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The Isolation, Immobilization, and Characterization of Urease from The Seeds of Winged Bean (*Psophocarpus tetragonolobus* (L.) DC.

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ABSTRACT. Urease has been utilized in the field of health and industry. Urease is commonly used in the form of free enzyme, so that the utilization is limited. Urease efficiency can be improved using immobilization enzyme. This research aimed to do the urease isolation, immobilization, and characterization from the winged bean seeds. This research was started by determining the amino-acid content of winged bean seeds using the Liquid chromatography-mass spectrometry (LCMS). The winged bean seeds were germinated and extracted. The obtained crude extract's activity was determined using Nessler reagent and measured using UV-Vis spectrophotometer with the wavelength of 500 nm. The urease of winged bean seeds was immobilized using the alginate matrix. The optimization of urease-immobilized beads could be made through the variations of natrium alginate concentration and beads formation periods in solution $CaCl_2$. Characterization free and immobilized urease were made using the variations of urea substrate concentration, pH, temperature, and also the repeated utilization of immobilized urease. Winged bean seeds are rich with essential amino acid, such as leucine, isoleucine, histidine, phenylalanine, and valine. The urease obtained from the winged bean seeds had the optimum activity in the germination period of 8 days. The urease immobilization showed the optimum condition in the natrium alginate concentration of 5% (w/v) and beads formation period in solution $CaCl_2$ for 60 minutes. The characterization results of free urease and immobilization had the optimum condition at the urea substrate of 0.2 M, and pH 7. Free urease had the optimum temperature of 35 °C, while the immobilized urease had the optimum temperature of 40 °C. The immobilized urease had the utilization stability up to 5 times with the relative activity of 48%. The EDX analysis results showed that the alginate did not contain N, while alginate urease beads contained N as much as 12%.

Keywords: alginate, immobilization, urease, winged bean seeds

INTRODUCTION

One element essentially required by plants is nitrogen. Urea is a source of nitrogen for plants obtained from soil (Cantarella et al., 2018). Nitrogen in urea cannot be directly utilized, yet should be hydrolyzed into ammonia and carbon dioxide. Urease is enzyme which has the role to hydrolyze urea into ammonia and carbon dioxide (Singh et al., 2017). Urease is enzyme which has an important role in nitrogen metabolism during plant germination (El-hefnawy et al., 2014).

Urease also has an important role in nitrogen waste cycle with the rumen of domestic cattle (Sujoy & Aparna, 2012). Urease also has an important role in the process: hemodialysis, management of liquid waste contained in spacecraft, and management of industrial fertilizer waste that is rich in urea (El-hefnawy et al., 2014).

Urease has been isolated from bacteria, fungi, and plants. Seeds, such as *Pisum Sativum* (Iyer et al., 2018), *Vicia Faba* L (Bedan, 2020), and *Durio zibethinus* L (Zusfahair et al., 2021) are plants used as urease-resulting sources. Urease in this research was extracted from the winged bean seeds. The old winged bean seeds contain protein of 29.8-39 g in 100 g of fresh weight (Saptadi et al., 2016). High protein content in winged bean seeds may also have high urease content. The use-value of winged bean can be improved by utilizing the winged bean seeds as the urease sources.

Enzyme as free molecule dissolved in water is difficult to be separated from its substrate and product. This make enzyme mixed with the product and difficult to be separated in the final reaction, so that the enzyme utilization ability is limited. To overcome these weaknesses, enzyme immobilization is made. Immobilization makes the product separation is easier and prevents from protein or activity loss in the following processes (Daâssi et al., 2014).

Immobilization can be performed through several methods: cross-linking, adsorption, covalent bond formation, and entrapping (Geethanjali & Subash,

2013). This research used the entrapping method. Immobilization using entrapping method was more frequently used than the others since the enzyme in this method had no bond with the supporting matrix, so that the natural structure and catalytic function of enzyme were not disturbed (Zusfahair et al., 2020). The matrix used in this research was alginate modified in the form of beads. The advantage of immobilization with the entrapping method using alginate is related to its simplicity, environmentally-friendly characteristics, and cost-effective (Daâssi et al., 2014). Some researchers have conducted immobilization of enzyme using alginate matrix, such as laccase (Daâssi et al., 2014), peroxidase (Bilal & Asgher, 2015), urease from jack beans (Danial et al., 2015); Maltase from *Bacillus licheniformis* KIBGE-IB4 (Nawaz et al., 2015), and urease from *Cicer arietinum* (Tetiker & Ertan, 2017).

This research was started by determining the amino-acid content of winged bean seeds using the Liquid chromatography-mass spectrometry (LCMS) device as well as determining the standard curve for ammonium sulfate. The next step was isolating urease from winged bean seeds. Winged bean seeds were germinated and extracted. The obtained crude extract was then immobilized with Na-alginate concentration variation and formation period beads. The crude extract and immobilized urease were then characterized using the concentration variations of substrate, pH, and temperature. The Morphology of immobilized urease Beads was analyzed using the Scanning Electron Microscopy (SEM), while the existence of enzyme was analyzed using the Energy Dispersive X-ray (EDX).

EXPERIMENTAL SECTION

Research Materials

The old winged bean seeds were from the green winged bean type (Agriculture in Kembangan area, Purbalingga). All chemical materials used were the analytical class, purchased from Merck Chemical Company (Merck, Jerman), except the (technical) Na-alginate.

Research Procedures

Amino acid analysis

The amino acid compositions from winged bean seeds were analyzed using LCMS. The analysis was conducted in the Integrated Research and Testing Laboratory of Gadjah Mada University.

Determining winged bean seeds

The winged bean seeds were determined at the Biology Faculty of Unsoed under an acceptance test letter number: 128/HP.LL/V/2022.

Determining the standard curve for ammonium sulfate

The measurement of standard solution of ammonium sulfate was started by determining the linear range of standard curve. The linear range determination was conducted with concentration variations from 1 ppm to 60 ppm. The standard solution of ammonium sulfate was taken as much as 1.5 mL, and then added with 0.25 mL of Nessler reagent. The blank was made with 1.5 mL of distilled water added with 0.25 mL of Nessler reagent. The solution absorbance was measured at λ 500 nm using the UV-Vis spectrophotometer.

Urease enzyme isolation from winged bean seeds

The urease isolation from winged bean seeds was similarly conducted with the urease isolation method (Zusfahair et al., 2022). The sprouts of winged bean seeds which have the highest activity value were then characterized and used to make the immobilized enzyme.

Optimizing urease immobilization with alginate (Zusfahair et al., 2020)

Urease was mixed with various percentage of sodium alginate solution (3, 4, 5, and 6% (w/v) of sodium alginate in 0,2M at pH 7 of phosphate buffer). The alginate solution was taken as much as 4 mL mixed with 1 mL of urease from the crude extract of winged bean and then extruded dropwise using a micropipette with 1000 mL sized tip into 0.2 M of $CaCl_2$ for various contact times 20, 40, 60, 80, and 100 min at 4 °C to produce beads. The beads was filtered using filter paper and stored in 0.03 M of $CaCl_2$ at 4 °C

Free and immobilized urease activity test

Free urease activity test was conducted by entering 1 mL of urea solution 0.2 M in the phosphate buffer at pH 7 into the sample reaction tube, and then added with 1.90 mL of buffer phosphate solution at pH 7. 0.1 mL of urease obtained from crude extract of winged bean was added into the sample reaction tube. The next process is the same as the method used by (Zusfahair et al., 2022)

The immobilized urease activity test was made by taking 5 mL of urea solution 0.2 M in the phosphate buffer at pH 7 added with the immobilized urease beads as many as beads formed in 4 mL of alginate with the most optimum variation of Na-alginate concentration and formation period beads. The solution was then incubated for 15 minutes at the temperature of 35 °C. The beads were then filtered and the solution activity was measured using Nessler reagent. The control was then similarly conducted using the beads made without the addition of urease enzyme.

The solution absorption was measured using the UV-Vis spectrophotometer at the wavelength of 500 nm. The urease estimation was made using the standard curve of ammonium sulfate (Zusfahair et al., 2022). One activity unit (U) is defined as the number of ammonias formed in 1 ppm and per minute from the urea hydrolyzed by urease in the sample.

Free and immobilized urease characterization

Free and immobilized urease characterization were similarly conducted, such as activity test. Characterization was conducted in the influence of

variations of substrate concentration, pH, and temperature on urease activity. The obtained optimum conditions were used for the following tests. The concentration variations of urea substrate used: 0.1; 0.15; 0.2; 0.25; and 0.3 M. The Variations of phosphate buffer 0.2 M used to dissolve the urea substrate were pH 6, 7, 8, and 9, while those of incubation temperature were 30, 35, 40, 45, and 50 °C. In the immobilized enzyme, the repetitive utilization test, Scanning Electron Microscopy (SEM) analysis, and Energy Dispersive X-ray (EDX) were conducted.

RESULTS AND DISCUSSION

This research uses winged bean seeds as the source of urease. These winged bean seeds are determined first to ensure that its species is correct. The determination result showed that the winged bean seeds used belonged to the *Fabaceae* family and *Psophocarpus* genus. The species name of the winged bean seeds used in this research was *Psophocarpus tetragonolobus* (L.) DC.

Amino Acid Analysis

The amino acid compositions from the winged bean seeds were analyzed using LCMS (Liquid chromatography-mass spectrometry) (**Table 1**). The data presented in **Table 1** showed that the most amino acid found in the winged bean seeds was L-arginine. Arginine is one compound classified into the semi-essential amino acid (Willson, 2009). Winged bean seeds are rich with essential amino acid, such as leucine, isoleucine, histidine, phenylalanine, and valine. Based on the research data, it can be concluded that winged bean seeds are classified into alternative food to improve people's nutrition.

Determining the Standard Curve of Ammonium Sulfate

The standard curve making aimed to figure out the relationship between the standard solution concentrations and absorbance values, so that a sample concentration can be revealed. A sample concentration can be determined by substituting the absorbance value obtained from the sample measurement to the linear regression equation obtained from the standard curve. The standard used in this research was ammonium sulfate solution.

The obtained standard curve was in accordance with the Lambert Beer law stating that absorbance will be directly proportional with concentration. The obtained linear regression was $y = 0.0539x - 0.3438$ with the value of R^2 (determination coefficient) of 0.989. The linier relationship from the standard curve was stated in the form of r resulted from the root of R^2. The correlation coefficient value was within the range of $-1 \leq r \leq 1$. The r-value obtained from the standard curve above was 0.994. The obtained r-value above 0.9 yet less than 1.0 showed that the obtained linear correlation was adequately strong.

Urease Isolation from Winged Bean Seeds

The urease isolation from winged bean seeds started with germination stage. Germination is the embryo growing process and grain component which has the ability to grow normally into plant (Girsang et al., 2019). This process requires the enzyme's role as the catalysis of various biochemical reactions in the related gains. The germination process started by immersing the old winged bean seeds in the water for 6 hours to absorb water to the cavity of plant tissues, so that cells in the plant tissues actively grow.

Table 1. Amino acid compositions from the winged bean seeds

Number	Test Parameters	Content (mg/Kg)
1	L-Arginine	18,914.2
2	L-Histidine	9,577.4
3	L-Lycine	4.3
4	L-Phenylalanine	8,552.3
5	L-Isoleucine	10,618.3
6	L-Leucine	12,620.1
7	L-Tyrosine	760.9
8	L-Methionine	229.8
9	L-Valine	2,646.0
10	L-Proline	200.9
11	L-Glutamic acid	328.9
12	L-Aspartic acid	201.8
13	L-Cysteine	NDD
14	L-Threonine	108.0
15	L-Serine	69.7
16	L-Alanine	163.4
17	L-Glycine	49.9
18	L-Thryptophan	18.9

After the immersion of old winged bean seeds, wet cotton was placed as growing media which were then sealed using black plastic. Germination is performed in a dark place at room temperature, so that the auxin hormone performance was not disturbed by the sunlight. The auxin hormone has the role in the cell-lengthening process and is found in the growing point of plant buds, such as roots and stem ends (Purwanti et al., 2013). The dark condition in this research can optimize the plant germination processes.

The sprout of winged bean seeds was then extracted every two days using the phosphate buffer at pH 7. The utilization of phosphate buffer at pH 7 was made to maintain the condition of urease at neutral pH. The enzyme was then centrifuged in cold condition to prevent from denaturation caused by heat. The obtained supernatant was the urease crude extract which activity was further tested to figure out the influence of germination time to the urease enzyme activity.

During the germination stage, the urease activity increased starting from Day-2 and optimum on Day-8 with the activity value of 21.027 U/mL. The urease activity decreased on Day-10 (not presented data). Germination on Day-8 was further used for experimental purposes.

Urease Immobilization with Alginate

The urease immobilization in this research was conducted using the entrapping method with alginate matrix. Entrapping method was selected due to its simple process with relatively small damage in the enzyme original structure. The entrapping method in the alginate calcium gel offered many benefits due to its simplicity, environmentally friendly characters, and cost-effectiveness (Daâssi et al., 2014).

Figure 1. Ca-alginate urease beads

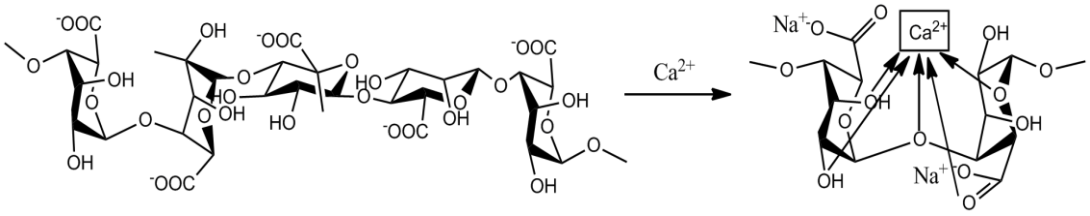


Figure 2. Alginate reaction with Ca^{2+} (Mazumder, 2013)

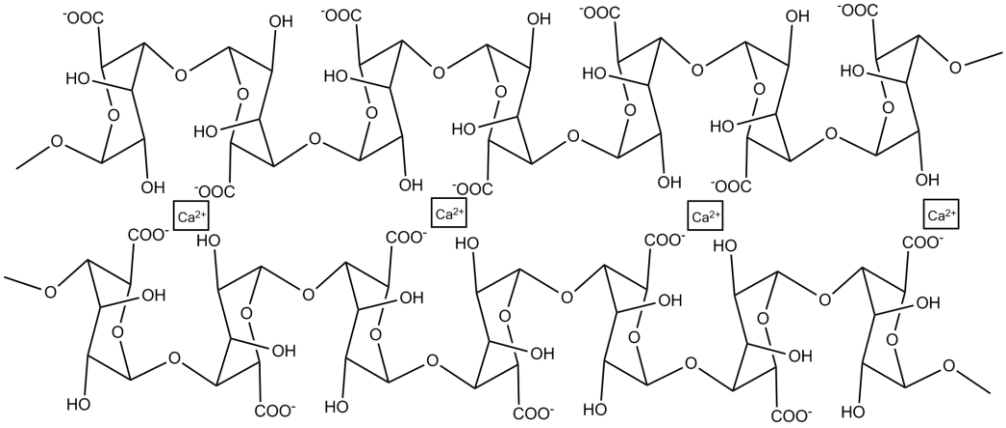


Figure 3. Alginate structure as ligan (Panggabean et al., 2012).

When the alginate solution was mixed with urease and dropped to solution $CaCl_2$, ion Na^{+2} from Na-alginate was replaced by ion Ca^{+2} from $CaCl_2$, the Ca-alginate urease beads were formed and gave the gel-like characters (**Figure 1**). The pores were formed on Ca-alginate beads due to the cross-linking between bivalent ion Ca^{2+} resulted from $CaCl_2$ with anion carboxyl (COO^-) from guluronate acid on alginate (**Figure 2**) (Danial et al., 2015). The interaction between Ca^{2+} ions with COO^- groups from alginate was made in both inter dan intra molecules (Panggabean et al., 2012).

Ion Ca^{2+} had empty orbital d, so that alginate as ligan can donate its electrons to Ca^{2+} (**Figure 3**). Ion Ca^{2+} as alginate inter-molecule connecting bridge can only receive 5 oxygen ligands, while alginate has the potential to donate 10 oxygen ligands from two parallel chains in which each from OH^- in C2 and C3. The O bond connects 1-4, a carboxyl group and O ring from the neighbor's residue (Panggabean et al., 2012).

Urease Immobilization with Variations of Na-alginate Concentration

The curve of relationship between Na-alginate concentration and immobilized urease activity can be seen in **Figure 4**. The cross-linking level from the gel-forming agent influenced the beads' pore size, and therefore, various natrium alginate concentrations were used to reach the optimum urease activity. The research results presented in **Figure 4** showed that urease activity continuously increased along with the increasing Na-alginate concentration from 3 to 5%. Low natrium alginate concentration caused the formation of big beads' pores, so that the trapped enzyme could easily leave the matrix and resulted in low activity. Urease reached its optimum activity at the Na-alginate concentration of 5% with the activity value of 14.760 U/mL. At the optimum concentration of 5%,

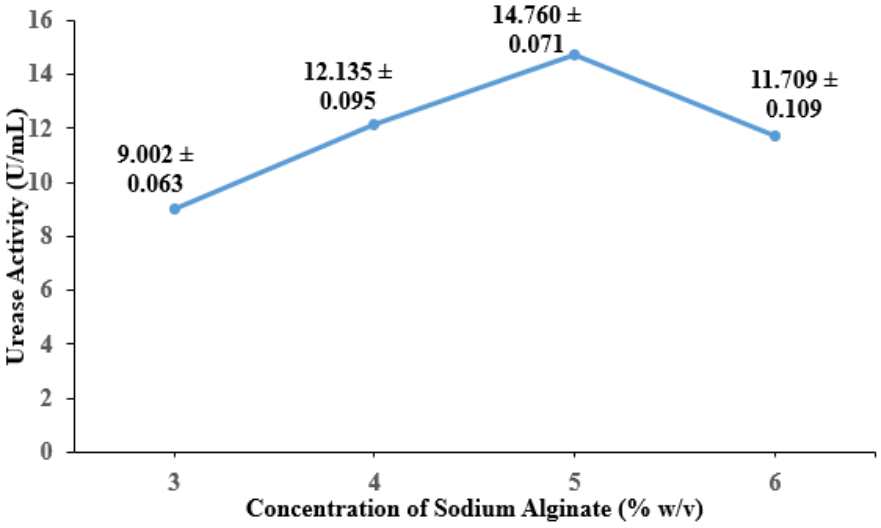


Figure 4. Curve of relationship between variations of Na-alginate concentration and immobilized urease activity

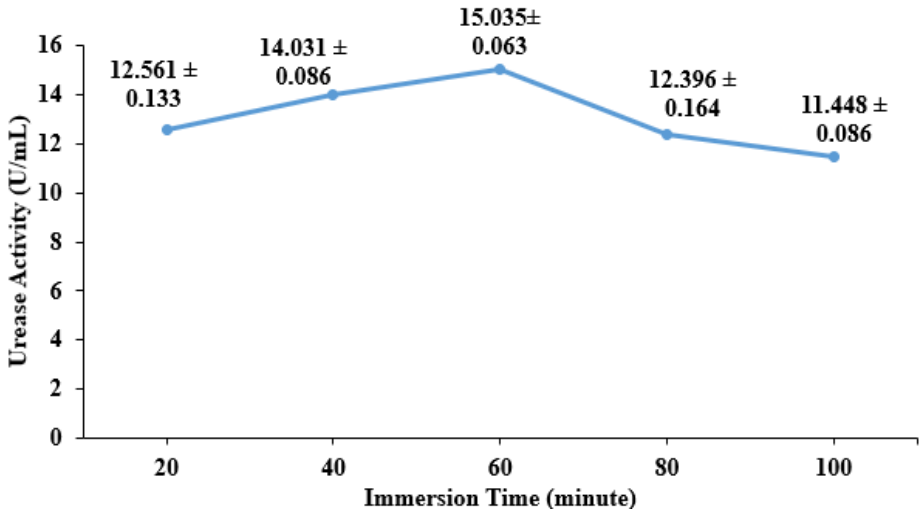


Figure 5. The curve of the relationship between variations in the formation time of alginate beads and the activity of immobilized urease

the pores of alginate beads were in ideal condition and eventually eased the diffusion process of substrate into enzyme. This caused more products were formed. Urease activity then experienced decrease at the concentration of 6%. This decreasing activity was caused by the denser pores of alginate beads due to the numerous formed cross-linking, so that the substate diffusion process was inhibited and the products were difficult to form. The higher the concentration of natrium alginate, the lower the gel porosity, so that the enzyme will stuck inside the gel (Bilal & Asgher, 2015). The increasing concentration of natrium alginate also increased the solution viscosity, so that the enzyme encapsulation became more complicated (Geethanjali & Subash, 2013).

Urease Immobilization with Variations of Formation Period Beads

The curve of relationship between alginate formation period beads and immobilized urease activity can be seen in **Figure 5**. The research results presented in **Figure 5** showed that urease activity increased along with the increasing immersion periods using $CaCl_2$ 0.2 M. Urease had the optimum activity within 60 minutes with the activity value of 15.035 U/mL. the cross-linking formation increased between matrix and enzyme when beads were exposed with calcium chloride solution for a relatively long period of time. This caused the homogenous-formed and strong beads as well as urease activity increased. The immersion time exceeded the optimum time and caused the activity decrease due to the leakage from the enzyme (Nawaz et al., 2015).

Free and Immobilized Urease Characterization

The Influence of substrate variations on urease activity

The measurement of free and immobilized urease activity on substrate concentration variations can be seen in **Figure 6**. The research results presented in **Figure 6** showed that free and immobilized urease activity continuously increased along with the increasing substrate concentration. Free and immobilized urease reached the optimum activity at the substrate concentration of 0.2 M with the activity value of 21.040 U/mL for free urease and that of 14.774 U/mL for immobilized urease. The complex enzyme substrate was obtained due to the existence of contact between enzyme and substrate. This contact happened in the enzyme active side. When the substrate concentration is low, the resulted enzyme activity is also low since only some enzyme active side binds the substrate. If the substrate concentration is increased, the enzyme activity will also increase since the enzyme active side will bind more the substrate, so that more complex enzyme substrate is resulted. Free and immobilized urease activity then decreased at the concentrations of 0.25 and 0.3 M. The decreasing activity could be caused by the inhibition of substrate in higher urea concentration (El-hefnawy et al., 2014).

In the research conducted by (Sujoy & Aparna, 2012), the urease isolation from the seeds of pigeon pea (*Cajanus cajan*) resulted optimum activity at the urea concentration of 0.3 M. The differences in this optimum substrate concentration were due to different enzyme-resulting sources used. This indicated that each enzyme resulted by each different source will have different characteristics.

The influence of pH variations on urease activity

The measurement of free and immobilized urease activity on pH variations can be seen in **Figure 7**. The research results presented in **Figure 7** showed that free and immobilized urease reached optimum activity at pH 7 with the activity value of 22.497 U/mL for free enzyme and 15.351 U/mL for immobilized enzyme. Enzyme activity is closely related to the enzyme conformation. Enzyme at optimum pH had the ideal conformation, because at optimum pH, the enzyme active side had the shape in accordance with that of its substrate. At less optimum pH, the enzyme activity decreased probably due to the environmental changes of enzyme pH. These changes could influence the intramolecular hydrogen bond resulting in non-accurate enzyme conformation which possibly reduced the enzyme activity (Kaushal et al., 2018).

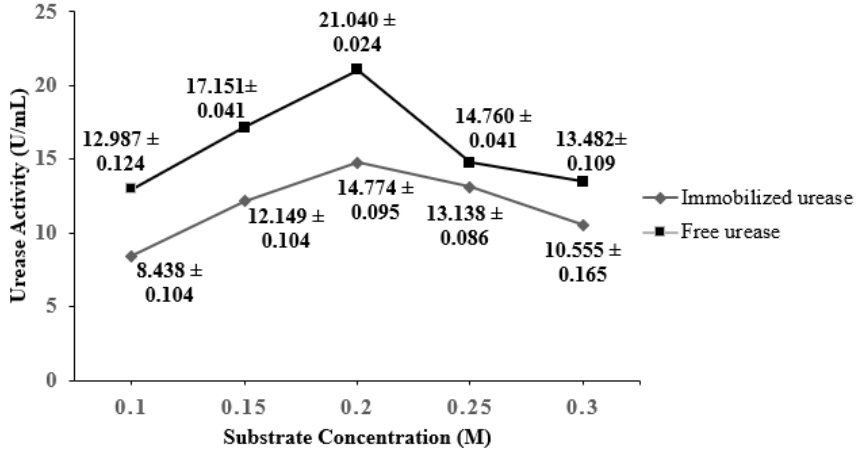


Figure 6. Curve of the influence of urea substrate concentration variation on free and immobilized urease activity

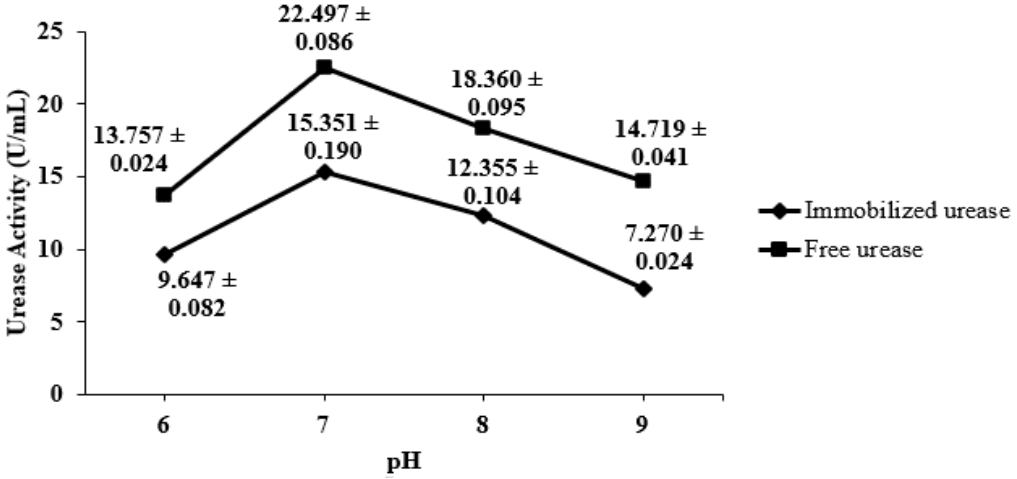


Figure 7. Curve of the Influence of pH variations on free and immobilized urease activity

In the previous research, the urease isolated from the seeds of chickpea (*Cicer arietinum* L.) had the optimum pH of 7.2 (Pervin et al., 2013), while that isolated from the seeds of pigeon pea (*Cajanus cajan*) had the optimum pH of 7.5 (Sujoy & Aparna, 2012).

Influence of temperature variations on urease activity

The measurement of free and immobilized urease activity on temperature variations can be seen in Figure 8. The research results presented on Figure 8 showed that free and immobilized urease had optimum activity respectively at the temperature of 35 °C and 40 °C with the activity values of respectively 22.071 U/mL and 16.024 U/mL. The optimum temperature of urease enzyme from the winged bean seeds increased after immobilized using alginate. This showed that alginate matrix could protect the enzyme, so that the immobilized enzyme could last longer to the increasing temperature. The alginate matrix could absorb heat and protect enzyme from denaturation (Kumar et al., 2009). According to (Danial et al., 2015), the shifts of enzyme optimum temperature after immobilization could be caused by the possibility of immobilized enzyme protection from high temperature through the formation of molecular cages around the enzyme protein. Most operations in industry were well performed at room temperature or higher temperature, so that the utilization of immobilized enzyme was more beneficial and economical.

Repeated utilization of immobilized urease enzyme

The measurement results of immobilized urease enzyme repeated utilization can be seen in Figure 9. A repeated utilization is one advantage of immobilized enzyme when compared to its free form since reducing costs and considered as an important factor for commercial application. The research results presented on Figure 9 showed the immobilized urease activity in optimum condition maintained up to 48% from the initial activity after five-time utilization. Urease from chickpea immobilized with alginate also can be used with five-time repetition (Tetiker & Ertan, 2017). The decreasing immobilized urease activity after the repeated utilization was caused by enzyme leakage and denaturation (Kaushal et al., 2018).

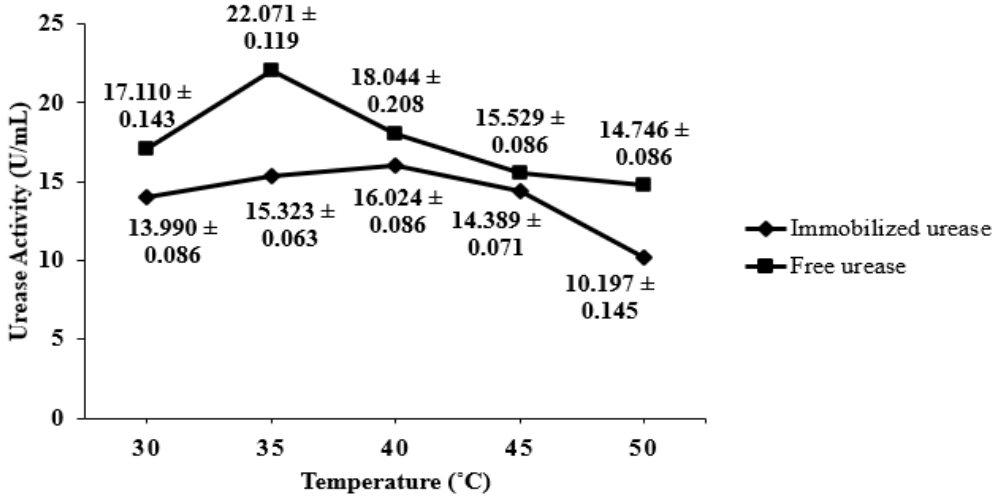


Figure 8. Curve of the influence of temperature variations on free and immobilized urease activity

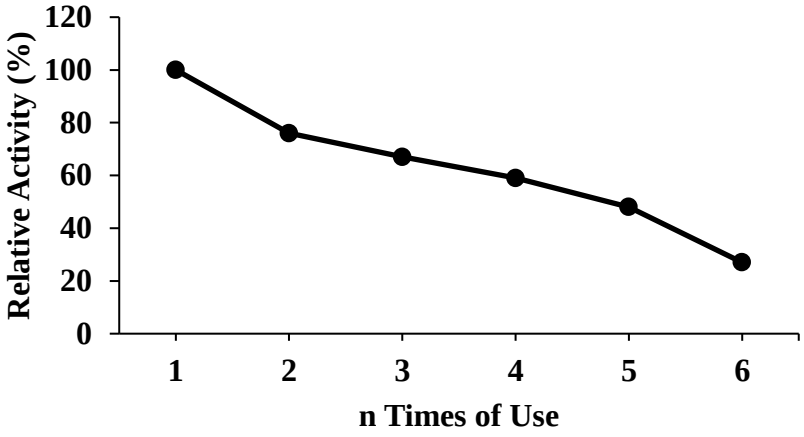
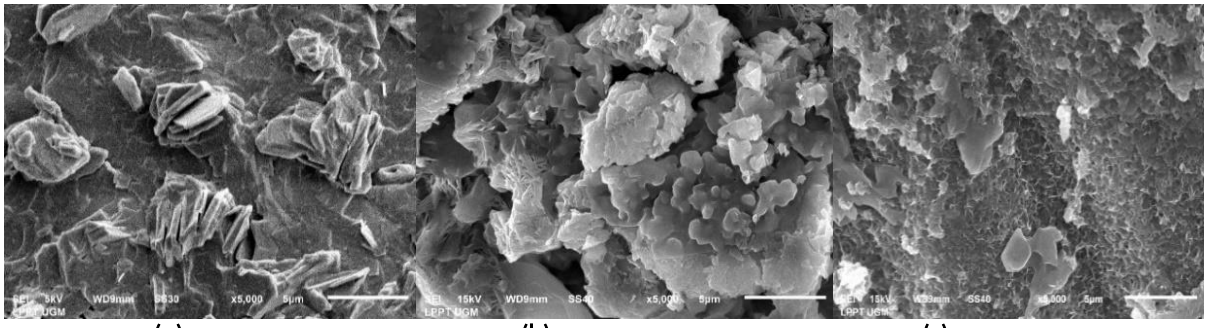


Figure 9. Curve of the influence of Immobilized Urease repeated utilization

Scanning electron microscopy (SEM) and energy dispersive x-ray (EDX) analysis

Immobilized urease beads analyzed using SEM-EDX (**Figure 10**). The Analysis using SEM was found in **Figure 10** showing the surface structure of alginate without enzyme (**a**) after 1x utilization (**b**) and after 5x utilization (**c**). Seen from **Figure 9**, this matrix disintegration occurred due to the repeated utilization.

Urease immobilized with alginate was also analyzed by EDX spectrum (**Figure 11**). The EDX analysis results showed that the alginate did not contain N (**A**), while alginate urease beads contained N as much as 12% (**B**). This was possibly due to the presence of enzyme on surface (Krishna et al., 2011).



(a) (b) (c)

Figure 10: (**a**) Control. (**b**) 1x Utilization. (**c**) 5x Utilization at magnification 5000 x.

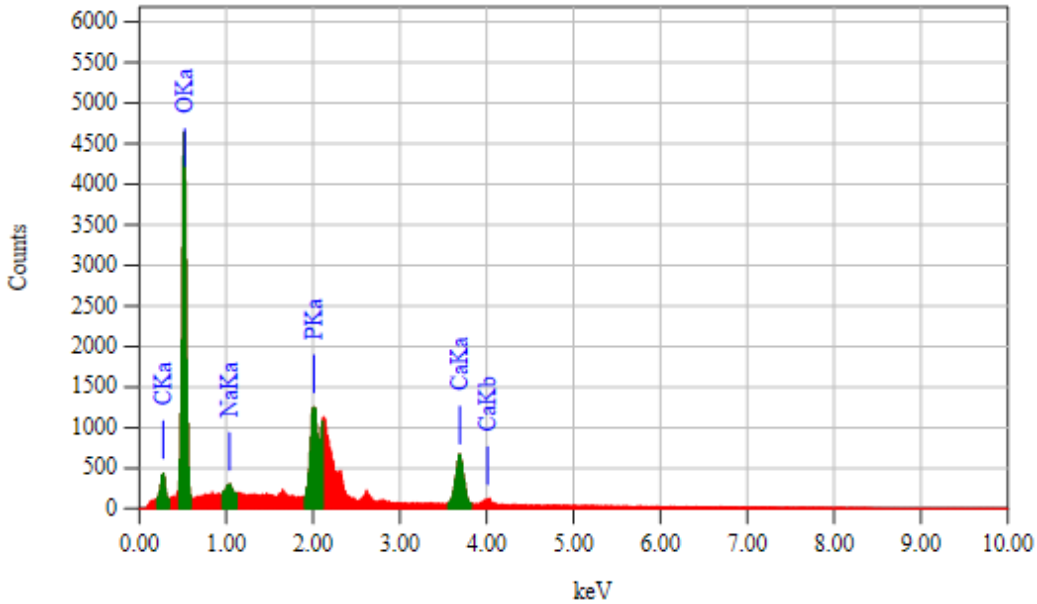


Figure 11 (**A**). Alginate beads without urease

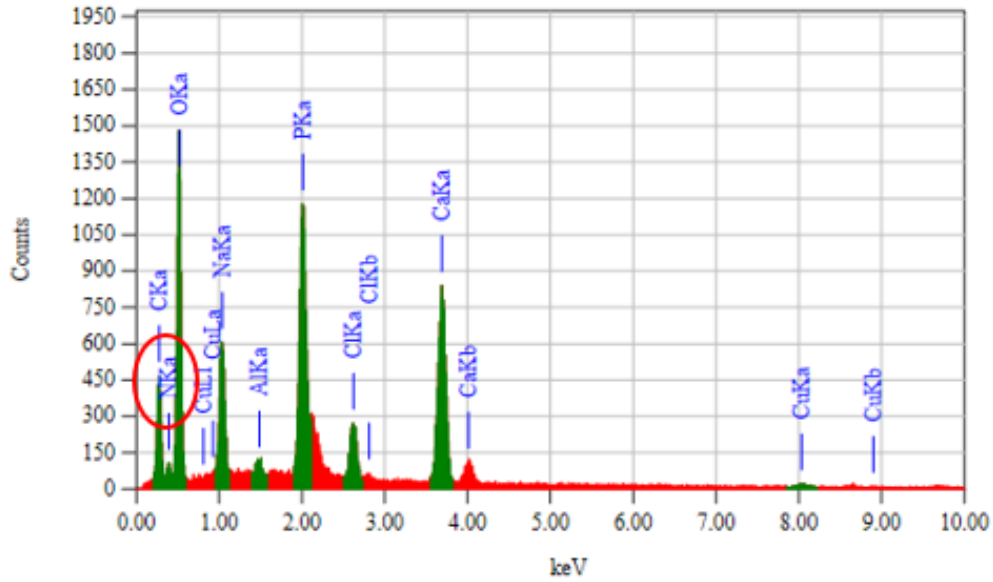


Figure 11 (**B**). Urease beads immobilized with alginate

CONCLUSIONS

Winged bean seeds are rich with essential amino acid, such as leucine, isoleucine, histidine, phenylalanine, and valine. The urease crude extract from winged bean seeds was obtained from the germination process. The urease from winged bean seeds was successfully immobilized using the alginate-supporting solid. The immobilized urease optimum temperature shifted up to 40 °C and had the utilization stability repeated 5 times with the remaining relative activity as much as 48%.

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