

Purification of Chromate(VI) Reductase from Lichen photobiont, *Trebouxia erici* and its Effect on Wastewaters.

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Abstract

Chromate (VI) reductase (CR) was isolated and purified from soluble extracts obtained from lichen photobiont, Trebouxia erici by salt gradient and sequential chromatographic methods. The soluble extract was fractionated by ammonium sulphate precipitation, dialysed and applied onto CM-Sephadex ion-exchange column. The active fractions were pooled and chromatographed on a SephadexG-75 column .Electrophoresis on SDS-PAGE analysis of the partially purified chromate(VI) reductase revealed two bands with molecular weights of 39 kDa and 63 kDa . The enzyme had maximum activity at pH 6 and an optimum temperature of 40^{o}C. The enzyme was stable between pH 4 and 7. Double reciprocal plots of initial velocity data, using NADH as substrate, gave a K_M value of 118 µM and V_{max} of 0.173 µmol/min/mg respectively . Studies on the effect of chromium reductase on synthetic wastewater shows that chromate(VI) reductase (CR) was effective in the reduction of over 65% of Cr (VI) to Cr (III) in 4h. The bioremediation potential of chromate(VI) by lichen was discussed.

Keywords: Chromate(VI) reductase, purification, bioremediation, wastewater.

1. Introduction

Hexavalent chromium (Cr (VI); chromate) is produced as a by-product of many industrial processes, including leather tanning, chrome plating, stainless steel welding, pigment production and as a corrosion inhibitor in conventional and nuclear power plants (Donmez and Kocberber, 2005; Thacker *et al*, 2006;Parveze et al.,2015). Chromium (III) occurs naturally in the environment and is an essential nutrient required by human system to promote the action of insulin in body tissues so that the body can use sugar, protein and fat. Chromium has no known taste or odour (Zayed and Terry, 2003). Chromium is the second most abundant heavy metal contaminant, ranging between 0.008 to 173 µM in groundwater (Cetin et al.,2008 ; Mishra et al.,2012). Chromium, Cr (VI) is toxic, mutagenic and carcinogenic, and has been implicated in birth defects and diminution of reproductive health (Kaniojia *et al*., 1998;Augustynowicz et al.,2010).

In addition, chromium is difficult to contain and spreads rapidly through acquatic systems and subterranean waterways (Gonzalez *et al*., 2003). Due to its high solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids makes it easily absorbed in living cells (Sultan and Hasnain, 2005; Liu *et al*., 2006; Mabrouk, 2008).

Lichen is a group of plants made up of fungi and algae co-existing in a symbiotic relationship. The new thallus is a single organism. The algae (photobiont) are responsible for photosynthesis while the fungi (mycobiont) make up the bulk of the plant body (Nash, 1996).Lichen has been shown to play major role in the environment and some of these includes: Regulation of the water cycle of the ecosystem (Schiedegger, 1995), utilization by animals as a source of food as well as a protective covering and an agent of biological weathering in rocks (Culberson *et al.*, 2000). In addition, Lichen has been shown to produce secondary metabolites that are implicated in the manufacture of drugs and perfumes (Scott *et al.*, 1997). It has also been shown to be a very good environmental indicator for pollution monitoring around cities and factories (Sochting, 1999). These properties of Lichens could be explored in the bioremediation of chromium (VI) from the environment.
Chromates are strong oxidizing agents that are capable of reacting with nucleic acids. Cr (VI) poses greater threat to public health, the environment and ecosystems, compared to Cr (III) (Gibb, 2000; Casadevall and Kortenkamp, 2002; Sedman *et al*., 2006). The reduction of Cr (VI) to Cr (III) is therefore an attractive and useful process for the remediation of chromium pollution. Cleaning up of chromium-contaminated sites is a

challenging task because removal of Cr(VI) in aqueous solution is difficult (Singanan et al.,2007; Ghachtouli et al.,2013). Hence, proper treatment of tannery wastewater is essential before releasing it into the recipient environment The use of chemical methods for the remediation of chromates is prohibitively expensive for large scale environmental decontamination, and frequently also have damaging consequences of their own (Blowes, 2002,Liu et al.,2013). As such, the development of an effective system for bioremediation of Cr (VI) is highly desirable. The use of lichens as an environmental indicator for pollution monitoring in cities and factories has been reported (Denison and Carpenter, 1973). The implication is that lichen photobiont contains enzymes that are insensitive to the presence of heavy metals. There is therefore a possibility of using lichens for bioremediation.

Preliminary work have shown that lichen photobiont is capable of converting Cr (VI) as $K_2Cr_2O_7$ to Cr (III) supporting the presence of a reductase that is capable of degrading chromium and hence clean up our environment from the toxic effect of Cr (VI). Also, it was found to reduce Mn^{7+} as $KMnO_4$ to Mn^{2+} however, at considerably lower efficiency compared to Cr (VI). Although a number of studies have been reported on chromate bioreduction (Kleiman and Cogliatti, 1998; Wang *et al.*, 1989; Rajkumar, 2005; Sultan and Hasnain, 2006; Poilti *et al.*, 2007; Mabrouk, 2008;Prabu et al.,2012; Augustynowicz et al.,2015). To date, no work has been reported on chromate(VI) reductase from *T.erici*.

It is in the light of this background that the present work was designed to isolate ,purify and characterized chromate (VI) reductase from *T. erici* and to further to assess the potential use of *T.erici* in the bioremediation of Cr (VI) from the environment.

2. Materials and Methods

2.1 Materials

2.1.1 Plant

The lichen photobiont, *T.erici* used for the experiment was harvested from the wall of Biochemistry department, Ahmadu Bello University, Zaria. It was authenticated at the herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria. It was washed with water to remove earthy impurities before being prepared for use.

2.2 Chemicals

Nicotinamide adenine dinucleotide reduced, citric acid, molecular weight markers, bovine serum albumin and 1,5-diphenylcarbazide were products of Sigma chemical company, USA. Tris (hydroxxylmethyl) amino methane, glycine, , Acrylamide, N, N, methyl bisacrylamide (Bis), ammonium persulphate, glycine, bromophenolblue, coomasie brilliant blue, N,N,N'N' tetramethylethylene diamine (TEMED), sodium dodecylsulphate (SDS), sodium borohydride and Sodium periodate were products of British Drug House (BDH) Chemical, Poole, England. EDTA, mercaptoethanol, sodium hydroxide, sulphuric acid, potassium dichromate, sodium, dihydrogen phosphate, disodium hydrogen phosphate were products of Pharmacia fine Chemicals, Upsala, Sweden. All other chemicals were of analytical grade and were supplied by reputable chemical manufacturers.

2.3 Methods

2.3.1 Isolation Of Chromium Reductase

Cell free extract of lichen plant was prepared by homogenizing the lichen plant with 0.1M phosphate buffer, pH 7.0 in a fast speed blender and the homogenates were filtered and then centrifuged at 3000xg to obtain a supernatant that served as the crude enzyme extract. The cell free extract was used for the partial purification.

2.3.2 Ammonium Sulphate Precipitation

The pooled supernatants were then subjected to ammonium sulphate precipitation according to the method of Bae *et al*. (2000).

2.3.3 Dialysis of Pooled Fractions

The pellet recovered by centrifugation at 10,000 g for 15 min was resuspended in 30ml of the extraction buffer. The fractions from ammonium sulphate precipitation was poured into a dialysis bag and dialyzed for 24-72 hours in 100mM phosphate buffer pH 7.2.

2.3.4 CM-Sephadex Chromatography

The dialyzed extract from was applied directly to a CM-Sephadex column previously equilibrated with phosphate buffer. The column was developed at a flow rate of 20ml/h and 3ml fractions were collected.

2.3.5 Size Exclusion Chromatography

The pooled fractions were applied unto Sephadex G-75 column previously equilibrated with 100mM phosphate buffer. The column was developed at a flow rate of 20ml/hr and 3ml fractions were collected and assayed for protein and chromium reductase activity. Fractions with high specific activity were pooled.

2.3.6 Chromium Reductase Assay

This was carried out by measuring decrease in Cr (VI) concentration as described by method of Park *et al* 2000. Extract of the chromium reductase will be incubated for 2 hours at 30^{o}C in a reaction mixture containing 0.1mM NADH, (40µl) and 600µl of 0.2% 1.5-diphenylcarbazide. Absorbance was read at 540 nm against a reference sample incubated without the enzyme extract.

2.3.7 Total Protein Determination

The protein content of the enzyme was determined according to Bradford (1976) using bovine serum albumin as standard.

2.3.8 SDS-PAGE Analysis

Electrophoresis was carried out for fractions with high specific activity due to Chromium reductase in 12% polyacrylamide disc gel according to the method of Davis (1964) using Tris-glycine buffer, pH 8.3. Protein bands were viewed by staining with Coomassie Brilliant blue R-250 (Sigma). Subunit molecular weight was also estimated by SDS-polyacrylamide gel electrophoresis (Davis, 1964).

2.3.9 Optimum pH Determination

The activity profile of chromium reductase was determined as a function of pH using NADH as substrate. The buffers were prepared at different pH values in the range of pH 4-9[100mM, Citrate (4.0-6.0), phosphate (6.0-7.0) and Tris (7.0-9.0)] and the activity of the enzyme was determined A plot of Chromium reductase activity was made against pH to determine the optimum pH.

2.3.10 pH Stability Studies

The enzyme was stored at various pHs using the following buffers; 100mM, Citrate (4.0-6.0), phosphate (6.0-7.0) and Tris (7.0-9.0) and 4^{o}C for 40 hrs, and after adjusting the pH to 7.0, the residual activity assayed as described in previous studies(Park *et al*., 2000; Bae *et al*., 2005). The use of two buffers compensated for the buffer associated effects.

2.3.11 Optimum Temperature Determination

Chromium reductase activity was assayed at 4°C, 20 °C, 40 °C, 50 °C, 60 °C and 70 °C. A plot of Chromium reductase activity was made against temperature to determine the optimum temperature.

2.3.12 Temperature Stability Studies

Aliquots of enzyme(0.1ml) was incubated in 100mM KH_2PO_4 for 14 hrs at various temperature (4 °C, 12 °C, 20 °C, 28 °C, 37 °C, 50 °C and 60°C), and the residual enzyme activity was assayed as described by Bae *et al* (2005).

2.3.13 Kinetic Parameters

The substrate NADH was prepared at the concentration range of 0.2-3mM in borate buffer pH 6.0. Chromium reductase activity was determined and the inverse of activity was plotted against the inverse of substrate concentration. The K_M and V_{max} of the enzyme were determined from a double reciprocal Lineweaver-Burk's plot (Lineweaver and Burk, 1934).

2.3.14 Determination of the effect of CR on wastewater

This was done according to the method of Greenberg *et al* (1981) and modified by Camargo *et al* (2003). Briefly, 100µl and 200µl of CR was added to each 100ml of synthetic wastewater[1.5g Sodium Chloride;0.5g Tannic acid and 0.2g Potassium dichromate dissolved in 1 litre of distilled water] .The CR-wastewater mixture was incubated in a shaker at 30°C and the reduction of Cr(VI) monitored spectrophotometrically at every 30 minute interval until all the Cr(VI) have been completely reduced.

3. Results

Table 1: Purification scheme for purified chromate(VI) reductase from *T.erici*

Step Number	Purification Steps	Total protein (mg)	Total activity (µmoles/ min)	Specific activity* (µmoles/mg/min)	Yield (%)	Purification fold
1	Crude extract	250	1.20	0.0048	100.0	1.00
2	$(NH_4)_2SO_4$ fractionation	128	0.90	0.0070	75.0	1.46
3	Dialysis	115	0.83	0.0072	69.2	1.50
4	CM-Sephadex	95	0.70	0.0074	58.3	1.54
5	Sephadex G-75	40	0.45	0.0110	37.5	2.29

*One unit of enzyme was defined as the amount of enzyme that converts 1 nmol of Cr (VI)/min under standard assay conditions.

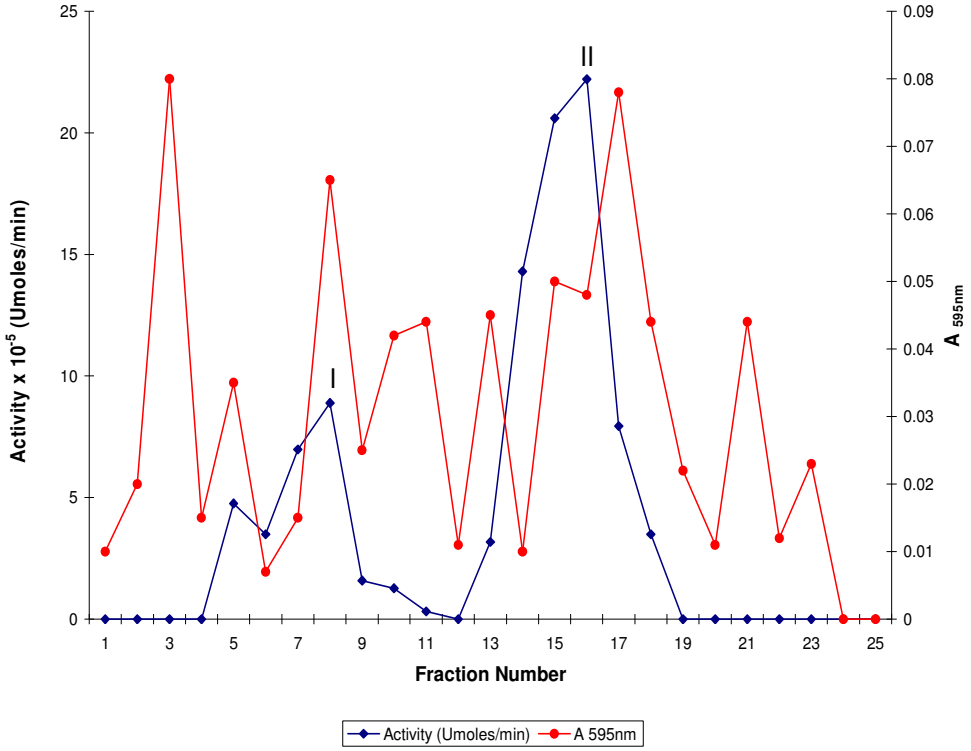

Fig.1: A typical elution profile for the chromatography of CR on CM-Sephadex Column (1 cm x 50cm) previously equilibrated with 100mM phosphate buffer at a flow rate of 20 ml/h and 3 ml fractions were collected.

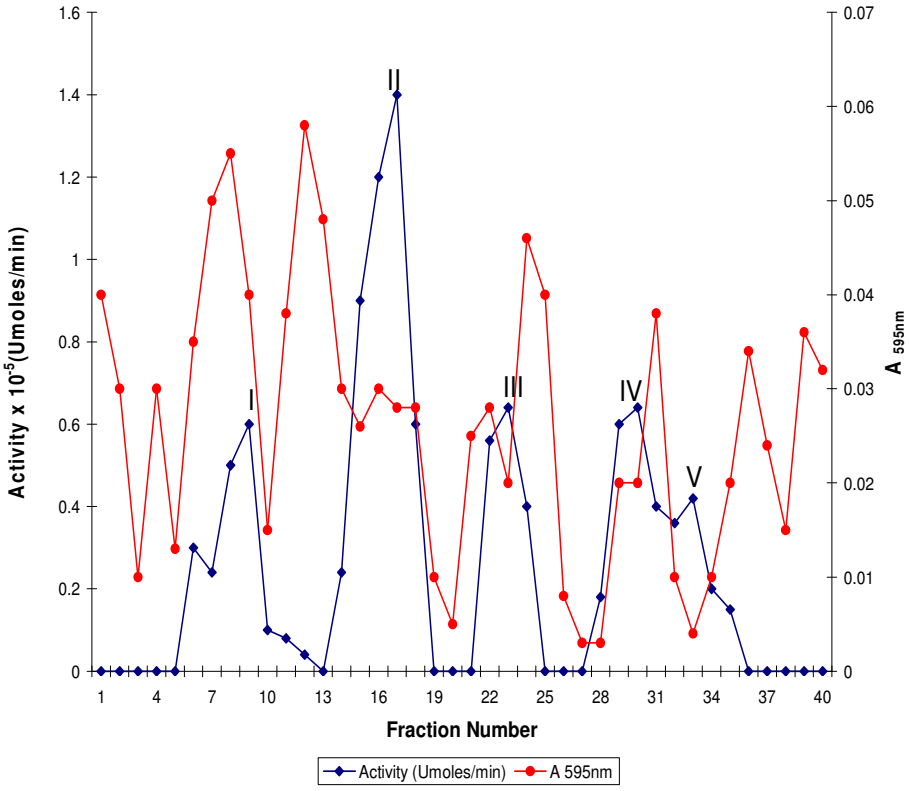

Fig.2: elution profile for gel filtration of *T. erici* CR on Sephadex G-75 Column (1 cm x 50cm) previously equilibrated with 100mM phosphate buffer pH 7.2 at a flow rate of 20 ml/h and 3 ml fractions were collected.

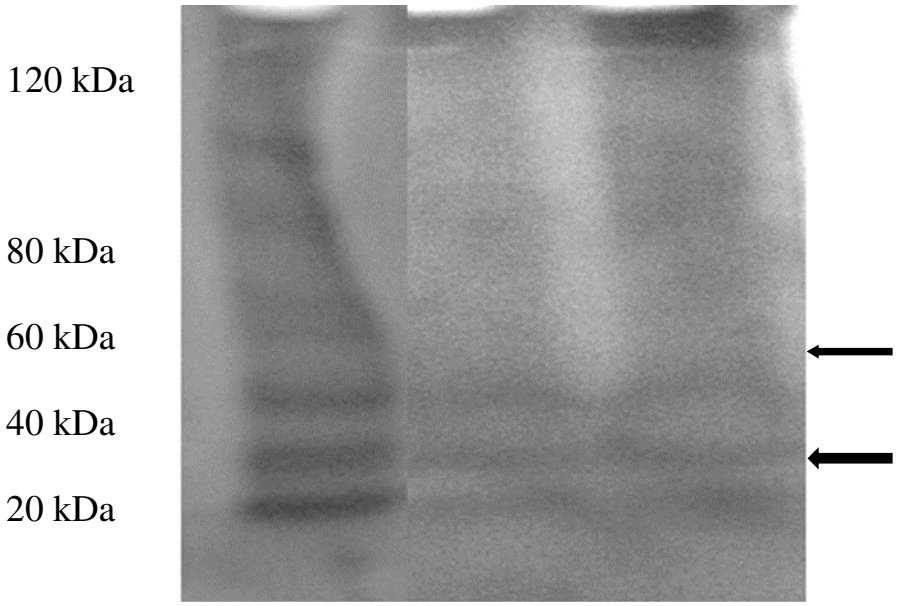

Plate.2: SDS-PAGE analysis of purified CR from *T. erici* on 12% acrylamide gels. Lane I: Molecular weight* standards (20 – 120 kDa). Lane II & III: Purified Chromate reductase.

* **Molecular Weight** standards Albumin, Bovine serum(66 kDa), Glutamine dehydrogenase, Bovine liver (55kD), Ovalbumin, chicken egg (45 kDa) glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36 kDa) Carbonic anhydrase, Bovine erythrocyte (29 kDa), Trypsinogen, Bovine pancrease (24kDa), Trypsin inhibitor, Soybean (20kDa), α-lactalbumin, bovine milk (14kDa) (Sigma).

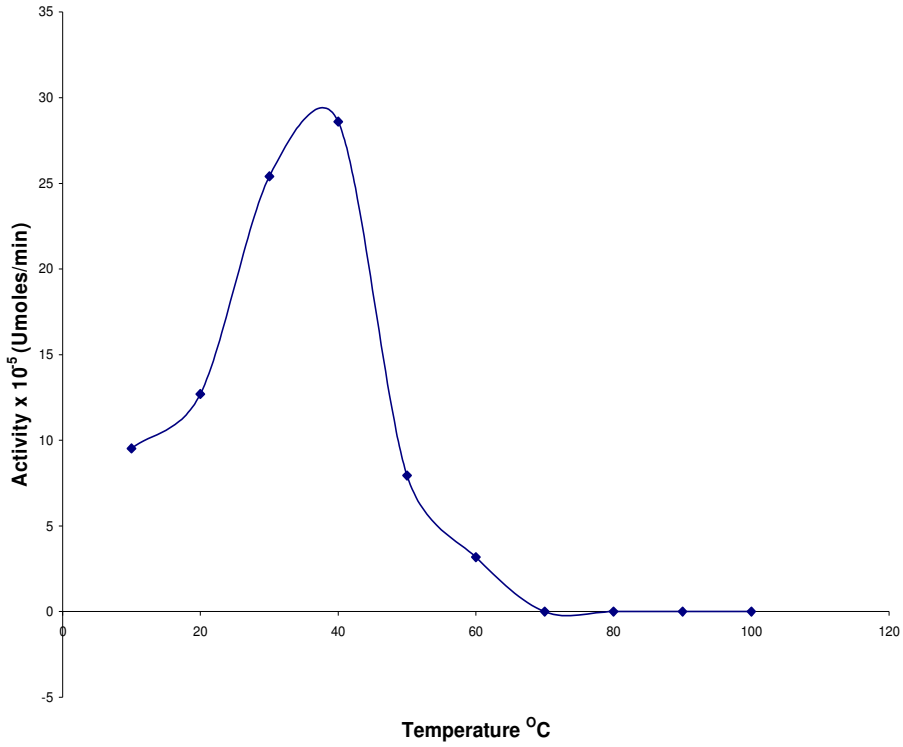

Fig.4: Optimum temperature determination for the activity of CR .Results are averages of three experiments

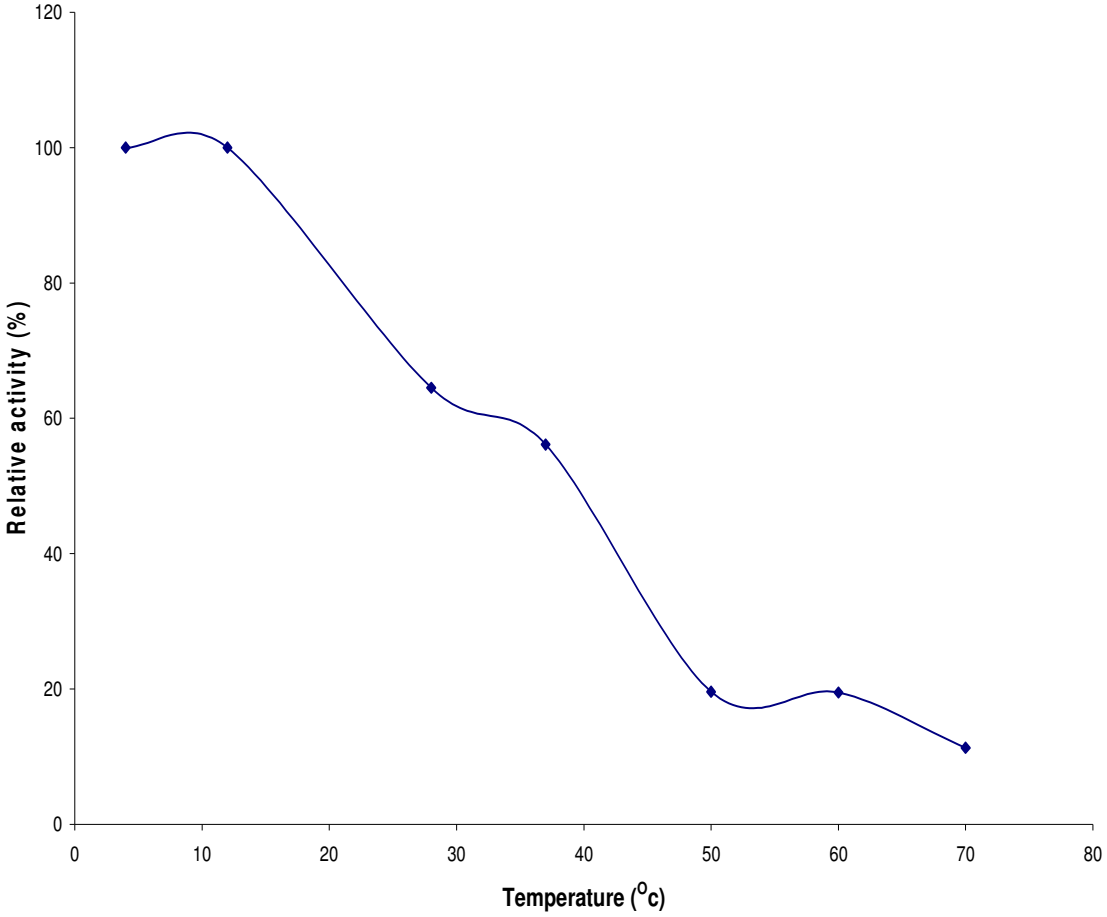

Fig. 5: Thermostability of CR at various temperatures. Results are averages of three experiments.

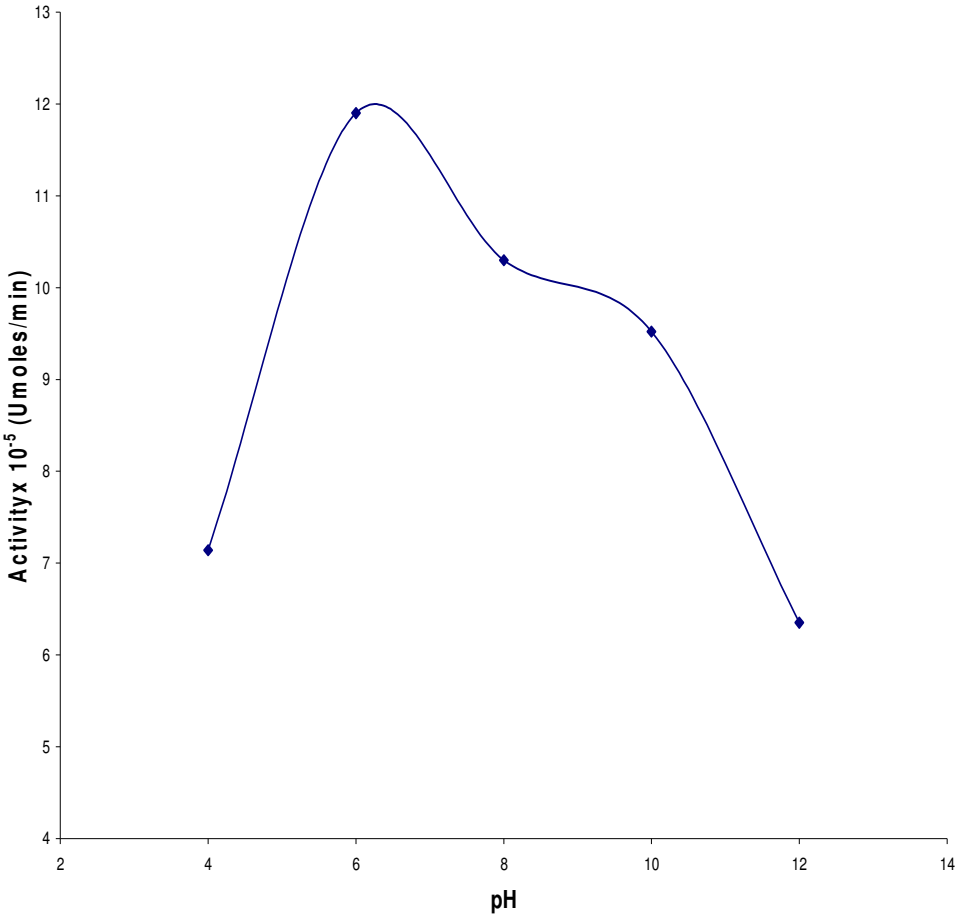

Fig.6: Optimum pH determinations for the activity of CR. Results are averages of three experiments.

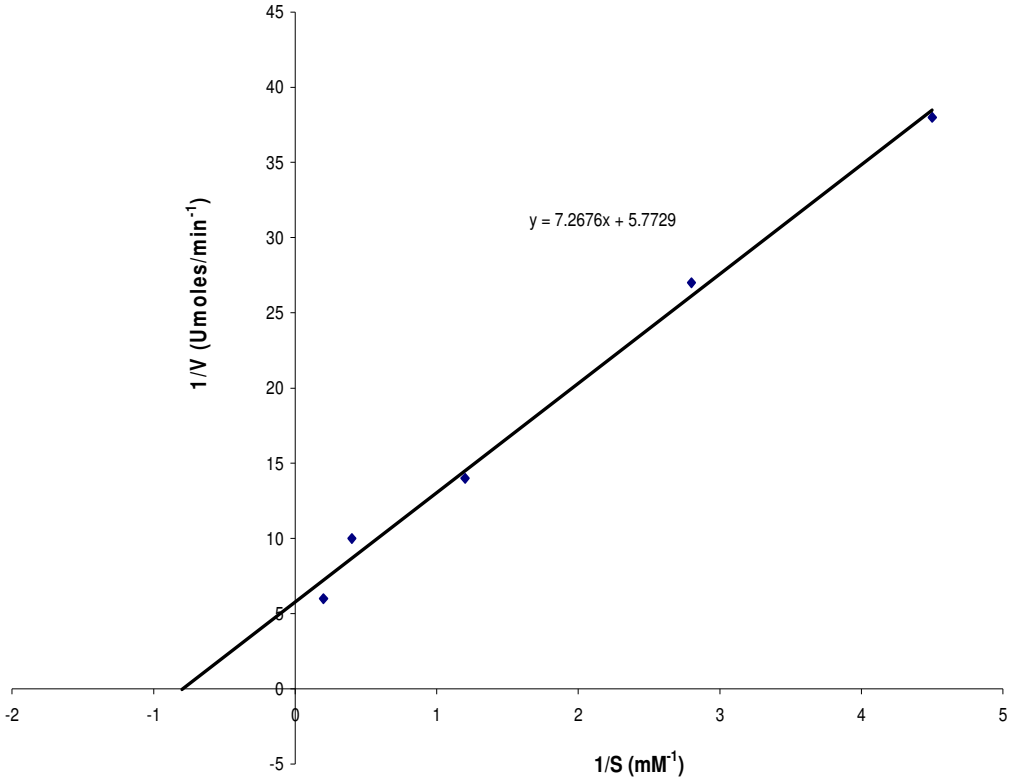

Fig. 7: Lineweaver-Burke plot relating CR velocity to NADH concentration. Each point represent the average of three experiments.

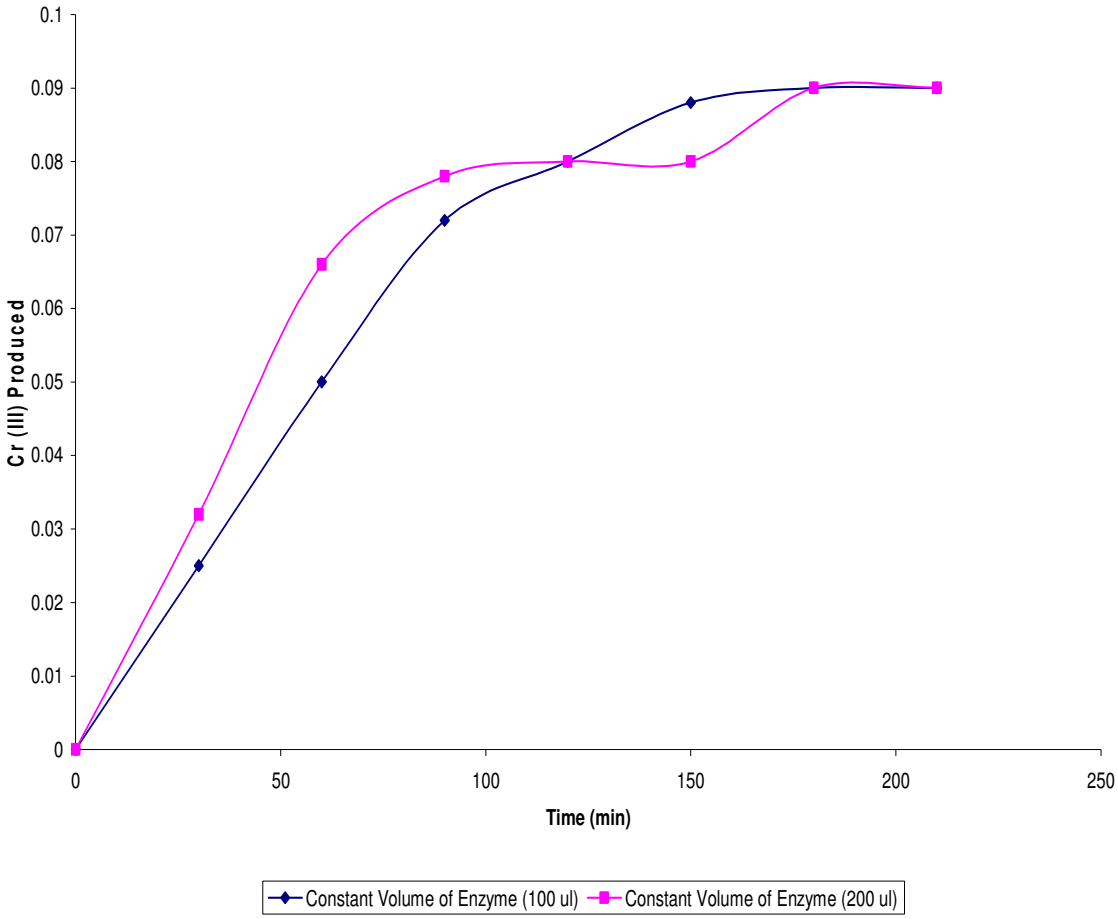

Fig. 8: Effect of Chromium Reductase on Synthetic Waste Water

4. Discussion

The purification scheme showed that the purification fold after ammonium sulphate fractionation increased by 1.46 fold compared to CM-sephadex and Sephadex G-75 steps which increased by 1.54 and 2.29 respectiviely.This suggests that ammonium sulphate fractionation step may have affected the CR activity. The enzyme was purified to about 2.29-fold with 37.5% recovery. The purified extract had a relatively higher CR activity than the crude extract. This observation can be attributed to the removal of endogenous inhibitors and other contaminating constituents by hydrophobic interaction with the column. That the crude enzyme extract contains activity modifying compounds was found by Suzuki *et al* (1992) for *P. ambigua*. This was also observed by a number of investigators for *P. putida* (Park *et al*., 2000) and *E. coli* (Bae *et al.*, 2005) respectively. The purified CR requires NADH as an electron donor for Cr (VI) reduction.

The single active peak obtained in ion exchange purification step indicates that the enzyme was purified at one cycle of purification step which could be attributed to non interaction of the enzyme with other protein components of *T. erici*. This is in contrast with CR from other sources that required several purification steps to attain purity (Suzuki *et al.*, 1992; Bae *et al*, 2000; Matin *et al*, 2003; Desai *et al*, 2008). The 58.3% yield showed that CR is not a major protein by composition in *T.erici*. This is also reflected by the presence of other peaks which had no CR activity.

The two peaks obtained from Sephadex G-75 elution profile suggest that CR exist in different forms. The protein electrophoregrams of the purified CR on SDS-PAGE showing subunits of 39 kDa and 63 kDa respectively further supports the possible existence of the enzyme in different forms. The molecular weight of the enzyme

determined on SDS-PAGE electrophoresis under denatured conditions gave two bands with an apparent molecular mass of 39 kDa and 63kDa respectively. This is consistent with most CRs from other sources. Park *et al* (2000) could not ascertain whether the enzyme is a dimer or a trimer as they reported a molecular weight of 20kDa for CR purified from *P. putida* on SDS-PAGE and 50kDa on gel filtration while Suzuki *et al* (1992) obtained a value of 65 kDa for CR purified from *P. ambigua*. However, Bae *et al* (2005) reported a value of 42kDa and 84kDa on SDS-PAGE and gel filtration respectively for CR purified from E. *coli* indicating a dimeric structure. Matin *et al* (2003) reported a molecular weight of 20kDa for *P. putida* with a broad single band on SDS-PAGE. The findings of Bae *et al* (2000,2005) suggest that there is a strong possibility that the enzyme exist as a dimer for *E.coli*. This however differs from the findings of Park *et al (2000)* that obtained a value of 20kDa on SDS-PAGE and a native protein molecular mass of presence of 50kDa on gel filtration for CR isolated from *Ps. putida.* Therefore it is not known whether the enzyme is a dimer or a trimer.The *Ps ambigua CR* exhibited a similar behaviour,with a molecular mass of 25 kDa on SDS-PAGE and a mass of 65 kDa on gel filtration(Suzuki *et al*,1992).These findings suggests that proteins can exhibit nonproportionate movement upon SDS-PAGE and gel filtration for several reasons. One possibility is that both reductases possess intrasubunit cross-linkages that, by producing altered conformation and Stokes radii, influence protein movement in gels differently (Smith, 1994). As to whether CR from lichen sp is a monomer or a dimer remains open for further investigation.

In the characterization of CR, the pH activity profile reveals optimal activity at pH 6.0 in the direction of Cr (VI) reduction. The pH and not the chemical composition of the given buffer determine the activity suggesting the fact that different buffers gave very similar activities at pH 6.0. However, considerable amount of activity by CR is observed at the pH range of 4-7. This indicates that CR is capable of effecting the release of H+ which is typical of most reductases (Song *et al.*, 2007) into the physiological milieu. Moreover, most studied CRs have been shown to be active within a broad pH of 5-9 (Donmez and Kocberber, 2005; Rajkumar *et al*, 2005; Bae *et al*, 2005; Thacker *et al*, 2006; 2007 and Mabrouk, 2008). The observed difference in CR optimal pH may be due to differences in sources, substrate type and concentration used for assaying CR activity. Also a profile of pH against time with various concentration of Cr (VI) show maximum activity at pH 6.0 in the narrow range of pH 6-8. This data indicate that CR is active under physiological conditions with a strong potential for Cr (VI) bioremediation *in vivo. Lichen spp*, being a plant that grows in harsh environment especially in places with heavy metal concentration, there a strong possibility that an acidic pH will greatly favour and contribute to the bioremediation of toxic Cr (VI) to less toxic Cr (III).

The effect of temperature on the activity of the purified CR was analyzed using NADH as substrate. The relationship between temperature and activity was a typical denaturation (bell-shaped) curve. The purified CR of *T. erici* exhibited a high relative activity over a broad range of temperature between 10 -50^{o}C and showed maximum activity at 40^{o}C.It was completely inactivated after 30 min incubation at 50^{o}C. Both findings indicate that CR from *T. erici* has the capacity to remain stable even beyond physiological temperature and still maintain its bioremediative property. As it is true for most chemical reactions, the rate of enzyme-catalyzed reactions generally increases with temperature, within the temperature range in which the enzyme is stable and retains full activity. The rate of most enzymatic reactions approximately doubles for each 10^{o}C rise in temperature (White *et al*, 1978). However, the temperature coefficient varies somewhat from one enzyme to another depending on the energy of activation of the catalyzed reaction. Although enzyme catalyzed reactions often appear to have an optimum temperature, the peak in such a plot of catalytic activity against temperature results because enzymes being proteins are denatured by heat and become inactive as the temperature is raised beyond a certain point. The apparent temperature optimum is thus the resultant of two processes (1) the usual reaction rate with temperature and (2) the increasing rate of thermal denaturation of the enzyme above a critical temperature (White *et al*,1978).

Most reports on the thermo stability of CR showed that optimum temperatures varied between 30 – 40^{o}C and findings from present study is consistent with previous report (Suzuki *et al*, 1992; Bae *et al*, 2005; Mabrouk, 2008).Such a low temperature suggest that CR isolated from *Lichen spp* will have a strong potential for chromium bioremediation at physiological temperature. However, exceptional cases have also been reported from various sources. Park *et al* (2000) reported an optimum temperature of 80^{o}C for CR activity purified from P. *putida* while Matin *et al* (2003) reported an optimal temperature of 70^{o}C with significant activity across a broad temperature range of 30 – 80^{o}C. Under low temperatures, inactivation of enzyme is low and extremely negligible (Patel, 1985).

From the Lineweaver-Burk plots for the evaluation of kinetic parameters of CR with NADH as substrate, the K_M and V_{max} values of 118 µM and 0.173 µmol/min/mg were obtained respectively. The K_M obtained from this study is comparable to those of CR from other sources. Its K_M was higher than those of *P. putida* (Park *et al*, 2000) and lower for those of *E. coli* (Bae *et al*., 2005). The low K_M is indicative of high substrate affinity of the enzyme, CR of *T. erici*. Furthermore with a V_{max} of 0.173umol/min/mg it shows that the enzyme can release 10.38 µmol of Cr (III) in 1h into the physiological milieu thereby potentiating the remediation of Cr (VI) from the environment. The findings of Park *et al* (2000) revealed that Cr isolated from *P. putida* gave K_M and V_{max} values of 374µM and 1.72 µMol/ min/mg protein respectively. Suzuki *et al* (1992) recorded a value of K_M and V_{max} for Cr isolated from *P. ambigua* as 23.5µM and 13.4 µM/min/mg protein respectively.

Based on tested physico-chemical properties, including the molecular weight, V_{max}, K_m, electron donor usage, optimum temperature and pH, there is a strong possibility that the purified CR in this study may be different from other known CRs (Suzuki *et al*., 1992; Park *et al*., 2000; Ackerley *et al*., 2004; Bae *et al*., 2005). This is however open for further investigation.

Studies on the effect of the native CR on waste water revealed that 100µl of CR of *T. erici* was effective in converting 100ml of Cr(VI) (as wastewater) to Cr(III) in 4h. *In-situ* capacity to remediate, necessitates the ability of a remediating organism and its key enzymes to function in the presence of mixed waste. Park *et al* (2002) studied the effect of department of energy(DOE) contaminants on CR purified from *Ps putida* and found that though the enzyme activity was inhibited as a result of mixed pollutants, there was however an appreciable remediation of chromate from the site. Matin *et al* (2003) in a separate study also observed enhanced effectiveness of chromate reducing enzyme at low growth rate and a decrease in enzyme sensitivity due mixed pollutants at contaminated sites.

5. Conclusion

The findings from present study have shown that *Lichens* contains CR and this enzyme has been purified through chromatographic method and characterized. The native CR tested on wastewater shows an appreciable conversion of Cr(VI) to Cr(III) thereby suggesting the novel role of purified CR from *T. erici* in bioremediation.

6. Recommendation

Further work in this area will require biophysical studies of CR as well as the cloning and DNA sequence analyses of the genes as this will provide additional information regarding the mechanism and function of CR activity by *T. erici*. Amino acid sequencing and determination of the 3-dimensional structure of the enzyme will also provide information that will greatly facilitate the use of protein and genetic engineering to enhance the CR remediation potential of *T. erici*.

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