Heavy metal ion detection using Immobilized ALP enzyme

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Abstract: Environment pollution by toxic heavy metals (HM) presents a real life threat for human health. Accumulation of heavy metals like Cadmium, Mercury, Cobalt and Copper in water causes toxic actions if the tolerance level is exceeded. Alkaline Phosphatase (ALP) activity was used for estimation of heavy metal ions in drinking water. It was based on inhibition of Alkaline Phosphatase enzyme activity exerted by metal ions. Kinetics of ALP was performed and maximum activity was found to be at using 2U/ml concentration of enzyme and hence was chosen for further studies. ALP immobilization was carried out by sol-gel method and sodium alginate method on two different surfaces: glass and stainless steel. Inhibition characteristics of ALP were tested using different concentrations of individual heavy metals ranging from 10mM to 10^{-5}mM and also using combination of heavy metals of concentration 10^{-4}mM.The reaction showed uncompetitive inhibition. Amongst the four heavy metals used, the amount of inhibition was found to be more in mercury compared to other metals.

Index Terms— Alkaline Phosphatase, Sol-Gel, Inhibition, meal ions

1. INTRODUCTION

The accumulation of toxic substances in the environment continuously increases due to diverse pollutants from the industries. Heavy metals in drinking water pose a serious threat to human health if the respective tolerance level is exceeded. Populations are exposed to heavy metals primarily through water consumption, but few heavy metals can accumulate in the human body (e.g., in lipids and gastrointestinal system) and may induce cancer and other risks. Hence, fast and accurate detection of metal ions has become a critical issue. Due to the high toxicity caused by the heavy metal ions there is an obvious need to determine them rapidly at trace levels. The present investigation aims determining heavy metal ions based n the inhibition studies of ALP on different metals.

2. MATERIALS AND METHODS

2.1 ENZYME KINETIC STUDIES

2.1.1 PNP STANDARD CURVE:

Enzyme kinetics was studied by plotting the pNP Standard Curve and carrying out the Enzyme Activity Assay by using 0.05M p-nitro phenyl phosphate (pNPP) as the substrate. The release of p-nitrophenol (pNP) in the reaction mixture (2.5ml) was continuously measured at 405nm spectrophotometrically (Thermo-Fischer), over the linear period. Different concentrations of standard pNP ranging from 0µg to 50µg were taken in a series volume of different test tubes. 1ml of distilled water is added to the test tubes. 2ml of 0.1M Sodium Hydroxide was added to make up the final reaction mixture to 4ml. Absorbance readings were taken at 405nm.

2.1.2 INHIBITION STUDIES OF FREE ALP WITH DIFFERENT HEAVY METAL IONS:

The effect of Hg^{2+}, Cu^{2+}, Co^{2+} and Mn^{2+} on ALP activity was studied. Various concentrations of the chosen heavy metals were added to the 0.1M Sodium Carbonate-Bicarbonate buffer followed by the addition of 0.1ml

enzyme. 1ml of 0.05M p-NPP substrate was then added to study the inhibition of the enzyme activity. 1ml of 0.2M NaOH was added to the final reaction mixture and the absorbance was measured at 405 nm spectrophotometrically.

2.2 IMMOBILIZATION OF ENZYME

2.2.1 SOL GEL METHOD

The stock gel solution for immobilization was prepared by adding 570µl of methanol 50µl of TEOS and 10ul of 3.8% CTAB (Cetyl Tri methyl Ammonium Bromide) solution in a small test tube at room temperature The solution was vigorously mixed. It was then cooled to 4^{o}C immediately after mixing. The enzyme stock solution was prepared by dissolving a known quantity of ALP in 50ml 0.02Mm phosphate buffer (pH- 7.0).The enzyme solution was cooled at 4^{o}C.

2.2.2 SODIUM ALGINATE METHOD (SA):

The sodium alginate method was performed by adding 0.6gms of Sodium alginate to 20ml of distilled water(solution 1) and 50µl of ALP (2U/ml) (solution 2). The Solution 1 and 2 were mixed and stirred for 30 minutes covering the electrode.

2.3 ACTIVITY OF IMMOBILIZED ENZYME ON DIFFERENT SURFACES

Immobilization was performed on two different types of surfaces (Glass and Stainless Steel) and by two different methods (Sol Gel and Sodium Alginate). 1ml of pNPP substrate was added to 0.4ml sodium carbonate-bicarbonate buffer .The enzyme immobilized glass beads, stainless steel fork prangs and stainless steel plates were added and incubated at 37^{o}C for 15minutes. glass beads, stainless steel fork prangs and stainless steel plates were removed. 1ml NaOH was then added and absorbance was measured at 405nm. Enzyme was not immobilized on the glass surface using the Sol Gel method as it specifically requires a conducting medium

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2.4 pNPP SUBSTRATE KINETICS

The primary function of the inhibitor is to reduce the rate of reaction. Hence the need to study the rate of the reaction is highly necessary to study the properties of inhibition.

2.4.1 pNP ASSAY

Various volumes of standard pNP (0, 0.2, 0.4, 0.6, 0.8, 1.0 ml) were taken in a series of test tubes. The Volume was made upto 1ml using distilled water. 2ml of 0.1M NaOH was then added to all the test tubes and absorbance was measured at 405nm

2.4.2 pNPP ASSAY

1ml of different concentrations of pNPP was added to 0.4ml buffer followed by the addition of 0.1ml enzyme. It was then incubated at 37^{o}C for 5minutes. 1ml of 0.2M NaOH was then added to the test tubes and the absorbance was measured at 405nm.

2.4.3 MM PLOT FOR pNPP SUBSTRATE

The Michaelis-Menton plot was plotted for the substrate to find the rate of the reaction. It is a plot with concentration on x-axis and activity on y-axis. The formula used to calculate the activity is:-

Activity = (concentration of pNP x dilution factor) in µmol/min

(mol. wt of pNP x Vol. of enzyme x Incubation time)

2.4.3 LB PLOT OF pNPP

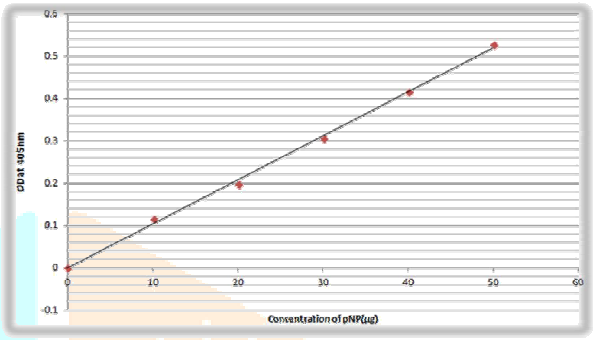
The Lineweaver-Burk plot is widely used to determine important terms in enzyme kinetics, such as Km and Vmax. The y-intercept of such a graph is equivalent to the inverse of Vmax. The x-intercept of the graph represents -1/Km. It also gives a quick, visual impression of the different forms of enzyme inhibition. The LB plot was plotted for the substrate to use as a reference to fine the type of inhibition exhibited by the heavy

metals.

3. RESULTS AND DISCUSSION

3.1 STANDARDIZATION OF pNP

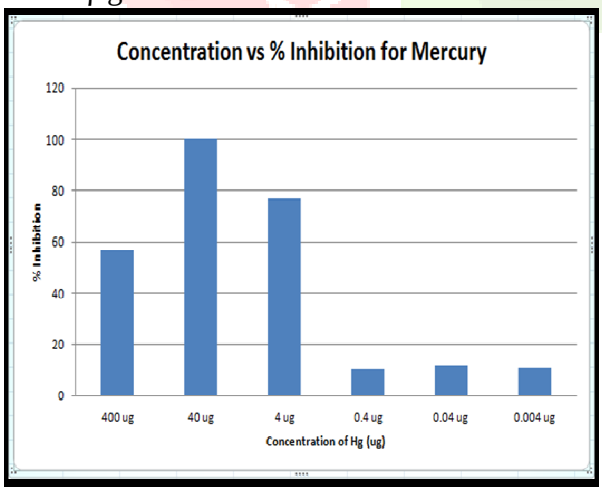
A graph was plotted with concentration of pNP taken on the x-axis and absorbance on y-axis. From the results it was observed that there was a linear increase in the absorbance level against increasing concentrations of pNP



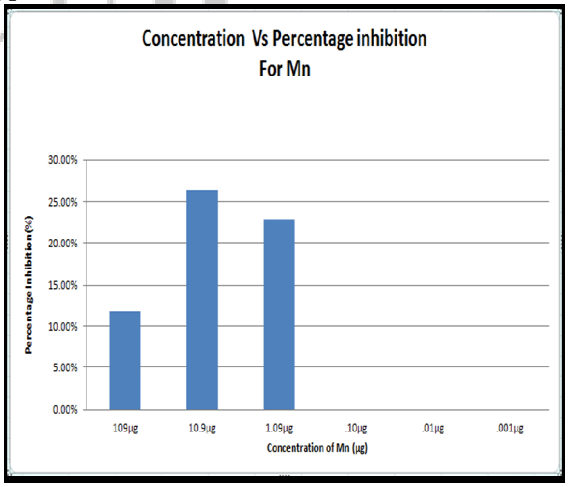
Graph 1: Standard Curve for pNP

3.2 INHIBITION STUDIES OF THE FREE ENZYME ALP

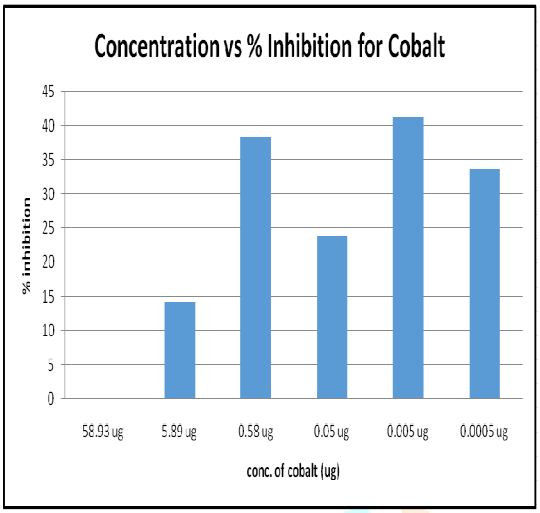
The compounds $HgCl_2$, $MnSO_4$, $CoSO_4$ and $CuSO_4$ were used as inhibitors and the percentage inhibition exerted by the respective individual heavy metals i.e. Hg^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+} is given below. The percentage inhibition was calculated for different concentrations of Mercury, Manganese, cobalt and copper.. It was found that maximum inhibition was exhibited by 40µg of Mercury whereas minimum inhibition was shown by 0.4µg of Mercury and maximum inhibition was exhibited by 10.9µg of Manganese whereas no inhibition was seen after concentrations of 0.10µg. It was found that maximum inhibition was exhibited by 0.005µg of Cobalt whereas no inhibition was seen in 58.93µg of Cobalt and it was found that high inhibitions were seen in 6.35µg and 0.06µg concentrations of Copper, lesser inhibition at 63.5µg whereas no inhibition were seen in the by 0.4µg of Copper



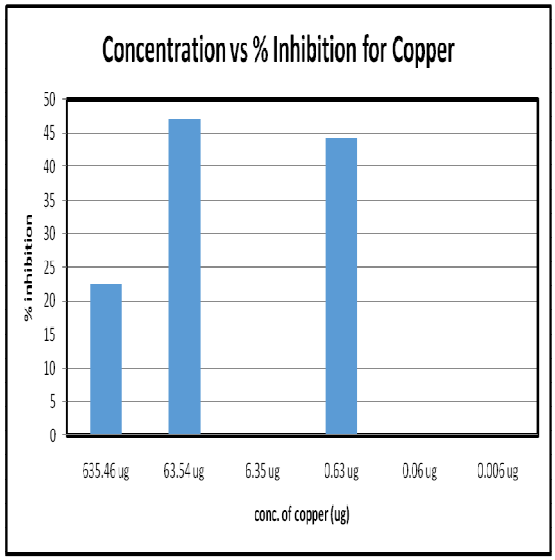
Graph 2: Concentration of Mercury vs. Percentage Inhibition



Graph 3: Concentration of Manganese vs. Percentage Inhibition



Graph 4: Concentration of Cobalt vs. Percentage Inhibition



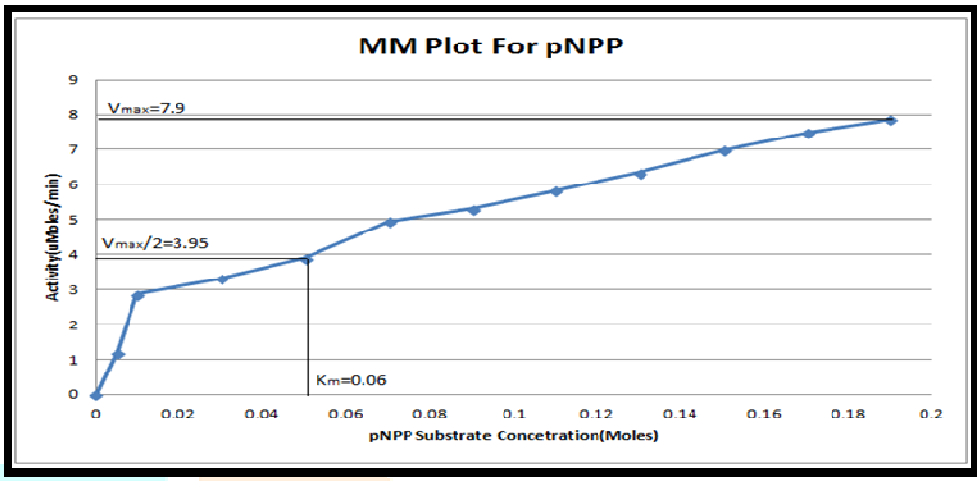
Graph 5: Concentration of Copper vs. Percentage Inhibition

3.3 RESULTS OF ACTIVITY OF IMMOBILIZED ENZYME ON DIFFERENT SURFACES

Immobilization was performed on two different types of surfaces (Glass and Stainless Steel) and by two different methods (Sol Gel and Sodium Alginate).The test was performed to find the activity of the immobilized enzyme on the glass beads. The sample (S) which had the enzyme immobilized glass beads showed negligible activity while the left over Sodium Alginate Extract (E) of the glass beads comparatively showed some activity. This showed that the enzyme had not been immobilized on to the glass surface using this method. The ALP immobilized prongs were tested for enzyme activity for a period of one week. The Immobilized enzyme showed high activity on the 1st day, which steadily decreased on the 2nd day and was almost negligible on the 7th day. This concluded that the enzyme showed stability for 1 week when it was immobilized with Sodium Alginate. For the Sol Gel Immobilized Surface, activity was found to be maximum on the 1st day which decreased over the 2nd and the 7th day. Moreover the activity for Stainless Steel Fork Prongs using Sodium Alginate Method had considerable good activity on day 2 whereas for the Sol Gel Immobilized prongs lost a greater amount of activity on the day 2 itself. Here the Sodium Alginate Immobilized Surface was tested for enzyme activity. This experiment showed considerably good activity on the 1st day which was rapidly lost on the 2nd. The results did not require us to proceed with testing the activity further ahead. The sol gel immobilized enzyme surface was then tested for enzyme activity. For this type of immobilization, the activity had reduced drastically from the 1st day to the 2nd. Moreover the activity for Stainless Steel Plates using Sol Gel method seemed to exhibit higher activity than that of the Sodium Alginate Method. The inhibition characteristics for four different metals i.e. Mercury, manganese, cobalt, copper were tested. All the metals were found to produce 100% inhibition.

3.4 MM PLOT FOR pNPP SUBSTRATE

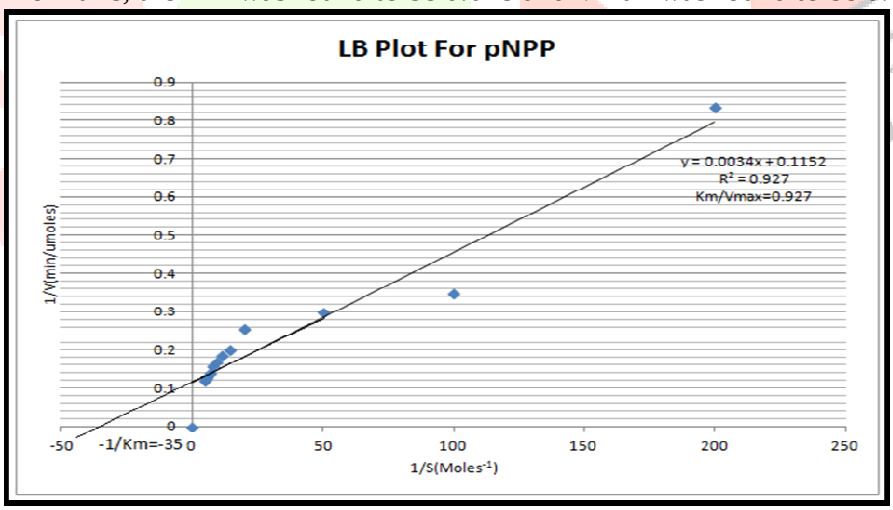
In the Michaelis-Menton curve obtained, it was seen that the rate of the reaction was found to increase with increasing concentrations of the substrate. This would tell us about the amount of the product pNP formed from the substrate pNPP. From the graph theVmax was found to be 7.9 and the Km value was found to be 0.06



Graph 7: MM plot for substrate

3.5 LB PLOT OF PNPP

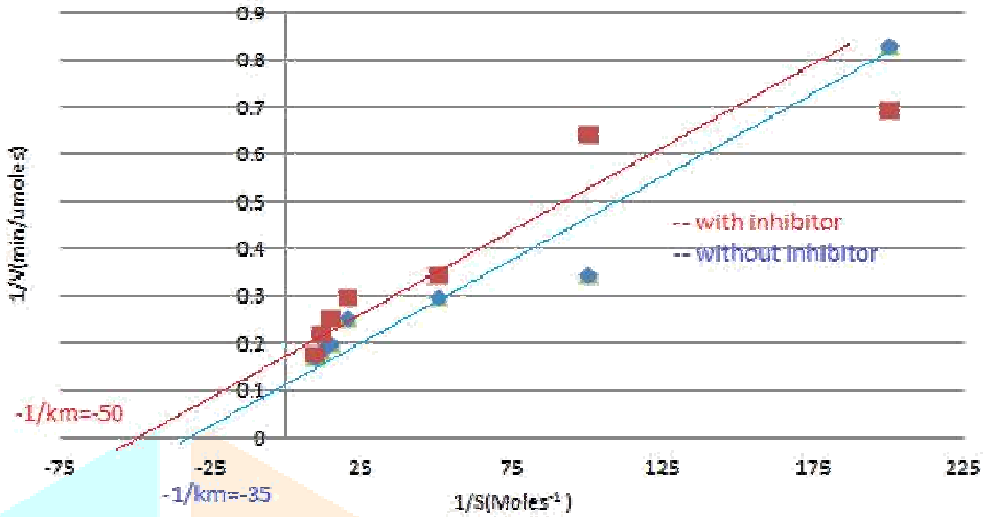
From the resulting LB plot, the substrate was used as a reference to find the type of inhibition exhibited by the heavy metals. From this, the Km was found to be 0.028 and Vmax was found to be 0.0302.



Graph 8: LB plot of pNPP

3.6 TYPE OF INHIBITION

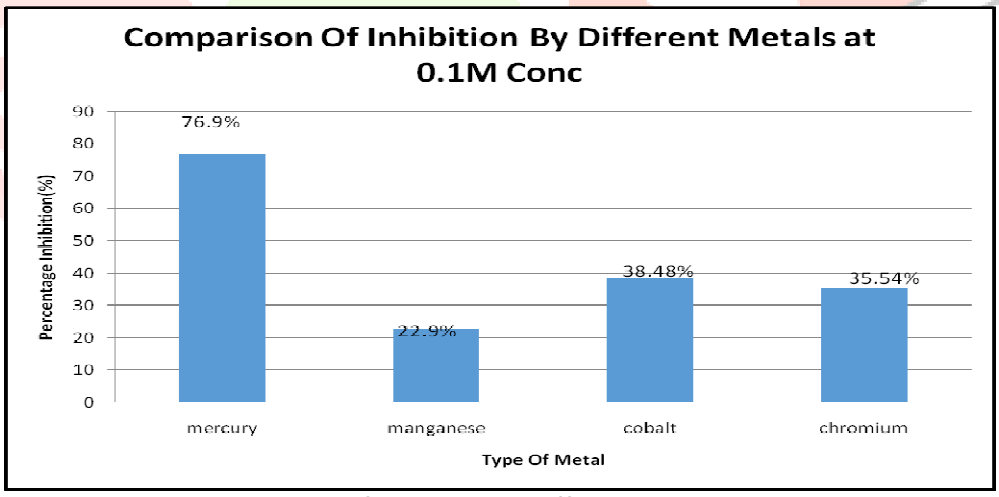
The Lineweaver-burk plot was plotted for the substrate with and without the inhibitor and the inhibition was found to be of uncompetitive type. Uncompetitive inhibition implies that the inhibitor can bind only to the substrate-enzyme complex. This was concluded due to the parallel slopes obtained for the respective curves.



Graph 9: LB plot with inhibitor

3.7 COMPARISON OF INHIBITION BY DIFFERENT METALS AT 0.1 M CONCENTRATIONS

The results obtained were plotted on a graph. It was found that Mercury exhibited maximum inhibition of 76.9% while Manganese seemed to show the least inhibition of 22.9%.



Graph 10: Comparison of Inhibition by Different Metals

4. CONCLUSION

The present study concluded that maximum activity for Alkaline Phosphatase enzyme was exhibited at 2U/ml concentration .Hence, this concentration was chosen for further work .On comparing the immobilization by the two techniques used, we observed that Sodium Alginate immobilized surface retained higher activity compared to Sol gel immobilized surface. By studying the effect of various

heavy metals on the free and immobilized enzyme, it was found that Mercury exhibited maximum inhibition on the activity of the enzyme Alkaline Phosphatase and Manganese was found to have the least inhibition. The heavy metals Copper and Cobalt showed unstable inhibition readings due to the inherent color exhibited by them which caused hindrance in Absorbance readings. The concentration range of heavy metals that can be detected by using this sensor is 10-4mM to 10mM .A Line weaver – Burk plot was plotted for the pNPP substrate with and without the inhibitor. The graph obtained showed that the inhibition was of uncompetitive type. This implies that the enzyme inhibitor can bind only to the complex formed between the enzyme and the substrate. This could imply that the binding site for the inhibitor is accessible only after the enzyme has bound to its substrate.

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