

13. Determination of Cu and Pb concentrations

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Determination of Cu and Pb concentrations based on urease activity inhibition of *Durio zibethinus* L. seeds

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ABSTRACT. The determination of heavy metal concentrations has been carried out using sophisticated instruments, and therefore a simple and reliable alternative method is needed as a comparison. The study aimed to determine Cu and Pb concentration of standard solution using the urease activity inhibition method of *Durio zibethinus* L. seeds. The research started with urease extraction from *D. zibethinus* L. seeds. The activity of the obtained extract was determined using the Nessler method. The optimum substrate concentration was also determined. Urease activity inhibition was carried out using various metal solution concentrations, which continued by plotting a log graph of urea concentration vs. %inhibition. The obtained graph would then determine the metal concentration in a synthetic water sample. The data was then compared to the measurement, determined by the Atomic Absorption Spectrophotometry (AAS) method. Results of the study showed that the urease activity of *D. zibethinus* L. seeds was 296.774 U/mL. Urease activity was optimum at a urea concentration of 0.3 M. The comparison Cu, and Pb concentration determination using the urease inhibitory activity and AAS methods showed no significant difference at 95% confidence level. This research showed that urease of *D. zibethinus* L. seed could be used to determine Cu and Pb's concentration based on its inhibiting activity.

Keywords : *Durio zibethinus* L. seed, urease activity inhibition, Cu and Pb concentration

INTRODUCTION

Heavy metals can cause environmental pollution, and this is a big problem because they affect human health. Even at low concentrations, heavy metals pose a threat to the environment and human health because they are not biodegradable, causing more severe pollution problems (Gumpu, Sethuraman, Krishna, & Rayappan, 2015).

Various methods have been developed to determine the concentration of heavy metals, such as atomic absorption spectrometry (Souza, Zanatta, da Silva, & da Veiga, 2018), inductively coupled plasma mass spectrometry (Djedjibegovic, Larssen, Skrbo, Marjanović, & Sober, 2012), anodic stripping voltammetry (Bernalte, Sánchez, & Gil, 2011) and X-ray fluorescence spectrometry (Aranda, Colombo, Perino, De Vito, & Raba, 2013); (Ozer, Çimenoğlu, & Güçer, 2011). These tools are expensive and need a reliable operator to operate. Therefore, fast and straightforward detection of heavy metals at deficient concentration levels is necessary.

In this study, heavy metal detection was carried out using enzymes. In recent years, enzymes received

considerable attention in searching for simple, fast, and inexpensive methods for determining compounds. Enzyme as a catalyst has excellent properties due to their selectivity and specificity. Molecules inhibit enzyme reaction with the substrate, called an inhibitor. The inhibitor's binding stops the substrate from entering the enzyme's active site or inhibit the enzyme from catalyzing its reaction. Each enzyme has a specific inhibitor. The inhibition of this specific enzymatic system has been applied as an analytical method to detect toxic pollutants such as heavy metals (Budnikov et al. 2015); (Ahmad, 2013); (Kaur, Kumar, & Verma, 2014); (Do, Lin, & Ohara 2011) and pesticides (Gan, Yang, Xie, Wu, & Wen, 2010); (Braham, Barhoumi, & Maaref, 2013).

The urease enzyme (urea amidohydrolase, EC 3.5.1.5) has been considered an enzyme model for determining heavy metals in industrial waste, drinking water, surface water, wastewater, groundwater, and soil extract. Various studies have reported the inhibition of urease by heavy metals such as cadmium, nickel, and silver (Gumpu et al. 2015), cobalt (Jing et al. 2016), copper (Pan et al. 2016), mercury (Do and Lin 2016), and zinc (Wieczorek, Wyszowska, & Kucharski, 2015).

Urease sensitivity towards heavy metal ions caused by several cysteine residues becomes the urease enzyme's active sites (Pervin et al., 2013). Heavy metal ions inhibit urease catalytic activity by binding to sulfhydryl groups in the enzyme's active center (Upadhyay, 2012). After the metal atoms are attached, the sulfhydryl groups cannot function in catalysis resulting in a significant decrease in the catalytic activity. At constant substrate concentrations and varying metal ion concentrations, inhibition causes the enzyme catalytic activity to decrease. The reduction in enzyme catalytic activity is proportional to the number of heavy metal ions as the sample's inhibitor (Hermanto, Kuswandi, Siswanta, & Gjudasir, 2019).

Urease is used for the hydrolysis of urea to carbon dioxide and ammonia. Urease catalyzes urea's hydrolysis reaction at a rate of 10^{14} times faster than the reaction without enzymes (Krishna, Singh, Patra, & Dubey, 2011). The urease enzyme can be found in plants, animals, and microorganisms (Cantarella, Otto, Soares, De, & Silva, 2018). Urease enzyme has been isolated from various grains such as in green pea bean (El-Hefnawi, Sakran, Ismail, & Aboelfetoh, 2014), black-eyed pea bean (Zusfahair, Ningsih, Putri, & Fatoni, 2018a), long bean seed (Zusfahair, Ningsih, Fatoni, & Santri, 2018b), jackfruit seed (Chouhan, and Gayathri, 2018), and bitter melon seed (Krisna et al., 2011). *D. zibethinus* L. seed contains protein 2.6 g / 100 g (Nurfiana, Mukaromah, Jeannisa, & Putra, 2009). Protein in *Durio zibethinus* L. seed would contain enzymes; one of them is urease. The use of *D. zibethinus* L. seed as a source of urease can overcome environmental pollution, increase the economic value of *D. zibethinus* L. seed, and increase farmers' income. To the best of the authors' knowledge, there is only a little information about the interaction of heavy metal ions with the urease enzyme from *D. zibethinus* L. seeds.

EXPERIMENTAL SECTION

Tools and Materials

The tools used are UV-Vis spectrophotometer (Shimadzu UV-1800), Atomic Absorption Spectrophotometer (Perkin Elmer model 3110), centrifuges (Quantum), cuvettes, incubators, analytical balance (Ohaus), micropipette, tip, mortar and pestle, rod stirrer, magnetic stirrer, pH meter (Hanna Instruments), and refrigerator (LG). Materials used are *Durio zibethinus* L. seeds locally purchased from farmers *Durio zibethinus* L. in District Kemranjen Banyumas, urea (Merck), Nessler reagent (Merck), NaH_2PO_4 (Merck), Na_2HPO_4 (Merck), HCl, ammonium sulfate (Merck), CuCl_2 (Merck), $\text{Pb}(\text{NO}_3)_2$ (Merck), distilled water, and wrapping.

Urease Extraction from *D. zibethinus* L. Seed (Zusfahair et al., 2018a)

Durio zibethinus L. seeds germinated for three days were weighed as much as 20 g, then mashed using a cold mortar and pestle. Mashed *D. zibethinus* L. seed sprouts were soaked in an 80 mL cold phosphate buffer solution of 0.2 M at pH 7 and then homogenized using a stirrer for 3 hours. Stirring using a stirrer was carried out in a cold state. The mixture results of the stirring process were centrifuged at 4 °C for 15 minutes at a speed of 12000 rpm. The supernatant obtained was a crude extract of urease enzyme from *D. zibethinus* L. seed used for activity testing.

Determination of Urease Activity (Magomya, Barminas, & Osemeahon, 2017)

The urease activity was determined using a slightly modified method in which the ammonia yield was determined by spectrophotometry. For 0.5 mL test buffer (0.2 M phosphate buffer, pH 7), 0.5 ml of the enzyme extract was added to 0.5 mL of urea solution (0.25 M in phosphate buffer), where the mixture was incubated at 30 °C. After 10 minutes, the reaction was stopped by the addition of 0.5 mL of 0.1 M HCl. The reaction mixture was made up to 25 ml with distilled water and added with 0.5 mL of Nessler reagent. The absorbance of the resulting solution was determined against the blank at 443 nm on a UV-Vis spectrophotometer. Urease activity was estimated (as μM ammonia released) from a standard ammonium sulfate curve. One enzyme unit is defined as the amount of enzyme required to release one μM ammonia per minute under test conditions. The urease activity of the sample was calculated using the equation.

$$\text{Urease activity (U / mL)} = \frac{A(\text{sample}) \times \text{dilution factor}}{\text{Slope} \times T \times v}$$

Where: A (sample) = the absorbance of the sample measured against the

T = incubation time (10 minutes for standard test). V = sample volume

Effect of Substrate Concentration on Urease Activity

The enzyme activity was studied by varying the amount of substrate (urea) of 0, 1; 0.2; 0.3; 0.4; and 0.5M at a constant enzyme concentration.

Inhibition Test of Urease Activity using Pb and Cu (Magomya et al., 2017)

A set of solutions containing Pb^{2+} dan Cu^{2+} with concentrations ranging from 0.001 - 10 mg / L was prepared using serial dilutions from 1000 ppm stock. The procedure was as follows: 0.2 mL metal ion solution, 0.5 mL enzyme extract, and 0.3 mL buffer (pH 7) were incubated at 30 °C for ten minutes to allow sufficient time for metal/enzyme interaction. A 0.5 mL of

urea (0.3 M) was then added, and after 10 minutes, the reaction stopped by adding 0.5 mL of 1 M HCl. The reaction mixture was prepared to 25 mL with distilled water, and 0.5 mL of Nessler's reagent was added. The absorbance of the resulting solution was measured against the blank at 443 nm on a UV-Vis spectrophotometer. The level of inhibition for each metal concentration tested was obtained using the equation as follow:

$$\% \text{ inhibition} = [A_o - A_i] / A_o \times 100$$

A_o = absorbance without metal

A_i = Absorbance obtained after preincubation of metal

The obtained data were plotted to a graphical log of concentration against % inhibition for each metal.

Determination of Pb and Cu Concentrations in Synthetic Water Samples using the Urease Activity Inhibition Method

Six water samples were prepared for each Cu^{2+} and Pb^{2+} by adding an unknown amount of the metal solution to the distilled water. All samples were prepared, and an inhibition test was performed using the same procedures mentioned previously. The concentrations of various metals in the sample were estimated from each inhibition calibration curve's regression equation.

Determination of Pb and Cu Concentrations in Synthetic samples Using AAS

Analysis of Cu and Pb from water samples was carried out using Atomic Absorption Spectrophotometer (AAS).

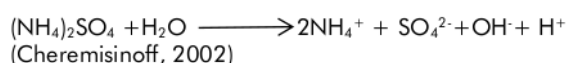
Comparison between Inhibition Testing Method and AAS Method

The synthetic water analysis results obtained by the inhibition test of urease activity and AAS were compared via a normality test preceded by a T-test.

RESULTS AND DISCUSSION

Urease Activity Test

The urease activity test initiation is the plot of a standard curve for ammonium sulfate. The basis for choosing ammonium sulfate as the standard solution is that ammonium sulfate solution will produce the same ammonium ion similar to the hydrolysis of urea. The following is the reaction of ammonium sulfate in water:



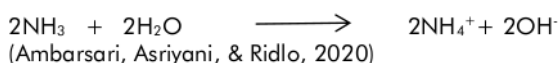
Urease (EC 3.5.1.5 urea amidohydrolase) is a hydrolase enzyme-containing nickel in its structure (You

et al., 2018). Urease will hydrolyze urea to ammonia and carbon dioxide. Hydrolysis of one urea molecule will produce two ammonia molecules and a carbon dioxide molecule (Singh, Singh, & Verma, 2017).

Hydrolysis of urea by urease:



Nitrogen in the ammonia solution will be in two species of (NH_4^+) and ammonia (NH_3). The following is the equilibrium reaction of ammonia in water:



The level of ammonium released is detected using the Nessler method. The Nessler method's principle is that the ammonia ion will give a yellowish-brown color when reacting with Nessler's reagent (K_2HgI_4). The produced color intensity will be directly proportional to ammonia in the sample (Jeong, Park, & Kim, 2013).

The standard curve is obtained from the linear regression equation results resulting from the absorbance measurement of the standard solution of ammonium sulfate. The ammonium ion produced by the above reaction was measured by the Nessler method using a UV-Vis spectrophotometer at a maximum wavelength of 443 nm. The regression equation value obtained is $y = 0.0093x - 0.0589$ with a value of $R^2 = 0.9978$. The slope value obtained is used to calculate the value of enzyme activity.

The urease activity test is carried out using a crude extract of urease from *D. zibethinus* L. seeds. The urease enzyme's crude extract is prepared by growing *D. zibethinus* L. seed sprouts with a germination time of 3 days. The germination process is carried out in dark conditions, which functions to maintain the auxin hormone. The auxin hormone is a growth hormone for plants sensitive to sunlight intensity. Sprouts exposed to sunlight with high intensity will cause stunting growth and further death due to the auxin hormone has interfered work, and therefore germination should be done in dark conditions (Utami, 2018).

The *D. zibethinus* L. seed sprouts are then extracted in cold conditions and centrifuged at cold temperatures, preventing denaturation due to the centrifugation process's hot temperatures. The supernatant obtained is a crude urease extract, which then is tested for activity. The urease activity of *D. zibethinus* L. seeds obtained in this study was 296.774 U / mL.

Effect of Substrate Concentration on Urease Activity

The effect of the given substrate concentration on urease activity is presented in Figure 1.

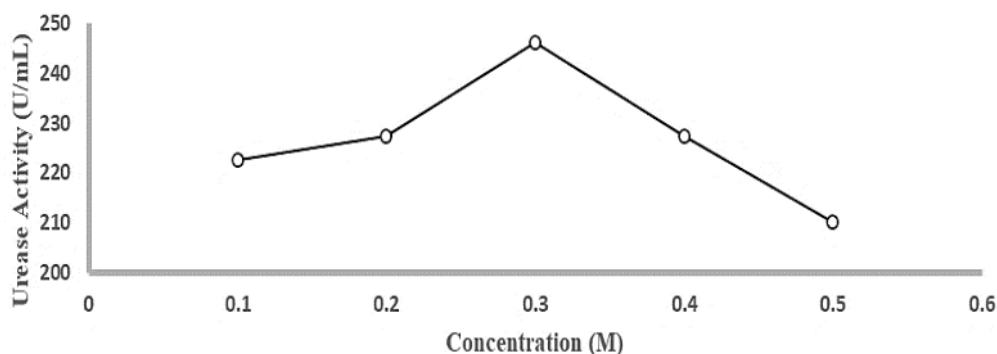


Figure 1. The effect of substrate concentration on the activity of urease enzyme in *D. zibethinus* L. seed

Figure 1 shows that the enzyme activity is low at the substrate concentrations of 0.1 and 0.2 M. This happens because the enzyme's active site only accommodates a small amount of the substrate, so the product is also small. Increasing the substrate concentration will increase the enzyme activity until it reaches the optimum condition or saturation point. The optimum activity obtained from the *D. zibethinus* L. seed urease enzyme is at a substrate concentration of 0.3 M, 246.237 U/mL. This optimum condition indicates that all enzymes are saturated with the substrate so that no active site of the enzyme is free to obtain the product (Kumari, Jain, & Malhotra, 2013). An excess concentration substrate, such as a concentration of 0.4 - 0.5 M, will reduce enzyme activity. This reduction occurs because the substrate at a higher concentration inhibits the enzyme's action (Pervin et al., 2013). The urease enzyme from different sources has different optimum substrate

concentrations, such as 25 mM for green pea beans (*Cicer arietinum* L) (Pervin et al., 2012) and 200 mM for legumes (*Pisum sativum* L) (El-Hefnawy et al., 2014).

The Urease Activity Inhibition Test on Pb and Cu

The urease activity inhibition test uses various concentrations of aqueous Pb and Cu solution. It aims to obtain a curve of the relationship between variations in metal concentration and percent inhibition. In this study, the metal ions used are Pb^{2+} and Cu^{2+} obtained from $Pb(NO_3)_2$ and $CuCl_2$ with various concentrations of 0.001; 0.01; 0.1 ; 1; and 10 ppm. Each metal concentration was reacted with urease, after which the urea substrate was added. The activity test was calculated based on the produced ammonia by the Nessler method. Data on the relationship between variations in the concentration of Pb and Cu to % inhibition is presented in Figure 2.

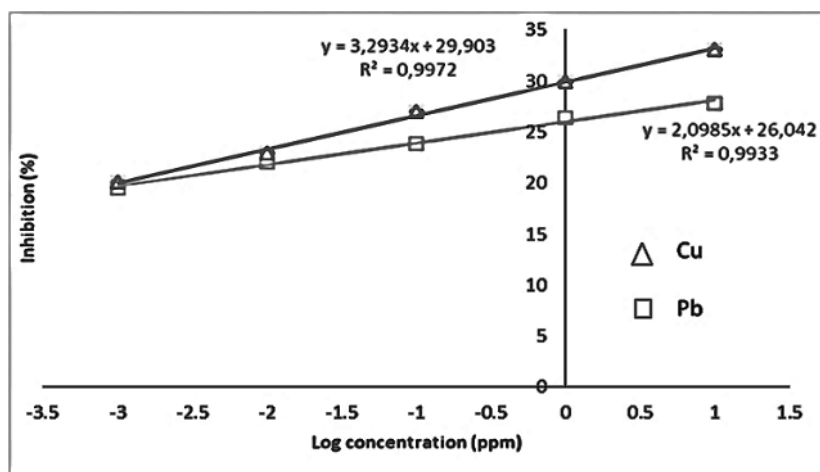


Figure 2. The relationship between urea log concentration and the inhibition of urease activity from *D. zibethinus* L. seed by Pb and Cu ion.

Figure 2 shows a linear relationship between log urea concentration and the inhibition urease activity of *D. zibethinus* L. seed by Pb and Cu ion. The regression equation obtained is $y = 2.0985x + 26.042$ with R^2 value of = 0.9933 for Pb ion. Meanwhile, the regression equation obtained for Cu ion is $y = 3.2934x + 29,903$ with R^2 value of = 0.9972. The study results show that the percentage of inhibition of the urease activity of *D. zibethinus* L. seed increases with increasing metal ion concentration. The addition of Pb and Cu ions has a different effect on the urease activity of the *D. zibethinus* L. seed. The inhibition of urease activity of *D. zibethinus* L. seed by Cu ion was stronger than Pb ion. All tested Cu concentrations showing a higher percentage of inhibition. The same result is also obtained on the inhibition of urease activity of watermelon seed (Upadhyay, 2012). The reaction of heavy metals with ligands containing N and O groups is why they inhibit urease activity. Heavy metals such as Cu, Zn, Ni, and Pb showed high affinity towards N and O groups containing ligands (Gabrovska & Godjevargova, 2009). The copper ion may coordinate with nitrogen- (histidine) and oxygen- (aspartic acid and glutamic acid), which present in the urease structure (Saboury, Pourakbar, & Rezaei, 2010). The urease activity decreases drastically with Cu addition, possibly due to energy blockage in the urea transport process (Banerjee & Aggarwal, 2012).

The inhibition of heavy metals against enzyme activity results from the reaction between metal molecules and the active part of the enzyme, namely thiol (-SH). The inhibitor binds to the enzyme prevents the enzyme from binding to the substrate on its active site (competitive inhibitor). The competition that occurs depends on the concentration of the substrate and inhibitor. The inhibitor works to bind the enzyme's active site and other parts of the enzyme, further change the three-dimensional conformation of the enzyme-forming protein. This condition causes the substrate-enzyme

complex not to form so that urease activity decreases (Fopase, Nayak, Mohanta, Kale, & Paramasivan, 2019). For this report, the type of inhibition is undetermined. However, the inhibition of Cu and Pb ions on the urease activity of *Citrullus vulgaris* seed showed a non-competitive inhibition (Prakash and Vishwakarma, 2001). The same type of inhibition was also found on the urease activity of jack bean by Cu ion (Mishra & Bahadur, 2010).

Determination of Pb and Cu concentrations in synthetic water samples using urease and AAS activity inhibition methods

Determination of Pb and Cu concentrations in synthetic water samples using the urease activity inhibition method initiated by adding Pb and Cu with unknown concentration to the urease extract, continued by the incubation process. When the incubation process is complete, urea addition takes place. At that time, the possibility of Pb or Cu ions bound to the active site of urease replaces the metal, which functions as a urease activator (Pervin et al., 2013). An activator is a compound or a metal that function to increase enzyme activity. Metal ion replacement causes a change in the urease active site's conformation, making it a mismatch to the substrate. This conformational change decreases the urease activity.

The measurement of urease activity inhibition is using a spectrophotometer. The absorbance value obtained from the measurement of urease activity inhibition was then entered into the equation to calculate the percentage of inhibition value. The inhibition percentage value is then plotted in the linear regression equation as obtained in Figures 2 and 3 to obtain the metal concentration value. Metal concentrations obtained by the urease activity inhibition method were compared with AAS measurement data. Data from the determination of Cu and Pb metal concentrations using the urease and AAS activity inhibition method, as presented in **Tables 1 and 2**.

Tabel 1. The concentration of Cu in synthetic water samples measured using the urease activity inhibition method compared to AAS

Sample No	Cu concentration measured using the urease activity inhibition method (ppm)	Cu concentration measured using AAS (ppm)
1	0.135 ± 0.000	0.133 ± 0.016
2	0.744 ± 0.079	0.757 ± 0.024
3	1.262 ± 0.000	1.283 ± 0.027
4	2.525 ± 0.190	2.595 ± 0.024
5	4.120 ± 0.796	4.151 ± 0.024
6	1.977 ± 0.586	1.982 ± 0.047

Table 2. The concentration of Pb in synthetic water samples measured using the urease activity inhibition method compared to AAS

Sample No	Pb concentration measured using the urease activity inhibition method (ppm)	Pb concentration measured using AAS (ppm)
1	1.718 ± 0.174	1.726 ± 0.083
2	6.346 ± 0.000	6.316 ± 0.068
3	14.917 ± 0.000	14.326 ± 0.117
4	24.902 ± 0.000	25.682 ± 0.541
5	33.203 ± 3.163	32.949 ± 0.000
6	49.330 ± 0.000	48.967 ± 0.895

Analysis data used to compare the determination of Cu and Pb concentrations using the urease activity inhibition method of *D. zibethinus* L. seeds and the AAS method starting with a normality test analysis. The normality test is carried out to see the level of normality of the data used, whether the data is in a normal distribution or not. The normality of the data is critical because, in normally distributed data, the data is appropriate as a representative of the population. The test used is the Kolmogorov Smirnov test calculated by the SPSS program. Based on the Kolmogorov-Smirnov data normality test, it is found that the significance value (p) is 0.782 (Cu) and 0.584 (Pb) ($p > 0.05$). The significance value (p) signifies that the Kolmogorov-Smirnov results show that the data is normally distributed (Dahlan, 2011). The data analysis continues with the T-test (Marliani, Hasanuddin, & Nurmaliyah, 2017).

Paired Sample T-Test shows whether paired samples experience significant changes. Their significance value determines the result of the Paired Sample T-Test. This value then determines the decisions taken in the study. The significance value (2-tailed) < 0.05 indicates a significant difference between the two methods. A low significance value shows a significant influence on the differences in the treatment given to each variable. The significance value (2-tailed) > 0.05 indicates no significant difference between the two methods. The high significance value shows that there is no significant effect on the differences in the treatment given to each variable. The result of data analysis using the T-test obtained the Asymptotic significance value. (2-tailed) of 0.081 (Cu) and 0.714 (Pb).

These results indicated no significant difference in the 95% confidence level for the determination of Cu and Pb concentrations using the urease activity inhibition method of *D. zibethinus* L. seeds using the AAS method because of the Asymptotic significance value. Sig. (2-tailed)) is greater than 0.05.

CONCLUSIONS

Cu and Pb ions inhibited urease activity. The higher the levels of Cu and Pb ions, the percentage of inhibition against urease activity increase. Determination of Cu

and Pb standard solution (0.001 - 10 mg/L) using the urease activity inhibition method and the AAS method showed no significant difference at the 95% confidence level.

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13. Determination of Cu and Pb concentrations

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