MOLEKUL
eISSN: 2503-0310

Articles
https://doi.org/10.20884/1.jm.2024.19.1.7335

Immobilization of Urease from *Psophocarpus tetragonolobus* L. DC. using Natrium Alginate Supporting Matrix

Zusfahair*[1], Dian Riana Ningsih[1], Puji Lestari[1], Bilalodin[2], Eva Aryanti[1], Niken Istikhari Muslihah[1]

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

*Corresponding author email: zusfahair@gmail.com

Received December 21, 2022; **Accepted** July 22, 2023; **Available online** March 20, 2024

ABSTRACT. Urease is an enzyme that has the role to hydrolyzes urea into ammonia and carbon dioxide. Immobilization is one of the most efficient strategies to improve its activity recovery and properties of urease. This research started with the germination of winged beans for 8 days. The winged bean was extracted by grinding using a mortar and pestle and then added with phosphate buffer at pH 7. The solution was homogenized using a stirrer and then centrifuged in cold conditions so that an extract of urease was obtained. Urease extracts were immobilized using a chitosan-supporting matrix. Optimization of the immobilization process of urease extract includes the concentration of chitosan and sodium tripolyphosphate (TPP) and contact time. The obtained was free and immobilized urease activities then tested using the Nessler method and measured using a UV-Vis spectrophotometer with a wavelength of 500 nm. The obtained data were then statistically tested using ANOVA. Urease-chitosan beads were further tested in repeated use and analyzed with SEM-EDX (Scanning Electron Microscopy-Energy Dispersive X-ray). The results showed that the optimum conditions for making urease-chitosan beads were a concentration of 4% (w/v), 2.5% (w/v) TPP, and 60 minutes of contact time, resulting in an activity value of 15.076 U/mL, which can be used 5 times with 46% activity from the initial activity. The EDX analysis results after the addition of the enzyme showed atom composition changes leading to increasing carbon and nitrogen contents. The existence of phosphor showed that TPP was a chitosan cross-link compound.

Keywords: Chitosan, immobilization, TPP, urease, winged bean

INTRODUCTION

Urease is an enzyme which has the role of a urea hydrolytic catalyst and then becomes ammonia and carbon dioxide. Urea and its hydrolytic product (ammonia) has an important role in both analytical and clinical chemistry, such as urine and blood analyses, kidney failure, and artificial kidney control, as well as in water waste management in food and drug analyses (Lahiri, 2015).

Urease is widely found in various bacteria, fungi, and plants. The most commercially available urease is from jack bean (*Canavalia ensiformis*), which is the plant with the best characteristics (Saem et al., 2015). Other researchers isolated urease from broad beans (*Vicia faba* L.) (Bedan, 2020), pea seeds (*Pisum sativum)* (Iyer et al., 2018), jackfruit (Chouhan et al., 2018), and asparagus beans (*Vigna unguiculata subsp sesquipedalis* L.) (Zusfahair et al., 2018). In this research, urease was isolated from winged beans. Winged beans contain protein in flowers at 2.8-5.6; leaves at 5-7,6; young pods at 1.9-4.3; fresh seeds at 4.6-10.7; dry seeds at 29.8-39 and tubers at 3-15, each calculated per 100 grams of fresh weight (Saptadi et al., 2016). High protein content found in the dry seed of winged beans can be used as urease source.

In its application, urease is used in its immobilized form. Enzyme immobilization can be made using various methods. Some effective methods for enzyme immobilization include surface absorption, encapsulation, or trapping. However, most immobilization processes result in the decreasing enzyme activity or changing enzyme structure. The main benefit of immobilization method is enzyme activity stability after immobilization and reversibility processes, which enable the supporting enzymes and matrixes reused. Enzyme immobilization is also essential for more efficient utilization. Immobilized enzymes are stronger and more resistant to environmental changes than free enzyme (Al-Garawi et al., 2022).

Urease immobilization has been made in various matrixes for analytical and clinical applications. The matrixes used for urease immobilization include ZnO

(Eghbali et al., 2015); sodium alginate (Danial et al., 2015), (Tetiker & Ertan, 2017), (Saxena et al., 2017); cellulose (Lv et al., 2018); Polyethersulfone (Zhang et al., 2019); and chitosan (Krishna et al., 2011), (Lahiri, 2015), (Al-Garawi et al., 2022).

Chitosan is known as a supporting material ideal for enzyme immobilization due to its characteristics, such as increasing mechanical strength, avoiding enzymes from the metal-ion disturbance, being resistant to chemical degradation, and having antibacterial characteristics. Chitosan, the poly-N-acetyl glucosamine and also the derivative of N-deacetylase from chitin, is the second most common biopolymer after cellulose. Chitosan has a primary amine in the position of C-2 from glucosamine residue. The existence of an amine group is a unique feature that causes chitosan to have important functional characteristics (Al-Garawi et al., 2022).

This research used the immobilization process with a trapping method. In trapping method, enzymes were immobilized by trapping gel, fiber, or microencapsulation. The enzyme was maintained in tissues while substrate and product were freed to pass. Those reduced enzyme washes, increased stability, and enabled enzymatic reaction to happen. There was no covalent bond between enzymes and the supporting matrixes, so that enzyme conformation was maintained and ensured high catalytic activity (Liu et al., 2018).

Exploration of urease-producing plants needs to be carried out considering that urease is still imported. Until now, there has been no research on urease extraction from winged beans. In this research, urease was isolated from winged beans. The urease extract obtained was immobilized with chitosan using a trapping method. Some factors influencing the formation of urease-chitosan beads were optimized, such as chitosan concentration, TPP concentration, and TPP contact time with urease-chitosan beads. Urease-chitosan beads were further tested in repeated use and analyzed with SEM-EDX.

EXPERIMENTAL SECTION

Equipment and Materials

The equipment used in this research included glasses commonly used in biochemical laboratory, centrifuge (Quantum), spectrophotometer UV-Vis (Shimadzu UV-1800), incubator (Memmert), analytical balance (O'Haus), pH meter (Hanna Instrument), and Scanning Electron Microscopy (JSM-6510LA). The materials used in this research included winged bean (from Kembangan, Purbalingga), acetate acid, urea, chitosan, Nessler reagent, ammonium sulfate, $Na_2HPO_4.2H_2O$, Na-TPP, $NaH_2PO_4.2H_2O$, H_2SO_4, Natrium wolframite, and distilled water. All chemical materials were obtained from Merck Chemical Company (Merck, Jerman), except (technical) chitosan.

Research Procedures

Urease isolation from winged bean

Urease isolation was made using the same method previously used by Zusfahair et al. (2022). The obtained urease extract was immobilized using chitosan.

Optimization of immobilized urease making with chitosan

The immobilized urease beads made with chitosan matrixes were created using three optimization parameters: chitosan concentration, TPP concentration, and contact time variations.

Urease immobilization with chitosan concentration variations

Chitosan solution with concentration variations of 3, 4, 5, and 6% (b/v) was made by dissolving chitosan powder into acetate acid of 2% (v/v). 4 mL of chitosan solution with each variation was mixed into 1 mL of urease from winged beans and then stirred with a magnetic stirrer at a temperature of 4 °C. The homogenous solution was then dropped with 30 mL of TPP 2% (b/v) solution. The formed urease-chitosan beads were left for 60 minutes in the TPP solution. The activity of urease-chitosan beads was measured using the Nessler reagent. The urease-chitosan beads with chitosan concentration variations having the optimum activity were used for immobilization with TPP concentration variations.

Urease immobilization with TPP concentration variations

Urease immobilization using TPP concentration variations was similarly made using chitosan concentration variations. The TPP concentration variations included 1.5, 2, 2.5, and 3% (b/v). The urease-chitosan beads with optimum activity were used for immobilization using time contact variations.

Urease immobilization with contact time variations

The urease immobilization using time contact variations was similarly made with that using TPP concentration variations. Urease-chitosan beads were left for 20, 40, 60, 80, and 100 minutes. Urease-chitosan beads with optimum activity were then used for further analysis.

Free and immobilized urease activity testing

1 mL of urea solution at 0.2 M was put into a sample test tube and then added with 1.9 mL of buffer phosphate at pH 7. Around 0.1 mL of urease extract from winged bean seeds was added to the sample test tube. The control test tube was contained with 2 mL of buffer phosphate and 1 mL of urea solution 0.2 M. The solution was then incubated for 15 minutes at a temperature of 35 °C. A solution in the sample and control test tubes that have been cold was added with 1 mL of H_2SO_4 2/3 N and 1 mL of Na-wolframite and further centrifuged for 15 minutes. The formed supernatant was taken as much as 1.5 mL added with 0.25 mL of Nessler reagent.

The immobilized enzyme activity test was made using the most optimum urease-chitosan beads. A

total of 5 mL of 0.2 M urea solution in phosphate buffer pH 7 was added with chitosan urease beads, as much as the number of beads formed in one making time. The solution was then incubated for 15 minutes at a temperature of 35 °C. Urease-chitosan beads were then separated from the solution and measured using a Nessler reagent. The control solution was similarly made without using urease-chitosan beads. The solution was then measured using spectrophotometer UV-Vis at the wavelength of 500 nm. The determination of urease activity from winged beans was conducted using the ammonium sulfate standard curve (Zusfahair et al., 2018). One activity unit is stated by the number of ammonium ions formed in µmol per mL per minute from urea hydrolysis by urease in the sample.

Repeated use of immobilized urease

Urease-chitosan beads were repeatedly used until the urease activity decreased. Besides, Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) were used to analyze the immobilized urease. The analysis was conducted at the integrated research and testing laboratory of Gadjah Mada University.

RESULTS AND DISCUSSION

Urease Activity Test

Urease is a metalloenzyme containing nickel on its active side. Urease is included in hydrolase. Urease hydrolyzes urea, resulting in ammonia and carbon dioxide (El-hefnawy et al., 2014). The reaction is presented as follows:

Urease activity is measured by the amount of the formed ammonia. There are two common methods to detect the formed ammonia: Berthelot and Nessler. The Berthelot method is based on two-step reaction of ammonia with two reagents: hypochlorite and a phenolic compound (non-toxic salicylate):

$NH_3 + ClO^- \rightarrow NH_2Cl + OH^-$ (1)

$NH_2Cl + C_7H_5O_3^- \rightarrow C_7H_7O_3N + Cl^-$ (2)

$C_7H_7O_3N$ (5-amminosalicylate) is a reaction product with a blue-green color that absorbs light at approximately 620 nm. In this method, the reaction runs in two stages, and color development is not a quick process, so the sensitivity of this method is greatly dependent on time (Bzura & Koncki, 2019).

The second method frequently implemented in analytical practices is ammonia reaction with the Nessler reagent (tetraiodomercurate in kalium hydroxide alkali solution):

$NH_3 + 2HgI_4^{2-} + 3OH^- \rightarrow OHg_2NH_2I + 7I^- + 2H_2O$ (3)

The principle of Nessler method is the reaction between NH_3 and the Nessler reagent forming complex compounds of yellow color following Beer-Lambert's Law. The apparent color intensity is directly proportional to the concentration of ammonia contained in the samples. The enzyme activity test as a control is urea solution without enzyme addition so that ammonia is not produced. Yet, when added with the Nessler reagent, the solution has a clear color. The absorbance of the apparent color intensity is measured at the wavelength of 500nm (Zusfahair et al., 2022a). This method has the strengths related to its easy operation and high sensitivity (Wu & Cao, 2013). The Nessler method is significantly faster and only needs one inexpensive and stable reagent. On the other side, the Nessler reagent is more poisonous than the hypochlorite dan phenolic compounds used in the Berthelot method. Considering these pros and cons, it is clear why the Nessler method is frequently implemented in routine and research laboratories (Bzura & Koncki, 2019).

Urease Isolation from Winged Bean

Urease was isolated from winged beans following steps similar to those developed by Zusfahair et al. (2022b). The obtained urease extract was further immobilized using chitosan matrixes.

Optimization of Immobilized Urease Making with Chitosan

Urease immobilization with chitosan concentration variations

Chitosan is a cheap and multi-functional biomaterial (Verma et al., 2020). Chitosan has inert characteristics as well as supporting material that is cheap, hydrophilic, bio-compatible, biodegradable, and non-poisonous, making it an appropriate material for enzyme immobilization (Kaushal et al., 2018). Literature shows that immobilization can be in the form of enzyme trap in chitosan beads or covalent bond with film-transparent chitosan. Enzyme immobilization in chitosan beads is one technique frequently used due to its simplicity and inexpensiveness.

Figure 1. Urea with a urease catalyst can be broken down into ammonia and carbonic acid (Zhang et al., 2019).

The enzyme immobilization process on chitosan beads was started by dissolving chitosan in acetate acid 2% (v/v). The existence of carboxyl group (-COOH) in acetate acid can ease chitosan dissolution since there is hydrogen interaction between the carboxyl group and the amine group ($-NH_2$) from chitosan forming cationic amino (NH_3^+) (Setyaningtyas et al., 2021). Chitosan solution is made with concentration variations of 3%, 4%, 5%, and 6% (b/v). Chitosan solution is added with urease from winged beans and homogenized, as well as dropped into sodium tripolyphosphate (TPP) solution using micropipettes. TPP as a cross-linking agent, possibly resulting in hydroxyl ions (OH^-) and phosphate ions when dissolved in water. Chitosan will form a cross-link with TPP. The formed cross-link is due to the existence of a reaction between chitosan positive charges ($-NH_3^+$) and negative charges in natrium tripolyphosphate ($H_3P_3O_{10}^{2-}$) (Kadri et al., 2018), so that the enzyme will be trapped within the cross-link between chitosan and TPP (Tantowidjojo et al., 2013). The cross-link occurring between chitosan and TPP is presented in **Figure 2.**

The activity of the formed urease-chitosan beads was measured at pH 7 and a temperature of 35°C. The obtained data were in the form of a relationship curve between chitosan concentration (%) and urease activity (U/mL) as presented in **Figure 3**. At constant urease concentration, the immobilized urease activity shows a typical curve with increasing chitosan concentration (**Figure 3**). When chitosan concentration increases up to 4%, immobilized urease activity also increases up to 13.660 U/mL. This result shows that the optimum chitosan concentration is 4% efficient for immobilization processes. The optimum chitosan concentration has a positive effect to strengthening the interaction with the enzyme and stabilizing the enzyme conformation (Cao et al., 2016).

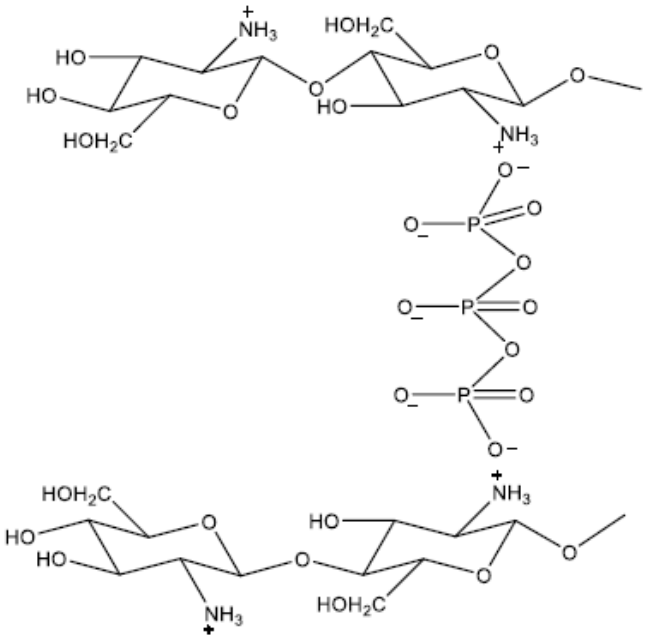


Figure 2. Structure of chitosan-TPP (Kurniasih et al., 2022)

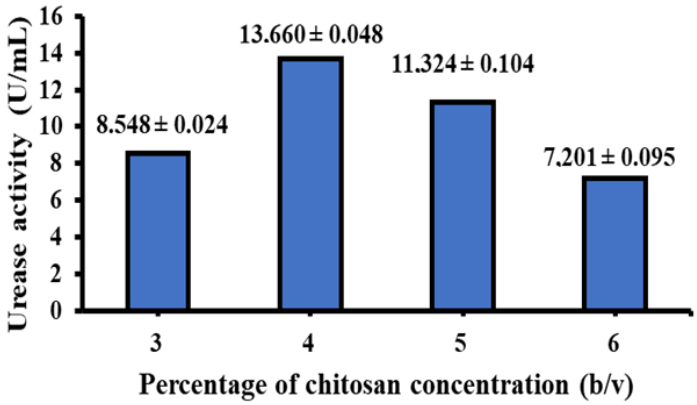


Figure 3. The relationship between chitosan concentration variations and immobilized urease activity. Values obtained are an average of three repeats.

The increasing chitosan concentration of > 4% shows the decreasing urease activity. This is predicted due to the increasing solution viscosity, which can inhibit the movement of enzyme molecules in the surrounding chitosan molecular chains (Gan & Wang, 2007). According to Cao et al. (2016), high supporting matrix concentrations can increase steric inhibition and enzyme diffusion resistance, causing some inhibitions in catalyzing reaction so that enzyme activity decreases. Urease from *Schizzosaccharomyces pombe* has the optimum activity at the chitosan concentration of 2.5%, with activity value of 0.29 Unit (Fatmawati et al., 2013). Different optimum chitosan concentrations are caused by different enzyme sources. Enzymes from different sources will also have different characteristics.

Urease immobilization with TPP concentration variations

The relationship data between TPP concentration variations and immobilized urease activity can be seen from **Figure 4**. Chitosan can be used as the appropriate supporting matrix for enzyme immobilization since it has two active groups: amino ($-NH_2$) and hydroxyl (-OH) (Oliveira et al., 2017). Chitosan, as an enzyme immobilization supporting matrix whose structures can be changed by TPP has the function as a cross-link compound (Bahreini et al., 2014). The benefits of using TPP as a cross-link are due to its stability, simplicity, and security. TPP addition can change chitosan pore size and porosity influencing the number of enzymes restrained in the matrix.

Based on the data presented in **Figure 4,** it showed that the highest urease activity was obtained from the use of TPP concentration 2.5% with the activity value of 14.444 Unit/mL. The most appropriate TPP concentration 2.5% was for cross-linking. TPP can intermolecularly and intramolecularly link with chitosan. Intermolecularly, TPP causes many pores to be created, while intramolecularly, TPP will form particular pores causing urease-chitosan beads become more rigid. Excessive TPP can also cause the enzyme lose its flexibility and activity (Qian et al., 2020). During cross-linking the negatively charged groups on the enzyme surface may be neutralized by the positively charged amine groups of chitosan. In addition, the $-NH_3^+$ group on the enzyme surface may be neutralized by polyanionic TPP molecules (Kadri et al., 2018).

Urease immobilization with contact time variations

The data related to contact time variations with immobilized urease activity can be seen on **Figure 5**. The data presented in **Figure 5.** showed that the immobilized urease activity was initially low. This was because the number of cross-links created was small, so the pores of the beads were big. The big pores of beads caused enzymes to escape from the beads. The immobilized urease activity gradually increased due to the addition of contact time, significantly increased at the contact time of 60 minutes, and significantly decreased after it. The longer the contact time between the immobilized urease beads and TPP solution, the greater cross-link between chitosan and TPP. This made the formed beads densre, resulting in pores that ease the interaction between substrate and an enzyme. If the contact time is too long, the immobilized enzyme configuration can significantly change due to the excessive number of cross-links, causing the denaturation the of protein enzyme (Chen et al., 2013).

The Influence of Repeated Use on Immobilized Urease Activity

Immobilized enzyme can be used repeatedly. Repeated use can be made when beads are optimally made. After the first activity test was completed, the beads were then filtered using filter paper, washed using buffer, and added to new substrate for further activity testing. The curve obtained from the influence of repeated use on immobilized urease activity can be seen in **Figure 6**.

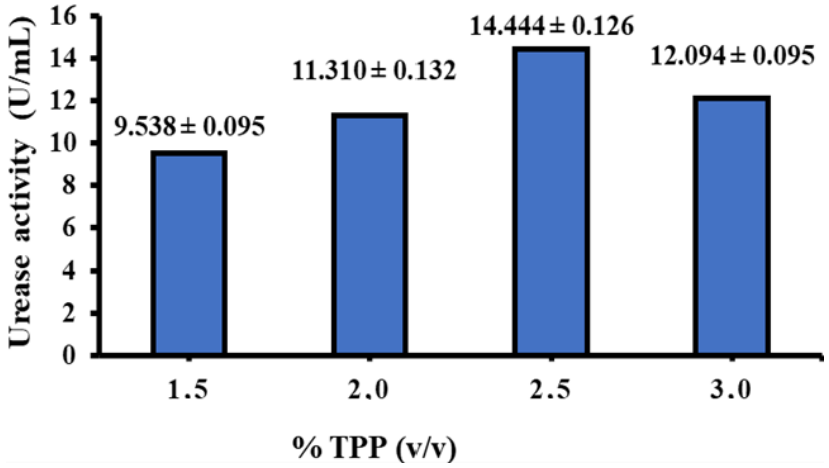


Figure 4. The relationship between TPP concentration variations and immobilized urease activity. Values obtained are an average of three repeats.

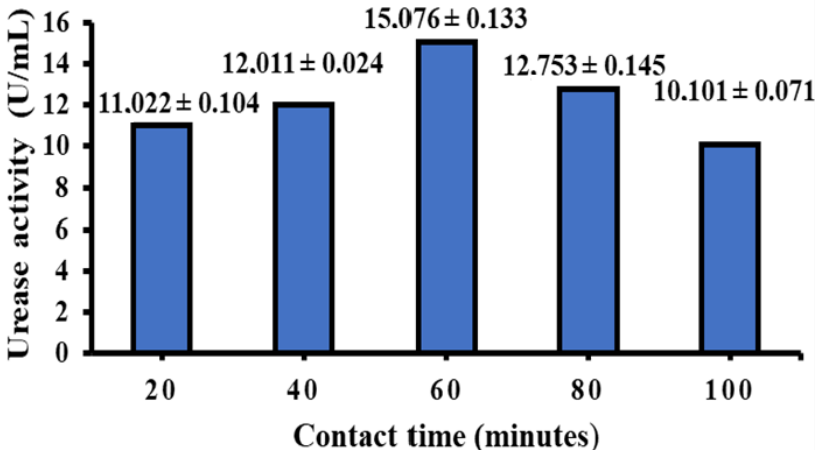


Figure 5. The relationship between contact time variations and immobilized urease activity. Values obtained are an average of three repeats.

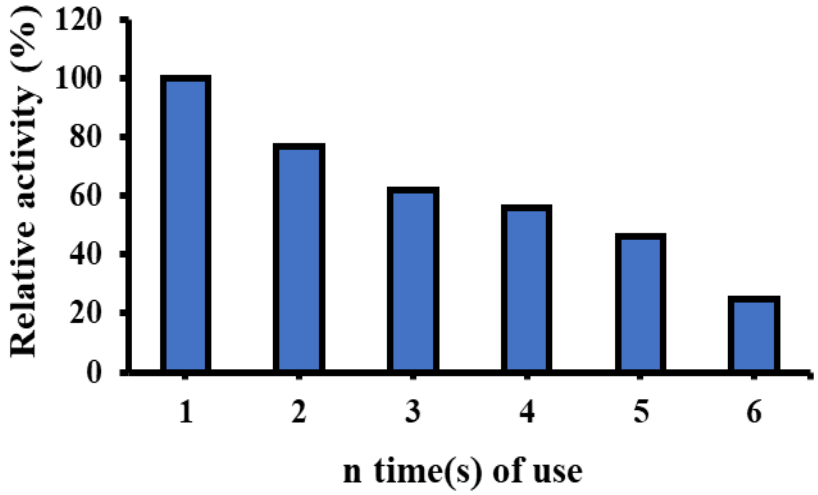
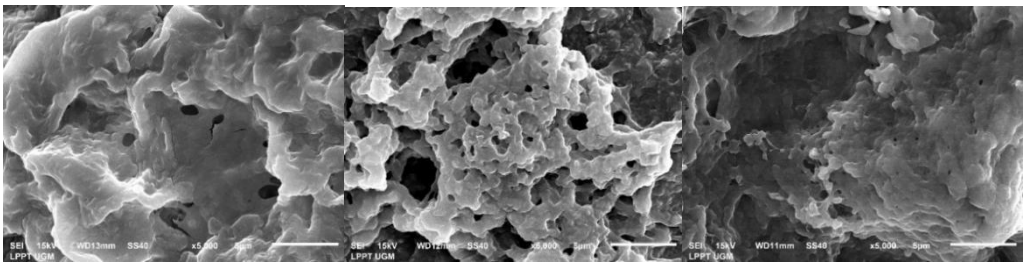


Figure 6. Repeated use of immobilized urease enzyme. Values obtained are an average of three repeats.

Immobilized enzymes are considered good to use when they have an activity value of approximately 50%. Based on the curve presented in **Figure 6**, it showed that the immobilized enzyme was still effective to use up to 5 times, with a remaining activity of 46%. Pithawala et al., (2010) used calcium alginate to trap urease, which had an activity of 60% after the third cycle. Urease from chickpeas immobilized with matrix-supporting alginate can be used up to 5 times and maintain activity at 60% of the initial activity (Tetiker & Ertan, 2017). β-D-Galactosidase immobilized with chitosan could only be used twice with the activity of 33.2% from the initial activity (Wahba, 2017).

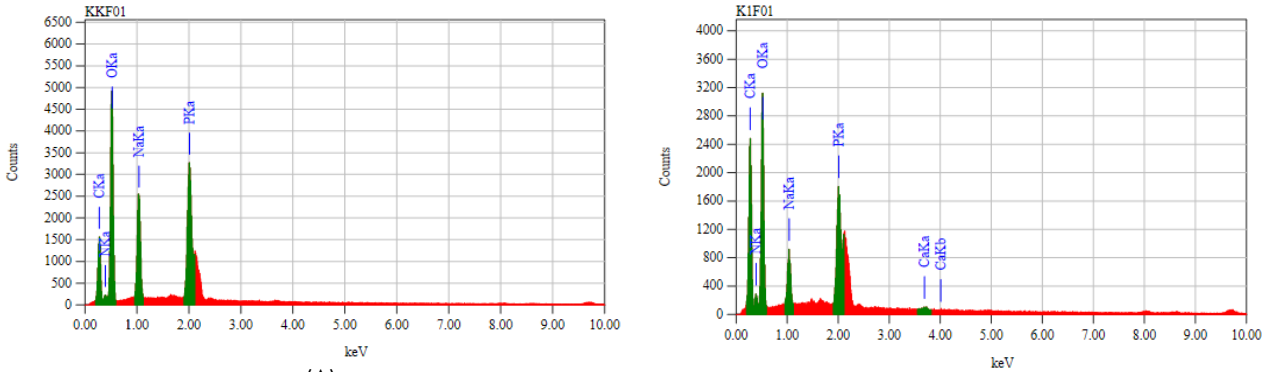
Enzyme activity decreased after repeated use. This was predicted because in the first use, the number of enzymes trapped in chitosan was still many so the activity was still high. In the fifth use, the number of enzymes trapped in chitosan continuously decreased, so that those enzymes were released after the repeated use. This statement was supported by the analysis conducted using SEM. SEM analysis can be seen in **Figure 7**. **Figure** 7A the pores seemed bigger as chitosan tended to agglomerate. **Figure 7B** when enzyme was added, the enzyme entered the pores and made them smaller when compared to those without enzyme. **Figure 7C)** the pores got bigger again when enzymes escaped due to the repeated use.

The urease immobilized with chitosan was also analyzed using the EDX spectrum (**Figure 8**). The EDX technique detected X-rays emitted from the samples during the bombing made by the electron beam to characterize the compositions of the sample elements (Malhotra & Basir, 2020). **Figure 8** showed that after the addition of enzyme, the atom compositions changed in which the content of carbon and nitrogen increased. This change is due to the interaction of the enzyme with chitosan which forms a composite where the enzyme is immobilized with chitosan. These results are in accordance with research conducted by Krishna et al., (2011), there was also an increase in the percentage of nitrogen, this might be due to the presence of enzymes on the surface. The presence of phosphor showed that TPP was the cross-link compound for chitosan.



(A) (B) (C)

Figure 7. Scanning Electron Microscopy (SEM) analysis. (**A**) Control. (**B**) After used 1 time. (**C**) After used 5 times at the magnification of 5000 times. Scale bars = 5μm



(A) (B)

Elemen	Atomic % of elemen	
	Chitosan beads	Chitosan-urease beads
C	30.57	38.35
N	11.36	17.42
O	47.74	40.07
Na	5.74	1.90
P	4.59	2.18
Ca	-	0.09

(C)

Figure 8. (**A**) Chitosan beads; (**B**) Urease-chitosan- beads; (**C**) Composition of elements in beads. EDX analysis using the standardless ZAF quantification method

CONCLUSION

Urease from the winged bean has been successfully immobilized using chitosan matrix. Immobilized urease is made with a concentration of chitosan 4% (b/v), Na-TPP 2.5% (b/v), and 60 minutes of contact time with an activity value of 15.076 Unit/mL, as well as repeatedly used for 5 times with the activity reaching 46% from the initial activity.

ACKNOWLEDGEMENT

The authors would like to thank LPPM Unsoed for funding this research through *'Riset Dasar Unsoed'* scheme of 2022.

REFERENCES

Al-Garawi, Z. S., Taha, A. A., Abd, A. N., & Tahir, N. T. (2022). Immobilization of urease onto nanochitosan enhanced the enzyme efficiency: Biophysical studies and in vitro clinical application on nephropathy diabetic Iraqi patients. *Journal of Nanotechnology, 2022*. https://doi.org/10.1155/2022/8288585

Bahreini, E., Aghaiypour, K., Abbasalipourkabir, R., & Mokarram, A. R. (2014). Preparation and nanoencapsulation of L-asparaginase II in chitosan-tripolyphosphate nanoparticles and in vitro release study. *Nanoscale Research Letters*, *9*(1), 1–13.

Bedan, D. S. (2020). Extraction, precipitation and characterization of urease from *Vicia faba* L. *Al-Mustansiriyah Journal of Science*, *31*(1), 9. https://doi.org/10.23851/mjs.v31i1.555

Bzura, J., & Koncki, R. (2019). A mechanized urease activity assay. *Enzyme and Microbial Technology*, *123*(November 2018), 1–7. https://doi.org/10.1016/j.enzmictec.2019.01.001

Cao, S. L., Huang, Y. M., Li, X. H., Xu, P., Wu, H., Li, N., Lou, W. Y., & Zong, M. H. (2016). Preparation and characterization of immobilized lipase from *Pseudomonas cepacia* onto magnetic cellulose nanocrystals. *Scientific Reports*, *6*, 1–19. https://doi.org/10.1038/srep20420

Chen, H., Zhang, Q., Dang, Y., & Shu, G. (2013). The Effect of glutaraldehyde cross-linking on the enzyme activity of immobilized β-galactosidase on chitosan bead. *Advance Journal of Food Science and Technology*, *5*(7), 932–935. https://doi.org/10.19026/ajfst.5.3185

Chouhan, S., Priya, V. V., & Gayathri, R. (2018). Extraction and partial purification of urease enzyme from jack fruit. *International Journal of Research in Pharmaceutical Sciences*, *9*(2), 321–327.

Danial, E. N., Hamza, A. H., & Mahmoud, R. H. (2015). Characteristics of immobilized urease on grafted alginate bead systems. *Brazilian Archives of Biology and Technology*, *58*(April), 147–153.

Eghbali, M., Farahbakhsh, A., Rohani, A., & Pour, A. N. (2015). Urea biosensor based on immobilization of urease on ZnO nanoparticles. *Oriental Journal of Chemistry*, *31*(2), 1237–1242. https://doi.org/10.13005/ojc/310284

El-hefnawy, M. E., Sakran, M., Ismail, A. I., & Aboelfetoh, E. F. (2014). Extraction, purification, kinetic and thermodynamic properties of ureasefrom germinating *Pisum sativum* L . seeds. *BMC Biochemistry*, *15*(15), 1–8.

Fatmawati, I., Prasetyawan, S., & Roosdiana, A. (2013). Optimasi amobilisasi urease dari *Schizzosaccharomyces pombe* menggunakan matrik kitosan-natrium tripolifosfat (Optimization of urease immobilization from *Schizzosaccharomyces pombe* using chitosan-sodium tripolyphosphate matrix). *Kimia Student Journal*, *2*(1), 407–413.

Gan, Q., & Wang, T. (2007). Chitosan nanoparticle as protein delivery carrier-Systematic examination of fabrication conditions for efficient loading and release. *Colloids and Surfaces B: Biointerfaces*, *59*(1), 24–34. https://doi.org/10.1016/j.colsurfb.2007.04.009

Iyer, P. ., Priya, V. V., & Gayathri, R. (2018). Assessment of urease activity in *Pisum sativum* seeds. *Drug Invention Today*, *10*(9), 1810–1813.

Kadri, T., Cuprys, A., Rouissi, T., Brar, S. K., Daghrir, R., & Lauzon, J. (2018). Nanoencapsulation and release study of enzymes from alkanivorax borkumensis in chitosan-tripolyphosphate formulation. *Biochemical Engineering Journal*, *137*, 1–10. https://doi.org/10.1016/j.bej.2018.05.013

Kaushal, J., Singh, G., & Arya, S. K. (2018). Immobilization of catalase onto chitosan and chitosan – bentonite complex : A comparative study. *Biotechnology Reports*, *18*, e00258. https://doi.org/10.1016/j.btre.2018.e00258

Krishna, B. L., Singh, A. N., Patra, S., & Dubey, V. K. (2011). Purification, characterization and immobilization of urease from *Momordica charantia* seeds. *Process Biochemistry*, *46*(7), 1486–1491. https://doi.org/10.1016/j.procbio.2011.03.022

Kurniasih, M., Riapanitra, A., Riyanti, R., Kartika, D., & Rositasari, D. (2022). Reducing levels of methyl orange dye using crosslinked chitosan–tripolyphosphate. *Proceedings of the Soedirman International Conference on Mathematics and Applied Sciences (SICOMAS 2021)*, *5*(Sicomas 2021), 17–22. https://doi.org/10.2991/apr.k.220503.004

Lahiri, P. (2015). Effective immobilization of jack bean (*Canavalia ensiformis*) urease onto chitosan. *Journal of Chemistry and Chemical Sciences*, *5*(7), 2319–7625.

Liu, D. M., Chen, J., & Shi, Y. P. (2018). Advances on methods and easy separated support materials for enzymes immobilization. *TrAC - Trends in Analytical Chemistry*, *102*, 332–342. https://doi.org/10.1016/j.trac.2018.03.011

Lv, M., Ma, X., Anderson, D. P., & Chang, P. R. (2018). Immobilization of urease onto cellulose spheres for the selective removal of urea. *Cellulose*, *25*(1), 233–243. https://doi.org/10.1007/s10570-017-1592-3

Malhotra, I., & Basir, S. F. (2020). Application of invertase immobilized on chitosan using glutaraldehyde or tris (hydroxymethyl) phosphine as cross-linking agent to produce bioethanol. *Applied Biochemistry and Biotechnology*, *191*(2), 838–851.

Oliveira, T. S. De, Almeida, C. M. S., Jesus, J. N. De, Ferreira, R. D. M., & Cavalcante, P. A. W. (2017). Immobilization of amylase using chitosan beads as support. *Scientia Plena*, *13*, 1–7. https://doi.org/10.14808/sci.plena.2017.114201

Pithawala, K., Mishra, N., & Bahadur, A. (2010). Immobilization of urease in alginate, paraffin and lac. *Journal of the Serbian Chemical*

Society, *75*(2), 175–183. https://doi.org/10.2298/JSC1002175P

Qian, J., Zhao, C., Ding, J., Chen, Y., & Guo, H. (2020). Preparation of nano-enzyme aggregates by crosslinking lipase with sodium tripolyphosphate. *Process Biochemistry*, *97*, 19–26. https://doi.org/10.1016/j.procbio.2020.06.026

Saem, A. S. ., Hossain, M. T., Chandan, D., Mottaleb, M. A., & Alam, M. T. (2015). Single-step purification of urease from *Canavalia gladiata* seeds. *International Journal of Pharmaceutical Sciences and Research*, *6*(2), 845–849. https://doi.org/10.13040/IJPSR.0975-8232.6(2).845-49

Saptadi, D., Ardiarini, N. R., Budi, W., & Kuswanto. (2016). Berkelanjutan keanekaragaman hayati Indonesia dan perannya dalam menunjang kemandirian bangsa (Sustainability of Indonesian biodiversity and its role in supporting national independence). *Proceeding Seminar Nasional Biodiversitas VI, Surabaya*, *September*, 978–979.

Saxena, A., Bhattacharya, A., Kumar, S., Epstein, I. R., & Sahney, R. (2017). Biopolymer matrix for nano-encapsulation of urease – A model protein and its application in urea detection. *Journal of Colloid and Interface Science*, *490*, 452–461. https://doi.org/10.1016/j.jcis.2016.11.030

Setyaningtyas, T., Riyani, K., Kurniasih, M., Purwati, & Masruroh, S. (2021). Synthesis, characterization, antioxidant activity, and toxicity properties of tripolyphosphate crosslinked chitosan. *Molekul*, *16*(3), 251–259. https://doi.org/10.20884/1.jm.2021.16.3.815

Tantowidjojo, R. V., Roosdiana, A., & Prasetyawan, S. (2013). Optimasi amobilisasi pektinase dari *Bacillus subtilis* menggunakan kitosan-natrium tripolifosfat (Optimization of pectinase immobilization from *Bacillus subtilis* using chitosan-sodium tripolyphosphate). *Kimia Student Journal*, *1*(1), 91–97.

Tetiker, A. T., & Ertan, F. (2017). Investigation of some properties of immobilized urease from *Cicer arietinum* and its using in determination of urea level in some animal feed. *Journal of Innovations in Pharmaceutical and Biological Sciences (JIPBS)*, *4*(2), 2–7.

Verma, M. L., Kumar, S., Das, A., Randhawa, J. S., & Chamundeeswari, M. (2020). Chitin and chitosan-based support materials for enzyme immobilization and biotechnological applications. *Environmental Chemistry Letters*, *18*(2), 315–323. https://doi.org/10.1007/s10311-019-00942-5

Wahba, M. I. (2017). Porous chitosan beads of superior mechanical properties for the covalent immobilization of enzymes. *International Journal of Biological Macromolecules*, *105*, 894–904. https://doi.org/10.1016/j.ijbiomac.2017.07.102

Wu, H. Z., & Cao, A. (2013). Preparation and adding methods of Nessler's reagent having effects on determination of water quality ammonia nitrogen. *Advanced Materials Research*, *726–731*, 1362–1366. https://doi.org/10.4028/www.scientific.net/AMR.726-731.1362

Zhang, J., Wang, Z., He, C., Liu, X., Zhao, W., Sun, S., & Zhao, C. (2019). Safe and effective removal of urea by urease-immobilized, carboxyl-functionalized PES beads with good reusability and storage stability. *ACS Omega*, *4*(2), 2853–2862. https://doi.org/10.1021/acsomega.8b03287

Zusfahair, Ningsih, D. R., Fatoni, A., & Pertiwi, D. S. (2018). Determination of urease biochemical properties of asparagus bean (*Vigna unguiculata ssp sesquipedalis* L.). *IOP Conference Series: Materials Science and Engineering*, *349*(1). https://doi.org/10.1088/1757-899X/349/1/012073

Zusfahair, Z., Ningsih, D. R., Fatoni, A., & Setiawan, E. (2022a). Immobilization of urease from *Phaseolus vulgaris* L. seeds using calcium alginate as a support matrix. *Jurnal Natural*, *22*(3), 135–140. https://doi.org/10.24815/jn.v21i3.19953

Zusfahair, Z., Ningsih, D. R., Fatoni, A., & Nuraini, A. N. (2022b). The Isolation, immobilization, and characterization of urease from the seeds of winged bean (*Psophocarpus tetragonolobus* (L.) DC. *Molekul, 18* (1), 70 – 79. https://doi.org/10.20884/1.jm.2023.18.1.5932