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Research Article

Kinetic Behavior and Stability of Alginate-Immobilized Urease from Green Bean Seeds (*Phaseolus vulgaris*)

Zusfahair[1]*, Sifa Nurfadillah Setiawan[1], Dian Riana Ningsih[1], Bilalodin[2], Amin Fatoni[1], Niken Istikhari Muslihah[1]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Science, Jenderal Soedirman University, North Purwokerto, 53123, Indonesia*
[2]*Department of Physics, Faculty of Mathematics and Natural Science, Jenderal Soedirman University, North Purwokerto, 53123, Indonesia*

Email: zusfahair@gmail.com

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Abstract

The industrial application of urease is limited due to its low reusability, which can be overcome by immobilization. Furthermore, the utilization of urease from plants such as mung bean seeds, which is affordable and readily available, offers a sustainable alternative to commercial enzymes. This study aimed to immobilize urease extracted from green bean seeds using an alginate matrix and to evaluate its kinetic and stability characteristics. Urease activity was measured at 500 nm using the Nessler method. Kinetic analysis showed that immobilization decreased Vmax from 0.674 to 0.223 M min^{-1} and increased K_M from 0.005169 to 0.006755 M. After incubation at 35 °C for 180 min, immobilized urease showed higher relative activity (52.26%) than free enzyme (42.82%). Stored at 4 °C for 9 days, the enzyme preserved 56.68% of its relative activity. The immobilized urease displayed the ability to be reused up to five cycles. SEM analysis showed morphological changes in the beads after enzyme incorporation, while EDX confirmed an increase in the elemental composition of the immobilized beads. This study shows that urease from green bean seeds can be effectively immobilized in an alginate matrix to produce a stable, reusable, low-cost biocatalyst with potential for application in urea biosensors.

Keywords: Alginate, green bean seeds, immobilization, urease

1. INTRODUCTION

Urease has attracted considerable interest in environmental monitoring because of its ability to catalyze the hydrolysis of urea into ammonia and carbon dioxide, which then produces ammonium ions and increases the pH of the surrounding medium. These chemical changes can be detected and utilized in urease-based biosensors for the determination of urea in environmental samples [1, 2].

Urease has been isolated from grains and legumes. Urease was isolated from the seeds of broad beans [3], jack bean [4], winged bean [5], red lentils [6] and *Vicia sativa* [7]. In previous studies, legumes with potential as a source of urease were green bean seeds [8]. The extraction results were still in the form of free urease. However, the use of urease in its free form has been limited by low operational stability and lack of reusability, making it inefficient for sustainable industrial processes [9]. Therefore, enzyme immobilization has been widely developed as a strategy to improve stability, facilitate enzyme separation from the reaction medium, and enable enzyme reuse in biocatalytic systems [10].

Various methods of immobilization have been used for enzymes, which can be categorized into three types: binding of the enzyme to a carrier, enzyme crosslinking, and entrapment [11]. The entrapment method is one of the most efficient immobilization techniques for encapsulating enzymes into a matrix. Its advantage lays in the simple and affordable process without disturbing the original structure of the enzyme [12]. The entrapment technique in an alginate matrix is widely used because it is biocompatible, affordable, and able to maintain enzyme activity in relatively mild reaction conditions. The porous structure of alginate gel also allows substrate and product diffusion,

thereby supporting the catalytic process efficiently. Recent research shows that enzyme immobilization systems can improve thermal and storage stability, as well as enhance the reusability of enzymes in various modern biocatalytic applications [10].

Several studies have reported the immobilization of urease using various support matrixes, but studies specifically exploring urease derived from green bean seeds in immobilization systems are still relatively limited. Previous studies have only reported the isolation of urease from red beans immobilized using alginate matrixes without in-depth studies of the performance of the resulting enzymes. The research still focuses on one or two characterization parameters, such as enzyme activity. So that comprehensive studies integrating kinetic analysis, enzyme stability, and reusability in a single immobilization system are still rarely conducted. Furthermore, the relationship between the structure of the trapping material (alginate) and the biocatalytic performance of the enzyme has not been studied in depth. Studies combining morphological analysis using Scanning Electron Microscopy (SEM) and elemental composition through Energy-Dispersive X-ray Spectroscopy (EDX) to link changes in the alginate matrix structure with enzyme catalytic function are still very limited in immobilized urease research. In fact, this structural characterization approach is important for understanding the mechanism of increased stability and efficiency of biocatalysts in modern immobilization systems[10]. Consequently, research integrating urease sources from plants such as green bean seeds, alginate-based immobilization systems, and comprehensive analysis of kinetics, stability, reusability, and morphological characterization is still needed to expand our understanding of developing more efficient and sustainable urease-based biocatalysts.

2. RESEARCH METHODS

Instruments and Materials

The materials used in this study were green bean seeds, urea, Na-alginate, $CaCl_2$, $Na_2HPO_4.2H_2O$, $NaH_2PO_4.2H_2O$, ammonium sulfate, HCl, and Nessler reagent. All chemicals were obtained from Merck Chemical Company (Germany) except Na-alginate (Technical).

Research Procedures

Preparation of the ammonium sulfate standard curve

The standard curve was determined using the Nessler method [8].

Isolation and extraction of the urease enzyme from green bean seeds

Isolation was carried out using the germination method for 4 days, and urease extraction was conducted using the immersion method with a phosphate buffer [13]. The filtrate obtained was centrifuged for 15 minutes at a speed of 12,000 rpm at a temperature of 4 °C. The supernatant obtained was taken as a crude extract of the urease enzyme, then its activity was tested, and also used for the production of immobilized enzymes.

Immobilization of urease with alginate [14]

Variations in alginate concentration

Urease immobilization from green bean seeds was carried out with the entrapment method using alginate as a support material [15]. The alginate concentration variations used were 1, 2, 3, and 4% (w/v) in 0.2 M phosphate buffer, pH 7. The selection of the concentration range was based on the strength and porosity of the alginate beads, which do not inhibit substrate diffusion and the absorbance produced complied with Lambert-Beer's Law. The beads were then measured for activity, and the Na-alginate concentration with the highest activity was used for urease immobilization with variations in bead formation time.

Variation in bead formation time

The immobilization of urease with varying bead formation times was performed following the same procedure as the immobilization with varying Na-alginate concentrations. The distinction lay in the duration of the bead formation process during immersion in a 0.2 M $CaCl_2$ solution. The immersion times investigated were 15, 30, 45, 60, 75, and 90 minutes. The selection of the formation time range was based on the strength and porosity of the alginate beads, which do not inhibit substrate diffusion, and the absorbance produced complied with Lambert-Beer's Law.

Activity assay of free and immobilized urease

The activity test procedure was performed with modifications[15]. The urease activity test used the Nessler method. Urease enzyme activity was measured based on the ammonia formed, which was then reacted with Nessler's reagent to produce a yellow-orange color. The solution absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 500 nm. Urease estimation was performed using an ammonium sulfate standard curve [16]. One unit (U) of activity is defined as the amount of ammonia formed in 1 µmol per minute from the hydrolysis of urea by the urease enzyme in the sample.

Characterization of free and immobilized urease enzymes

Characterization included the maximum reaction rate (Vmax) and Michaelis-Menten constant (K_M), the effect of storage temperature, the effect of storage time on the activity of free and immobilized urease, the reusability of urease beads, and analysis using SEM-EDX

Effect of substrate concentration on urease activity

The determination of urease activity at varying substrate concentrations was in the same way as the activity assay, but conducted at concentration variations of 500, 750, 1.000, 1.250, and 1.500 ppm, followed by incubation for 15 minutes and determination of enzyme activity. The obtained data were then used to determine the maximum reaction rate (Vmax) and the Michaelis-Menten constant (K_M).

Effect of storage temperature

The test for the effect of urease storage temperature was performed by calculating the activity of free and immobilized urease enzymes stored at 25 °C and 35 °C for 180 minutes, from minute 0 (before incubation) to minute 180 with 30-minute intervals. The urease enzyme activity assay was performed in the same way as the urease enzyme activity assay. The relative residual activity was calculated using the following equation (1).

$$\text{Relative residual activity (\%)} = \frac{A_t}{A_0} \text{x } 100\% \quad (1)$$

Where:

- A_0= enzyme activity at time 0
- A_t= enzyme activity at time t

Effect of storage time on the activity of free and immobilized urease

The test for the effect of urease enzyme storage time was conducted by storing free and immobilized urease enzymes for 10 days at 4 °C until a 50% decrease in urease activity was obtained. Immobilized urease beads were stored in a wet state with the addition of a small amount of distilled water. The urease enzyme activity assay was performed in the same way as the urease enzyme activity assay.

Effect of repeated use of immobilized urease enzyme

The repeated use of immobilized urease enzyme was performed by repeatedly using the immobilized urease beads until a decrease in urease activity was observed. The reaction was carried out at optimum conditions and stopped by filtering the beads using filter paper, then the beads were reused for urea hydrolysis. The urease enzyme activity assay was performed in the same way as the urease enzyme activity assay. The repeated use of immobilized urease was further analyzed using SEM-EDX.

3. RESULTS AND DISCUSSION

Determination of the Standard Curve of Ammonium Sulfate

Based on the standard curve, the regression equation obtained was y = 0.0437x – 0.0234 with an R^2 value of 0.9996. The regression equation obtained was then used to determine the urease enzyme activity value.

Extraction of Urease from Green Bean Seeds

The extraction method was used to isolate urease from green bean seeds in this study. The purpose of extraction is to extract enzymes from plant tissue cells [17]. Urease enzyme extraction from green bean seeds was carried out by soaking them in distilled water. During the soaking process, imbibition occurs, where water was absorbed by the dry beans. Water absorption caused the beans to expand and enlarge [18]. The soaked green bean seeds were then ground using a mortar and pestle that had been stored in a freezer. The grinding process was carried out to break down the tissue and cell walls so that the cell contents could be extracted [19]. During the grinding process, the temperature had to be kept cool so that the enzymes were not denatured, by storing the mortar and pestle in the freezer before use and working in an air-conditioned room. The ground green beans were then suspended in phosphate buffer and stirred in an ice bath using a magnetic stirrer. The filtrate was separated using a muslin cloth. The filtrate obtained was centrifuged. The centrifugation resulted in a precipitate and supernatant. The obtained yellowish-white supernatant was used as a crude extract and was subsequently tested for activity.

The urease activity test was conducted using urea as the substrate and ammonia as the reaction product. The ammonia produced was reacted with Nessler's reagent, resulting in a yellow-orange color. The absorbance of the solution was measured. This absorbance was then plotted on the standard ammonium sulfate curve to determine the activity of the crude urease extract from green bean seeds. Based on the results, the activity value was 0.537 U/mL.

Optimization of Urease Immobilization with Alginate

Optimization of the process of producing immobilized urease from green bean seeds using alginate is necessary to obtain strong beads (not easily broken) that still allow substrate and product diffusion to occur properly, enabling high urease activity.

Enzyme immobilization with alginate begins by dissolving Na-alginate powder into a pH 7 phosphate buffer. The solution is then added to the enzyme and

dripped into a $CaCl_2$ solution using a syringe to form Ca-alginate beads.

One unique property of alginate is its ability to form a gel in the presence of divalent cations such as Ca^{2+} and Ba^{2+}. This property, known as gelation, results from ionotropic cross-linking of alginate chains [20]. The Na^+ ions in Na-alginate will be replaced by Ca^{2+} ions tn the $CaCl_2$ solutions which will cross-link with the carboxyl groups (COO^-) of guluronic acid from different chains, producing a three-dimensional lattice that is insoluble in water and thermally irreversible. This structure is often called the "egg box." [21]. The formation of Ca-alginate can be seen in **Figure 1**.

Factors affecting bead formation include Na-alginate concentration and contact time with $CaCl_2$. This study optimized the variations of Na-alginate concentration and the soaking time of beads in 0.2 M $CaCl_2$ solutions so that the enzyme immobilization process runs efficiently.

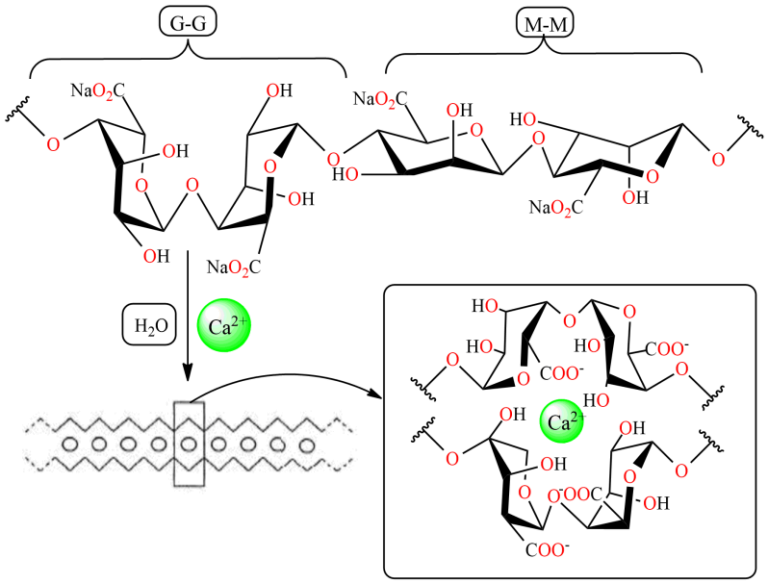

Figure 1. Reaction of Na-alginate with Ca^{2+} [22]

Variation of Na-alginate Concentration

The graph of the relationship between Na-alginate concentration and immobilized urease activity can be seen in **Figure 2**. The optimal alginate concentration must be determined so that the product and substrate can easily pass into the immobilized urease beads without enzyme leakage from the gel beads. Leakage can be prevented by increasing the alginate concentration. Too high an alginate concentration will cause difficulties in combining the enzyme with the substrate, while too low a Na-alginate concentration will cause enzyme leakage [23]. As shown in **Figure 2**, when the concentration of the Na-alginate solution is less than 2%, the pores of the resulting beads are large, making it easier for trapped enzymes to escape from the matrix, resulting in low activity. At a Na-alginate concentration of 2%, the pores of the beads formed are in an ideal condition, facilitating the diffusion of the substrate toward the enzyme and increasing the amount of product produced. If the concentration of the Na-alginate solution is above 2%, the pores of the formed beads will be smaller, because the cross-links formed also increase, thereby inhibiting the diffusion of the substrate and making it difficult for the product to form, resulting in decreased activity [15]. An illustration of the process can be seen in **Figure 3**. Endo (1→4) β-D-glucanase produced by *Bacillus licheniformis* KIBGE-IB2 was immobilized using calcium alginate beads with the entrapment technique. The result showed that optimum enzyme immobilization was achieved at a 2% Na-alginate concentration [24].

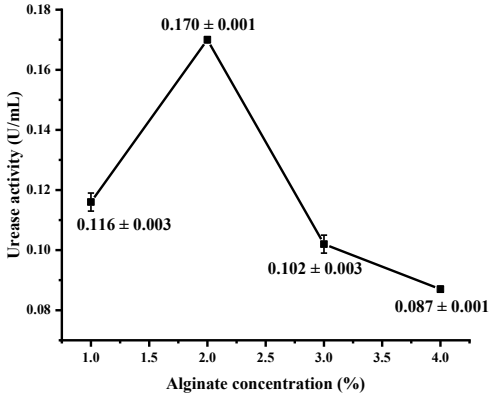

Figure 2. Effect of Na-alginate concentration on the activity of immobilized urease from green bean seeds

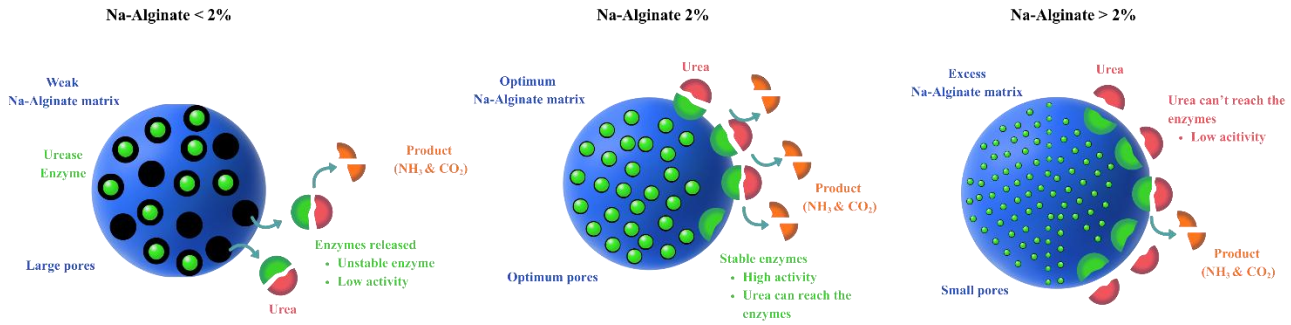

Figure 3. Illustration of the effect of Na-alginate concentration on immobilized enzyme activity

Variation of Beads Formation Time

The relationship between bead formation time and immobilized urease activity can be seen in **Figure 4**. Based on **Figure 4**, it shows that urease activity increases as the soaking time with 0.2 M $CaCl_2$ increases from 15 to 60 minutes because the formed beads become firmer. A short soaking time causes the beads to be thin and not firm enough, resulting in a low activity value. Bead formation reached the optimum value at 60 minutes. Similar results were found for the optimum solution, after which the immobilized enzyme activity decreased. The decrease in enzyme activity after the optimum soaking time is caused by the beads forming pores that are too dense, which blocks the enzyme-substrate interaction and causes enzyme inactivation [23].

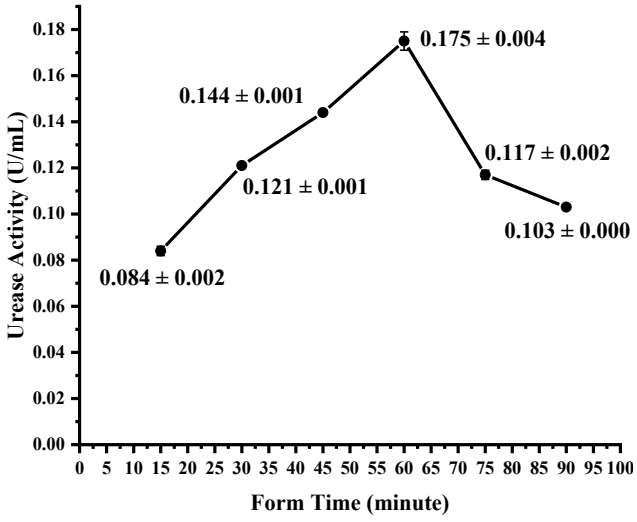

Figure 4. Effect of bead formation time on the activity of immobilized urease from green bean seeds

Characterization of Free and Immobilized Urease Effect of Substrate Concentration

The relationship between substrate concentration and urease enzyme activity can be seen in **Figure 5**. Based on **Figure 5**, it shows that as the substrate concentration increases, the urease activity also increases. At a substrate concentration of 500 – 750 ppm, urease activity increases along with the increase in substrate concentration; this is because more substrates are binding to the active site of the enzyme. Free and immobilized urease reach their optimum at a concentration of 1,000 ppm with activity values of 0.538 ± 0.004 U/mL and 0.168 ± 0.002 U/mL, respectively.

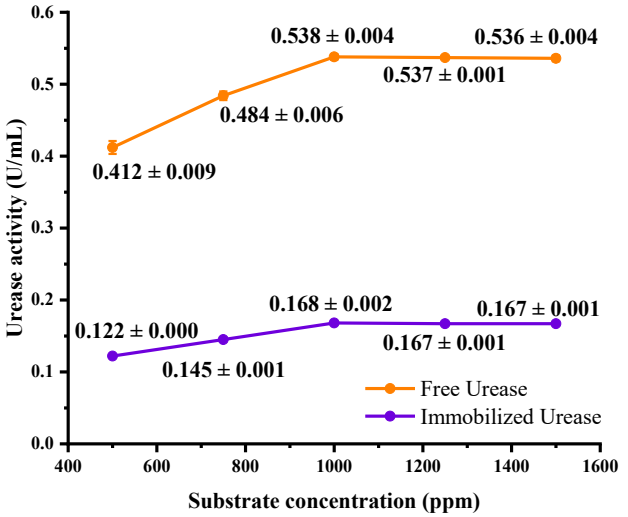

Figure 1. Effect of substrate concentration on the activity of free and immobilized urease

The activity of immobilized enzymes is lower than that of free enzymes because immobilized enzymes are trapped in a matrix or solid support, which can inhibit the movement of urease to interact with the urea substrate [25]. At a substrate concentration of 1.250 – 1.500 ppm, urease activity was stable; this is because under that condition, the enzyme had reached a saturated state with its substrate [26]. The data obtained from the effect of substrate concentration variations are then used to determine Vmax and K_M. The curve of the relationship between 1/V and 1/[S] for the free and immobilized crude urease enzyme extract can be seen in **Figure 6**.

Based on **Figure 6**, linear equations were obtained for free urease, which is y = 459.79x + 1.4826, and for immobilized urease, which is y = 1820.7x + 4.4923; from these equations, Vmax and K_M values can be determined. The Vmax values obtained for free urease were 0.674 M/min and for

immobilized urease were 0.223 M/min, while the K_M values obtained for free urease were 0.005169 and for immobilized urease were 0.006755 M. The results show a decrease in Vmax and an increase in K_M for the immobilized enzyme. The increase in K_M value in immobilized urease indicates that the enzyme's affinity for the urea substrate has decreased. This phenomenon is not only caused by changes in the enzyme structure, but also by the diffusion inhibition of the urea substrate in the alginate matrix. In the trapping method using Na-alginate, the enzyme is trapped in the gel network formed through cross-linking between alginate and calcium ions from $CaCl_2$. The gel structure forms pores that serve as diffusion pathways for the urea substrate and reaction products (ammonia). The urea substrate must diffuse from the solution into the matrix before reaching the enzyme's active site. This process causes mass transfer limitation. As a result, the K_M value obtained increases in immobilized enzymes [27]. This mechanism is illustrated in **Figure 7**.

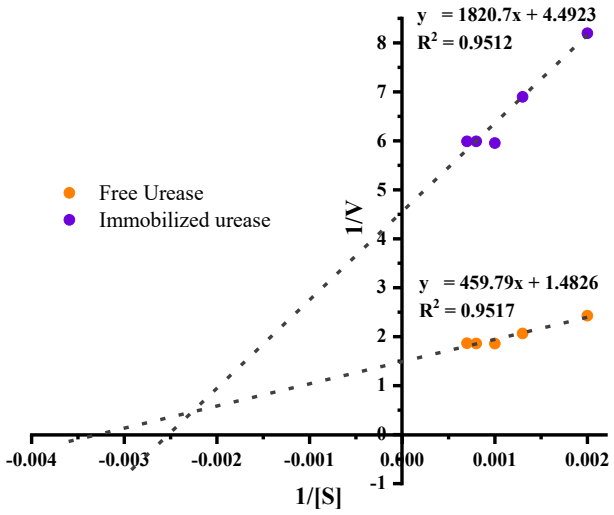

Figure 2. Curve of the relationship between 1/V and 1/[S].

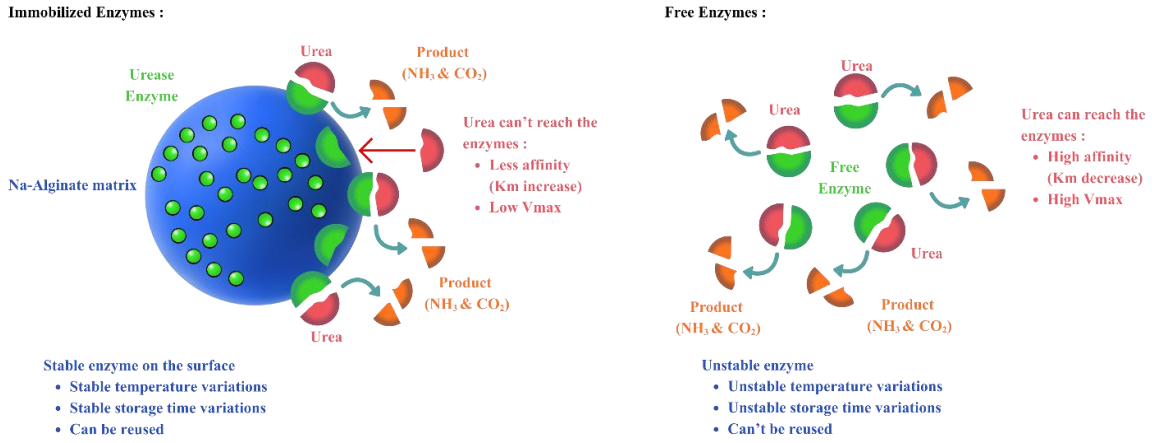

Figure 7. Illustration of the effect of Na-alginate mobilization on enzyme kinetics.

The microenvironment within the alginate matrix also plays a role in influencing enzyme activity. The alginate matrix can create conditions that differ from the surrounding solution, such as changes in the electrostatic interaction between the enzyme and the carboxylate groups in alginate. These conditions can affect the stability of the enzyme's three-dimensional structure and the orientation of the enzyme within the matrix, thereby altering the interaction between the enzyme and the substrate. Several studies have reported that the alginate matrix can form a microenvironment that is separate from the solution phase, thereby affecting the reactivity and diffusion of molecules around immobilized enzymes [28].

Effect of Storage Temperature

Previous research showed that the optimum temperature of the urease enzyme from green bean seeds is 35 °C [8]. The temperature variation used in determining the effect of storage temperature was 25 °C and 35 °C. The relationship between storage time and urease activity can be seen in **Figure 8**.

Based on **Figure 8**, free and immobilized urease enzymes maintained their activity until 180 minutes of storage time. At temperatures of 25 °C and 35 °C, the values for free urease are 0.210 ± 0.008 U/mL (39.58%) and 0.230 ± 0.001 U/mL (42.82%), while for immobilized urease, they are 0.066 ± 0.000 U/mL (48.62%) and 0.088 ± 0.001 U/mL (52.26%). The stability of free and immobilized urease increased from 25 °C to 35 °C; this is because 35 °C is the optimum temperature for the urease enzyme. Immobilized urease could maintain half of its activity up to 180 minutes and is more stable than free urease, even though its activity is lower. This is because the immobilized enzyme is more protected from external influences, such as temperature, which can cause enzyme denaturation [29].

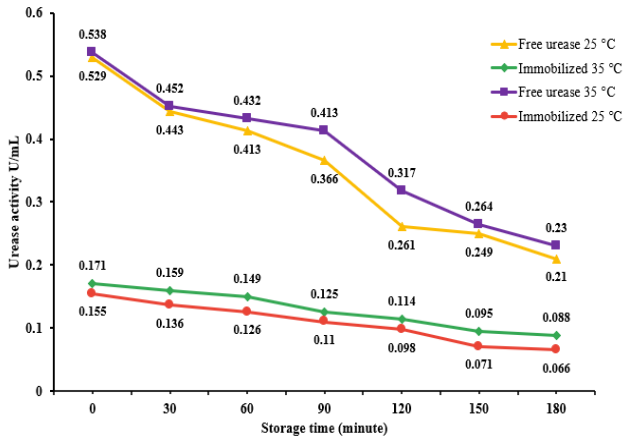

Figure 8. Effect of storage temperature on free and immobilized urease enzymes at 25 °C and 35 °C.

Effect of Storage Time

Free and immobilized urease enzymes were stored at 4 °C for 10 days to evaluate the effect of storage time on urease activity. The relationship between storage time and urease activity can be seen in **Figure 9**.

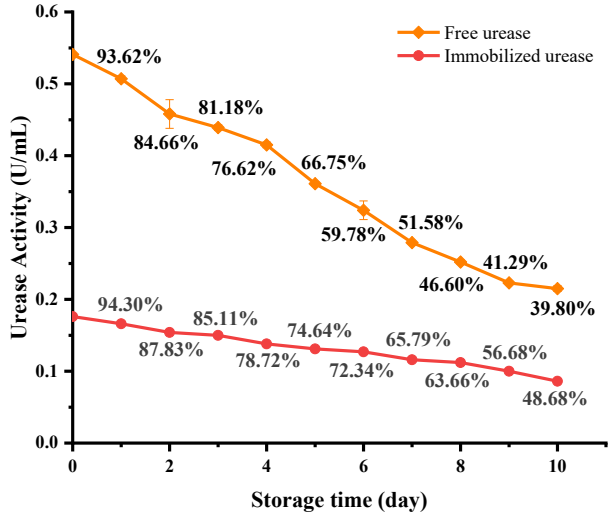

Figure 9. Effect of storage time on the activity of free and immobilized urease enzymes.

Based on **Figure 9**, it was found that immobilized urease is more stable than free urease. Immobilized urease has an initial activity of 0.176 ± 0.000 U/mL (100%) and can keep its activity until day 9 at 0.100 ± 0.003 U/mL (56.68%). On the other hand, free urease has an initial activity of 0.541 ± 0.002 U/mL (100%) and a remaining activity of 0.223 ± 0.002 U/mL (41.29%). Free urease lost all its activity by day 4, while immobilized urease could keep it until day 7 [30].

Based on the data, the longer the storage time, the lower the enzyme activity becomes. Free urease showed changes in color and odor from day 8 to day 10, while the immobilized enzyme only showed odor change on day 10. These results indicate that urease immobilized using an alginate matrix has better storage stability than free urease.

The increased stability in immobilized urease is related to the role of the alginate matrix in limiting the mobility of enzyme molecules. In free enzymes, enzyme molecules are in a more flexible state, making them more susceptible to structural changes due to environmental effects such as temperature and pH. In enzymes immobilized using a Ca-alginate matrix, the enzymes are trapped in a three-dimensional network, thereby limiting their conformational mobility. This restriction of mobility can reduce the possibility of unfolding in the protein structure, so that the denaturation process occurs more slowly than in free enzymes[31].

In addition, the Ca-alginate gel structure also functions as a microenvironment that protects enzymes from external conditions. The alginate polymer network forms a porous matrix that can stabilize the tertiary structure of enzymes through non-covalent interactions such as electrostatic interactions and hydrogen bonds. This microenvironment helps maintain the active conformation of enzymes so that the active site structure remains stable longer during storage [27].

Reusability of Immobilized Urease

The reusability profile of immobilized urease is shown in **Figure 10**. Based on these data, urease beads could be reused up to five times with a residual activity of 56.23%. Endo (1→4) β-D-glucanase produced by *Bacillus licheniformis* KIBGE-IB2, immobilized using calcium alginate beads via the entrapment technique, showed reusability for up to five cycles[24]. Lipase immobilized using Fe-alginate through the entrapment method retained 50% of its activity after four reuse cycles [27].

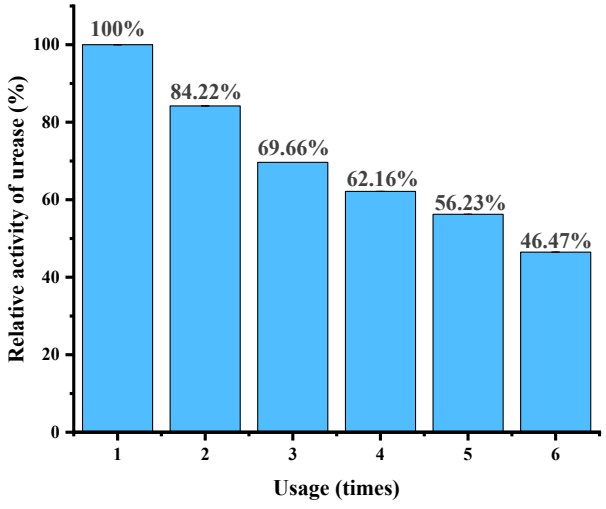

Figure 10. Effect of reusability on immobilized urease.

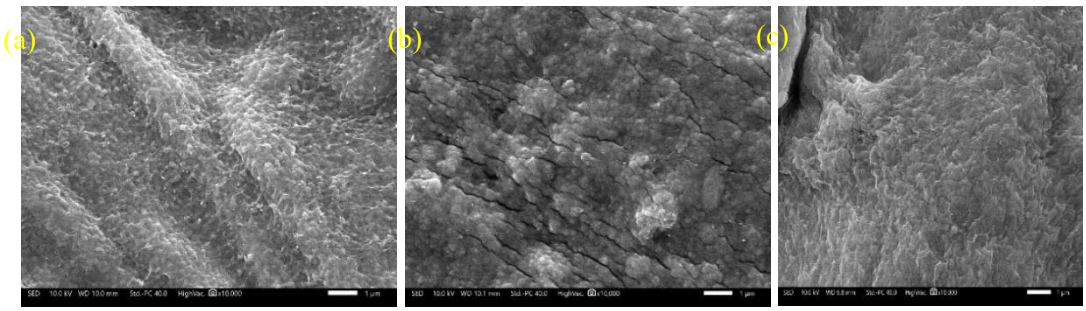

Figure 11. SEM analysis of immobilized urease beads from green bean seeds at 10.000× magnification [(a) alginate, (b) alginate-enzyme, (c) alginate-enzyme after five uses].

Table 1. Elemental composition of immobilized beads in EDX analysis. EDX analysis was performed using the standardless Zaf quantification method.

Element	(%)Na-alginate	(%) Alginate-enzyme	(%) Alginate-enzyme (5x use)
C	31.75	36.25	19.69
Ca	10.10	10.43	10.89

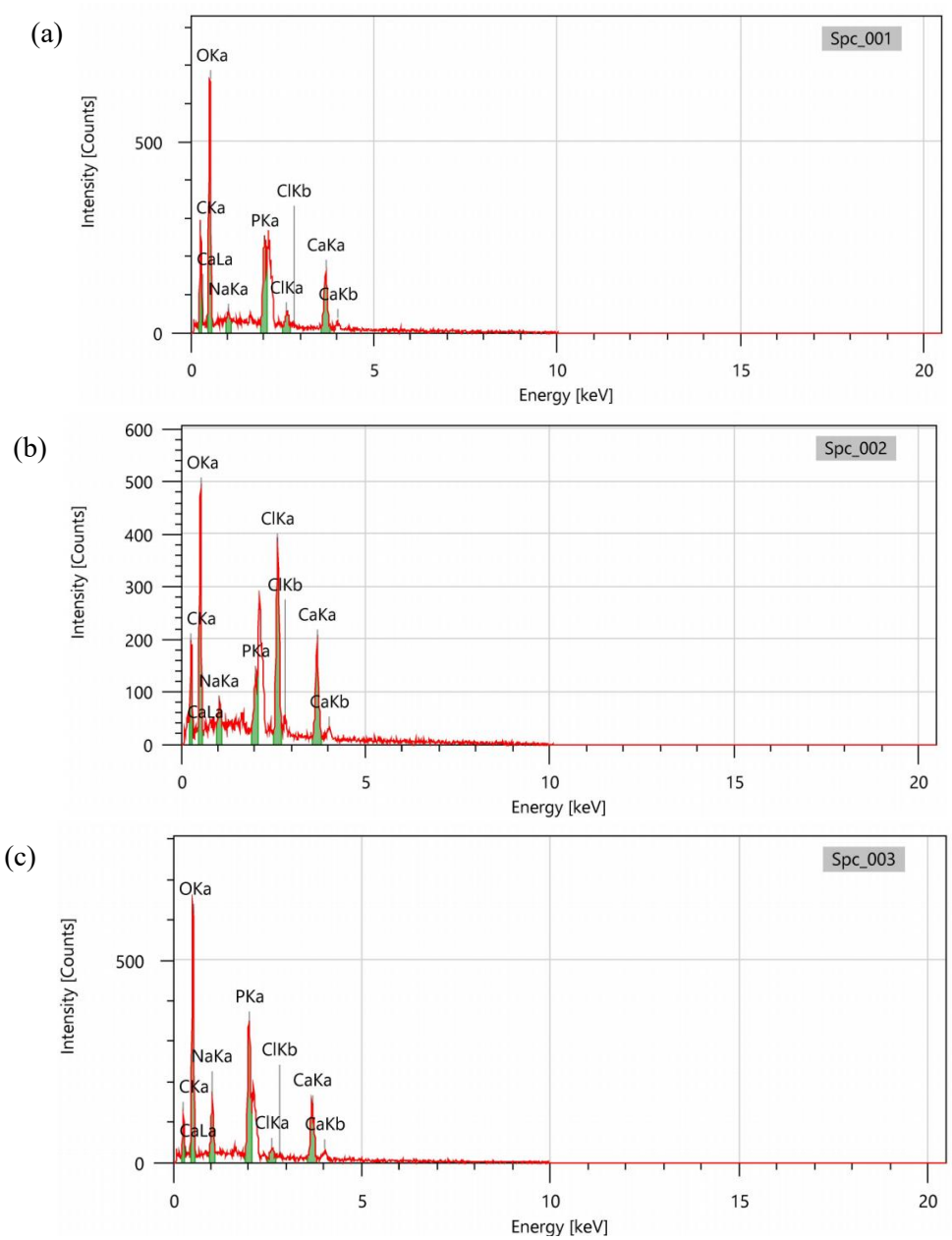

Figure 12. EDX analysis of immobilized urease beads from green bean seeds [(a) alginate, (b) alginate-enzyme, (c) alginate-enzyme after five uses].

The findings in **Figure 10** are further supported by the SEM-EDX analysis results presented in **Figures 11, 12,** and **Table 1**. The results of morphological and elemental composition analyses using SEM–EDX showed evidence of a structure that supports changes in activity and kinetic parameters in enzymes immobilized using Ca-alginate. The porous and pointed surface of the enzyme-free beads indicates that the Ca-alginate network has a structure that allows the diffusion of the urea substrate towards the enzyme trapped in the matrix. SEM-EDX analysis showed a smoother morphology and an increase in carbon content after the addition of the enzyme to the alginate matrix. This indicates the successful trapping of urease in the Ca-alginate matrix. This condition is also related to the observed changes in kinetic parameters, namely an increase in the K_M value and a decrease in Vmax, which indicate the presence of urea substrate diffusion inhibition and limited substrate access to the active site of the enzyme within the alginate matrix [31,27].

After several cycles of use, SEM images show deformation of the alginate matrix structure, characterized by a change in pore shape to a flatter, sheet-like appearance. Based on EDX analysis, this change was accompanied by a decrease in carbon content, indicating enzyme leaching from the alginate matrix. Some of the enzymes lost from the matrix directly caused a reduction in the number of active sites available for catalysis, so that during repeated use, enzyme activity decreases. In addition, changes in the alginate matrix structure could also affect the diffusion pathways of substrates and products, which ultimately increased mass transfer resistance in the immobilized enzyme system [32].

4. CONCLUSIONS

Immobilization of urease from green bean seeds using a calcium-alginate matrix successfully produced a more stable biocatalytic system. Kinetic analysis showed a lower Vmax and a slightly higher K_M for immobilized urease compared to free urease, indicating diffusion limitations within the alginate matrix. Although activity was reduced, immobilization significantly increased enzyme stability under variations in incubation temperature and storage time. SEM-EDX analysis confirmed successful enzyme entrapment, evidenced by a smoother bead surface and increased carbon content. After repeated use, structural deformation of the beads and a decrease in carbon content indicated enzyme dissolution, which correlated with a gradual decrease in activity, although 56.23% of activity remained after five cycles. These results indicate that alginate immobilization enhances enzyme stability and reusability. However, further research is needed to improve matrix stability, reduce enzyme dissolution, and evaluate performance in large-scale or continuous systems.

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