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Effects of Chemical Inhibitors on Partially Purified Chromate (VI) Reductase Activity in *Trebouxia erici*

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Abstract

Hexavalent chromium [Cr(VI)] is a widespread industrial pollutant that poses severe environmental and health risks due to its high solubility, mobility, and carcinogenicity. Bioreduction of Cr(VI) to the less toxic and more stable trivalent form [Cr(III)] offers a sustainable alternative to physicochemical detoxification methods. The green alga *Trebouxia erici*, a lichen photobiont frequently inhabiting metal-stressed environments, has emerged as a promising candidate for biological chromium reduction. In this study, chromate (VI) reductase activity from *T. erici* was partially purified using ammonium sulphate precipitation and dialysis, and its sensitivity to chemical inhibitors was examined. Enzyme activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm in the presence of respiratory inhibitors, thiol-blocking agents, chelators, and metal ions. The enzyme exhibited NADH-dependent Cr(VI) reduction with measurable baseline activity. Sodium azide and potassium cyanide strongly inhibited activity (<50% residual), while mercury ions nearly abolished it (<90% residual). Iodoacetate caused moderate inhibition (~65% residual), indicating involvement of thiol groups. EDTA produced concentration-dependent inhibition, reducing activity to ~40% at 5 mM, suggesting a requirement for divalent metal cofactors or metal-associated electron transfer. However, Mg^{2+} and Ca^{2+} had negligible effects, implying tolerance to non-disruptive cations. Collectively, these results indicate that *T. erici* chromate (VI) reductase depends on redox-active cofactors and is highly susceptible to heavy metals and chelating agents, yet retains activity under less competitive ionic conditions. The enzyme's inhibition profile highlights both its promise as a biocatalyst for Cr(VI) detoxification and its limitations in chemically complex environments. Further studies on cofactor interactions, structural stabilization, and kinetic optimization are warranted to enhance its bioremediation potential.

Keywords: *Trebouxia erici*, chromate (VI) reductase, enzyme inhibition, EDTA, heavy metals, bioremediation.

Introduction

The hexavalent chromium (Cr(VI)) problem that poses serious threat to health and environment has been the subject of numerous studies, reflected in an extensive body of scientific literature [1]. Hexavalent chromium (Cr(VI)), a highly toxic and carcinogenic substance, is one such legacy, and its presence in ecosystems is now a global problem

[2]. The widespread use of chromium compounds in industries such as electroplating, leather tanning, pigment production, metallurgy, wood preservation, and many others has led to massive discharges of chromium-containing wastewater over decades [3][4][5]. As a result, Cr(VI) is a common and dangerous pollutant in industrial effluents, soils, and alarmingly, in drinking water sources worldwide [6][7][8].

In its two most prevalent oxidation forms, trivalent (Cr(III)) and hexavalent (Cr(VI), Cr has completely different toxicity and essentiality in human health, as well as in the soil. In humans, Cr(III) is a necessary micronutrient for metabolism of glucose, lipid, and proteins. Cr(III) is a component of glucose tolerance factor (GTF), which is generated in vivo from absorbed dietary Cr and regulates the rate of glucose elimination from the bloodstream through an insulin-boosting mechanism [7,9,]. Cr(III) ions play a role in activating the insulin receptor tyrosine kinase, which enhances insulin activity and potentiates it three-fold. Cr(III) ions deficiency can thus contribute to carbohydrate-related diseases and weight loss [10][11].

On the contrary, Cr(VI) has no recognized biological functions and is a potent carcinogen [12]. Chromium(VI) [Cr(VI)]—the hexavalent form is far more toxic, mobile, and carcinogenic [13]. Cr(VI) readily penetrates biological membranes, induces oxidative stress, and damages cellular macromolecules [14]. Toxic amounts of Cr(VI) ions in humans can damage the skin and cause irritations, rashes, skin lesions, nasal and skin inflammation, hearing impairment, lung cancer, and respiratory diseases [15]. Cr(VI) is also known to accumulate in the placenta of the mammal fetus, leading to developmental disorders [16]. In the soil, Cr(VI) pollution poses a danger to earthworms' gut epithelium, gut membrane, nuclear membrane, and mitochondrial function, as well as having an effect on metabolic processes such as osmoregulation, NA metabolism, and energy metabolism [17].

Chromate (VI) reductase is a microbial enzyme system that catalyzes the reduction of toxic Cr(VI) to the less soluble and less toxic Cr(III) [18]. This biotransformation has significant ecological relevance since it provides a biological means of detoxification and environmental restoration. However, the catalytic efficiency of this enzyme can be influenced by various chemical inhibitors, such as metal ions, metabolic intermediates, or synthetic compounds, which may alter enzyme native conformation, electron transfer pathways, or substrate binding [19]. Studying inhibitor effects is crucial for understanding the mechanistic properties of chromate reductases and for designing bioremediation strategies under realistic, contaminant-rich conditions.

Trebouxia erici, a green microalga that functions as a lichen photobiont, has recently gained attention for its remarkable resilience in heavy metal–polluted environments. Reports indicate that it harbours enzymes capable of reducing toxic metal ions, making it a potential candidate for eco-

friendly chromium detoxification [20][21]. However, limited information exists on the enzymology of its chromate(VI) reductase, particularly regarding its susceptibility to inhibitors. Since naturally contaminated sites are rarely free from other chemical agents, evaluating inhibitor interactions is essential to determine the enzyme's scientific and practical applicability.

Scientifically, it provides biochemical evidence on the inhibition kinetics of chromate (VI) reductase, expanding knowledge on algal enzymology. Practically, it offers baseline data for assessing the feasibility of deploying *T. erici* in real-world chromium-polluted sites, where inhibitors may compromise detoxification efficiency. Therefore, an understanding of the enzyme's inhibition profiles is critical in optimizing environmental conditions to improving its bioremediation potential.

Despite increasing recognition of microalgae in heavy metal detoxification, the mechanistic understanding of chromate(VI) reductase in *Trebouxia erici* remains poorly defined. Specifically, the influence of different chemical inhibitors on its activity is yet to be fully documented in pollution chemistry. This knowledge gap limits our ability to predict how effectively *T. erici* can function in complex, inhibitor-rich polluted environments. Without such insights, efforts to harness this organism for bioremediation remain a mirage.

Therefore, there is the need to investigate the effects of chemical inhibitors on partially purified chromate(VI) reductase activity from *Trebouxia erici* which can bridge the knowledge gap into optimizing bioremediation processes, enhancing Cr(VI) removal, and mitigating environmental pollution through contribution to the development of efficient bioremediation strategies.

Materials and Method

Plant material collection and preparation

Treboxia erici samples were harvested from naturally occurring lichen thalli collected at the Mahuta area in Kaduna South, Nigeria. It was authenticated by a botanist at the Department of Biological sciences, Kaduna State University, Kaduna, Nigeria. Voucher specimens of the lichen were deposited at the University Herbarium Samples were manually separated from fungal components under a stereomicroscope and thoroughly washed with sterile distilled water to remove debris. The algal biomass was air-dried at room temperature, homogenized with liquid nitrogen using a chilled mortar and pestle, and stored at –10 °C until use.

Chemicals and Reagents

Potassium dichromate ($K_2Cr_2O_7$), Tris-HCl, NADH, EDTA, DTT, and inhibitors (e.g., sodium azide, cyanide, iodoacetate, heavy metal salts) were obtained from Sigma-Aldrich (St. Louis, MO, USA, 2020). Distilled water was used in all preparations. All chemicals used were of analytical

grade and supplied by reputable chemical manufacturers.

Instruments and Equipment

UV–Vis Spectrophotometer (Shimadzu UV-1800, Japan, 2020) for enzyme activity assays, Refrigerated centrifuge (Eppendorf 5810R, Germany, 2019) for protein fractionation, pH meter (Mettler Toledo SevenCompact S210, Switzerland, 2020) for buffer preparation, Analytical balance (Sartorius Entris 224-1S, Germany, 2021) and Incubator shaker (New Brunswick Innova 44, USA, 2018). Other instruments and equipment used were of laboratory standard.

Enzyme Extraction and Partial Purification

Algal homogenates were suspended in 50 mM Tris-HCl buffer (pH 7.2) containing 1 mM EDTA and 1 mM DTT. Crude extracts were clarified by centrifugation at 12,000 × g for 20 min at 4 °C. The supernatant was subjected to ammonium sulphate precipitation (30–70% saturation) followed by dialysis against the same buffer overnight. Protein concentration was determined by the Bradford method [22] using bovine serum albumin as standard.

Stock Solution Preparation

The following stock were prepared for the study:

Chromate stock: 100 mM potassium dichromate was prepared in distilled water and stored at 4 °C.

NADH stock: 10 mM NADH solution was prepared fresh in ice-cold buffer before each assay.

Inhibitor stocks: Each inhibitor was prepared at 10–50 mM depending on solubility, sterilized by filtration, and stored at recommended conditions.

Enzyme Activity Assay

Chromate (VI) reductase activity was assayed by monitoring the decrease in absorbance of NADH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) as described by Park[23] using a Shimadzu UV-1800 spectrophotometer. Reaction mixtures contained 50 mM Tris-HCl (pH 7.2), 0.2 mM NADH, and varying concentrations of Cr(VI) in a final volume of 1.0 mL. Enzyme activity was expressed as μmol of NADH oxidized per min per mg protein.

Inhibitor Analysis

To determine inhibitor effects, enzyme assays were performed in the presence of selected inhibitors (Sodium azide, EDTA, ethyliodoacetate, mercaptoethanol, potassium cyanide, Hg^{2+}.Mg,Ca, Cu} at different concentrations as prescribed by Park *et al.*, [23]. Residual activity was compared to control assays lacking inhibitors. Data were analyzed using GraphPad Prism (v9.0, GraphPad Software, USA, 2021).

Results and Discussion

Fig 1: Lichen photobiont*

*Source: Picture taken beside one of River Kaduna tributaries, Mahuta, Kaduna South, Kaduna State, Nigeria

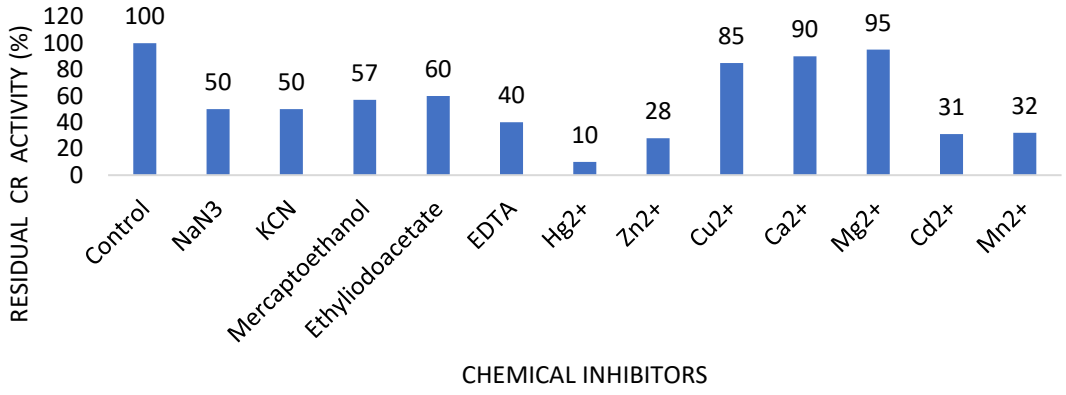

Fig. 2: Effect of Chemical Inhibitors on CR Activity

Discussion of Results

Partial Purification of Chromate(VI) Reductase

Chromate(VI) reductase was partially purified from *Trebouxia erici* cell extracts by ammonium sulfate fractionation (30–70% saturation) followed by dialysis. The purification process resulted in a 2.23-fold increase in specific activity with a recovery yield of 33.3%. The Bradford assay confirmed protein concentration within the range of 3.0–12.0 mg/mL.

Baseline Enzyme Activity

Under standard assay conditions (50 mM Tris-HCl, pH 7.2; 0.2 mM NADH; 100 µM $K_2Cr_2O_7$),

the partially purified enzyme exhibited a specific activity of 115 µmol NADH oxidized min^{-1} mg^{-1} protein]. Activity increased proportionally with substrate concentration up to 65 µM Cr(VI)], after which saturation was observed. The apparent Km and Vmax values for **Cr(V**I) were estimated as 128 µM and 0.105 µmol/min/mg, respectively.

Effect of Chemical Inhibitors

The addition of chemical inhibitors resulted in varying degrees of enzyme inhibition (Fig. 2).

- **Sodium azide (NaN_3) s**trongly inhibited enzyme activity, with residual activity reduced to 50% at 1 mM concentration.
- **EDTA showed moderate inhibition with residual activity reduced to 50% at 1mM concentration**
- **Potassium cyanide (KCN)** caused significant inhibition, reducing activity to 50% at 1 mM.
- **Iodoacetate** moderately inhibited enzyme activity, leaving 40% residual activity at 1 mM.
- **Heavy metal ions s**howed potent inhibitory effects, with Hg^{2+} nearly abolishing CR activity .
- **Mg^{2+} and Ca^{2+} which are non-disruptive cations h**ad negligible effect, maintaining >90% activity.

The current study explored the impact of selected chemical inhibitors on the partially purified chromate(VI) reductase from *Trebouxia erici*, illuminating key biochemical characteristics and implications for chromium bioremediation.

The partial purification of chromate(VI) reductase from *Trebouxia erici* using ammonium sulphate precipitation, dialysis, ion exchange, and molecular sieve chromatography resulted in a 2.23-fold increase in specific activity with an overall yield of 33.3%. This level of purification indicates that the applied procedures were effective in enriching the enzyme while retaining a substantial proportion of its catalytic activity. The moderate purification fold also suggests that the enzyme is not present in high abundance within the algal cell extract, and further purification steps such as affinity chromatography may be required for homogeneity as characterized in previous reports [24][25].

The estimated kinetic parameters, a Km of 128 µM and a Vmax of 0.105 µmol/min/mg, provide useful insight into the enzyme's catalytic efficiency. The relatively low Km reflects a reasonable affinity of the enzyme for chromate(VI), consistent with its physiological role in detoxification. The modest Vmax value suggests that while the enzyme can effectively

bind Cr(VI), the turnover rate is not very high, which is typical of reductases involved in stress response rather than primary metabolism.

In the context of inhibitor studies, these kinetic values serve as a baseline for evaluating how specific inhibitors alter enzyme performance. For instance, inhibitors targeting electron transfer proteins or cofactors (such as cyanide or EDTA) are expected to increase the apparent Km or decrease the Vmax, thereby reducing the efficiency of Cr(VI) reduction[26]. Thus, the observed purification and kinetic parameters highlight the functional characteristics of the enzyme and provide a framework for interpreting inhibitory effects, especially in relation to the ecological role of *Trebouxia erici* in chromium bioremediation.

The enzyme was greatly inhibited by Zn^{2+}, Hg^{2+}, Mn^{2+}, and Cd^{2+}. severely inhibited enzyme activity with Hg^{2+} nearly abolishing activity. Comparable trends were reported in a *Pseudomonas* sp., where Hg^{2+}, Cd^{2+}, and Zn^{2+} significantly inhibited chromate reductase, whereas Cu^{2+} surprisingly enhanced it [27].

Additionally, in *Penicillium* sp., Cd^{2+} and Hg^{2+} decreased reductase activity by as low as 30 and 12%, respectively, with the inhibition by Hg^{2+} attributed to its strong affinity for sulfhydryl(–SH) groups, hinting at thiol involvement in the enzyme's active site. This aligns with earlier studies showing that heavy metals can bind to sulfhydryl groups, causing enzyme inactivation [28][29]. The strong effect of Hg^{2+} underscores the vulnerability of the enzyme to toxic metals commonly present in polluted environments, which could restrict its efficiency *in situ*.

The inhibition pattern exhibited suggest that the enzyme activity is highly sensitive to electron transport chain disruptors and thiol-reactive agents, consistent with its dependence on NADH and cysteine residues [29]. The observation that the thiol-reactive inhibitor iodoacetate modestly suppressed CR activity further lends credence to the hypothesis that cysteine residues are integral to the enzyme's active site and by extension its catalytic function. Pal [30] in a study on reduction of hexavalent Cr by cell free extract of *Bacillus spharicus* AND 303 reports that heavy metal ions such as Ni (II) and Cd (II) were strong inhibitors of CR activity unlike of 100µM Co (II) which retained 93% activity over control. Studies on Cr (VI) reductase activity in cytosolic fraction of *Pseudomonas sp* isolated from Cr (VI) contaminated land fill reveal that CR activity was enhanced in the presence of metal ions like Cu^{2+}, Mg^{2+}, Na^{+} and electron donors like citrate, succinate, acetate, and was significantly inhibited in the presence of metal ion like Hg^{2+}, Cd^{2+}, Ag^{+}, and disulphide reducers like mercaptoethanol, while respiratory inhibitors had minute effect on CR activity [31][32].

Bae [33] reported the possibility of a sulfhydryl group in the active site of CR characterized from *E.coli* as a result of NEM inhibition, another

well-known thiol group inhibitor. Brown [34] in their findings indicated the presence of a sulfhydryl group in the active site of CR while Elangovan [*35*] showed that CR forms a mercaptide bond with sulfhydryl group thus suggesting the presence of sulfhydryl bond in the active site of CR

However, the non- disruptive ions, Mg^{2+} and Ca^{2+} had minimal effect on reductase activity in *T. erici*. This aligns with observations in other systems—such as *Penicillium* sp.—where certain benign divalent ions had less deleterious effects, reinforcing that not all metal ions perturb enzyme activity. This suggests that the enzyme maintains stability in saline or ion-rich environments lacking toxic contaminants. Findings from present study are consistent with previous report [36][37][38].

The chelating agent, EDTA, inhibited the CR activity, indicating that divalent cations may be required for enzyme activation. The strongest inhibition by EDTA suggests a metallo-dependency, where one or more divalent metal ions are required for catalytic activity or structural stabilization. This is consistent with many biological Cr(VI)-reducing systems involving electron flow through redox cofactors [38]. As to whether CR from lichen is a metalloenzyme or a metal dependent is open for further investigation.

Sodium azide and potassium cyanide are known respiratory inhibitors that bind to metal centres, particularly heme and non-heme iron, and copper-containing enzymes. Report has shown that cyanide is a metabolic inhibitor that affects chromate reduction in bacteria [40]. In the case of *Exiguobacterium sp,* the impact of cyanide on CR activity can be profound [41]. Similar studies on bacteria such as *S.maltophilia* ZA-6 and *S.gallinarum* W-61 have shown that sodium cyanide along with sodium azide, severely affects chromate reduction [40]. Therefore, the comparable inhibition exhibited by both azide and cyanide suggests that the enzyme contains an accessible metal site that can be ligated by small anions that supports the presence of an adjacent or auxiliary metal site or a tightly coupled metal-containing partner.

Mercaptoethanol, a disulphide reducer significantly inhibits CR activity in *T. erici*. Studies showed the potential of the compound to bind sulfhydryl bond [42]. This suggests that the enzyme's activity is sensitive to reducing conditions, potentially due to the disruption of disulphide bonds. This inhibition implies that the enzyme's structure and function are maintained by a delicate balance of redox states, and excessive reduction can lead to destabilization.

The moderate inhibition shown by Ethyl iodoacetate, an alkylating agent, suggests that one or more cysteine residues are involved in the enzyme's catalytic function or structure. However, the partial inhibition implies that no single cysteine residue is essential for enzyme activity, and the cysteine(s) may play a modulatory or auxiliary role.

Perhaps the interesting development observed in this study, particularly in the inhibition patterns of high sensitivity to electron transport inhibitors and heavy metal indicated that *T. erici* chromate reductase may operate via a Class I "tight" or Class II "semi-tight" electron transfer mechanism, similar to known chromate reductases, which rely on NAD(P)H and possibly flavin cofactors [43,44,45,46]. The strong suppression by thiol-reactive and heavy metal inhibitors implies the presence of essential cysteine residues at the active site, and perhaps a dependence on structural integrity modulated by redox-sensitive groups.

For practical bioremediation, these findings underscore both the enzyme's potential and its limitations. In environments contaminated with respiratory chain inhibitors or heavy metals, reductase effectiveness may be severely compromised. However, the enzyme's resilience to innocuous ions suggests it could still function in moderately polluted water. To improve applicability, approaches such as protective immobilization, directed evolution for inhibitor resistance, or co-application with metal-sequestering agents could be explored.

Conclusion

This study demonstrated that *Trebouxia erici* possesses a chromate(VI) reductase capable of reducing toxic Cr(VI) to the less harmful Cr(III) in an NADH-dependent manner. The enzyme was successfully partially purified and exhibited measurable activity under baseline conditions. However, inhibition assays revealed strong susceptibility to respiratory blockers (sodium azide, potassium cyanide) and heavy metals (Cu^{2+}, Hg^{2+}), while thiol-reactive compounds caused moderate inhibition. In contrast, benign divalent cations such as Mg^{2+} and Ca^{2+} had negligible impact. These findings suggest that the enzyme's catalytic function depends on intact sulfhydryl group and electron transfer pathways, but that its activity may be compromised in chemically complex polluted environments containing inhibitory agents.

The study is yet another avenue to enhance the basic enzymological data of algal chromate reductases and highlights *T. erici* as a potential candidate for eco-friendly chromium detoxification. Nonetheless, its effectiveness in real-world applications may be limited by co-existing chemical inhibitors.

Further work in this area will require not only molecular studies that involves cloning and sequencing of the gene encoding CR in T. erici but also detailed enzyme kinetics by determining the IC50 and Ki values for major inhibitors for better understanding of the inhibition mechanisms as well as cofactor characterization, environmental simulations and enzyme stabilization strategies involving protein engineering approaches to enhance additional information regarding the mechanism and

function of CR activity that will enhance its remediation potential and resistance to inhibitors.

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