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PURIFICATION AND CHARACTERIZATION OF CHROMATE REDUCTASE FROM *Lactobacillus fermentum* IFO3956 ISOLATED FROM TANNERY EFFLUENT

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Abstract

Chromate reductase was isolated from *Lactobacillus fermentum* IFO3956, purified and its properties characterized. The bacteria were isolated from chromium-rich tannery effluent, identified using Grams staining technique and 16S rRNA gene sequencing. The purification of the enzyme involved gel filtration chromatography using Sephadex G – 75, ion-exchange chromatography using Dowex 50Wx8 and molecular weight estimation using Sodium Dodecyl Sulphate – PolyAcryamide Gel Electrophoresis (SDS - PAGE) analysis. The bacteria were found to be cylindrical rods, positive to Gram's reaction and showed 92% identity with *Lactobacillus fermentum* IFO3956 on National Center for Biotechnology Information (NCBI) database. The kinetics studies on the enzyme revealed K_M of 0.25 mM and V_{max} of 10.0 µmol/min. In addition, the enzyme activity was optimum at pH 4.5 and temperature 40°C and was found to be stable in the presence of aprotinin and EDTA with no apparent increase in activity. SDS – PAGE analysis showed two bands revealing a molecular weight of 35 and 70 kDa, suggesting a dimeric protein or an enzyme with two subunits. The results of this study have revealed the properties of a novel chromate reductase that can be employed in the bioremediation of toxic hexavalent chromium in industrial effluents.

INTRODUCTION

Chromium is an element that exists in several chemical forms displaying oxidation numbers from 0 to 6^+. However, only two of these oxidation states are stable enough to occur in the environment, Cr^{3+} and Cr^{6+} [1]. Both Cr (III) and Cr (VI) enter the environment as a result of effluent discharge from industries [2]. Furthermore, Cr^{6+} is more toxic than Cr^{3+} and high levels of exposure cause liver and kidney damage, skin ulceration and also affect the central nervous system [3]. Cr^{6+} pollution due to its discharge in high concentrations onto the environment by a number of industries is of great concern all over the world.

Chromate (Cr^{6+}) is a widespread environmental contaminant. Therefore, it is important to control this element since it is toxic, carcinogenic, and mutagenic and causes tissue damage in plants and animals [4]. It is produced as a bye-product of many industrial processes which include leather tanning, chrome plating, stainless steel wielding, pigment production and nuclear weapons generation among others [5]. The majority of tanneries worldwide use chromium salts that are highly toxic and pose a serious threat to the environment upon improper disposal of their wastewater. Tannery effluent wastes are ranked as high pollutant among all other industrial wastes [6]. The high solubility of chromate allows its leaching and eventual contamination of surface and underground water supplies, and thus chromate contamination does not remain confined to site of initial contamination [7]. The

permissible limit of Cr in drinking water is 0.05 mg/L according to the World Health Organisation [8].

Chromium in the effluents may exist as Cr^{6+}. Oxidation of Cr^{3+} to Cr^{6+} by dissolved oxygen without any mediate species has been reported to be negligible [9, 10] whereas mediation by Manganese oxides was found to be the effective oxidation pathway in environmental systems [1]. Conventionally, physico-chemical processes such as precipitation, ion-exchange, reverse osmosis, electrochemical treatment and the use of various types of adsorbents are used in the removal of Cr from industrial effluents to reduce the concentration to levels that comply with regulatory standards. However, the cost to set up the required equipment to operate these processes is prohibitively high for large-scale treatment [11, 12]. Moreover, conventional methods find difficulty in the removal of highly soluble metals such as Cr from effluents and leachates [4]. Therefore, the aim of this research is to investigate the application of biological means to reduce the concentration of toxic Cr^{6+} by converting it to less toxic Cr^{3+} via chromate reductase enzyme system. The characteristics and kinetic properties of the enzyme are therefore being studied.

MATERIALS AND METHODS

Materials

Spectrophotometer (Cary 300 UV – Vis, Agilent Technologies), USA, Atomic Absorption Spectrophotometer (AA 280 FS) USA, SDS Electrophoresis machine (Cleaver Scientific), UK, Microscope (Thermo Fisher Scientific), UK, Centrifuge (Heraeus Labofuge 300), Autoclave (Astell Scientific ASB 300). NADH, Sephadex G-75, Dowex 50Wx8, Tris, Aprotinin were product of Sigma Aldrich Co. Ltd. All other chemicals were of analytical grade.

Isolation and Characterization of Bacteria from Tannery Effluent

Seven bacterial strains were isolated from tannery effluent, identified as *Bacillus species* and tested for chromate reduction. One of the strains that showed highest chromate reduction was selected and further identified as *Lactobacillus fermentum* IFO3956 using molecular characterisation. *Lactobacillus fermentum* IFO3956 was then employed for extraction and studies of chromate reductase.

The isolation of bacteria from the tannery effluent was done by serial dilution technique as described by Chaturvedi [13]. The tannery effluent was plated on Nutrient Agar (NA) media previously sterilized at 121ºC for 15 minutes before use and incubated at 37ºC for 24 hours. After the incubation period the plates were observed for growth on the media. Discrete colonies have grown and been subcultured into fresh NA plates to obtain pure isolates. The plates were also incubated at 37ºC for 24 hours. The pure bacterial isolates were Gram-stained and observed under a microscope for identification. The bacterial isolates were then inoculated into the tannery effluent to investigate their bioremediation potentials.

Bioremediation studies of tannery effluent using the bacterial isolates for chromate reduction were carried out using the method of Aneez *et al*., [6]. Approximately 50 ml of sterilized tannery effluent sample was transferred to 7 different conical flasks containing 50 ml each of the prepared minimal media. The media had 5g yeast extract, 5g $(NH_4)_2SO_4$, 1g KH_2PO_4, 1g K_2HPO_4, 1g $MgSO_4$, 1g NaCl, 1g $CaCl_2$ dissolved in one liter distilled water. Then 3 ml (containing 3.0 x 10^8 CFU/ml) each of standardized pure bacterial isolates (1 – 7) were added to the mixture. Another conical flask containing 100 ml of sterilized effluent sample was also set up to serve as control. They were kept in an orbit shaker for 72 hours and maintained at 30ºC. The level of chromium was analysed in the effluent before (control) and after the bioremediation experiment. Furthermore, the bacterial isolate that showed highest chromate reduction in the effluent was subjected to molecular characterization (DNA extraction, Polymerase Chain Reaction (PCR) and sequencing) and chromate reductase extraction.

Extraction of Chromate Reductase from *Lactobacillus fermentum* IFO396

Crude chromate reductase from *Lactobacillus fermentum* IFO396 was extracted using the method described by Conceicao *et al*., [14]. Bacterial cells were grown in Luria – Bertani (LB) media at 30ºC with orbital shaking (150 rpm) for 24 hours. The media contained 10 g tripton, 5 g yeast extract and 5 g NaCl dissolved in one liter distilled water. Thereafter, cells were harvested by centrifugation 280 x g for 5 minutes at 4ºC and washed twice with Tris – HCl buffer (50 mM, pH 7.0). Cells were then resuspended in the same buffer and chilled in an ice bath to disrupt the cells. Bacterial cells were then further centrifuged at 4,800 x g at 4ºC for 45 minutes and filtered to obtain the clear soluble extract which serves as the crude chromate reductase extract. Aprotinin was added to a final concentration of 1 microgram % (w/v) to stabilize the enzyme. EDTA was also separately added to a final concentration of 1 mM to stabilize the enzyme.

Purification of Chromate Reductase

Chromate reductase from *Lactobacillus fermentum* IFO396 was purified by method described by Sallau *et al*. [5]. The purification steps involved the extraction of the crude chromate reductase (crude enzyme), Ammonium sulfate fractionation (35% - 90%), Gel filtration chromatography using Sephadex G-75 and Ion Exchange Chromatography

using Dowex 50Wx8 resin. Assay of chromate reductase activity and protein concentration was carried out at each purification step. For ammonium sulfate fractionation, crude chromate reductase extract was precipitated with 35% - 90% saturation of solid ammonium sulfate at 4ºC overnight. The precipitate was then centrifuged at 4,000 x g for 5 minutes and dissolved in 5 ml, 50 mM Tris – HCl buffer pH 7.0. The dissolved precipitate was then assayed for chromate reductase activity and protein concentration. The dissolved crude enzyme was then dialyzed against 50 mM Tris – HCl buffer pH 7.0 at 4ºC for 24 hours with the buffer changed after 12 hours. The dialysate (ammonium sulfate-free crude chromate reductase) was then subjected to a gel filtration column containing settled Sephadex G – 75, which was pre-equilibrated with 50 mM Tris – HCl buffer pH 7.0. The column was then eluted with the same buffer to collect 30 fractions at the rate of 0.25 ml/min that were also assayed for chromate reductase activity and total protein concentration. Fractions with the highest chromate reductase activity were pooled together and then subjected to ion-exchange chromatography in a column containing settled Dowex 50Wx8 ion–exchange resin which was pre-equilibrated with 50 mM Tris – HCl buffer pH 7.0. The column was loaded with 3.0 ml of the chromate reductase active fraction and eluted with NaCl solution (0.00 M to 1.00 M gradient) in a continuous passion for collecting 30 fractions (5.0 ml each) at the rate of 1.0 ml/min, which were assayed for chromate reductase activity and total protein.

Assay for Chromate Reductase Activity

Chromate reductase activity was assayed by method described by [7] and modified by [5]. The activity was assayed after incubating a reaction mixture at 30ºC for 60 minutes. The reaction mixture contained 100 µl of sample (enzyme extract), 300 µl of 0.05 mM potassium dichromate (substrate), 300 µl of 0.1 mM NADH and 300 µl of 50 mM Tris – HCl buffer pH 7.0 in a 1.0 ml final volume. The reaction was stopped by adding 1.0 ml of 0.2 % of 1,5 – diphenylcarbazide, allowed to stand for 10 minutes and absorbance was read at 540 nm against a reference sample (where enzyme extract was replaced with the buffer) using a spectrophotometer. One unit of chromate reductase activity was defined as the amount of enzyme required to reduce 1 µmole of chromate VI to chromate III under defined assay conditions.

Determination of Total Protein Concentration

Total protein concentration was determined using the Biuret test. The sample (containing the protein), 1.0 ml was placed in a test tube and 4.0 ml of biuret reagent was also added and then mixed, allowed to stand for 30 minutes and read at 540 nm using a spectrophotometer. Protein concentration was obtained from a reference standard curve prepared for Bovine Serum Albumin (BSA).

Kinetic Studies for Chromate Reductase isolated from *Lactobacillus fermentum* IFO3956

Initial velocity studies were carried out on the partially purified chromate reductase and activity measured at various concentrations (0.01 – 0.05 mM) of the substrate (potassium dichromate). A plot of reciprocal of the enzyme activity against reciprocal of substrate concentrations was plotted (Lineweaver – Burk plot), K_M and V_{max} were then determined from the plot.

Effect of pH on the Activity of Chromate Reductase Isolated from *Lactobacillus fermentum* IFO3956

Chromate reductase activity was assayed for at different pH using the following buffers: 50mM Acetate buffer (pH 3.5 – 6.5) and 50mM Tris – HCl buffer (pH 7.0 – 8.0) and potassium dichromate as substrate. A graph of enzyme activity versus pH range was then plotted to determine the optimum pH.

Effect of Temperature on the Activity of Chromate Reductase Isolated from *Lactobacillus fermentum* IFO3956

Chromate reductase activity was assayed at varying temperature ranging from 10ºC to 80ºC in order to establish the optimum temperature for the enzyme activity. A graph of enzyme activity versus temperature was then plotted to establish the optimum temperature.

Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis (SDS PAGE)

This was carried out according to method of Laemmli [22]. The sample (35 µl) and 7.5 µl Bromophenol blue as loading dye were treated with 0.5 ml of treatment buffer (1% SDS, 1% mercaptoethanol and 0.1 M phosphate buffer, pH 6.8) and glycerol (10 µl) was also added in an Eppendorf tube and heated at 95ºC for 5 minutes to denature the proteins. The mixture (40 µl) was then loaded onto the gel (12% running gel and 5% stacking gel). The electrophoresis was performed at 100 volts for 2 hours and trisglycine buffer pH 6.8 was used as the running buffer. After the electrophoresis, the gel was removed and then stained with Comassie Brilliant Blue for 2 hours. The gel was then rinsed and also destained many times in 7% acetic acid and then viewed for the presence of bands. The molecular weight of the partially purified enzyme was then estimated in comparison with the bands of the molecular weight marker used as described by Bashir *et al*., [23].

RESULTS AND DISCUSSION

Seven *Bacillus* strains were isolated from the tannery effluent and were tested for chromate reduction. They were found to be medium rods and positive to Gram's reaction. *Bacillus* is a genus of Gram positive and rod shaped bacteria that may be facultative anaerobes and resistant to stress condition in the environment [15]. Bacterial isolate number 5 was found to reduce chromate by 71% in 72 hours from the initial concentration of 7.36 mg/l (Figure 1). This isolate was further subjected to molecular characterization. The genomic DNA extracted from the isolate, amplified using PCR and the product subjected to agarose gel electrophoresis. Thereafter the amplified gene product was sequenced in a gene sequencer (ABI Prism, 310 – Genetic Analyzer) and revealed a nucleotide sequence of 550 bases that were subjected to Basic Local Alignment Search Tool (BLAST) software and indicated 92% identity with the organism *Lactobacillus fermentum* IFO3956 with Accession Number = NC 010610.1. *Bacillus* species have been extensively studied for chromium bioremediation [4, 13]. The mechanisms by which hexavalent Cr is reduced to nontoxic trivalent Cr is of renewed interest to researchers. However, the exact pathways leading to intracellular/extracellular reduction still remain unclear [4]. Researchers have identified NADH, NADPH, flavoproteins and other heme proteins to play a key role in reducing Cr (VI) to Cr (III), the reducing component being reductases existing inside the bacterial cells [4].

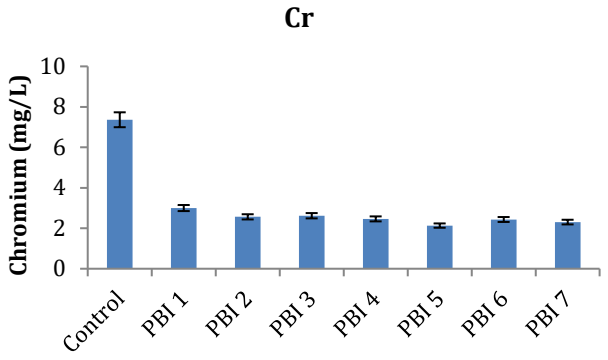

Figure 1: Cr Concentration of Tannery Effluent Before and After 72 Hours of Bioremediation Using Pure Bacterial Isolates (PBI)

Chromate reductase extracted and partially purified from *Lactobacillus fermentum* IFO3956 revealed purification profile for the enzyme as presented in Table 1. From the table, it is deducible that three purification steps were carried out; ammonium sulfate fractionation, gel filtration chromatography using Sephadex G – 75 and ion–exchange chromatography using Dowex 50Wx8. The increase in specific activity for the enzyme after the purification steps could be due to the removal of other interacting compounds in the crude protein as they may exist. This further buttresses the fact that the enzyme has been partially purified even though the purification factor is low, however, the percent recovery of 20% is commendable since it is higher than 4.61% reported by Sallau *et al*. [5] for *Aspergillus niger* chromate reductase.

Table 1: Purification Profile for *Lactobacillus fermentum* IFO3956 Chromate Reductase

Purification Steps	Vol. (ml)	Total Protein (mg)	Total Activity (µmole/min)	Specific Activity (µmole/min/mg)	% Yield	Purification Fold
Crude Enzyme	120.00	0.29±0.01	3.41±0.93	11.76±3.70	100.00±27.03	1.00±0.31
Ammonium Sulfate Fractionation (35% - 90%)	5.00	0.27±0.14	3.38±0.72	12.52±3.50	99.12±21.09	1.07±0.30
Gel Filtration Chromatography (Sephadex G-75)	3.00	0.09±0.04	2.53±0.36	28.11±3.74	74.19±11.35	2.39±0.32
Ion Exchange Chromatography (Dowex 50Wx8)	3.00	0.02±0.00	0.68±0.38	34.00±11.67	19.94±2.64	2.89±0.99

The elution profile of chromate reductase from sephadex G – 75 gel filtration column with Tris – HCl buffer pH 7.0 is described in Figure 2. Thirty fractions 5 ml each were collected from the column at the rate of 0.25 ml/min. Fraction 29 had the highest chromate reductase activity and had highest total protein concentration. In Figure 3, the elution profile of chromate reductase through dowex 50Wx8 ion – exchange column is being described. Sodium chloride gradient (0.00 – 1.0 M) was used in the elution. Thirty fractions were also collected, 5 ml each at the rate of 1.0 ml/min. It can also be seen from the figure that only two fractions had chromate reductase activity with fraction 12 having the highest enzyme activity and then fraction 13.

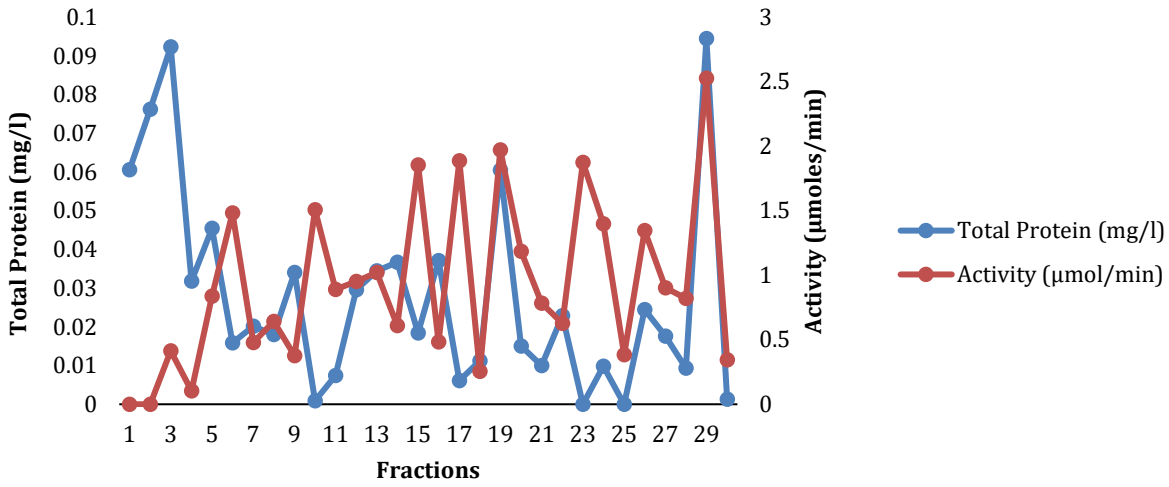

Figure 2: Elution Profile of Chromate Reductase Isolated from *Lactobacillus fermentum* IFO3956 from Sephadex G – 75 Gel Filtration Column

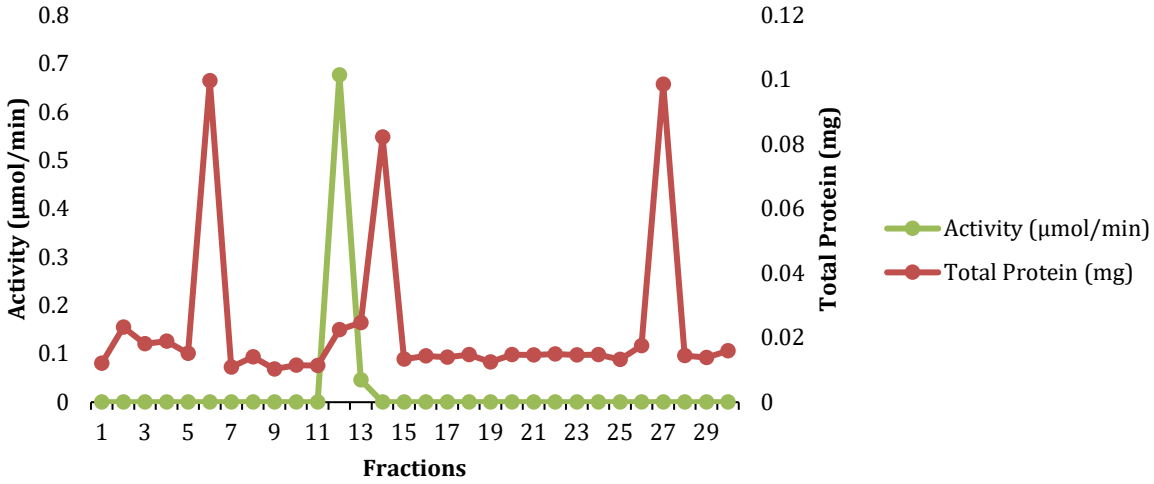

Figure 3: Elution Profile of Chromate Reductase Isolated from *Lactobacillus fermentum* IFO3956 from Ion - Exchange Chromatography (DOWEX 50Wx8) Column

The initial velocity studies carried out on the enzyme using potassium dichromate as the substrate is described in Figure 4. From the figure, it is deducible that a K_M and V_{max} of 0.25 mM and 10.0 µmol/min were obtained for the enzyme. The K_M value for this enzyme is lower than 374 µM for chromate reductase isolated from the bacterium *Pseudomonas putida* reported by Park *et al*., [7]. This result, suggest that the enzyme analyzed in this research had higher affinity for the substrate chromate than the one reported by Park *et al*., [7]. Other researchers have also reported a K_M value for chromate reductases from different bacterial species that is lower than the one obtained for chromate reductase isolated from *Lactobacillus fermentum* IFO 3956 [16, 24]. The maximum velocity (V_{max}) for chromate reductase isolated from *Lactobacillus fermentum* IFO 3956 (10 µmol/min) obtained is higher than 1.72 µmol/min reported by Park *et al*., [7] and 27 nmol/min reported by Suzuki *et al*., [16], which implies that chromate reductase isolated from *Lactobacillus fermentum* IFO 3956 had higher turnover and could therefore, convert higher concentrations of Cr^{6+} to Cr^{3+} than chromate reductases reported by previous researchers.

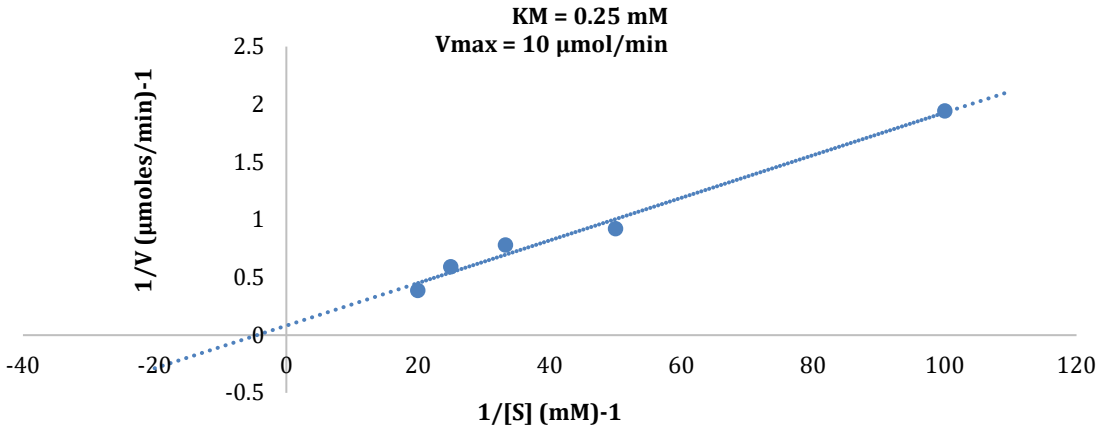

Figure 4: Lineweaver – Burk plot showing activities of Chromate Reductase Isolated from *Lactobacillus fermentum* IFO3956 at different substrate concentrations

The activity of the chromate reductase as a function of pH showed that the enzyme had an optimum pH for activity at 4.5 (Figure 5). This suggests that Acetate buffer pH 4.5 may be the best buffer for the activity of chromate reductase from *Lactobacillus fermentum* IFO3956. Figure 6 described the temperature dependence studies on the activity of the enzyme and it can be seen that the enzyme had 40ºC as the optimum temperature for its activity. A close optimum temperature for chromate reductases of 50ºC was reported by Suzuki *et al*. [16] and Sallau *et al*. [5], while Kwak *et al*., [17] reported an optimum temperature of 55ºC.

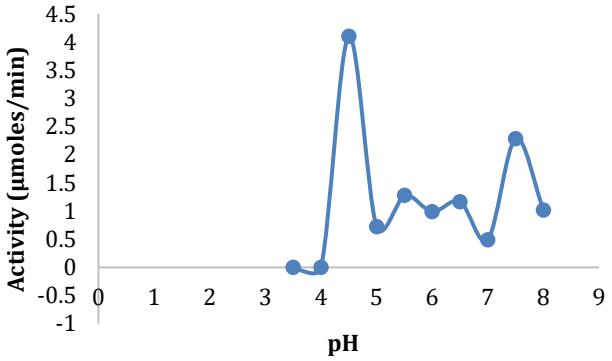

Figure 5: Activities of Chromate Reductase Isolated from *Lactobacillus fermentum* IFO3956 at varying pH

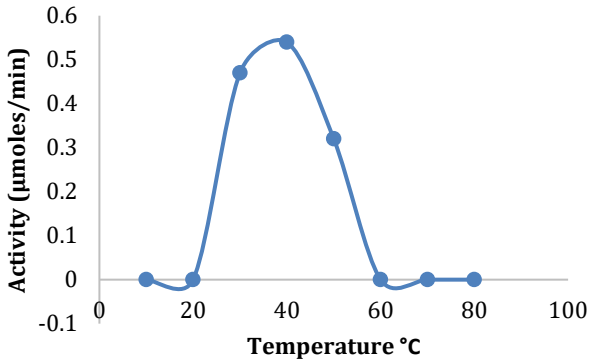

Figure 6: Effect of Temperature on the Activity of Chromate Reductase Isolated from *Lactobacillus fermentum* IFO3956

SDS PAGE analysis of the denatured purified chromate reductase gave two bands (Plate 1) with apparent molecular weight of 70 and 35 kDa, suggesting a dimeric protein or enzyme with two subunits. This therefore, confirms the purity of the isolated chromate reductase. However, this finding is in consistent with the reports of other researchers for chromate reductase from other sources. Bae *et al*. [18] reported a value of 84 kDa and 42 kDa for chromate reductase purified from *E. coli* which also indicates a dimeric structure. In addition, Omwirhiren and Amlabu [19] also reported chromate reductase purified from lichen photobiont, *Trebouxia erici* to have two subunits with molecular weight of 63 and 39 kDa. Enzymes were before, used in crude or not well – characterized form [20]. However, current technology makes it possible to isolate, purify and to even immobilize (bind to fixed support) specific enzymes needed for desired function [21].

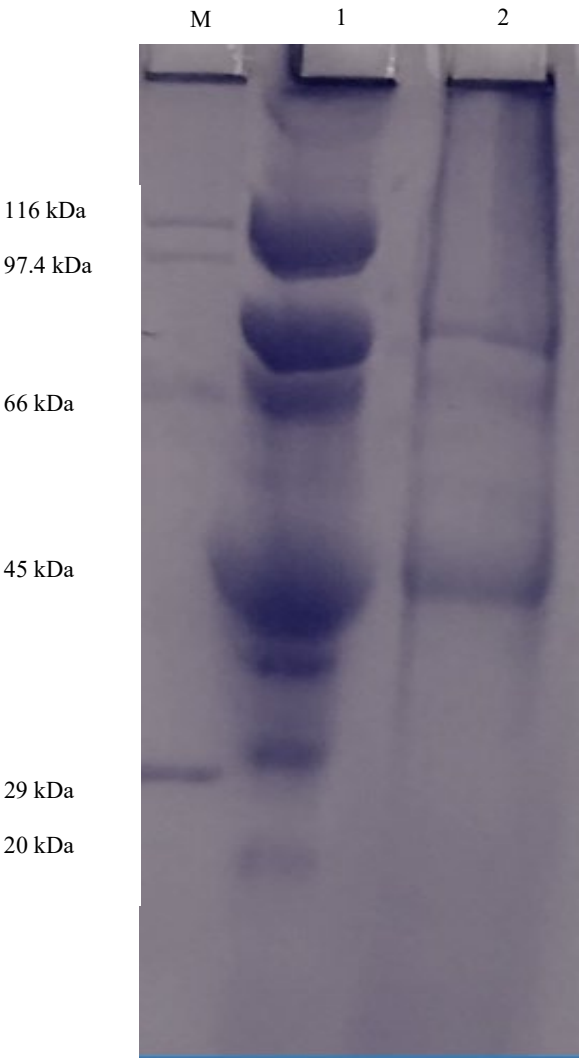

Plate 1: Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoregram of Chromate Reductase Isolated from *Lactobacillus fermentum* IFO 3956: Lane M = Marker, Lane 1 = Crude enzyme, Lane 2 = Partially purified enzyme

CONCLUSION

Chromate reductase, an enzyme that catalyse the conversion of toxic and highly soluble hexavalent chromium ion (Cr^{6+}) to less toxic and less soluble trivalent chromium ion (Cr^{3+}), was isolated and partially purified from *Lactobacillus fermentum* IFO3956. The characteristics of the enzyme revealed highly remarkable properties that could be used in remediating Cr^{6+} polluted environment via an enzyme immobilization system.

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CONFLICT OF INTEREST

The authors wish to declare that there is no conflict of interest regarding the publication of this manuscript.

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