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Role of the enzyme chromium reductase in detoxification of potassium dichromate (Cr^{+6}) by bacteria

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Abstract

Nine dichromate resistant bacteria were isolated: *Bacillus cereus*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Acinetobacter radioresistens*, *Acinetobacter venetianus*, *Ochrobacterum sp*, *Massilianiabensis*, and *Leucobacter chromiireducens*. All of the isolates absorbed and reduced potassium dichromate (Cr^{6+}) from the growth medium amended by dichromate Cr^{6+} at concentration of 100 ppm at higher percentages in the presence of dehydrogenase $NADH^+$ compared to bacteria growth in medium without dehydrogenase $NADH^+$. Absorption of potassium dichromate Cr^{6+} from the growth medium by the bacteria increased with increasing incubation time over 96 hrs. The nine bacteria species isolated from the leather tanning factories in Riyadh were shown to produce both intracellular and extracellular chromium reductase, with the percentage of precipitate of the internal enzyme is always being higher than the external precipitate except in the case of *A. radioresisten* which showed values of 26% for the intracellular enzyme and 52% for extracellular enzyme.

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Introduction

Bioremediation can be employed both to concentrate metals (including chromium in order to avoid toxicity and to recover metals for reuse (Yilmaz, 2003). There are a number of ways by which bacteria can reduce the impact of free-metal toxicants, including a) metal-binding and chelation to various media components b) the formation of complexes, and c) sorption or chelation of metals to unspecified organic compounds which are present in most growth media. Heavy metals and metalloids can be involved in a series of complex chemical biological interactions, the most important factors which affect their mobility being pH, the nature of the sorbent, the presence and concentration of organic and inorganic ligands, notably humic and fulvic acids, root exudates and nutrients (Gadd, 2008).

Heavymetals (such as chromium) are soluble and have a high capacity for movement; and as a result, are particularly damaging because they can cause contamination of the groundwater and also become accessible to plants where they can accumulate, be magnified and then enter the food chain, finally reaching humans (Wu, L., 2004), Such metals usually originate from a variety of industrial wastes, especially leather tanning waste, which is considered one of the most important hazardous waste worldwide (Doble and Kumar, 2005). Studies suggest that bacteria (e.g. *Pseudomonads, Aeromonads*, *Providencia* sp) can be used to detoxifying polluted environments. For example, the last named species can grow in high concentrations of hexavalent chromium, ranging between (100 - 400 mg L^{-1}, 37^{o}C, pH 7), (Thacker *et al.,* 2006; Srivastava *et al.,* 2007; Congeevaram, *et al.,* 2007).Such detoxification involves soluble chromate reductases which have been reported to be present in numerous bacteria; only a few however, have been purified and characterized.

The followings facultative lyanaerobic bacteria reduce Cr^{6+}: *P. dechromaticans; P. chromatophila; Aeromonas dechromatica; Microbacterium* sp. MP30; *Geobacter metallireducens; Shewanella putrefaciens* MR-1; *Pantoea agglomerans* SP1; *Agrobacterium radiobacter* EPS-916 and a consortium which is capable of simultaneously reducing Cr^{6+} and degrading benzoate (Pattanapipitpaisal *et al* 2001; Myers *et al* 2000). Other examples of bacteria which can reduce Cr^{6+}include: *B. cereus; B. subtilis, P. aeruginosa; P. ambigua; P. flourescens; E. coli; Achromobacter Eurydice; Micrococcus roseus; Enterobacter cloacae; Desulfovibrio desulfuricans* and *D. vulgaris* (Lovley, 1994). Generally, the thickness of any bacterial biomass layer will increases, mass transport and inevitably reduce the rate of chromium detoxification (Ganguli and Tripathi, 2002).

In this study,nine dichromate resistance bacteria, isolated from the leather tanning factories from the El-Riyadh industrial area were used to study the role of the enzyme chromium reductase in the detoxification of potassium dichromate (Cr^{+6}) .

Materials and methods

Preparation of Bacteria Isolates

The following nine bacteria were isolated from highly potassium dichromate (Cr^{+6}) contaminated soil, solid wastes and discharged water associated with leather tanning factories: *Bacillus cereus*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis, Acinetobacter radioresistens*, *Acinetobacter venetianus, Ochrobacterum* sp, *Massilia niabensis*, and *Leucobac-terchromiireducens.*

Assay of Enzymatic Activity

In order to measure the activity of the enzymes, 500 ml of bacterial cultures in L.B broth were amended with 50 ppm of Cr^{+6} for 5 days (in order to insure the maximum yield of bacterial cells). The cells were then harvested by centrifugation at 600 rpm for 10 min and any pellets deposited at the bottom of the centrifuge tubes were washed twice with sterile 0.85% NaCl solution. The washed pellets were then re-suspended at 2-3 gram wet weight per 10 mL sterile 0.85% NaCl. Re-suspended cells were disrupted using a 3mm diameter micro tip mounted to the Ultra High Intensity Ultrasonic Processor 500 watt model (VCX 500, SONICS and Materials, Inc., Newtown, CT).

Medium (5ml) was then transferred into tubes and stored at -20°C for later analysis. The bacterial cells were transferred to tubes aseptically and also stored at-20°C. Frozen cells were allowed to thaw in a water bath set to 37°C. The tubes containing concentrated cells were placed inside an ice container to avoid overheating during sanitation. The tip was previously cleaned with ethanol and dried thoroughly before use. Cells were the exposed to four cycles of 15 min with 5 min rests between cycles. Disrupted cells were centrifuged at 1200 rpm for 20 min to remove un-disrupted cells and any large pieces of cellular debris. Two 100 mg/l Cr^{6+} batches were prepared, one containing undisrupted cells and cellular fragmented membranes and the other supernatant and cytoplasmic materials. Cr^{6+} reduction was then monitored and compared with Cr^{6+} reduction rates in intact cells. All experiments were conducted in duplicate. The supernatant suspected of containing enzymes was finally separated in freezing tubes and stored at -20°C. The concentration of hexavalent chromium was determined calorimetrically (at OD_{540}) using the diphenylcarbazide method (Spectronic 1001, Milton Roy Co., Rochester, NY). Estimation of enzymatic activity whether intracellular or extracellular involved the use of a Cr^{+6} stock concentration 1000ppm and a $NADH^{+}$ concentration 10% or 20% (depending on the required amount, i.e. 0.1 or 0.2).

Preparation of standard curve

In order to measure hexavalent chromium, the1,5-diphenylcarbazide reaction was used (Pflaum and Howick, 1956). A linear Cr^{+6} standard curve (Fig. 4) was generated by plotting absorbance (at 540 nm). The standard curve for Cr^{+6} measured demonstrated a high degree of reproducibility (R^2 = 99.5 %) for a composite data set from predetermined points. This standard curve was then used to determine the Cr^{+6} concentration at OD_{540} using a spectrophotometer. Using the standard curve, results were converted from OD_{540} to ppm. The line equation is of our spectrophotometer is:$x_{ppm} = 0.0122x_{OD} + 0.0255$, and conversion to mM by:$x_{mM} = 0.0027x_{ppm}$.

Results

Percentage of potassium dichromate reduction in minimal medium in the presence of NDAH+ and without NDAH+

Chromium reductase can be either a primary or secondary product inside bacterial cells and if the bacterium produces reductase in the log phase it can play an important role in their survival by using reduction of Cr^{+6} either as an energy source, or to coexist with toxicity. If the enzyme is produced in the stationary phase (essentially the enzyme seen in the medium) it is a secondary product and is therefore not used as a main energy source. Although bacteria, withstand or resist, Cr^{+6} without producing reductases as a primary product, they also use other resistance mechanisms. The diphenylcarbazide method (APHA, 1989) was used for the detection of potassium dichromate Cr^{6+}. The percentage of potassium dichromate reduction by bacteria in minimal medium amended with $NDAH^{+}$ clearly exceeded dichromate percentage reductions achieved by bacteria in the medium without $NADH^{+}$ dehydrogenase (Table 1). The rate of dichromate reduction by bacteria increased over the growth period from 24 h up to 96 h, to reach complete dichromate reduction in seven of the species in the medium amended with $NDAH^{+}$ compared to a maximum Cr^{+6} reduction of only 62% by only one bacteria species (*A. radioresistens)* in medium lacking $NDAH^{+}$.

The following bacteria can secrete enzymes both internally intracellular enzymes (In) and externally, i.e. "chromate reductase" (Ex) or supernatant in broadly semi equal amounts and reduce potassium dichromate at 100 ppm concentration (Table 2 and Figs. 1-3), *B. cereus* (In 41%, Ex 38%), *B. subtilis* (In 47%, Ex 37%), *B. licheniformis* (In 34%, Ex 28%), *B. pumilus* (In 43%, Ex 36%). Other bacteria were seen to secrete chromate reductase internally more than they do externally (supernatant), i.e., *Massilia niabensis* (In: 37%, Ex:19%), *Leucobacter chromiireducens* (In:48%, Ex:14%), *Ochrobacterum sp* (In:33%, Ex:15%), *Acinetobacter ventianus* (In:35%, Ex:19%). *Acinetobacter radioresistens* was

the only species found to produce the enzyme extracellularly in the culture supernatant more than intracellulary and as a result led to reductions of about (In: 26%, Ex:52%). The percentage of dichromate Cr^{+6} reduction increased continuously with increase in inoculation period, and reduced 100% at 96 h by all species except *B. pumilus, B. licheniforms* and *M. niabensis* which reduced Cr^{+6} at rates of 86, 73 and 91% respectively; *A. radioresistens* and *A. rventianus* reduced dichromate at 100% at 72 h. in the presence of $NDAH^{+}$.

Table 1. Percentage potassium dichromate reduction assay in minimal medium with NDAH+ and without NDAH+.

100 ppm Concentration of Cr^{6+} OD 540 at pH = 7, 37°C with ($NADH^{+}$) Bacteria cells = 6µl										
Species	% ohr		24 h % 24hr		48hr % 48hr		72 h % 72hr		96 h % 96hr	
	without	NDAH	without	NDAH	without	NDAH	without	NDAH	without	NDAH
B. cereus	0	0	19	56	23	76	47	98	50	100
B. pumilus	0	0	23	53	31	73	42	86	42	86
B. licheniforms	0	0	16	26	19	56	41	69	42	73
B. subtilis	0	0	19	53	23	79	45	94	46	100
A. radioresistens	0	0	23	56	32	76	58	91	62	100
A. radioresistens	0	0	22	64	25	85	50	100	54	100
A. rventianus	0	0	16	61	19	80	38	100	39	100
Ochrobacterumsp	0	0	12	50	16	70	34	89	36	100
M. niabensis	0	0	22	41	23	64	46	89	46	91
L.chromiireducens	0	0	19	56	23	76	47	91	50	100
Control without $NADH^{+}$	0.35	0	0.35	00	0.35	00	0.35	00	0.35	00
Control with $NADH^{+}$	0.34	0	0.34	00	0.34	03	0.34	03	0.33	06

$NADH^{+}$ is a dehydrogenase enzyme

Table 2. Enzymatic activity assay of intracellular and supernatant with $NADH^{+}$.

Result of Enzymatic activity assay when 100 ppm of Cr^{6+} at 540 nm (OD_{540})								
Species	Con. $NADH^{+}$	%R Con. IN	Precipitate Enzymes in Intracellular	%R IN	Con. with $NADH^{+}$	%R Con.EX	Supernatant Enzymes in Extracellular	%R EX
B. cereus	1.97	0	1.18	41	1.65	0	1.03	38
A. ventianus	1.97	0	1.29	35	1.65	0	1.34	19
B. pumilus	1.97	0	1.16	43	1.65	0	1.07	36
M. niabensis	1.97	0	1.24	37	1.65	0	1.34	19
B. licheniformis	1.97	0	1.30	34	1.65	0	1.20	28
L. chromiireducens	1.97	0	1.04	48	1.65	0	1.43	14
A. radioresistens	1.97	0	1.46	26	1.65	0	0.80	52
Ochrobacterumsp	1.97	0	1.33	33	1.65	0	1.41	15
B. subtilis	1.97	0	1.05	47	1.65	0	1.04	37
Control with $NADH^{+}$		0	1.97			0	1.65	00
Control without $NADH^{+}$		0	2.09			0	1.70	00

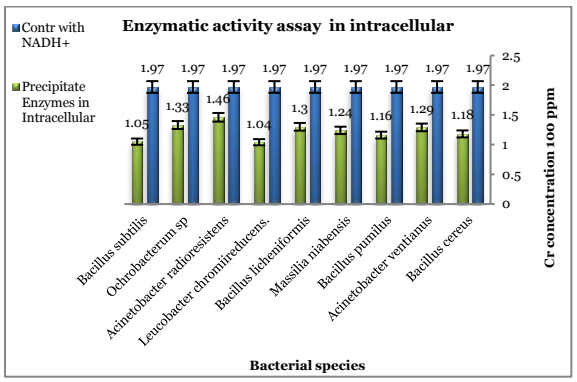

Fig. 1. Overall rate of enzymatic activity assay in intracellular.

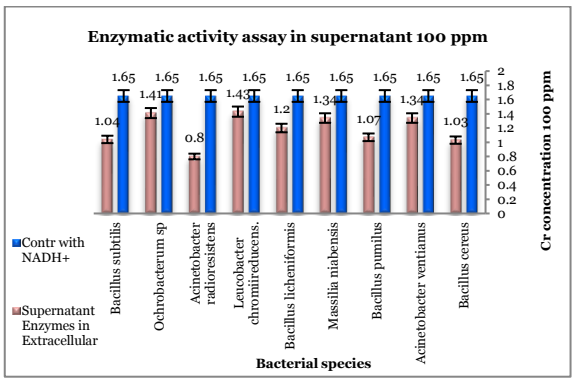

Fig. 2. Overall rates of enzymatic activity assay in supernatant.

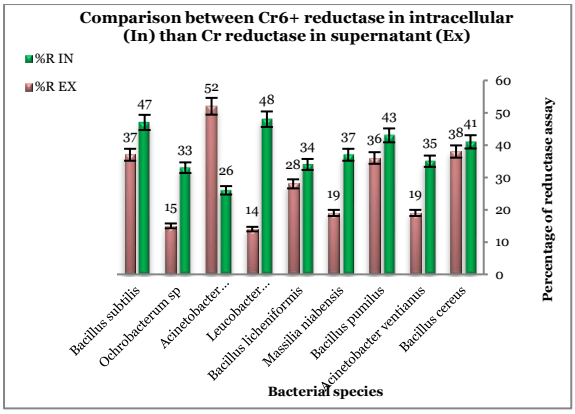

Fig. 3. Bacterial intracellular reductase activity and Supernatant reductase activity.

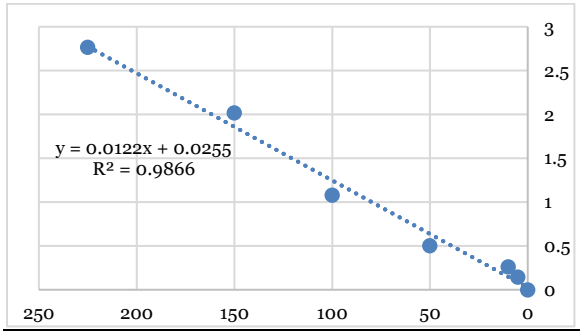

Fig. 4. A linearized Cr^{+6} standard curve.

Discussion

Some bacterial species can secrete chromate reductase that has the ability to reduce potassium dichromate both intracellularly and extracellularly (i.e. in the supernatant) in roughly semi- equal amounts, as was show here for *Bacilluss,* while *Acientobacter radioresistance* was the only bacterium shown to secrete chromate reductase externally (in the supernatant) more than it did internally. Other bacteria secrete intracellular enzymes more than they do extracellular ones. In this study, *B. cereus* brought about potassium dichromate reduction by secreting intracellular (41%) or extracellular (38%) enzyme. *Bacillus pumilus* also induced potassium dichromate reduction using intracellular (43%) and extracellular (36%) enzyme, as did *B. subtilis* (intracellular 47% and extracellular 37%) and finally, *B. licheniformis* (intracellular 34% and extracellular 28%). *Acinetobacter radioresistence* was the only bacterium that was found to secrete more extracellular (52%) than intracellular enzyme (26%).

The diverse characteristics of some ancient enzymes together with their widespread distribution supports the hypothesis that the reduction of chromate is a secondary role for chromium reductase (Cervantes *et al* 2002). Dichromate Cr^{6+} may be reduced either as a response to Cr^{6+} toxicity or as a result of a physiological need to conserve energy in the cell through a dissimilatory pathway reaction. In extracellular, dichromate Cr^{6+} can be released into the supernatant from the cytoplasm in the form of $Cr(OH)_3$. The formation of $Cr(OH)_3$ under the higher pH intracellular environment is expected and represents a physiological reaction which protects cells by forming a barrier to Cr^{6+} toxicity and confers a low cell membrane permeability to the ion (Chen and Hao, 1998). Chromate Cr^{6+} reduction under aerobic condition is commonly associated with soluble chromate reductases that use $NADH^+$ or $NADPH^+$ as cofactors (Cervantes *et al.,* 2007).

$NADH^+$ was used in this study as a co- enzyme; the most important mechanism observed involving detoxification of the toxic cation or anion enzymatically through converting it from a more toxic to a less toxic form. In intracellular processes, dichromate Cr^{6+} is reduced in the cytosol via cytoplasmic soluble reductase. In general, Cr^{6+} reduction maybe achieved via a chromium reductase, or as a two or three step process with Cr^{6+}initially reduced to the short-lived intermediates Cr^{5+} and /or Cr^{4+} before further reduction to the thermodynamically stable end product $Cr^{3+}NADH^+$/; electrons from endogenous reserves were implicated as electron donors in the Cr^{6+} reduction process by Appenroth *et al* (2000).

The electron donors implicated in a dichromate Cr^{6+} reduction is $NADH^+$; which is active over a wide range of temperatures above (40°C) and pH (6 to 9) (Ackerley. *et al.,* 2004). In an earlier study, McLean and Beveridge (2001) found that Cr^{6+} reduction by a *Pseudomonad* (CRB5) was largely contained within in soluble cell fractions.

Since the reduction of chromate is associated with the soluble fraction, the enzyme responsible could be either cytoplasmic or periplasmic in origin.

Shen and Wang (1993) using mass balance studies showed that Cr^{6+} reduction pathways occurred both inside and outside the cells and that Cr^{6+} reduction can take place through the membrane electron chain respiratory pathway. It has also also suggested that the reductase may be exported to the medium, and Cr^{6+} is reduced in the external environment. In membrane-associated enzymes for Cr^{6+} reduction, a constitutive enzyme mediates the transfer electrons from intercellular electron donors such as $NADH^{+}$ to Cr^{6+} is the terminal electron acceptor.

$NADH^{+}$ - dehydrogenase enzyme catalysis the chemical reaction:

$NADH^{+} + H^{+}$ + acceptor ↔ NAD^{+} + reduced acceptor

At the molecular level, dichromate Cr^{6+} reduction has been shown to be encoded on the *ChrR* which produces the reductase *ChrR*. This enzyme catalyzes the transfer of electrons from $NADH^{+}$, and other endogenous electron reserves to Cr^{6+}. Some 70% of total chromium tends to remain in the supernatant and 30% is generally attached to the cell surface. (Appenroth *et al.,* 2000). Smith and Gadd (2000) established the extra-cellular Cr^{6+} reduction pathways in sulfate-reducing bacteria through a mass balance in which 90% of reduced Cr^{6+} was detected in the supernatant. This could be achieved through secretion or through cell lysis (McLean and Beveridge, 2001); dichromate Cr^{6+} reductase activity is associated with the soluble fraction of the cells and not extracellular (Thacker *et al.,* 2006).

Other studies have shown that Cr^{6+} reduction activity is enhanced following the addition of external electron donors such as $NADH^{+}$. The addition of $NADH^{+}$ was shown to enhance the reduction of Cr^{6+} by *Providencia* sp. (Thacker *et al.,* 2006), *Pseudomonas ambigua* G-1 (Suzuki *et al.,* 1992), and *E. coli* ATCC 33456 (Shen and Wang, 1993).

Conclusion

One of the major results obtained in this study is that the isolated bacteriacan reduce high concentrations of hexavalent chromium and thus detoxify hexavalent chromium Cr^{+6} to non-soluble, less-toxic Cr^{+3}; Such an ability can facilitate the bioremediation of industrial waste and reduce the environmental impact of hexavalent chromium. The nine bacteria isolated from the leather tanning factories in Riyadh industrial area were shown to secrete chromium reductase both intracellular and extracellular and the addition of dehydrogenase $NADH^{+}$ significantly enhanced chromium Cr^{+6} absorption and detoxification by the bacteria. Finally, potassium dichromate Cr^{6+} absorption from the growth medium by bacteria species was seen to increase over the 96 h incubation period.

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