Inhibition Assays of Urease for Detecting Trivalent Chromium in Drinking Water

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1 Introduction

Chromium is one of the most common heavy metals occurring in the soil with a wide range of industrial applications. Metallurgical industries use almost 90% of the total Cr production for making stainless steel and alloyed steel, while the remaining fraction utilizes in refractories and foundries. Leather tanning, pigment manufacturing, and plating industries use different Cr-based chemicals on a large scale [1]. Cr occurs in two primary oxidative forms in nature as trivalent (Cr^{3+}) and hexavalent (Cr^{6+}) [2]. Various hazardous effects are reported by the studies on human as well as on the ecosystem [3]. India contributes 14% of worldwide chromium production out of which the Sukinda Mines Valley in Odisha state produces 98% of total chromium. Sukinda valley is a known polluted area in the world due to the high amount of chromium leaching out in the surrounding water bodies [4].

The level of toxicity of chromium depends on the uptake and accumulation [5]. Cr^{6+} is highly water soluble and exists as chromate oxyanion (CrO_4^{-2}). The presence of excessive amount of this metal ion within the body causes hazardous effects on the individual's health. The solubility, high oxidation potential, and membrane permeability make the hexavalent chromium more toxic. Cr^{6+}, acting as the potential carcinogen, triggers the formation of reactive oxidation species and affects the DNA causing mutations [6]. Cr^{6+} is reported for detrimental effects on anti-oxidation genes resulting in increased oxidative stress in human placenta

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leading to apoptosis [7]. The effects of Cr^{6+} toxicity include respiratory tract irritation, gastrointestinal problems, dermatitis, and allergic skin reactions [8, 9]. Cr^{6+} toxicity affects the vital organs of freshwater fishes and declines the levels of proteins and glycogen in the body of fishes leading to the decrease of biodefence mechanism of fish [10].

However, Cr^{3+} cannot cross the biological membranes, and a significant amount of Cr^{3+} is bearable by mammals [11]. Cr^{3+} is relatively insoluble in water and thermodynamically stable. Cr^{3+} gets oxidized to Cr^{6+} in the presence of redox couples, for example, Mn(II)/Mn(IV) or H_2O/O_2, with redox properties [12, 13]. Transformation of Cr^{3+} into Cr^{6+} and vice versa results in the increased health hazard for humans and the environment [12]. Earlier studies reported the essentiality of Cr^{3+} as a cofactor for insulin action to regulate the blood glucose level [11]. However, recently some studies raised doubt on the requirement of Cr^{3+} as a cofactor for the action of insulin. Furthermore, Cr^{3+} showed the insignificant benefits in the patients with type 2 diabetes and impaired glucose syndrome [14]. Nevertheless, the excess amount of Cr^{3+} accumulation by these supplements may lead the formation of Cr-DNA adducts causing potential genotoxicity [15]. Thus, the controversy arises over the use of chromium as supplements.

The uncontrolled release or the excessive presence of chromium in the environment creates a non-negligible threat to the ecosystem. The threat demands to take some necessary steps for detection and elimination of chromium from the affected areas. Enzyme-based biosensors can apply for the detection of various heavy metals such as lead, arsenic, and cadmium. Urease (urea amidohydrolase; EC 3.5.1.5) is one of the well-studied enzymes for the inhibition by heavy metals due to its certain advantages. Urease has abundant sources: bacteria, algae, fungi, plants, and soil. The biological activity of soil is a direct indication of the presence of urease. Urease is sensitive toward heavy metals, for example silver, cobalt, nickel, cadmium, lead, chromium, and mercury. The metal ions interact with the cysteine residues present in its active site [17, 18]. Various urease-based biosensors are in use for the detection of heavy metals in the environment. Chromium was found to form an unstable complex with urease and thus reduces the overall enzyme activity significantly [19].

Urease is the first crystallized and known nickel-containing enzyme and can catalyze the urea into ammonia and carbon dioxide [20, 21]. The rate of catalysis may approach 10^{14} times the uncatalyzed conversion [22]. The structure and molecular weight of this enzyme vary with its source of origin such as plant, bacterial, fungal, and algal. Urease is a hexameric molecule with the active site located in the α-subunit of the molecule. The active site of the urease contains nickel and conserved amino acid sequence irrespective of the origin of the enzyme [23, 24]. Certain heavy metals found to inhibit the activity of urease by reacting with the sulfhydryl groups in the active site of urease [25].

The present work elaborates the study of jack bean urease kinetics and enzyme inhibition assays for the effect of Cr^{3+} on urease activity. The variation in the activity of the urease due to the presence of Cr^{3+} ions can apply for the detection of Cr metal present within the sample.

2 Materials and Methods

Urease-catalyzed reactions using urea as a substrate were carried out in the presence of different concentrations of Cr^{3+} for the study of the effects of Cr^{3+} ions on the enzyme activity. Comparison of inhibited enzyme activity with and without Cr gives the extent of the effect of Cr ions on the enzyme.

2.1 Materials

Commercial jack bean urease (EC 3.5.1.5) purchased from Sigma-Aldrich was used for enzyme assays. Laboratory grade urea was used as substrate obtained from Merck Chemicals. Trivalent chromium standard solution was prepared using chromium chloride obtained from Merck Chemicals. Reagents for the phenate method constituted phenol, absolute ethanol, sodium nitroprusside, trisodium citrate, and sodium hypochlorite. The reagents for phenate method include a phenate reagent (10 g of phenol to 100 mL of 95% ethanol), sodium nitroprusside solution (0.5 g in 100 mL of deionized water), and an oxidizing reagent. The oxidizing agent comprises a solution of alkaline reagent (10 g trisodium citrate and 0.5 g sodium hydroxide for 100 mL) and sodium hypochlorite in 4:1 ratio. All the chemicals were prepared using deionized water. Urease solution (0.25 mg/mL) was prepared in 0.2 M sodium phosphate buffer pH 7.0.

2.2 Determination of Enzyme Activity

Urease catalyzes the urea into ammonia and carbon dioxide as the reaction given in Eq. 1. Phenate method detects the amount of ammonia released by the action of urease.

$$(NH_2)_2CO + 2H_2O \rightarrow 2NH_4^+ + CO_3 \quad (1)$$

The volume of the enzyme assay was 10 mL. Urease solution (20 µL) was mixed with one mM urea solution and incubated for 15 min at 30 °C. After incubation, 0.4 mL each of phenate reagent and sodium nitroprusside, and 1 mL of an oxidizing reagent were added to the reaction mixture with gentle mixing. The

assay tubes were kept for incubation for 30 min in the dark at room temperature. Phenate reagent reacts with ammonium ions released and produces the blue-colored compound, indophenol blue. Sodium nitroprusside acts as a catalyst, while oxidizing reagent provides alkaline conditions. Amount of ammonia released determined by spectrophotometric analysis at 630 nm. The standard curve for ammonium ions was prepared using ammonium sulfate solution. Urease activity is calculated using Eq. 2.

$$\text{Urease activity} = \frac{\text{Absorbance of sample} * \text{Dilution factor}}{\text{Slope of standard curve} * \text{Time of assay} * \text{Volume of assay}} \quad (2)$$

2.3 Estimation of Enzyme Kinetics

Experiments were carried out to estimate the enzyme activity by plotting the experimental observations as suggested in Michaelis–Menten method. The hyperbolic curve is translated into a straight line to obtained the critical enzyme kinetic parameters, V_{max} (maximum velocity at the saturating substrate concentration) and K_m (the substrate concentration for which V_{max} is half of its value) as advocated by various researchers. Lineweaver–Burk plot, Eadie–Hofstee plot, and Hanes–Woolf plots were attempted in this study to find out the urease kinetics. Thus, enzyme activity was determined by varying the concentrations of urea from 0 to 4 mM at the constant enzyme concentration of 0.25 mg/L.

2.4 Inhibition Studies

The principle for the development of a biosensor to detect chromium in the potable water was based on inhibition studies of urease by Cr^{3+}. Urea of 1 mL (1 mM) was subjected to different concentrations of Cr^{3+} solutions ranging from 0.0001 to 1000 ppm. Enzyme solution (20 μL) was added to the 10 mL of the reaction mixture and incubated for 15 min at 300 C. To the mixture, 0.4 mL of phenate reagent and sodium nitroprusside were added and mixed well. Followed by the addition of 1 mL oxidizing reagent and incubation for 30 min in the dark, absorbance was measured at 630 nm using a double beam UV visible spectrophotometer. The degree of inhibition is calculated by relating the inhibited activity with the activity of pure enzyme solution as given in Eq. 3.

$$\% \text{ Inhibition} = \frac{\text{Activity without inhibitor} - \text{Activity with inhibitor}}{\text{Activity without inhibitor}} \times 100 \quad (3)$$

3 Results and Discussion

3.1 Enzyme Activity

The plot of enzyme activity and substrate concentrations for the definite enzyme concentration was obtained as a hyperbolic pattern (Fig. 1). This behavior can be explained by Michaelis–Menten equation as given in Eq. 4.

$$v = \frac{V_{\max} \times S}{K_m + S} \tag{4}$$

where v is the initial velocity, $V_{\max}$ is maximum velocity, and K_m is the Michaelis–Menten constant. K_m and $V_{\max}$ reveal the kinetics of the enzyme-catalyzed reaction for the definite set of experimental conditions. From Fig. 1, approximate values of K_m and $V_{\max}$ can be observed as 0.6 mM of urea and 23 mM/min, respectively. The hyperbolic curve was translated into a straight line as suggested in Lineweaver–Burk plot for precise determination of the kinetic parameters. The double reciprocal plot estimated the values of K_m and $V_{\max}$ as 0.7 mM of urea and 26.88 mM/min and represented in Fig. 2.

The Lineweaver–Burk plot gives the accurate estimation of the $V_{\max}$. However, for K_m, the error value might increase as the reciprocal data may not necessarily give the symmetrical values. The lower concentrations of substrate influence the slope of the plot to a great extent. Researchers have suggested the different plot to avoid the drawbacks of the double reciprocal plot for calculation of $V_{\max}$ and K_m. Eadie–Hofstee plot gives the relation between the substrate concentration and velocity of the enzyme action as given in Fig. 3. The plot has given the $V_{\max}$ and K_m values as 29.94 mM/min and 0.8152 mM, respectively, which are higher than

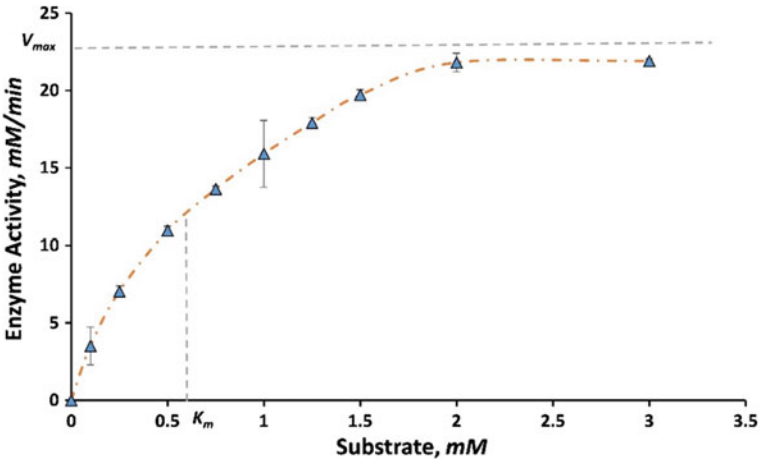

Fig. 1 Michaelis–Menten curve for urease enzyme activity

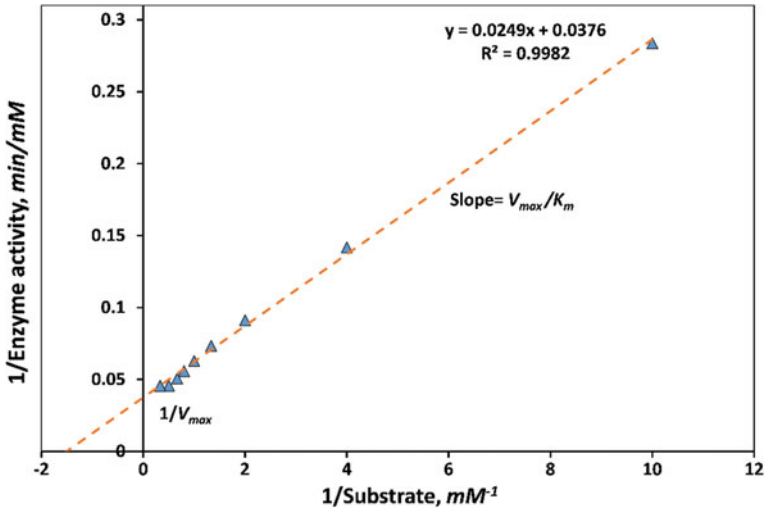

Fig. 2 Lineweaver–Burk plot for urease enzyme activity

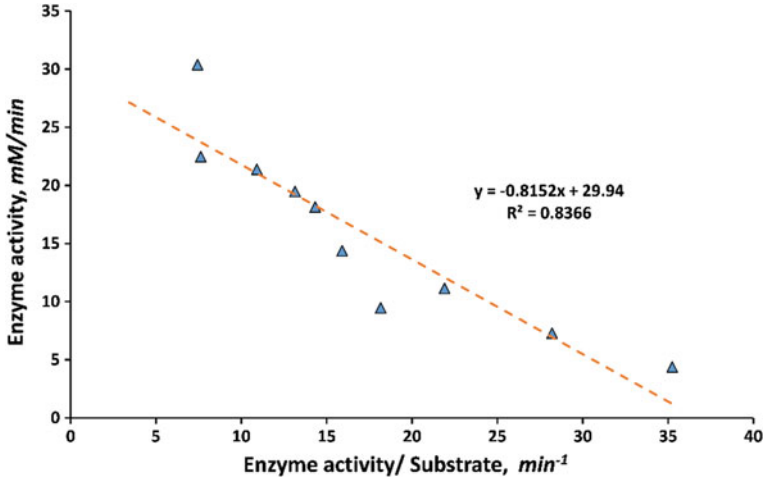

Fig. 3 Eadie–Hofstee plot for urease enzyme activity

the values obtained from Lineweaver–Burk plot. Eadie–Hofstee plot may subject to error as a parameter of enzyme activity used on both the axes. However, this approach reduced the biases based on substrate concentration while calculating the kinetic parameters.

Hanes–Woolf plot is the rearrangement of Eq. 4 for the calculation of $V_{\max}$ and K_m. The rearrangement gives the plot for the ratio of substrate concentration and velocity plotted against the substrate concentration. Hanes–Woolf plot gives the

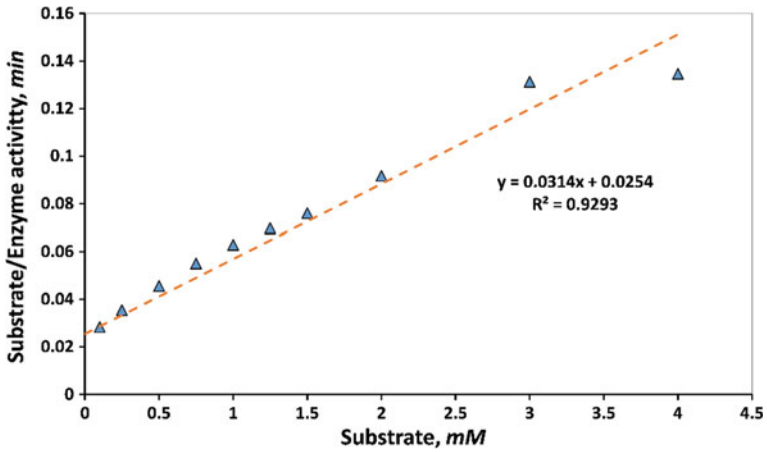

Fig. 4 Hanes–Woolf plot for urease enzyme activity

Table 1 Kinetic parameters of urease by different plots

Plot	V_{max} (mM/min)	K_m (mM)	R^2
Lineweaver–Burk plot	26.88	0.70	0.9982
Eadie–Hofstee plot	29.94	0.82	0.8366
Hanes–Woolf plot	31.85	0.81	0.9293

most accurate estimation of the enzyme activity. The calculated values of V_{max} and K_m were 31.85 mM/min and 0.81 mM of urea, and Fig. 4 represents the Hanes–Woolf plot.

Table 1 summarizes the values of V_{max} and K_m calculated from plots mentioned above. Among the three plots for determination of enzyme kinetic parameters, Lineweaver–Burk plot gives the direct relation of the rate of reaction with the inverse of the substrate concentration provided. Eadie–Hofstee plot and Hanes–Woolf plot gave similar values for both V_{max} and K_m. However, the results from Eadie–Hofstee plot might have a higher chance of error due to use of a parameter v on both of the axes. The Lineweaver–Burk plot shows the highest correlation (R^2) for the urease assay and could be preferred further.

3.2 Enzyme Inhibition Assays

Enzyme inhibition assays provide the extent of the inhibition of enzyme activity for the respective inhibitor concentration. The relation between the degree of inhibition and the inhibitor concentration gives the calibration plot to determine the inhibitor concentration.

For inhibition studies, assays were performed with the various initial concentrations of Cr^{3+} ions as they are known to react with the urease and influence the overall enzyme activity. The heavy metals ions react with the sulfhydryl groups of the active site of the urease and thereby decrease the catalytic activity. The amount of ammonia detected has given the activity of the enzyme in the presence of inhibitors.

Figure 5 shows the relationship between the inhibitor concentrations and a decrease in the enzyme activity. Urease has found sensitive for the low concentrations of Cr^{3+} and shown a decrease in the activity up to 44%. With the increased concentration of metal ion, the activity decreased further, and Cr^{3+} concentrations above 1 ppm showed the complete inhibition of the enzyme. Cr^{3+} (0.001 ppm) was the observed IC_{50} value of urease for which the activity reduces to half of its actual value.

Magomya et al. [26] studied the inhibition kinetics of soybean urease with the IC_{50} value for Cr^{3+} in between the range of 1–10 ppm [26]. Jung et al. [27] performed an inhibition assay of free as well as an immobilized urease using Cr^{3+}. The IC_{50} value of for free urease solution for Cr^{3+} was observed at 0.82 ppm, while for the immobilized enzyme the value was noted as 36.1 ppm [27]. Samborska et al. [28] studied the effect of different oxidation states of chromium on the activity of soil urease. Cr^{3+} caused a decrease in the soil urease activity to 50% for a Cr^{3+} concentration of 0.2 mg per kg of the soil solution. The soil urease was found more sensitive to the Cr^{3+} than Cr^{6+}. Cr^{6+} (0.4 mg) per kg of soil solution was the observed IC_{50} value of soil urease. Further, in the study, pure urease showed more sensitivity toward heavy metals compared to soil urease [28].

The observed decrement in the enzyme activity might be because Cr^{3+} tends to react with carboxyl and sulfhydryl groups in the active sites of the enzyme leading to changes in the enzyme structure [29]. Along with the chromium, other heavy

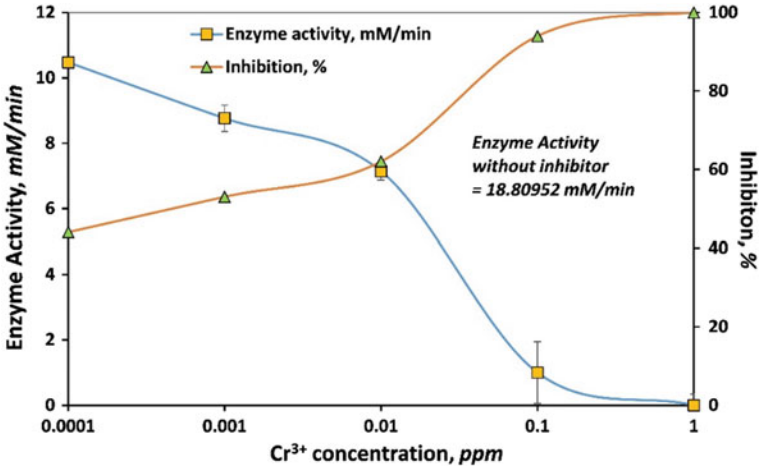

Fig. 5 Effect of Cr^{3+} on urease activity

metal ions, for example, silver, copper, mercury, nickel, cadmium, ferrous, are reported to inhibit the activity of the urease by forming insoluble metals sulfides. Non-competitive type of inhibition of urease is showed by heavy metals [30].

The sensitivity of urease to the other heavy metals is reported by various other researchers [31–33]. The order of degree of urease inhibitions while subjected to various heavy metals was as in the order as follows: Ag > Hg > Cu > Ni > Co > Cd > Fe > Zn > Pb in case of soil urease [31]. Another study suggested the inhibition order as $Cu^{2+} > As^{3+} > Cr^{3+} > Cd^{2+} > Zn^{2+} > Pb^{2+}$ in case of urease extracted from soybeans [26]. From these studies, it observed that the origin of the enzyme plays a crucial role in its activity and the tolerance for the inhibitors as chromium. The jack bean urease used for this study showed to have maximum sensitivity for the Cr^{3+} compared to the other resources.

4 Conclusion

Enzyme kinetics of the jack bean urease was studied in this work to understand the effect of trivalent chromium on urease activity. Kinetics parameters (V_{max} and K_m) are calculated using different kinetics plots (Lineweaver–Burk, Eadie–Hofstee, and Hanes–Woolf plots) relating the substrate concentration and the enzyme activity. Based on the regression analysis, Lineweaver–Burk plot was found to give reliable values (V_{max} and K_m of 26.88 mM/min and 0.7 mM, respectively) for the urease kinetic parameters. The low concentrations of Cr^{3+} affected the urease activity to considerably high level. The IC_{50} value is observed as 0.001 ppm of the metal ion. The relationship between the decreases in the enzyme activity with the inhibitor concentration can apply to determine the amount of heavy metal in the sample. The higher sensitivity of the enzyme can efficiently apply for the detection of the meager amount of chromium. Urease-based biosensor delineating the impact on enzyme function index may use for determining the chromium presence in potable water.

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