Partial purification of chromate reductase from an Alkaliphile Pseudomonas DL17 (JN595813.1)

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Abstract:
An attempt was made to purify the chromate reductase from an alkaliphile with 46 fold purification with 40 percent recovery. The molecular weight was found to be 20 kDa. The higher enzyme activity was observed at pH 7.5 to 8.5 and temperature 35^0C to 50 ^{0}C with $K_2Cr_2O_7$ as a substrate. The thermal stability of chromatereductase was up to 70^0C. Metal ions have no significant inhibitory action on chromate reductase. No activity was observed at 80 ^{0}C during enzyme stability experiments. It was an interesting thing observed in this regard was the enzyme also shown nitro reductase activity. Km value is 0.065 and the Vmax is 0.3 indicating its high affinity towards potassium dichromate.

Key words: Chromate reductase, Nitroreductase, Pseudomonas DL17, Purification, xenobiotics

1 Introduction:

Chromium is an essential trace element required nearby 200 mg/day to humans for health. It is involved in the metabolism of carbohydrates, fats, and proteins .Higher concentration of hexavalent chromium Cr(VI) is toxic. Glutathione reductase activity was inhibited by hexavalent chromate ion in erythrocytes. Cr (III) is impermeable to biological membranes hence Cr (III) generated inside the cell binds to protein and interacts with nucleic acid. Chromate toxicity is partly attributed to the flavozyme-catalyzed reduction and the formation of reactive oxygen species. In some species extracellular bio-surfactants found responsible for the tolerance towards hexavalent chromium and protects the cells from oxidative stress during reduction of Chromium. Alkaliphiles, and their enzymes are also potentially important for use in the degradation of xenobiotics, and they play a major role in the bio-geo cycling of inorganic compounds [1]. Thus they have an important, environmentally-friendly role in biocatalysts [2].

2. Methodology:

Chemicals: Bacterial media were purchased from Hi media Mumbai; other chemicals were purchased by SRL Mumbai

2.1 Cell mass harvesting and Preparation of cell free extract:

The culture of **Pseudomonas DL17 (JN595813.1)** was grown in presence of chromate ions in alkaline broth media for 24 hr as earlier [3] and harvested by centrifugation at 10000g using DuPont Soverall G. The harvested cell mass was washed thrice with phosphate buffer pH 8.0. These cells were re-suspended in phosphate buffer and sonicated by ultrasonic probe. The sonicated cell debris were washed thrice with

phosphate buffer and re-dissolved in 10 ml of same buffer. Among these (2 ml) of cell-free extract or membrane fraction used for enzymatic assay to know the chromate reduction activity is cytosolic or membrane-associated. The cell free extract obtained was then centrifuged at 15,000 × g for 30 min at 4 ºC. The supernatant of the cytosole obtained was used as source of enzyme after confirming the cytosolic fraction showing redox potential using N-N' dimethylamine.

2.2Isolation and Purification of chromate reductase: 3.5 g cell mass isolated by shaking culture method and after 24 hrs of cell induction with $K_2Cr_2O_7$ it was sonicated in lysis buffer for 5 cycles of 20 sec. The cell free extract isolated by Dupont Sorvall cold centrifuge at 15,000 g for 30 min.The crude cell free extract was subjected to ammonium sulphate precipitation. Solid ammonium sulphate $(NH_4)_2SO_4$ was added to the cell free extract to obtain 30 % saturation. The precipitated protein was separated by centrifugation in cold condition at 15000 × g for 30 min and discarded as it did not show any activity. In the resulting supernatant was followed with addition of ammonium sulphate to get 70% saturation. The precipitated protein was separated by centrifugation in cold condition and was dissolved in phosphate buffer pH 8.0, (0.05M). The activity of chromate reductase and protein concentration was determined[4]. Redissolved precipitate was dialyzed against the same buffer and stored at 4°C for further purification. The sample was then loaded to DEAE – cellulose column (2 cm×45 cm) equilibrated with 0.05M phosphate buffer (pH8.0). The column washing was continued till no protein was detected in the following fraction(measurement of '0' OD at 280 nm on Jasco Varian 630 spectrophotometer). The enzyme was eluted from DEAE- cellulose column by phosphate buffer pH 8.0 (0.05M) containing 0 to 0.5 M NaCl linear gradient. Similarly the eluted protein volume was reduced after reverce osmosis and further elutedon Sephadex G-50 column. The protein was reconcentrated by reverse osmosis again. This purified protein was having higher specif activity; pulled together for specific activity and Native polyacrylamide gel electrophoresis[5]. The molecular weight of the enzyme was determined by comparing the electrophoretic mobility of the enzyme with Std. markers of Hi media.

2.3 Characterization of oxido-reductase:

Fig.[1]Reaction with N-N'Dieithylene amine

As the enzyme belongs to oxidoreductase family; its activity was judged by reaction with N N$^-$ di ethylene amine showed faint blue color canged in dark blue suggesting the enzyme has redox potential showed in **Fig.[1]** The another test-tube contain distilled water as a control.

2.4 Chromate reductase assay:

Partially purified enzyme was used for analysis of chromium reductase activity. An enzymatic reaction system of 1 ml including (25 mM /L) NADPH; 0.2 ml, 2.5 mg/ Cr (VI; 0.1 ml), enzyme solution (0.3 ml) and 0.4 ml pH 8.0 PBS buffer was established at 37˚C. After 30 min, the Cr (VI) concentration was detected in the system. The blank control group had the same reaction system without NADPH. By comparing the Cr (VI) content in different groups, the reductase capacity of partially purified enzyme was analyzed little modification of Simin Zhou and others [6]. The chromium concentration was monitored by Annija Lace and others [7]. The specific enzyme activity was noted for 1 unit as amount of enzyme required to convert 1 mM Cr (VI) of substrate ($K2\,Cr_2O_7$) in 30 min at 37˚C temperature for the color development, thereafter. The complex was measured at 543 nm with spectrophotometer Jasco Varian 630 UV-Vis.

2.5 Native gel Electrophoresis:

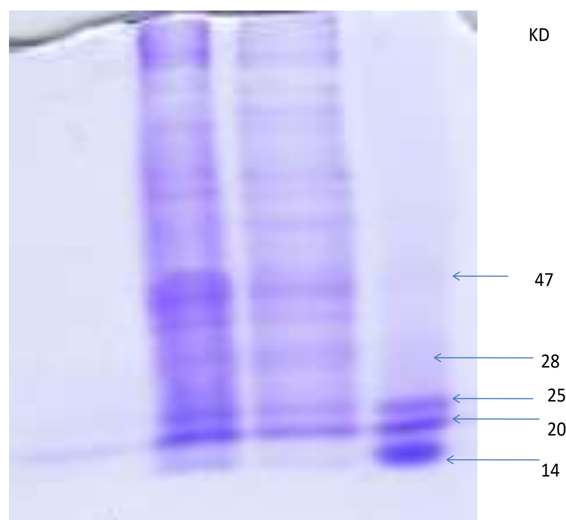


Fig [2] Native polyacrylamide gel electrophoresis pattern of chromate reductase

Native gel electrophoresis was done by usual procedure. By this **Fig [2]** it seems the purified protein having molecular weight 20 KD. Left side of the marker one can find the crowd of protein after 70% ammonium sulphate precipitation, DEAE ion exchange chromatography and last column from left side of the marker is the probable enzyme strip of isolated after G50 sephadex purification shown molecular weight 20 KD.

2.6 Effect of temperature:

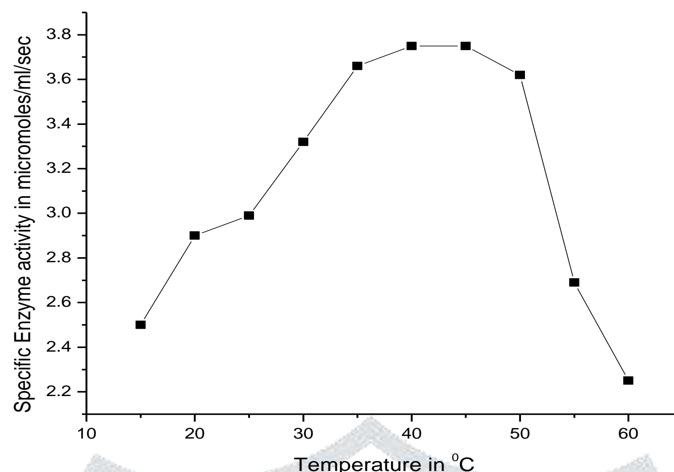
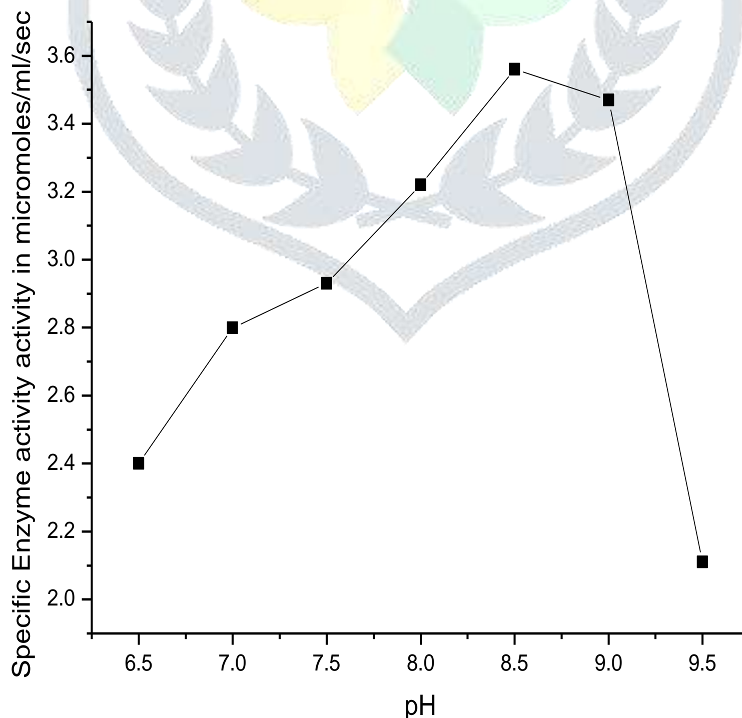


Fig [3] Effect of temperature on enzyme activity

Optimal temperature was determined wherein the chromate and quinone reductase activity was assayed at various temperatures similarly to Ochrobactrum intermedium (ranging 20–50 _C) BCR400 isolated from a chromium-contaminated soil[8]. The experimental bacteria also showed higher enzyme activity between 30 to 50 ^{o}C as shown in **Fig [3]**. Enzyme stability experiments were carried in sodium bicarbonate buffer.

2.7 Effect of pH:



Fig[4] Effect of pH on enzyme activity

Optimum pH and temperature for chromate reductase activity were determined for the partially purified enzyme by incubating the reaction mixture at pH values ranging from 6.5 to 9.5 at 37°C found optimum pH at 8.5, respectively for 30 min [9].

2.8 Transformation of o - nitro aniline

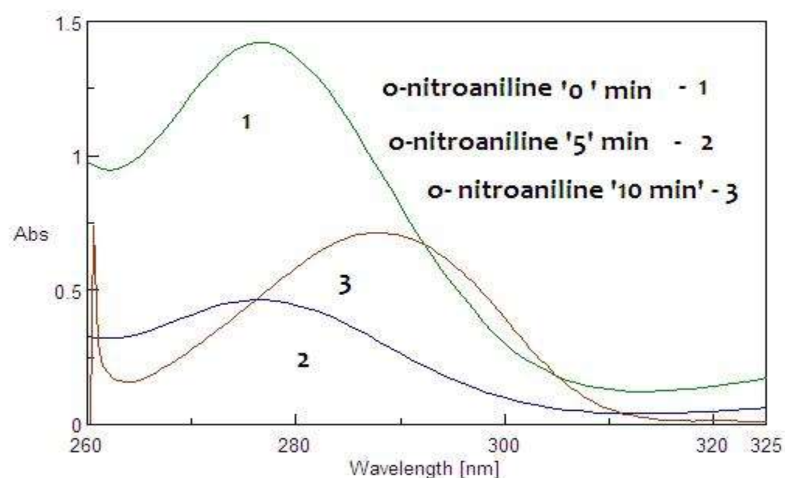


Fig [5] Nitro-reduction activity of the chromate reductase

The chromate reductase isolated showed an activity of nitro reductase. The purified product after enzyme reaction noticed that o-nitro-aniline transformed into o-phenylne-diamine. It was confirmed by FTIR, NMR and GCMS. The chromate reductase purified from *Pseudomonas ambigua* was found to be homologous with several nitroreductases [10]. Majority of nitroreductases reported are known to be flavozymes [11].

2.9 Kinetics of Chromate reductase:

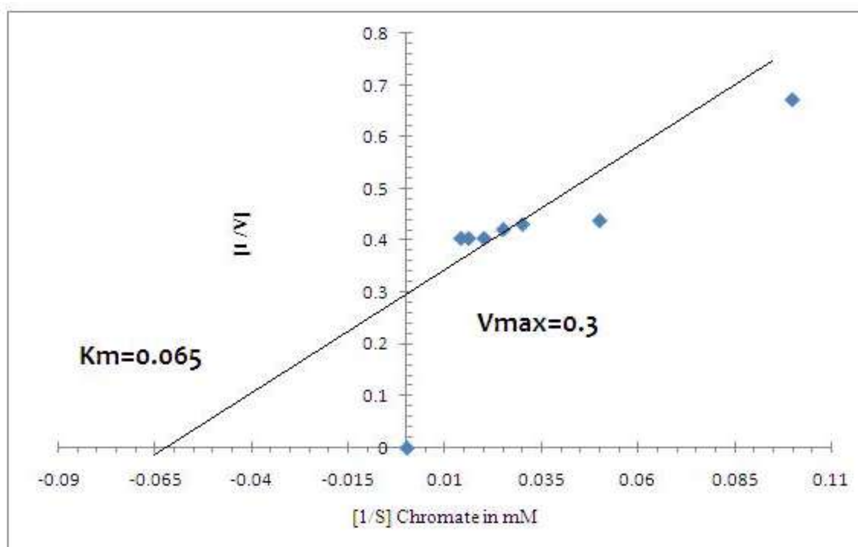


Fig [5] Line weaver- Burk plot of isolated chromate reductase.

The line weaver Burk plot was drawn after the chromate reduction reaction with the experi-mental concentration as shown indicated Km value is 0.065 and the Vmax is 0.3 indicating its high affinity towards potassium dichromate.

3. Result and Discussion:

Chromium (III) is biologically important which is required for proper carbohydrate and lipid metabolism. Hexavalent chromium has high mobility in soil and ground water which pollute the water and shows harmful effects on living organisms including humans. A maximum acceptable concentration of 0.05 mg^{-1} for chromium in drinking water has been established on the basis WHO and Indian standard parameters. Chromium in the trivalent form is considered to have low order of toxicity while long term exposer to hexavalent chromium can develop tissue necrosis. Reduction of Cr (VI) was mainly found in soluble fraction, indicating the presence of chromate reductase in cytoplasmic fractions similar to Thacker and others [12]. No significant activity of chromate reductase was observed in the membrane fraction used after sonicated cells debries or even heat killed cells or heated cell-free extracts. This has confirmed the presence of enzyme in crude cell-free extract. Cr(VI)] being highly notorious chemical species having mutagenic and carcinogenic effects on living systems. Many much bacteria and fungi have been found to adapt and colonize the toxic metal polluted environment. They have developed the mechanisms to evade metal toxicity, eg. metal efflux channels, metal resistant plasmids, adsorption, uptake of toxicants , DNA methylation by F- met tRNA, and or metal biotransformation by specific enzymes and cellular metabolites.

The ability of a chromate-reducing *Pseudomonas aeruginosa* strain, isolated from tannery effluent [13].carbon C , N ,P source supported the bacterial growth and chromate reduction. Cr (VI) reduction is important to study to know the contribution of microorganism's bioremediation of Cr (VI) into Cr (III) i.e.

the less toxic form [14]. Cr (VI)-resistant and reducing bacterium *Bacillus sphaericus* was reported useful in decontaminating the sites by immobilizing the cells[15]. Chromate reductase activity in *Acenetobactor haemolyticus* was detected in three fractions namely whole cells, membrane fraction and cytosolic fraction. Highest chromate reductase activity was noted in the whole cells[16]. This might be because of holozyme activity based on apoenzymes and their active site with particular cation. In case of whole cell physiological condition might be remain undistracted. Glucose might sometimes acts as enzyme activity enhancer. Methanotrophic microbe *Methylococcus capsulatus* and severl other organisms shown chromium reductase activity using methane as the carbon and energy source[17]. The enzymatic purification and reduction of Cr (VI) are scare in alkaline condition. The experimental organism has shown dual enzyme activity as chromate reductase and nitroreductase. The purification and characterization of chromate reductase lower (20 KD) molecular weight [18].

3.1 Mechanistic approach:

Reduction at cell surfaces may occur through a three-step process in which negatively charged dichromate ($Cr_2O_7^{2-}$) is bound to positively charged groups on cell surfaces; reduced by adjacent functional groups; and released as Cr(III) by electronic repulsion. Generally bacteria are negatively charged because of the presence of carboxyl and phosphate groups, which is a source of attachment to adsorbent. Formate-dependent Cr(VI) reductase activity was observed in anaerobic bacteria of *S. putrefaciens*MR-1, with high specific enzyme activity in the cytosolic plasma membrane. Formate and NADH might act as an electron donors for Cr(VI) reductase, while lactate or NADPH did notshown such effects. Inhibitors of Chromate reductase were found as *p*-chloromercuri-phenylsulphonate, azide, 2-heptyl-4 hydroxyquinolone-*N*-oxide,vantimycin A etc. suggesting the involvement of a multi-component electron system with cytochromes and quinine[19]. Many bacterial enzymes show one-electron reduction of chromate, lead in the formation of Cr (V), this produce more reactive oxygen species (ROS). Such enzymes are not appropriate for bio-remediation, as they harm the bacteria and their reaction product is not one step to convert Cr (VI) to Cr (III). Thus certain other way following bacteria are more useful for bio-remidiation[20]. Multielectron transformation is not so useful for bioremediation of chromate; the one electron transfer system of chromate reductase minimizes the generation of reactive oxygen species and ultimately it prevent the cellular damage. Comparatively four electron transfer system of chromate reductase is found better in which three electrons being given to chromate and one to the molecular oxygen. Enhancement of Cr(VI) reductase activity was noted on addition of NADH as an electron donor while it was highly inhibited by $Hg^{2+,}$ Ca^{2+} and $Mg^{2+,}$ and azide, EDTA as well as KCN [21].

Chromate reductase activity was parllally noticed with nitroreductase[22].Chromate reductase activity was dependent on the presence of the divalent metal Ca^{2+} or Mg^{2+}, which increase the activity 4 -fold, whereas Zn^{2+}, Mn^{2+}, and EDTA inhibit the reaction. The chromate reductase could accept electrons from both NADH and NADPH, with a preference to words NADPH [23]. Chromate reducing protein of 30 KD was islolated from periplasmic membrane fraction of a bacteria pseudomonas aerogenosa[24].Constitutive chromate

reductase enzyme activity was noted in Bacillus firmus. NADH or NADPH had been used as a redox process mediator [25].

Both in case of prokaryots and eukaryots Cr (VI) can pass through the cellular membrane and form Cr (III) as a reduction product in the cytoplasm. Eukaryots can additionally reduce Cr (VI) in mitochondria and nuclei [26]. The chromate ions caused bovine leukemia virus-transformation and lamb kidney fibroblasts formation[27]. Bioremediation of chromate contaminated sites have different mechanisms of biotransformation like of Cr (VI) to Cr (III) reduction, which may be enzymatic or non enzymatic, incorporation in cellular metabolites, bioaccumulation; biosorption, immobilization etc. Some microbes reduce Cr (VI) to Cr (III) by reducing power generated from carbon[28].

It also been noticed in one another study that chromate binds proximal to FMN, and regulate the structural change in FMN loop that enhance optimal chromate reduction rate. As a side chains proximal to the β3/α4 FMN binding loop 4 contributed both NADH and metal ion binding which leaded the structural changes around the FMN binding pocket and coupled the chromate and NADH binding sites during chromate reduction[29]. Several enzymes shown to have Cr(VI) reductase activity; eg.$FADH_2$-dependent metal reductase, nitroreductase , c-type cytochrome, hydrogenase etc. Extracellular chromate reductase is supposed more useful in case of industrial effluent treatment due to cost efficacy, simpler process and is non-toxic to DNA due to no intake of chromate in cell[30].

Cytosolic extracts from various E. coli analyzed for oxidoreductase activity. And found that number of these enzymes can reduce Cr (VI) indirectly, via redox intermediates present in the cytosolic crude extracts . It has been said by that cofactor regenerating enzyme partner can be employed to provide a renewable supply of reduced NAD(P)H which drive the Cr(VI) transformation[31].Similarly in case of experimental organism **Pseudomonas DL17 (JN595813.1)** cytosolic fraction showed oxidoreductase as well as chromate reductase activity. It has shown its activity under alkaline condition. There are various types of Cr (VI) reduction enzymes in bacteria like Cr (VI) reductase, prominent examples are aldehyde oxidase, cytochrome P450, DT-diaphorase and several other oxidoreductases with different metabolic functions have been reported to reduce catalyze Cr(VI) which include nitroreductase, iron reductase, quinine reductases flavin reductases etc. Enzymatic reduction of chromium (VI) using bacteria has recently received much attention [32].

Chromium reduction by thermophile has been studied and found that the enzyme chromate reductase involved in it was resistant under acidic and neutral condition but its stability was decreased under alkaline condition. insuch condition the enzyme of the experimental stain shows quite importance.The Cr(VI) reductase activity of Cytoslic fraction was enhanced when exposed to $Cu^{2+}$and Fe^{3+}while its activity found reduced in presence of Mn^{2+} and Ag+, respectively. The cations such as $Mg^{2+,}$ $Zn^{2+,}$ As^{3+} and electron acceptors like sulfate and nitrate didn’t affect on Cr(VI)reductase activity while; the external electron donors glucose, glycerol, citrate, malate, succinate, and acetate, activated the reaction but not found essential to improve the enzyme activity. The chromate reductase was mainly associated with the soluble fraction in the

cytoplasm of the bacterial cell. The molecular weight of the enzyme was 20 KD[33]. In another case also hexavalent chromium reduction was observed in soluble fraction when evaluated the cell-free extracts. The protein of molecular weight around 25 kDa was detected by SDS gel electrophoresis [34]. As the Chromate (CrO_4^{2-}) is a strong oxidizing agent it is reduced to Cr(V) at intracellular level. This might forms reactive species and free radicals resulting in damage of DNA molecules and other biomolecules producing a wide spectrum of genomic changes such as DNA strand breaks, alkali-labile sites, DNA-protein, and DNA-DNA crosslink's as well as Cr(III)-DNA adducts associated with the mutagenic effects[35-36]. The ChrT gene capable of producing chromium reductase was reported in the Serratia spp. S2 strain, and ChrT-engineered bacteria shown higher chromium reduction capacity. This gene belongs to the FMN red enzyme family [37]. It had also observed that both of the two GSH-synthatase and GSH reductase enzymes were involved in Cr(VI) resistance in HepG2 cells and the yieF gene expression might reduce the oxidative stress caused by Cr(VI)[38].

4 Conclusion: As this enzyme is having low molecular weight and dual enzymatic activity. Physico-chemical methods are involved in removal of chromium containing as well as nitro aliphatic and aromatic compounds from waste water. Use of alkaliphilic enzymes may be a good remedy for water purification in upcoming days. As the isolated enzyme having less molecular weight it can be immobilized and used for such contaminated places or even in society water purification where these contaminants are the substrates of this enzyme.

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