}) in the absence of Cr, and it drops significantly as the concentration of the metal increases. Thus, we note that the bacterial growth rate decreases from 0.1932 h^{-1} in the Cr-free medium, to reach 0.0048 h^{-1} when the medium was supplemented with 50 mg/L Cr.

These results are in agreement with those of Xu et al.[26], indicating that the inhibitory effect of Cr(VI) on cell growth increases with the increase in the concentration of Cr(VI) from 12.5 to 125 mg/L for *Bacillusanthracis* Cr-4. A slight reduction in cell growth was observed in the presence of 12.5 mg/L and 25 mg/L of Cr(VI), while a clear reduction in cell growth appeared in the presence of 50 mg/L of Cr(VI), and cell growth was significantly inhibited by 100 mg/L and 125 mg/L of Cr(VI).The results of Zahoor and Rehman(2009) [23], who studied *Bacillus* sp JDM-2-1 and *Staphylococcus capitis* growth curves in the absence and presence of 100 µg/ml of Cr, indicated that for the first strain no significant difference was noted, however for the second the growth rate is lower in the presence of Cr(VI).

III.3. Hexavalent chromiumremoval

The purpose of this test is to study the ability of *B. megateriumA3-1* to reduce the Cr(VI) in nutrient broth in the presence of an initial concentration of 30 mg/L. The removal was monitored by measuring its concentration in the culture medium over 72 h. In parallel, the bacterial growth was also followed by measuring the OD at 600 nm throughout the test (Figure 2). The percentage of final reduction of Cr(VI) by the strain is calculated for the times t0, t24, t48 and t72 (Table 2).

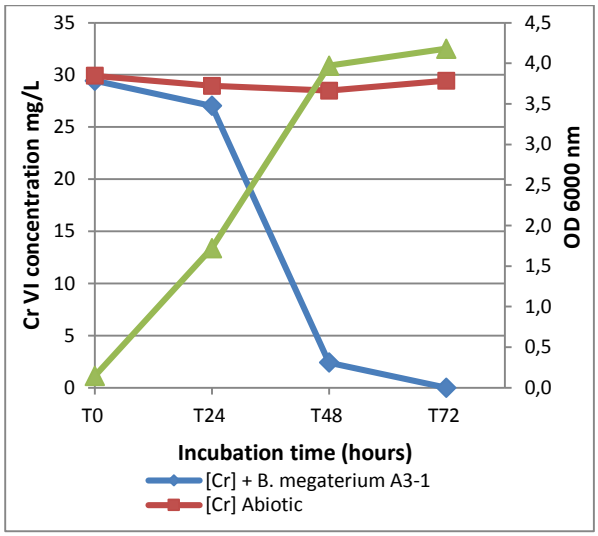

Figure 2. *Monitoring the concentration of Cr(VI) and B. megateriumA3-1 growth in nutrient broth supplemented of Cr at an initial concentration of 30 mg/L*

The Cr(VI) reduction rate is very slow between t0 and t24, and an increase in the reaction rate is visible starting from 24 h of incubation, which stabilizes and reaches its maximum at t72. The reduction of chromium is important when the *B. megateriumA3-1* growth enters the exponential growth phase. In the control, the concentration of Cr remains steady and relatively unchanged.

These results indicated that the Cr(VI) reduction activity and the bacterial growth are closely related and dependent on each other [26]. This may be due to the nature of Cr(VI)-resistant bacteria, which creates a reducing environment to detoxify the Cr(VI). Most likely, bacterial growth as well as the damages induced by Cr(VI) are two competing processes, and bacteria may face exposure to Cr(VI) as long as the metabolizable carbon sources are available [27, 28].

Tableau 2. *Cr(VI) reduction rate by B. megaterium A3-1 at different time intervals*

	Time (hours)			
	0	24	48	72
Cr(VI) reduction rate %	1.89	9.93	91.95	100

It was noticed that the percentage of Cr reduction after 24 h was only 9.93%, and reaches approximately 92% after 48 h to finally reach 100% after 72 h. In this context and inalmost the same experimental conditions, it was reported that*Bacillus* sp. XW-2 and *Bacillus* sp. XW-4 were separately capable of removing up to 100% of the Cr(VI) present in the culture medium with an initial concentration of 40 mg/L after 96 h of incubation[29]. Similarly, Shakoori et al. [14],reported that the Gram positive strain CMB-Cr1 (ATCC 700729) could reduceup to 87% of the Cr(VI) which was present at a concentration of 20 mg/L after 72h.Other bacterial strains belonging to the genus *Pseudomonas*, such as *P. aeruginosa*A2Chr and *P.aeruginosa* CCTCC AB91095, could eliminate 60% in 35h and 55% after 72 h of Cr(VI) starting from an initial concentration of 40 mg / L, respectively [30, 28].
In this case, the final removal rate of the chromium (VI) and the time needed to achieve it can be considered satisfactory, compared with the previously cited results.In addition; Cr(VI) is completely reduced in 72 h in samples where the presence of viable cells is not required (stationary phase). These results indicated that the mechanism involved in Cr(VI) reduction is not exclusivelyintracellular. Severalresearches were concerned by the localization of the reducing activity. Depending on the bacterial species, the chromate reductaseactivity can be attributed to a membrane or to a cytoplasmic enzyme.

III.4. Bioaccumulation of chromium (VI)

The ability of *B. megaterium* A3-1 to accumulate Cr(VI) after 72 h of incubation at 37°C in the presence of an initial concentration of 30 mg/L Cr(VI) was assessed and compared to the control test consisting of a culture under the same conditions but in the absence of the metal. The results are presentedbelow in Table 3. They showed that one gram of the dried biomass of *B. megaterium* A3-1 could accumulate up to 27.50 mg of Cr(VI).

Tableau 3. *Intracellular concentrations of Cr (VI).*

	mg Cr accumulated /g biomass dry weight
*B.megaterium*A3-1	0.483
*B.megaterium*A3-1 + Cr	27.50

The obtained results are similar to those reported by Srinath et al.[18], where *B. megaterium* presented a bioaccumulation of 32 mg/g biomass after 24 h for an initial concentration of Cr(VI) equal to 50 mg/L, while*B. circulans* achieved the accumulation of 34.5 mg/g biomass under the same experimental conditions.These same authors have studied the biosorption of Cr(VI) at an initial concentration of 100 mg/L by *B. coagulans*and *B. megaterium*, using living and dead cells. It has been shown that biosorption by dead cells was higher than that of living cells. The living cells accumulated 23.8 and 15.7 mg of Cr/g dry weight biomass of *B. coagulans* and *B. megaterium*; respectively, whereas with dead cells, 39.9 and 30.7 mg of Cr /g dry weight were accumulated by *B. coagulans* and *B. megaterium*, respectively.

III.5. Chromate reductase activity of the cell-free supernatant

The test is necessary to determine the chromate reductase specific activity in the supernatants collected from samples taken at time t0, t24, t48 and t72 (Table 4).

Tableau 4. *Chromate reductase activity of the cell-free supernatant.*

Incubation time (hours)	0	24	48	72
Total protein (g/l)	0.051	0.064	0.111	0.199
Enzyme activity(unit)	0.0224	0.3314	0.6410	0.6410
Specific activity(U/mg protein)	0.0004	0.0052	0.0058	0.0032

From the above results, it was noted that both the enzyme activity and the specific activity in supernatants increased significantly, with a peak at 48 h. Camargo et al.[19], compared chromate reductase activity of five bacterial strains isolated from soil ES 04, ES 23, ES 29, ES 32 and ES 39. The cell-free extract (cytosolic fraction) tested showed a chromate reductasespecific activity ranging from 0.38 to 0.48 unit/mg protein; they also showed that ES 29 and ES04 strains exhibited a Cr(VI) reduction

rate of 81.2% and 79.5%, respectively. In another report, Soni et al.[27], recorded an enzyme activity of 0.32 ± 0.109, 0.42 ± 0.098, 0.34 ± 0.080 and 0.28 ± 0.150 mol/min/mg protein for cytosolic fractions of *Bacillus*spJN674188 strain,*Microbacterium* sp. JN674183, *B.thuringiensis* JN674184 and *B.subtilis* JN674195, respectively, confirming the presence of soluble enzyme in the cytosolic fraction, suggesting that it has been released into the extracellular medium by the bacterial cells during the 48 h of growth.

Most of the aerobically produced chromate reductases, described so far, are located in the soluble fraction. There are two strains for which the direct reduction process under aerobic conditions has been more deeply described. These strains are*P.ambigua* G-1 and of *P.putida* MK1. The chromate reductase activity of the two strains is detected in the soluble fraction. In *P. ambigua*, the enzyme is a protein of 65 kDa. This enzyme is active in a fairly broad temperature and a pH ranges (40-70 °C, ideally 50 °C, pH 6-9, optimal 8.6). This enzyme allows the reduction of the Cr(VI) in the presence of NADH or NADPH and the reduction of 1 mole CrO_4^{2-} requires the use of 3 moles of NADH which are oxidized to NAD^+[11].

The processes by which microorganisms interact with heavy metals to reduce their toxicities are biosorption, bioaccumulation, and the enzymatic reduction. The reduction of Cr(VI) to Cr(III) by biological treatment is a cost effective approach for removal of Cr(VI). A number of studies have demonstrated the bioremediation using whole cells. However, few studies have demonstrated a reduction *in vitro* by microbial enzymes [31]. The use of enzymes has many advantages; because, unlike the latter, they are not affected by microbial growth inhibitors, toxins, predators or microbial competition in the environment. In addition, enzymes may be immobilized in reactors for the removal of chromium [19].

VI. Conclusion

In view of the obtained results, it appears that *B.megaterium* A3-1 is not only able to develop and persist in nutrient broth supplemented with 30 mg/L Cr (VI), but it shows a remarkable ability to bioaccumulate this metal and even better to reduce it completely from the culture medium after 72 h of incubation at 37°C. However, more in depth investigations are required to characterize the involved chromate reductase. These properties make it a potential decontaminating agent that can be efficiently used for the bioremediation of Cr (VI) and therefore the treatment of polluted sites moreecofriendly.

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