A Novel Chromate Reductase from Bacillus sp. ES29: Characterization and Partial Purification

Uma Nova Cromo Redutase de Bacillus sp. ES29: Caracterização e Purificação Parcial

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Abstract: The use of microorganisms for reduction of Cr(VI) to Cr(III), or their enzymes, is an attractive strategy that is cost-effective, safe and produces no secondary by-products. In the study of culture conditions and time sonication showed that using low concentrations of Cr (IV) in the medium, growing by 16 hours, provided the highest production of enzymes. Using 40 seconds of time sonication from 40 to 50 mM phosphate buffer, it was obtained the higher enzyme activity for chromium reductase of *Bacillus sp*. ES29 (CRB). Later, a partial purification was performed taking place with ammonium sulfate precipitation, gel filtration and hydrophobic interaction, where were obtained the purification factor of 11.22 times. Further studies are needed to obtain a purified enzyme, amino acid sequencing of the protein to better understand the mechanism of reduction of chromium and for the viability of this enzyme use in remediation processes.

Key words: chromatograghy; culture conditions; precipitation; sonication.

Resumo: O uso de micro-organismos para a redução de Cr(VI) para Cr(III), ou suas enzimas, é uma estratégia atrativa, segura, barata e que não produz subprodutos secundários. No estudo das condições de cultura e sonicação, foi observado que utilizando baixas concentrações de Cr (IV) no meio e cultivando por dezesseis horas, tem-se a maior produção de enzimas. Utilizando o tempo de sonicação de quarenta segundos e tampão fosfato 50 mM, obteve-se a maior atividade enzimática para cromo reducase de *Bacillus sp*. ES29. Após esse procedimento, foi realizada uma purificação parcial, realizando-se precipitação com sulfato de amônia, gel filtração e interação hidrofóbica. Neste caso, foi obtido um fator de purificação de 11,22 vezes. Estudos adicionais serão necessários para obter uma enzima purificada, além do sequenciamento dos aminoácidos da proteína. Outrossim, espera-se compreender melhor o mecanismo de redução do cromo e viabilizar o uso desta enzima em processos de remediação.

Palavras-chave: condições de cultura; cromatografia; precipitação; sonicação.

1 Introduction

Hexavalent chromium is widely used in industry including leather tanning, electroplating, paint pigment, dye manufacturing and steel production as well as at Department of Energy (DOE) facilities [1]. The hexavalent form of Cr is highly soluble, mobile and consequently widespread in polluted environments. Chromium in this form is a known carcinogen and mutagen in humans and animals. Besides its adverse health effects, Cr(VI) inhibits the growth of plants due to root damage [2]. On the other side, trivalent chromium is more insoluble, less bioavailable and less toxic to the cell as compared to hexavalent chromium [3].

The use of microorganisms for reduction of Cr(VI) to Cr(III) is an attractive strategy that is cost-effective, safe and produces no secondary by-products. Bioreduction of Cr(VI) can occur directly, as a result of microbial metabolism (enzymatic), or indirectly, through a bacterial metabolite such as H2S [4]. Two enzymatic mechanisms are reported to reduce Cr(VI), both through metabolic activities of the chromate reducing *Bacillus sp*. ES29 (CRB). In aerobic conditions, most of the enzyme chromate reductase activity reported is soluble in the cytosol and reduces Cr(VI) to Cr(III) inside or outside the plasma membrane [5, 6]. Under anaerobic conditions, CrO42- is used as a terminal electron acceptor and is reduced in the membrane during anaerobic respiration [7].

The main mechanism used for Cr(VI) reduction involves chromate reductases produced by Gram-negative strains like *Escherichia coli* [8], *Rhodobacter sphaeroides* [9], *Pseudomonas putida* [10] and *Pseudomonas ambigua* [11]. Purification and partial characterization of chromate reductase produced by a Gram-positive bacterium was only reported by [12]. More recently, [13] assessed *in vitro* reduction of hexavalent chromium using a cell-free extract of a CRB isolated from soil contaminated with dichromate. This Gram-positive bacterial isolate reduced Cr(VI) under aerobic conditions, using NADH as an electron donor and produced a soluble Cr(VI)-reducing enzyme stimulated by copper (Cu^{2+}). The cell-free extract (CFE) of the bacterial isolate reduced 50% of Cr(VI) in six hours. The Cr(VI)-reduction activity of the CFE presented a K_M of 7.09 M and a V_{max} of 1.171 M min^{-1} mg^{-1} protein. In this paper, we report the characterization of the enzyme chromate reductase produced by a *Bacillus sp* ES29 and partial purification.

2 Material and methods

2.1 Microorganism and culture conditions

A chromate-resistant bacterial isolate was isolated from soil samples obtained from a landfarming site in south Brazil contaminated with dichromate. The isolate reduced Cr(VI) under aerobic conditions and produced a soluble Cr(VI)-reducing enzyme stimulated by copper (Cu^{2+}) and was identified by 16S rRNA gene sequencing as a *Bacillus sp*.ES29 (ATCC: BAA-696) [13]. Cells were grown overnight in 500 mL of Luria-Bertani (LB) medium containing Cr(VI) at 30 ºC with orbital shaking (150 rpm). Thereafter, cells were harvested by centrifugation (4 800 x *g*), and washed twice with phosphate buffer (0.1 mM; pH 7.0). Cells were then re-suspended in 10 mL of the same buffer and chilled in an ice bath. Best culture conditions for enzyme production were evaluated, including time course of growth, induction by Cr(VI), disruption time (power was applied five times in 20, 40, 60 and 100 s pulses at 35 W each) and buffer concentration (50 and 100 mM at pH 7.0).

2.2 Chromate reducing enzyme assay

Hexavalent chromium was determined colorimetrically OD_{540} (Spectronic 1001, Milton Roy Co., Rochester, NY) using the s-diphenylcarbazide method [1] with a detection limit of 5 μg L^{-1}. Protein was determined using the Bradford method at 595 nm, with bovine serum albumin as standard [14]. The reaction mixture for the enzyme assay contained 1 mg L^{-1} of Cr(VI) as K2Cr2O7 in 0.8 mL of 50 mM phosphate buffer, pH 7.0. After 5 min of pre-incubation at 30 °C, the reaction was initiated by the addition of 0.2 mL of the enzyme (as a cell-free extract or purified fractions) and Cr(VI) reduction was measured after 10 min. One unit of enzyme activity was defined as the amount of enzyme that reduced one nM of Cr(VI) per minute at 30 °C.

2.3 Enzyme purification

Bacillus sp ES29 was grown in LB medium (500 mL) in a 1.5-liter flasks containing 2 mg L^{-1} of Cr(VI) and incubated by 16 h at 30 ºC with orbital shaking (150 rpm). Cells were centrifuged at 280 x g for 5 min at 4 ºC, suspended in phosphate buffer (50 mM, pH 7.0) and washed two times in the same buffer. Cells in ice bath were disrupted with an ultrasonic probe (Vibra Cell, Sonics & Nature, Inc. Danbury, CT) using two pulses of 20 s at 35 W each. Sonicated material of the bacterial crude extract was centrifuged at 1 120 x g at 4 ºC for 45 min and filtered (0.45 μm) to obtain the soluble extract. The protein present was precipitated with different concentrations of ammonium sulfate (45 to 75 %; 55 to 75 % and 65 to 75 %). After each stage of precipitation, samples were centrifuged at 1 120 x g by 45 min at 4 ºC and the precipitated re-suspended in 5 mL of phosphate buffer. Salt was removed from the precipitated fraction by dialysis using phosphate buffer with agitation at 4 ºC for 24 h. The fraction with the highest activity was applied to a gel filtration chromatography column (5 by 10 cm) containing Sephadex G-25 (Pharmacia®). Proteins were eluted with phosphate buffer (50 mM; pH 7.0) and the fractions containing chromate reduction activity were submitted to hydrophobic interaction chromatography column (3 by 8.5 cm) containing Octyl Sepharose 4 fast flow (Amersham®). The column was equilibrated with phosphate buffer (100 mM; pH 7.0) and unbound with this buffer and with a linear gradient of (NH4)2SO4 of up to 1.0 M and phosphate buffer. Selected fractions were evaluated for absorbance at 280 nm and for chromate reduction activity. Samples from the ammonium sulfate fractionation, gel filtration and hydrophobic interaction chromatography were submitted to SDS-PAGE. Samples containing 2 % SDS, 10 % glycerol and 10 % 2-mercaptoethanol were denatured by boiling at 100 ºC for 5 min. Proteins were separated by 7% SDS-PAGE according to [5] and were visualized with a 1 % Coomassie brilhant blue R-250 (Sigma) solution, 40 % methanol, 10 % acetic acid and 50 % distillated water. Proteins of known molecular mass like myosin (200KDa), β-galactosydase (150KDa), bovine serum albumin (66 KDa), ovalbumin (43 KDa), tripsine soybean inhibitor (20 KDa), lysozime (14

KDa) and aprotinin (6.5 KDa) were used as reference standards for native enzyme molecular mass determination.

3 Results

3.1 Growth and culture conditions for enzyme production

Time course of the *Bacillus sp*. ES29 was evaluated for growth and Cr(VI) reduction, where 50 and 100 mg of Cr(VI) L^{-1} were added (Figure 1). In the presence of 50 mg L^{-1} of Cr(VI) it was possible to observe a stationary phase of growth during the period of 24 h, at the same time occurring a sharp chromate reduction. After reduction of 80% of the chromate the curve entered the logarithmic phase of growth. Similar behavior was observed at the concentration of 100 mg L^{-1} of Cr(VI), demonstrating that the largest activity of Cr(VI) reduction occurs before the logarithmic phase. In both concentrations there was a reduction of approximately 100% of the Cr(VI) after 48 h incubation. During the same period the number of cells in both concentrations was practically the same.

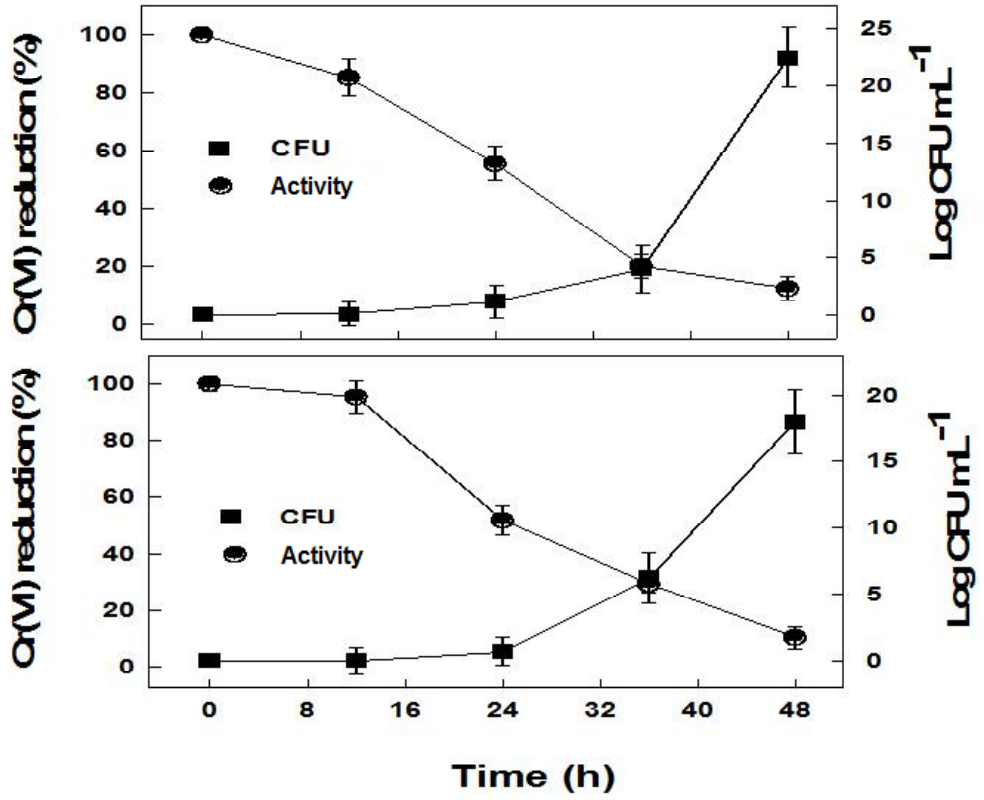

Figure 1. Cell growth and chromate (VI) reduction on LB broth at concentration of 50 mg L^{-1} (A) and 100 mg L^{-1} of Cr(VI) (B) by the Bacillus sp. ES29, were CFU is colony forming unit

Bacteria from soil samples obtained from a landfarming site contaminated with dichromate in south Brazil, that was capable to grow and to reduce Cr(VI) at concentrations ranging from 500 to 2500 mgL^{-1}. Bacterial isolate, named ES29 (ATCC: BAA-696), was identified by 16S rRNA gene sequencing and presented more than 98 % of similarity to the genus *Bacillus sp*. [1]. It was demonstrated that this isolate reduces the Cr(VI) toxicity before starting to growth. This isolate behavior is very important for bioremediation strategy. This activity of fast reduction and subsequent exponential growth was already noticed by [16]. Was reported that during the Cr(VI) reduction there was a decrease in the optical density of the isolates, and that the presence of high concentrations of the metal could be poisonous to the cells due to low cell metabolic capacity of reduction [17].

To evaluate if the enzyme it is stimulated by the presence of chromate and to verify the time of incubation for obtaining the highest enzyme production a growth curve was accomplished and the cells were lysed for determination of the Cr(VI) reduction activity (Figure 2). The cell growth tended to be smaller with the presence of chromate in the LB broth indicating that the presence of the metal reduced the growth of the cells. The largest activity of Cr(VI) reduction in the cell extract was obtained after 16 h incubation, the period used for further experiments.

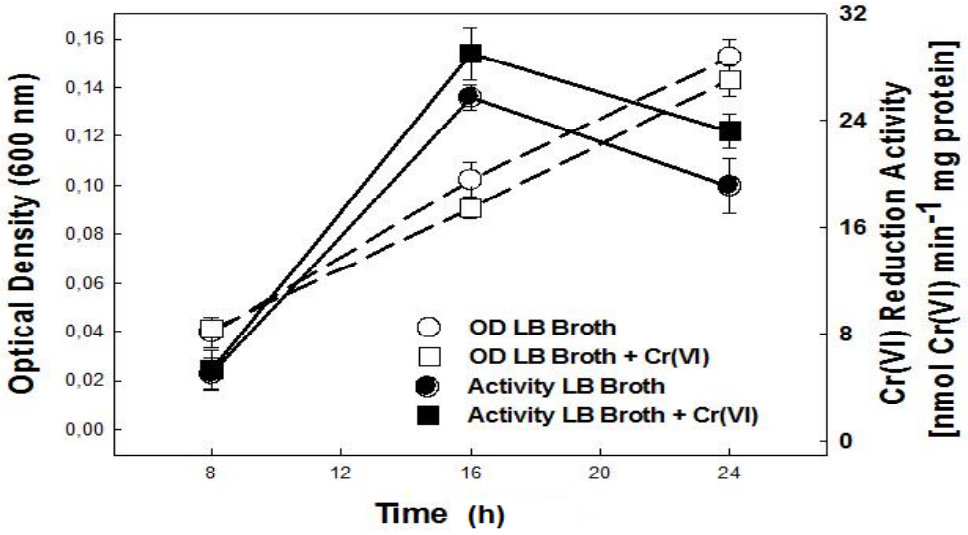

Figure 2. Chromate reductase activity during cell growth with 2 mg of Cr(VI) L^{-1} or without Cr(VI)

To obtain the cellular fraction with high enzymatic activity, cells were sonicated in ice bath at different times in order to evaluate the heating effect of sonication on enzyme denaturation. It was stipulated as 100 % the Cr(VI) reduction activity for the unbroken cells and the sonication time with the highest concentration of proteins as 100 % of soluble proteins (Figure 3). After 20 s of sonication, low enzyme activity and protein concentration were observed, probably due to the small amount of broken cells. Sonication for 60 s yielded an increase of the protein concentration, due to the efficient cell lyse, however, there was a reduction of the enzyme activity, probably due to protein denaturation by the heat. Thus, 40 s for sonication was chosen to carry out the experiments to obtain the highest enzyme activity.

The effect of phosphate buffer concentration on the Cr(VI) enzymatic activity was evaluated on the cell lyse and reaction mixture (Figure 4). In both tests it was possible to observe that the 50 mM concentration of phosphate buffer was more efficient to maintain the enzyme activity during the cellular lyses and greater activity in the reaction mixture. This fact could be due to lower concentrations of sodium and potassium ions present in this buffer in comparison to those in 100 mM buffer, where these ions might have interfered on the activity of the enzyme.

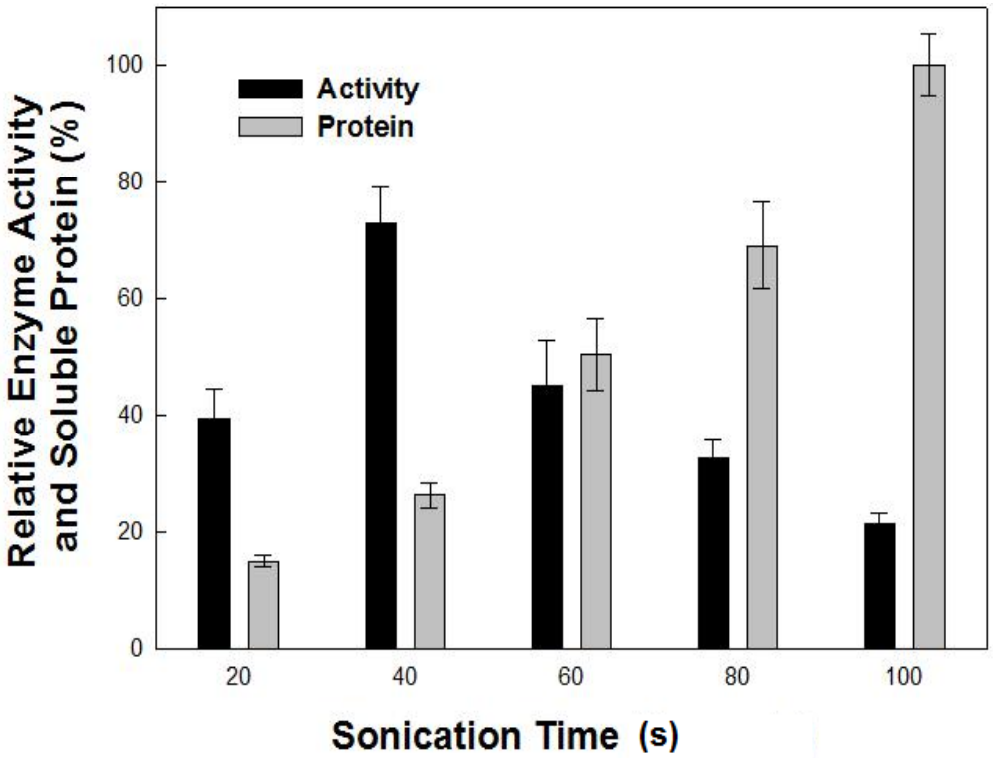

Figure 3. Chromate activity and protein content of the Bacillus sp. ES29 cell free extract after different times of sonication

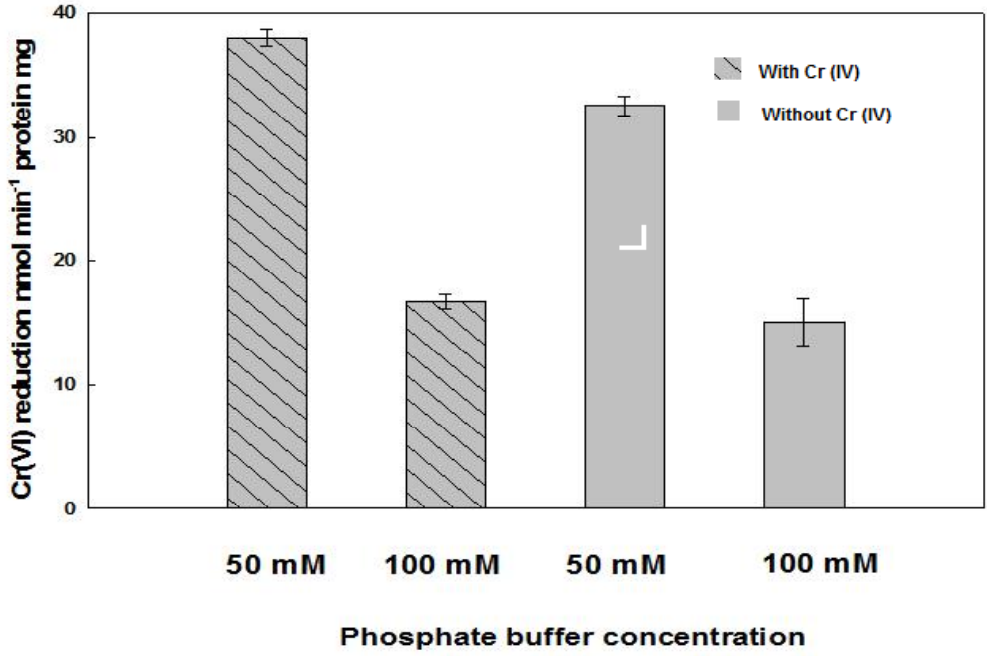

Figure 4. Chromate reduction at different buffer concentrations of the Bacillus sp. ES29 cell free extract

The tests revealed that the best conditions for the cell growth and enzymatic activity was reached after 16 h of incubation and 40 s of sonication, using 50 mM phosphate buffer. The increase on the reduction activity was verified when the cells were grown at low concentration of Cr(VI), as compared to the growth in absence of Cr(VI). The presence of the chromate might have stimulated the production of the enzyme, which probably is a substrate-induced enzyme. In the absence of Cr(VI) enzyme production could be explained by the fact that the inductive enzymes would be produced in basal level, even in the absence of the induction factor. Was evaluated the Cr(VI) reduction by some bacterial isolates described in the literature and they found an increase of the enzyme activity when the *Pseudomonas putida* KT2440 and the *Bacillus subtilis* were grown in the presence of Cr(VI), were the chromate reductase produced by the *Pseudomonas putida* KT2440 was induced by the presence of Cr(VI) and this finding was supported by Western blot analysis,

where an increase on the production of the protein ChrR (chromate reductase) was detected when the microorganism grew in the presence of Cr(VI) [3].

3.2 Chromate reducing enzyme characterization

Bacillus sp. ES29 was grown in optimized conditions to obtain the Cr(VI) reducing enzyme in LB broth (pH 7.0), with 2 mg L^{-1} of Cr(VI), at 30°C, for 16 h. The cell extract obtained was submitted to ammonium sulfate precipitation (Figure 5). The protein content and enzyme activity for each saturation step were expressed in percentage with 100 % representing the cellular extract. Precipitation at 45 to 75 % yielded up to 50 % of the activity and over 60 % of protein content; this saturation step was used for further purification stages. The other precipitation steps did not produce significant enzyme activity or protein concentration.

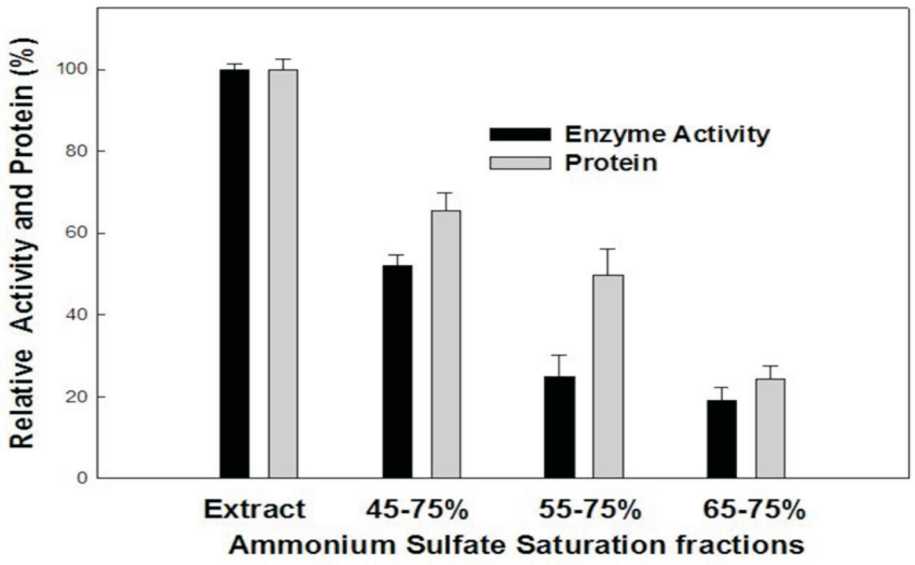

Figure 5. Chromate reductase activity and soluble protein at each stage of precipitation with ammonium sulfate

The effect of pH (5.0 to 10.0) and temperature (15 to 70 ºC) on the enzyme activity was tested in the ammonium sulfate precipitated fraction as well as the effect of non enzymatic reduction of Cr(VI) at high temperatures, low pH and in the presence of the NADH coenzyme. Both pH (Figure 6) and temperature (Figure 7) had great influence on the enzyme activity for reduction of Cr(VI). The ideal pH was pH 7.0 in phosphate buffer, with decrease in the activity at the same pH when the Tris-HCl buffer was used.

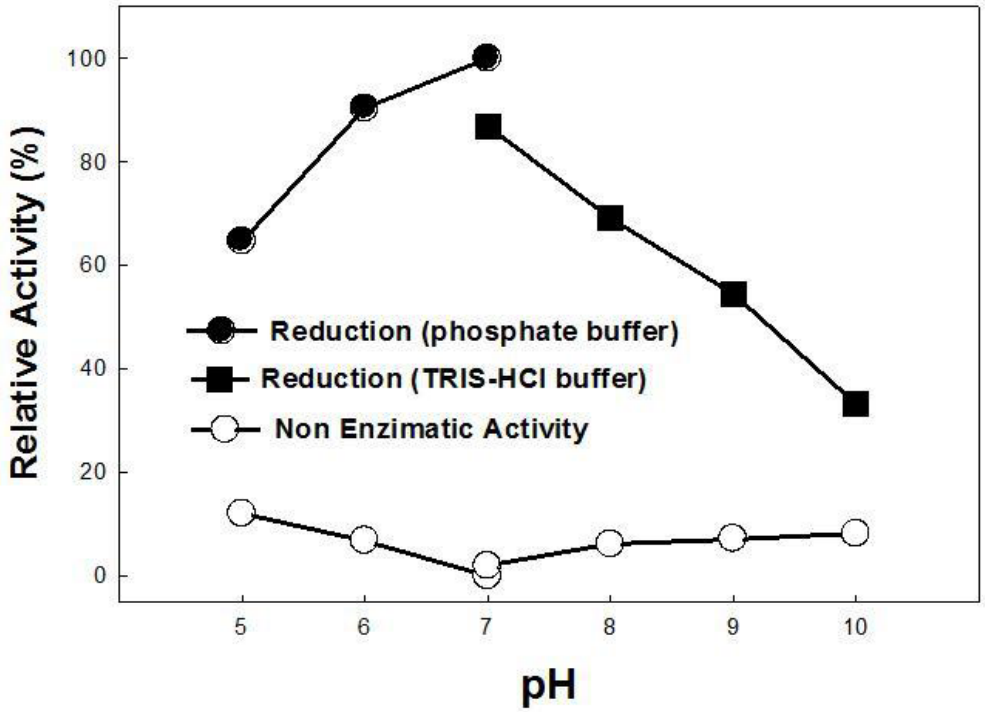

Figure 6. The pH effect on the chromate reduction activity present at the precipitated enzyme with ammonium sulfate and on the non-enzymatic reduction

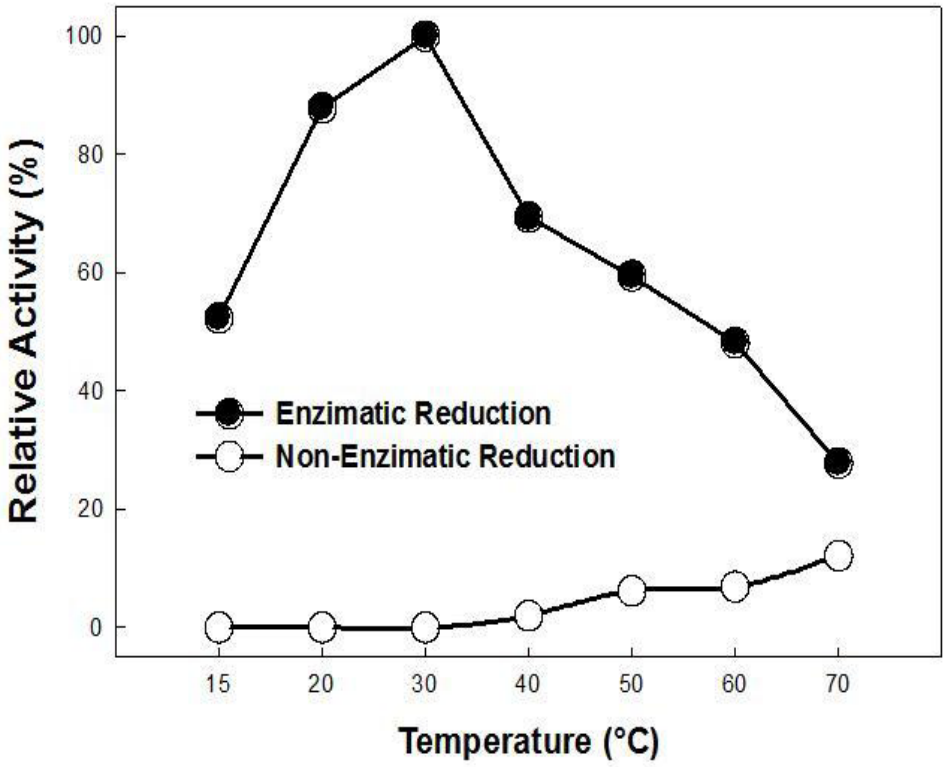

Figure 7. Temperature effect on the chromate reduction activity present at the precipitated enzyme with ammonium sulfate and on the non-enzymatic reduction

The speed of the reaction increased with the temperature until reaching maximum value, at 30 °C. This increase is probably due to the largest number of molecules with enough energy to form reaction products, like reduction of Cr(VI). Increasing the temperature up to 40 °C resulted in reduction of the activity, possibly due to the enzyme denaturation. At 20 °C it was detected 80 % of the enzyme activity, which is an important aspect to be considered for reactions occurring in environmental conditions.

Proteins precipitated on fractions by 45-75 % ammonium sulfate were submitted to a gel filtration chromatography using Sephadex G-25 column and 30 fractions of 1.8 mL were collected (Figure 8). It was observed that the fractions 3 to 9 presented the highest protein content but only the fractions 6 and 7 were mixed to be used in the next purification step, because they presented activity on Cr(VI) reduction. This procedure has demonstrated to be very useful since it was

possible to separate the chromate reductase enzyme (fractions 6 and 7) from other proteins (fractions 3, 4, 5, 8 and 9).

This precipitation technique was used by other authors, how [10] that found the highest reductase activity in the 55 to 70 % fraction during the purification of an enzyme produced by *Pseudomonas putida.* However, in our work, the use of this ammonium sulfate concentration could eliminate smaller proteins not precipitated. The purification of a NADPH-dependent Cr(VI) reductase from *Escherichia coli* and the protein precipitate was obtained at 35 to 60 % ammonium sulfate saturation [8]. Nevertheless, in spite of occurring precipitation of great part of the proteins present in the cellular extract at this concentration, it is more difficult the purification due to protein excess. Was verified by [11] that the best temperature and pH conditions for the purification chromate reductase enzyme were 6.0 to 9.0 and 40 to 70 °C, respectively. H+ concentration affects the enzymatic activity in several ways, so the enzyme analyzed here is possibly a neutral enzyme as the best activity was shown at pH 7.0.

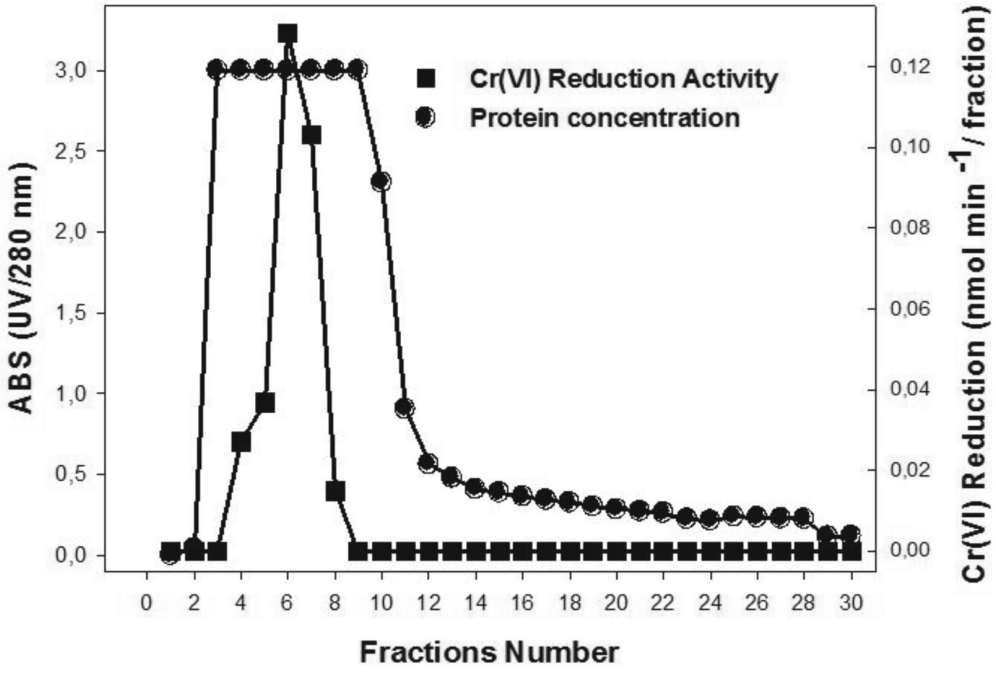

Figure 8. Gel filtration chromatogram of the ammonium sulfate fractions

The fractions 6 and 7 were mixed and applied to a. hydrophobic interaction chromatography column containing Octyl Sepharose 4 fast flow. The sample was applied into the column with 1 M ammonium sulfate and after elution of 20 mL of phosphate buffer, a gradient of ammonium sulfate (0 to 1M) was used for collection of twenty five of 1.5 mL fractions. As can be observed in Figure 9 the enzyme came out in the former fractions. A peak of proteins was observed between fractions 1 and 7 but only fractions 2, 3 and 4 were used since they presented reduction activity of Cr(VI).

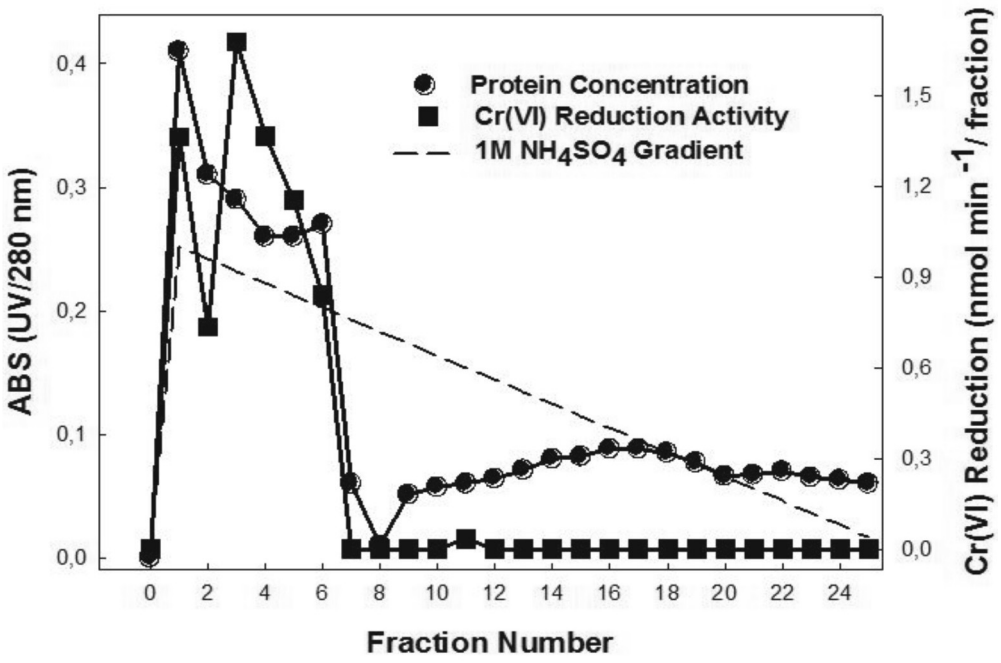

Figure 9. Hydrophobic interaction chromatography of the ammonium sulfate fractions

Partial purification steps of the CRB are presented in Table 1. The purification process showed an increase of 11.22 times in the purification factor (PF), with a yield of 2.6 %, after all stages of purification. However, these values are still low income, making sure that part of the enzyme was lost or denatured during the purification process. This could be associated with the use of ammonium sulfate, which is an agent for inhibiting the enzymatic activity or in small quantities in the eluted fractions were not used in these seeds, which were not quantified due

to limitations of the method used for measuring the activity enzyme. But, the PF found in our study is high for this type of purification and is a value widely used for commercial enzyme, where there is no need for a total purification, although more studies should be undertaken in order to optimize the process purification to increase yield, to allow its use in wastewater treatment.

Table 1. Purification steps of the CRB

Purification steps	**Total activity (U)1**	**Total Protein (mg/mL)**	**Specific activity (U/mg)**	**Yield (%)**	**Purification factor (PF)**
Cell free extract	8373.30	128.68	65.07	100.00	-
Precipitation	1156.60	5.92	195.11	14.00	3.00
Gel Filtration	800.00	1.48	537.93	9.60	8.27
Hydrophobic interaction	217.00	0.29	730.27	2.60	11.22

[1] One unit is defined as the amount of enzyme that converts 1 nM of Cr(VI) / min at 30°C

All fractions that displayed Cr(VI) reduction activity in the cell extract and after the steps including ammonium sulfate precipitation, gel filtration chromatography and hydrophobic interaction chromatography were submitted to SDS-PAGE. It was observed that the number of protein bands decreased as the purification steps were carried on. Following hydrophobic interaction chromatography three bands of 150, 43 and 20 kDa were visualized, with activity for that enzyme, demonstrating an effective partial purification of this enzyme. However, to determine its molecular weight and subsequent sequencing, additional purification steps should be performed to obtain a single and homogeneous band. The difficulty to obtain a purified chromate reductase enzyme in this work is also reported by many authors in the literature, due to the complexity of the purification process. The partial purification of an enzyme produced by *Pseudomonas putida* PRS 2000 did make for Ishibashi et

al. [18], while Suzuki et al. [11] achieved a 38-fold purification of chromate reductase from *P. ambigua* [10]. The only attempt to get a partial purification and characterization of a Gram-positive chromate reductase was carried out by Campos et al. [12] using four purification steps, obtaining a 40-fold purification and a 3% yield. Most of the chromate reductase enzymes had been shown to be composed of more than one subunit and there is no evidence if they are a dimeric or trimeric enzyme [10]. These authors described a NADH or NADPH-dependent enzyme with a 50 kDa molecular weight containing two subunits of 20 kDa. Other chromate reductases NADPH or NADH-dependent were purified and after analysis displayed two subunits of 42 kDa [8], two subunits of 25 kDa [11] and two subunits of 24 kDa [12].

Good purification level (> 600-fold) was achieved more recently by [10] who characterized a novel soluble chromate reductase from a *Pseudomonas putida* strain. Analysis of the N-terminal sequence of this protein showed the absence of methionine, which suggested to the authors to be a periplasmic protein. Further studies using this enzyme were performed and the gene *chrR* responsible for the enzyme synthesis was cloned and identified as a flavoprotein gene [3]. The chromate reductase enzyme obtained from *Rhodobacter sphaeroids* [9] was purified (40.6 fold) and the N-terminal sequence determined from a 35 kD band; no significant homology to sequences available from databanks was observed. Recently [8], purified and characterized an NADPH-dependent Cr(VI) reductase from the cytoplasm of *E. coli* ATCC 33456. After six purification steps a pure enzyme was obtained with purification fold of 967. The N-terminal analysis showed 80 % similarity with N-ethylamine reductase and no similarity with the enzyme purified by [10].

Characteristics as size, pH and temperature of action for most of the chromate reductases reported in the literature are different. Some authors attribute the presence of these enzymes in different organisms as a secondary activity with different primary roles or as enzymes with multiple metabolic functions [3]. Interestingly, great chromate reduction activity was verified in flavin reductases [19], flavoproteins [3] and soluble quinones [20], widely distributed in bacteria.

The actual classification of chromate reductases does not include those produced by Gram-positive microorganisms since there are no studies and results on purification, characterization and N-terminal sequencing.

4 Conclusion

In this study, the culture conditions and time sonication showed that using low concentrations of Cr (IV) in the medium, growing by 16 h, provided the highest production of enzymes. Using 40 s of time sonication from 40 to 50 mM phosphate buffer, was obtained the higher enzyme activity for chromium reductase of *Bacillus sp.* ES29 (CRB). The CRB was partially purified by ammonium sulfate precipitation and chromatography. Further experiments will be necessary in order to achieve a purified enzyme for protein sequencing to better understand the mechanism of chromate reduction and to improve the knowledge for the possible use of this enzyme in remediation processes.

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