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Extraction, Characterization and Inhibition Studies of Velvet Beans Urease Enzymes

[1]Buba Mohammed, [2]Abubakar Isa and [3]Isah Labaran

[1,2&3]Department of Science Laboratory Technology, Federal Polytechnic, Mubi Adamawa State, Nigeria
Corresponding author:-bubanenne58@gmail.com, +2348036823918

ABSTRACT

Urease enzymes was extracted and studied for possible application as biorecognition element in electrochemical biosensors for heavy metal analysis in water matrix. This research work was based on the fact that heavy metal ions inhibit urease enzymes activity and the amount of inhibition is directly proportional to the concentration of inhibiting species in the sample. Urease enzymes activity and the effects of temperature, pH, enzyme/substrate concentration and incubation time on the urease activity was examined. Maximum urease activity of 10.230 U/mol was obtained at 40 °C and pH 7.5 with 220 µg/L enzyme concentration after 120 min incubation period. The Vmax and Km were estimated as 0.31 mM/min and 125 µg/L respectively. All of the heavy metals tested showed evidence of inhibition on the extracted urease enzymes, highest inhibition 80.541 was observed with Pb^{2+} and the lowest was 9.42 with Fe^{2+} meanwhile, no inhibition was observed with low concentration of Cr^{2+}. This research revealed that Velvet beans urease enzymes can be used as biorecognition element in electrochemical biosensors for determination of heavy metal ions in water matrix. We therefore recommend for further electrochemical characterization with glassy carbon electrodes in electrochemical cells.

Keywords: - Enzymes activity, Heavy metals, Biosensors, Biorecognition elements, Water matrix

INTRODUCTION

Various types of enzymes have been utilized as recognition element in an enzyme-based biosensor, and they are immobilized on/within the support matrix on the transducer surface to maintain enzyme activity. The benefits of using enzymes such as the high specificity of enzyme-substrate interactions and the high turnover rates of biocatalysts (i.e., the product of catalyst activity and lifetime), makes enzyme-based biosensors to become one of the most extensively researched areas. Immobilized enzyme biosensors are a type of catalytic biosensor in which the transducer surface is immobilized with enzymes that act as a bridge between the transducer and the analytes. Immobilized enzyme-based biosensors have been widely used in a variety of applications, including biomedical applications [1], [2], environmental pollutant detection [3], [4], food safety monitoring [5], [6], and bioprocess monitoring in industries [7]. Examples of enzymes used in biosensors for heavy metal detection include, such as acetylcholinesterase, alkaline phosphatase, urease, invertase, peroxidise, L-lactate dehydrogenase, tyrosinase, and nitrate reductase among others. The inhibition of the immobilized enzyme can be detected via electrochemical (Amperometric, Potentiometric, and Conductometric) or Optical measurements. Enzymes are proteins that catalyze chemical reactions on a specific substrate depending on their chemical functions, The prospect of the use of enzymes as biosensor recognition agents is their ability to change the structure of the protein in response to environmental conditions (Urease activity), making them possible to detect a large number of analytes [8]. However, bearing in mind about how different environmental factors such as pH, temperature, ionic strength, and substrate concentration affect the enzyme activity is primordial. In this sense, it has been shown that increasing temperature favors the enzymatic activity and therefore the reaction rate. However, enzymes can denature at temperatures over 40 °C, and hence, at this point, the reaction rate decreases; in most cases, enzyme biosensors have an optimum performance at 25 °C. The tertiary structure of an enzyme can change at high temperatures, with the consequent loss of the enzymatic catalytic activity. In this sense, the effect of temperature on GOx has been extensively studied, [9], where it has been shown that the response of a glucose biosensor increases in the range of 25–50 °C, at higher temperature the biosensor response decays, due to the loose of enzyme activity. It is important to notice that the effect of the temperature on both free enzyme (in solution) and enzyme immobilized on an electrode's surface is almost the same.

Other factors such as PH, substrate concentration, Urease enzymes concentration, contact time with heavy metals were analysed.

MATERIALS AND METHODS

Materials

All reagents used are of analytical grade and were used without further purification these include among others; Phosphate buffer 0.1 M pH7 (this was prepared by dissolving 40 cm^3 of 2 M stock solution of potassium dihydrogen phosphate $KH_2PO_4.2H_2O$, 10 cm^3 potassium hydrogen phosphate $KHPO_4.2H_2O$ and 0.9 g NaCl in 100 cm^3 distilled water). Zinc acetate Gelatin, Urea, Nesler reagent, Chromium (III) standard solution (1000 ppm), Cd standard solution (1000 ppm), (1000ppm stock solutions were prepared by dissolving 1 g of the reagent grade chemical in 1 dm^3 of distilled water). $Pb(NO_3).3H_2O$, Ethanol, Absolute alcohol, $Pb(NO_3).6H_2O$, Ammonium sulphate, Uv-Visible spectrophotometer Janway model 7315, Refrigerated centrifuge Labnet Z 233 MK, weighing balance, Magnetic stirrer, Votex mixer Clever scientific limited XH-C.

METHODS

Collection and preparation of velvet beans (*Mucuna pruriens*) seeds

Freshly, harvested Velvet beans seeds Fig.1 were purchased from the local market at Maisamari Sardauna Local Government Area Taraba state, Nigeria. These were washed with distilled water, air dried and pulverized with a kitchen blender prior to use [10].

Figure 1. Velvet beans (*Mucuna pruriens*)

Extraction of bio-recognition element/ Acetone precipitation

Urease was extracted from velvet beans seeds according to a slightly modified method of [10]. Ten grams of powdered seeds were soaked in 100 mL extraction buffer (0.2 M phosphate buffer pH 8) and refrigerated at -4 °C for 3 – 4 hours. The mixture was sieved through four layers of muslin cloth and the resulting filtrate was centrifuged at 6000 rpm for 15 min. The clear supernatant was collected and used as the crude urease extract while the pellets was discarded. The "crude extract" was adjusted to 50% saturation by addition of acetone (chilled to −20°C) under constant and gentle stirring. The resulting precipitate was centrifuged, collected, dissolved in minimum volume of pre-cold 50 mM phosphate buffer (pH = 7.4), and finally dialyzed against the same buffer for 24 h [11].

Enzyme characterization

Determination of urease activity (nessler's reagent method)

The urease assay was performed following the method described by [11]. Enzyme extract 0.25 µL of was added to 10 mL of urea solution (0.4 g urea in 25 $cm^3$of phosphate buffer pH 7.5). 1 cm^3 of the solution was then added to the test tubes containing 5 mL of Nessler's reagent. The mixture was incubated at 30 °C for 10 min followed by the addition of 1.0 M HCl thus terminating the reaction. The absorbance was taken at 405 nm using a UV-Vis spectrophotometer. One unit of urease activity is defined as the amount of enzyme required to liberate 1.0 µM of NH_3 from urea per min at pH 8.0 and temperature 30 °C. The estimation of urease was carried out using the standard curve of ammonium sulphate.

Effect of temperature

The optimum temperature for urease activity was determined over the temperature ranges from 20 °C to 50 °C using the standard conditions of the assay [11], [10].

Effect of pH

The optimum pH was detected over the pH ranges 5.5 to 8.5 with an increment of 0.5 while keeping other parameters constant [11], [10].

Effect of incubation time

To determine the optimum incubation period for maximum urease activity, the estimation was done by incubating the enzyme at different time intervals ranging from 30 to 240 sec while keeping other parameters unaltered [11], [10].

Effect of different concentration of substrates (determination of kinetic parameters Km and Vmax of the extracted urease)

The kinetic parameters (Vmax and Km) of the extracted urease was evaluated by means of the Michealis -Menten model (as discussed in 2.7.2) which is a representation of the effect of substrate concentration on enzyme velocity. Urease activity was determined with varying amounts of substrate (40 to 280 µL urea solution) while keeping the enzyme concentration constant (10 U/mol). The Michaelis menten graph of enzyme velocity (µmol product formed per min) against substrate concentration was plotted and the Vmax (maximum velocity achieved by the system, at saturating substrate concentrations) and Km (the substrate concentration at which the reaction velocity is *50%* of the *Vm)* was estimated from the plot [11], [10].

Urease inhibition assays for heavy metals (spectrophotometric inhibition assay)

Standard solutions (0.5 – 2.0 ppm) of the heavy metals Fe^{2+}, Pb^{2+}, Cr^{3+} and Cd^{2+} were prepared by serial dilution from the respective 1000 ppm stock solutions and their inhibitory activities was estimated on the extracted urease. For each measurement, 0.2 cm^3 metal ion solution, 0.2 cm^3 enzyme extract and 0.6 cm^3 buffer were pre incubated for 2 mins followed by addition of 1 cm^3 urea *(O.25M)*. After another 2 mins the reaction was terminated by adding 0.1 M HC1 and the total mixture was made up to 50 cm^3 with distilled water. 2 cm^3 Nessler's reagent was then added and absorbance of the resulting solution was taken against a blank at *405 nm* on a Uv-Vis spectrophotometer. The level of inhibition for each tested metal concentration was obtained using the relationship:

$$\% \text{ inhibition} = \frac{Ao-Ai}{Ao} \times 100 \quad \text{---------- equation 1.}$$

Where; Ao = the obtained absorbance without the inhibitor (metal ion)

Ai = absorbance obtained after pre- incubation with metal ion.

A graph of % inhibition against concentration will be plotted for each heavy metal to obtain the linear range for estimation of the metal concentration [8], [10], [12]

RESULTS AND DISCUSSIONS

Urease Activity

Urease activity was determined using the standard curve of ammonium sulphate Figure. 2. the urease activity was estimated as 10.230 U/mol. Sevaral reports have presented urease activities of various urease enzymes within our obtained values, Urease enzyme activity of 15.065 U/cm^3 for crude urease was reported by [13], [14], [15], also reported 9.0 U/mol and 4.7 U/mol urease activity of crude and acetone purified urease extract of Cajanus cajan seed. One unit of urease activity is defined as the amount of enzyme required to liberate 1.0 µM of NH_3 from urea per min at pH 8.0 and temperature 30 °C, [11]. The biosensor intended for fabrication is based on the fact that heavy metal ions inhibits enzymatic activities of urease enzymes, estimation of urease activity is therefore, one of the initial steps in the development of urease enzymes based biosensors [16], [17], [18], [11], [15] , [10] and [12].

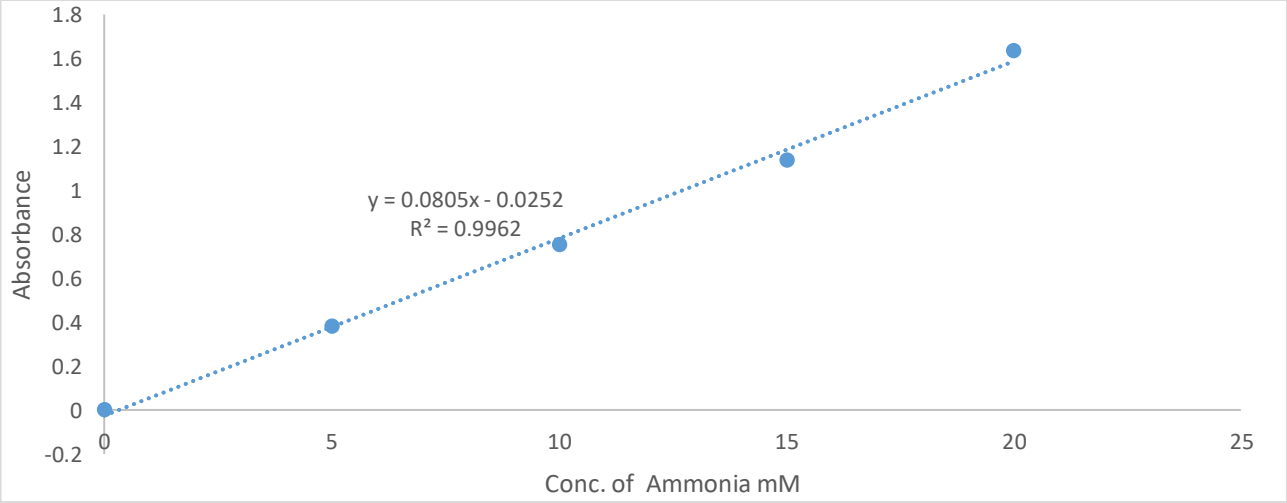

Figure 2. Standard Ammonium Sulphate Curve for Determination of Urease Activity

Effect of Temperature on Enzyme activity

Figure 3 showed the temperature optimum curve for the extracted urease. The complete assay of the enzyme was incubated at different temperatures from 20 to 50 °C for 2 min. Result obtained showed linear increase over the temperature range of 20 °C to 40 °C. The result also indicate that the urease has its highest activity at 40 °C. [11], also reported 22.9 U/mol urease activity at 40 °C, in their studies titled "Extraction, Purification, Kinetics and Thermodynamics properties of Urease of germinating *Pisum Sativum* L. Seeds". Urease activity value of 23.6 U/mol at 40 °C was also reported by [11] in their research on "Kinetics and Thermodynamic Study of Urease Extracted from Soybeans",, the result also corroborates the findings of [8], in their studies titled "Electrochemical Biosensors: Enzyme Kinetics and Role of Nanomaterials". From the results obtained, it can also be seen that increasing temperature favors the enzymatic activity just like most chemical reactions. However, very high temperatures (above the optimum temperature) can cause decreased or total loss of activity due to enzyme denaturation. Loss of enzyme activity at high temperatures can also be attributed to disruption of membrane structure in the enzyme. Results from various studies suggest that the tertiary structure of an enzyme can change at high temperatures, with the consequent loss of the enzymatic catalytic activity [8], [11], [10].

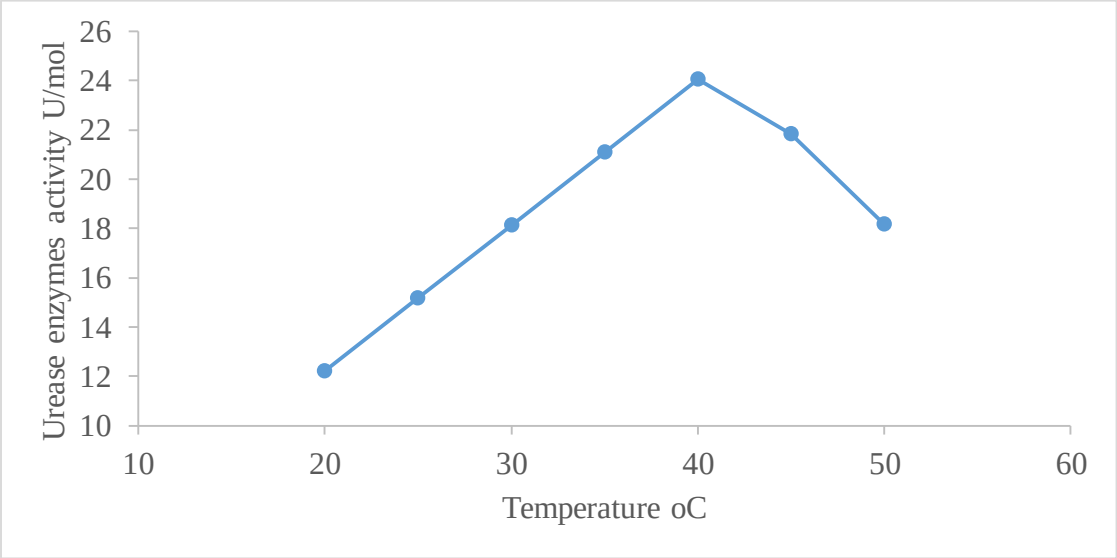

Figure 3. Effect of Temperature on Enzyme activity

Effect of pH of the Extracted Urease

The effect of pH on Urease enzyme activity studied over a range of pH 5.5 to 8.5 is presented in the pH optimum curve for the extracted Urease Figure 4. Maximum urease activity was obtained at the pH of 7.5, pH of the mixture lower or higher than 7.5, gave relatively less amount of urease activity, this was also reported by [10]. [13] also reported highest activity at the optimum pH 7.5 for urease extracted from soybeans. [11] however, reported optimum pH 7.5 for urease extracted from germinating *Pisum Sativum* L. pH is generally known to affect enzyme activity due to its effect on the structure of most enzymes, also it can affect the state of ionization of acidic or basic amino acids (Acidic amino acids have carboxyl functional groups in their side chains while basic amino acids have amine functional groups in their side chains). If the state of ionization of amino acids in a protein is altered, the ionic bonds that help to determine the 3-D shape of the protein would equally be affected and this can lead to altered protein recognition or inactivation of enzymes. Changes in pH may not only affect the shape of an enzyme but may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis. Extremely high or low pH values generally result in complete loss of activity for most enzymes [13], [19].

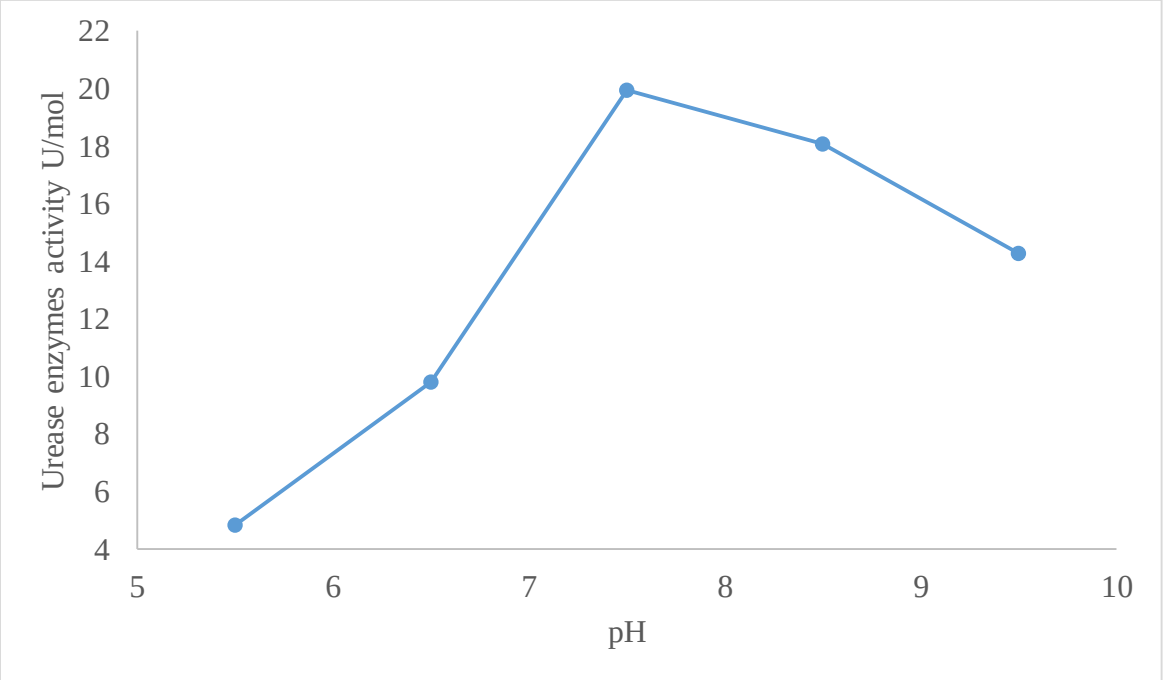

Figure 4 pH optimum curve for the extracted urease

Effect of enzyme Concentration on Urease Activity

The effect of urease enzyme concentration on urease activity studied over the enzyme concentration range between 10 to 220 µg/L is presented in the enzyme optimum curve for the extracted Urease Figure 5. The result showed rapid significant increase of urease activity by increasing the enzyme concentration until the highest enzyme activity value was attained. After then, the activity kept on increasing slowly insignificantly, the maximum urease activity reached was 10.711 U/mol at 220 µg/L enzyme concentration. This finding is in accordance with the result obtained by [11] who obtained maximum activity of 100 U/mol at 200 µg/L enzyme concentration for urease from germinating *Pisum Sativum L.* seeds. [20] and [18] also reported similar trend as indicated by our result.

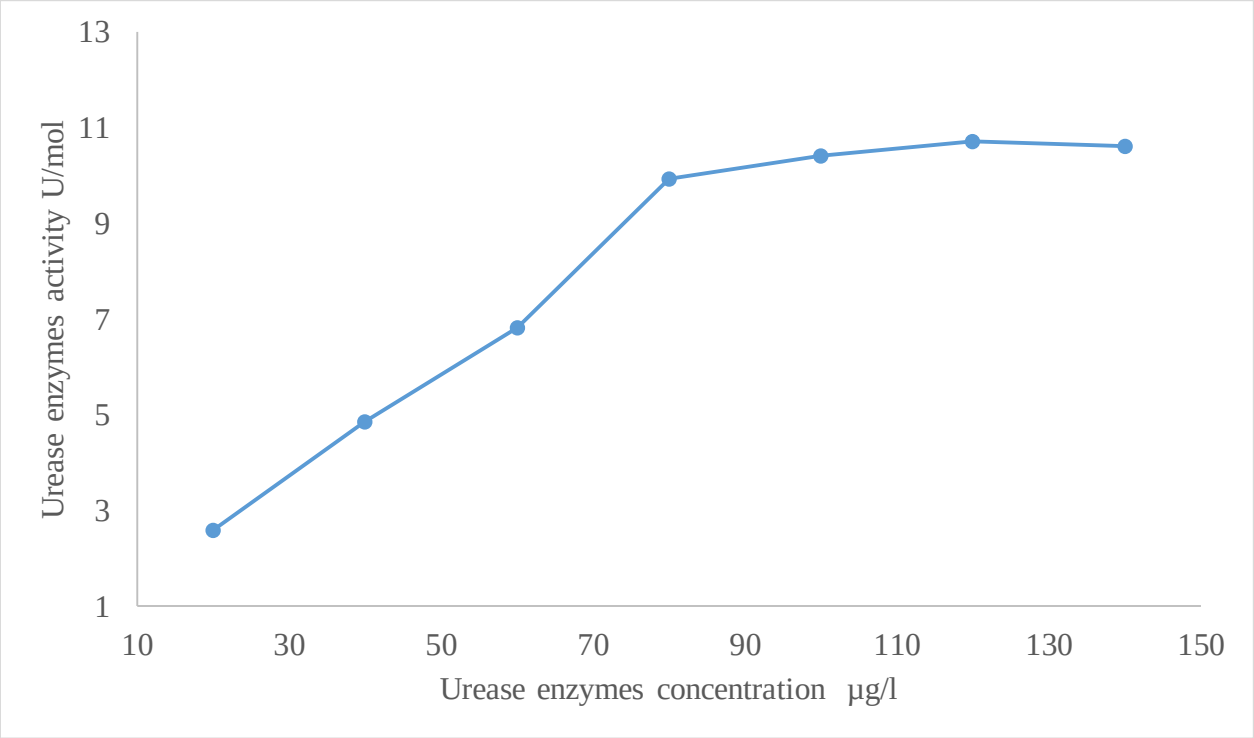

Figure 5. Enzyme optimum curve for the extracted urease

Effect of Incubation Time on Urease Enzymes Activity

The effect of Incubation period on the velvet beans urease enzymes activity studied over the period between 30 to 240 sec is presented in the incubation period curve Figure 6. The result obtained showed rapid increase in enzyme activity for the first 120 seconds. Further increase in time of incubation showed rapid decrease of the activity of urease. Similar trend was also reported by [20], studies on the effect of incubation period by [10] also showed that the maximum urease enzyme activity of soybeans (81.4 µg/l) was obtained at the incubation period of 120 seconds when incubated at 30 °C pH 8.0

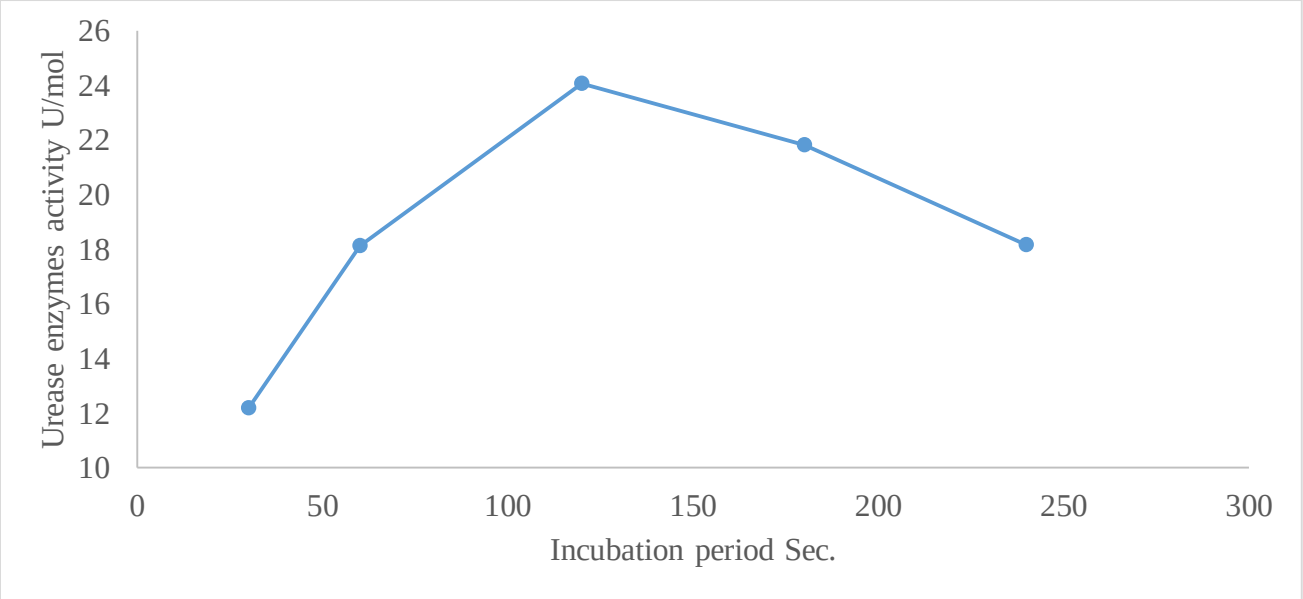

Figure 6. Effect of Incubation period on enzyme activity

Effect of Substrate Concentration (urea) on the Rate of Enzyme Reaction

The effect of substrate (urea) concentration on reaction rate studied over the substrate concentration range between 40 to 280 µg/L is presented in Michaelis-Menten plot of reaction rate against substrate concentration Figure 7. The result showed a simple Michaelis-Menten type of kinetic pattern as reported by several studies [11], [10], [13], [21]. From the plot obtained, it showed that the Vmax value which represents the highest velocity attained by the system at maximum (saturated) substrate (urea) for the velvet beans urease was 0.31 mM/min while the Km (Michaelis-Menten constant) is the substrate concentration at which the reaction velocity is 50 % of the Vmax which was found to be 125µg/L. The Km is a measure of how well a substrate complexed with an enzyme, also referred to as its binding affinity. Low Km value indicates a large binding affinity therefore the reaction will approach Vmax more rapidly while a high Km indicates that the enzyme does not bind efficiently with the substrate, and Vmax will only be reached if the substrate concentration is high enough to saturate the enzyme. Velvet beans urease in this study showed a higher affinity for its substrate similar to the G max reported by [13]. The findings here indicate that the extracted urease has good affinity for the substrate (urea) under the conditions tested.

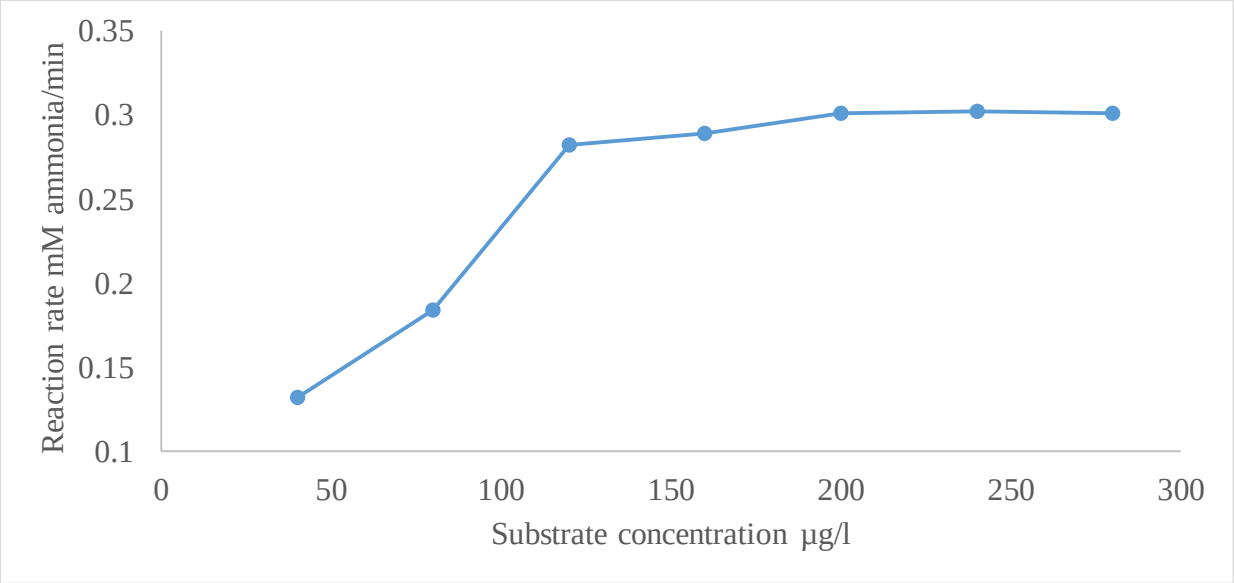

Figure 7. Michaelis-Menten plot for estimation of Km and Vmax of velvet beans urease

Uv-Visible spectroscopic inhibition assay of Fe^{2+}

Results for the inhibition assay of Fe^{2+} (0.5-2.0 ppm) standard solutions are presented in Table 1. The data obtained revealed decreased responses with increased concentrations of the inhibitor metal ion. This corresponds to increased inhibition with increasing concentration of the inhibitor metal ion. These values also reflect the quantity of ammonia released from the catalytic activity of urease i.e., the hydrolysis of urea to HCO_3^- and NH_4^+. The control represents the enzyme activity in the absence of Fe^{2+}. The result also showed a dose dependent inhibition of urease activity by Fe^{2+}. The lowest concentration of Fe^{2+} tested (0.5 ppm) resulted in 9.42 % inhibition of urease activity while the highest concentration (2.0 ppm) gave 75.07 % inhibition, the level of inhibition at this concentration showed more than LC_{50} meaning that Fe^{2+} is a strong inhibitor of the tested urease enzyme, this result agrees with the report of [22].

Table 1 Spectroscopic inhibition assay of Fe^{2+}

S/N	Concentration of Fe^{2+} (ppm)	Optical Density	% Inhibition (I%)
	Control	1.412 ± 0.13	0.00
1	0.5	1.279 ± 1.10	9.42
2	1.0	1.273 ± 0.11	9.84
3	1.5	1.163 ± 0.01	17.63
4	2.0	0.352 ± 1.23	75,07

Uv- Visible spectroscopic inhibition assay of Pb^{2+}

Results for the inhibition assay of Pb^{2+} (0.5-2.0 ppm) standard solutions are presented in Table 2, the data obtained revealed similar trend as with Fe^{2+}, however, the lowest I% (15.109) was found at 1.0 ppm concentration of the inhibitor metal ion, the percentage inhibition for the lowest concentration measured 0.5 ppm was out of range. The level of inhibition for 2.0 ppm inhibitor metal ion concentration (80.54), showed almost complete deactivation of urease activity. Comparing with the LC_{50} standard, it implies that Pb^{2+} is an excellent inhibitor of the urease enzyme, similar result was reported by [23], [22]. The high inhibition of urease enzyme activity by the Pb^{2+} is also an indication of its great affinity for urease thiol groups.

Table 2 Spectroscopic inhibition assay of Pb^{2+}

S/N	Concentration of Pb^{2+} (ppm)	Optical Density	% Inhibition (I%)
	Control	1.701 ± 1.11	0.00
	0.5	1.811 ± 1.24	NIL
	1.0	1.444 ± 1.10	15.109
	1.5	1.212 ± 0.10	28.748
	2.0	0.331 ± 0.01	80.541

Uv-Vis spectroscopic inhibition assay of Cr^{2+}

Data from the spectrometric inhibition assay of Cr^{2+} is shown in Table 3. The results revealed poor inhibition of the enzymes urease activity by Cr^{2+} at lower concentrations 0.5-1.0 ppm however, value complying to LC_{50} standard is observed at 2.0 ppm concentration of the inhibitor metal, this result suggest that the proposed biosensor may not be able to detect Cr^{2+} at relatively low concentration using pre-incubation method, however, the standard addition method in which the sample need to be spiked with the standard solution of the test metal ion will have to be adopted according to [23]. Several reasons may lead to poor inhibition of urease enzyme activity by metallic ion of which one of them is the low affinity to the thiol group of the enzyme by the inhibitor metal, low toxicity of metallic ion can also lead to poor inhibition [13].

Table 3 Spectroscopic inhibition assay of Cr^{2+}

S/N	Concentration of Cr^{2+} (ppm)	Optical Density	% Inhibition (I%)
	Control	1.649 ± 1.12	0.00
1	0.5	1.717 ± 1.43	Nil
2	1.0	1.681 ± 1.11	Nil
3	1.5	0.901 ± 1.10	45.36
4	2.0	0.579 ± 1.01	64.89

Uv-Vis. Spectroscopic inhibition assay of Cd^{2+}

Results for the inhibition assay of Cd^{2+} (0.5-2.0 ppm) standard solutions are presented in Table 4. The data obtained revealed decreased responses with increased concentrations of the inhibitor metal ion lowest concentration of the inhibitor metal 0.5 ppm achieved 10.08% inhibition of the urease enzyme activity while the highest concentration 2.0 ppm of the inhibitor metal tested showed 50.03 % inhibition of the urease enzyme activity. The result revealed that Cd^{2+} is a good inhibitor of the urease enzyme this may be due to its high toxicity or greater affinity to the thiol group in the mobile flap of the urease enzyme [13]. Several studies also reported excellent inhibition of urease enzymes by Cd^{2+} [8], [24], [23]. This suggest that the proposed biosensor can be able to detect Cd^{2+} even at low concentration.

Table 4 Spectroscopic inhibition assay of Cd^{2+}

S/N	Concentration of Cd^{2+} (ppm)	Optical Density	% Inhibition (I%)
	Control	1.643 ± 0.62	0.00
1	0.5	1.477 ± 1.21	10.08
2	1.0	1.299 ± 1.10	20.94
3	1.5	1.071 ± 0.71	34.81
4	2.0	0.821 ± 1.91	50.03

CONCLUSION

This work has shown the feasibility for the use of velvet beans urease as biorecognition elements in the development of biosensors for determination of heavy metal ions in water matrix. The result obtained showed convincing evidence of inhibition of urease enzymes activity by the heavy metal ions in water samples. Results of the urease enzymes characterization also quite agreed with the properties of most of the enzymes reported in the literature used as biorecognition elements. More so, the use of crude urease from Velvet beans as opposed to the expensive pure enzymes reported by previous findings would allow a sensible reduction in costs and ultimately result in a relatively cheap biosensing element.

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