International Journal of Technology 11(4) 754-763 (2020)
Received September 2019 / Revised March 2020 / Accepted June 2020

International Journal of Technology

http://ijtech.eng.ui.ac.id

Immobilization of Cholesterol Oxidase in Chitosan Magnetite Material for Biosensor Application

Meka Saima Perdani[1,2], Mohammad Didy Juliansyah[1], Dwini Normayulisa Putri[1], Tania Surya Utami[1], Chairul Hudaya[3], Masafumi Yohda[4], Heri Hermansyah[1*]

[1]*Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia, Kampus UI Depok, Depok 16424, Indonesia*
[2]*Department of Chemical, Faculty of Mathematic and Natural Sciences, Indonesia Defense University, Kawasan IPSC Sentul, Bogor 16810, Indonesia*
[3]*Department of Electrical Engineering, Faculty of Engineering, Universitas Indonesia, Kampus UI Depok, Depok 16424, Indonesia*
[4]*Department of Biotechnology and Life Science, Faculty of Engineering, Tokyo University of Agriculture and Technology, Tokyo 183-8538, Japan*

Abstract. Cholesterol oxidase, a bio-catalyst that can catabolize cholesterol, has proven applications in medicine. Here, a support material was used to enhance the characteristics of the enzyme. Magnetite (Fe_3O_4) is widely used as an enzyme support; however, the interaction between the enzyme and the support should be capped with another material, such as chitosan biopolymer-based material. In this study, chitosan-magnetite materials were synthesized by mixing both compounds and activating with glutaraldehyde. The materials were then characterized by Fourier Transform Infrared (FTIR) Spectroscopy. The enzyme kinetic parameters were studied by following the cholesterol oxidation reaction using high-performance liquid chromatography (HPLC) and comparing the results between the free and the immobilized enzyme. The substrate concentration was 2.5 mg/mL. The effect of enzyme concentration was tested using different concentrations of enzyme (0.5, 1, and 2 mg/mL) to determine the best operating conditions. The best conditions for the oxidation reaction were immobilized enzyme at a 2 mg/mL concentration. Enzyme immobilization significantly decreased the optimum substrate concentration to 0.1 mg/mL.

Keywords: Cholesterol; Cholesterol oxidase; Immobilized enzyme; Magnetite; Oxidation

1. Introduction

Cardiovascular disease is related to heart and vein malfunctions and can include coronary heart disease, heart malfunction, hypertension, and stroke. According to the World Health Organization (WHO), cardiovascular disease causes 17.9 million deaths every year, or 31% of all annual deaths worldwide, making this the number one cause of global death. The most significant cause of cardiovascular disease is cholesterol, which accounts for 56% of cardiovascular disease (Mackay et al., 2004). Cholesterol is needed by the human body but is dangerous when present in excessive amounts. For this reason, blood cholesterol amounts should be monitored periodically. Cholesterol is monitored by two methods: chemical and enzymatic.

*Corresponding author's email: heri.hermansyah@ui.ac.id, Tel.: +62-21-7863516; Fax: +62-21-7863515
doi: 10.14716/ijtech.v11i4.3484

The enzymatic method is more advantageous, as it is not corrosive and is run as a specific reaction. However, the enzymatic method still has disadvantages that include enzyme inactivation under abnormal conditions of temperature and pH. The specific enzyme used for enzymatic cholesterol is cholesterol oxidase. This enzyme is produced by several pathogenic and non-pathogenic microorganisms, such as *Mycobacterium, Brevibacterium, Streptomyces, Corynebacterium, Arthrobacter, Pseudomonas, Rhodococcus, Chromobacterium*, and *Bacillus* species. Cholesterol oxidase from *Streptomyces* sp. can also reportedly oxidize the substrate from fatty foods and can degrade up to 80% of the initial concentration of the substrate (Perdani et al., 2019a).

Cholesterol oxidase kinetic behavior has been investigated with a first order irreversible reaction model. The enzymatic reaction needs one or two enzymes to break the structure into a complex component (Perdani et al., 2019b). The enzyme acts as an electron donor to the CH-OH group in cholesterol. The final cholesterol enzymatic oxidation product is 5-cholesten-3-one, which is isomerized through three stages to produce 4-cholesten-3-one (Devi and Kanwar, 2017). In the first catalytic stage, dehydrogenation of the OH group results in the loss of two hydrogen molecules in the third steroid ring. The released hydrogen molecules are transferred to the FAD enzyme cofactor so that FAD is reduced. The reduced FAD cofactor then reacts with oxygen molecules to restore the initial condition of the enzyme, where the FAD is re-oxidized and the hydrogen atom reacts to form H_2O_2. The final stage of this process is the isomerization of the double-chain steroid ring and production of the final product, 4-cholesten-3-one (Devi and Kanwar, 2017).

Enzymatic methods have been applied in many research applications, such as renewable energy, bio-products, and pharmaceuticals. Enzymes can have multiple functions and can be used as catalysts, extraction agents, and capping agents. The process parameters of temperature, enzyme substrate concentration, and extraction time have the greatest effects on the enzymatic reaction. In addition, the reaction slows down unless enzyme is continuously added (Handayani et al., 2018). However, a study of enzymes used as biocatalysts to produce biodiesel has shown that immobilization of the lipase enzyme by an adsorption-crosslinking method stabilized the enzyme and kept it soluble in the reaction. Assays of the immobilized enzyme in the biodiesel synthesis reaction revealed that it retained 84% of its initial activity (Aliyah et al., 2016). Similarly, Hermansyah et al. (2018), examined a lipase enzyme from *Pseudomonas aeruginosa* by a fermentation method for biodiesel production from palm oil mill effluent (Hermansyah et al., 2018). Therefore, immobilization is a commonly used method to improve enzyme activity.

Enzyme immobilization requires a material that will act as a support to stabilize the enzyme. Various kinds of supports are used for immobilization, including polymers, carbon, metals, or metal-metal combinations. One common support used in enzyme immobilization is chitosan. Chitosan is a biopolymer with a number of advantages; it is renewable, nontoxic, and highly available in Nature (Peter, 1995). In industrial applications, chitosan is a source for composites of activated clinoptilote zeolite/chitosan used as a support for biogas purification (Kusrini et al., 2019). According to Ahmad and Goswami (2014), chitosan also can be used as a support for bioreactions. It can improve the characteristics of the cholesterol oxidase enzyme against changes in reaction temperature. For example, the activity of an immobilized enzyme can be maintained up to 50°C, where it can still show 77% activity after 12 repeated enzyme reaction cycles (Ahmad and Goswami, 2014).

Chitosan and agarose are the most common biopolymers used as enzyme immobilization supports. Biopolymers used as immobilization supports bind the enzyme by adsorption and covalent bonding as immobilization techniques. However, the ability of

biopolymers to form geometric structures, such as gel forms, makes them also useful for immobilization techniques involving encapsulation and entrapment (Zdarta et al., 2018).

In addition to biopolymers, inorganic and organic materials are also widely used in the preparation of enzymes, due to their specific characteristics. Magnetite particles have been attracting much interest for the development of many applications because of their unique properties, such as small size, superparamagnetism, low toxicity, good biocompatibility, and high surface area. Previous studies have also confirmed that bioenzymes can be immobilized with magnetic chitosan. The ability to undergo covalent binding produced strong enzyme activity and immobilization was confirmed by characterization of the sample with FTIR (Hamzah et al., 2019).

Magnetite nanoparticles dispersed in chitosan have been used as an electrochemical detector for the determination of the endocrine disruptor parathion. This composite showed good detection of the specific target compound. Chitosan has also been used to improve the stability of magnetite and to functionalize the surface of particles. The performance of a detector was improved with an electrode modified with magnetite and chitosan when compared with an unmodified electrode. The adsorption of magnetite was also increased after the addition of chitosan, as the matrix components of chitosan had a great influence on magnetite (Piovesan et al., 2018).

Magnetite-chitosan materials are widely used because of their easy separation and great support. A lipase enzyme covalently immobilized with magnetite chitosan was found to maintain 75.5% of its initial activity for 6 h. The magnetite chitosan has a great influence on the specific properties of enzyme, as it allows the creation of monolayers composed of different types of lipids, sterols, and their mixtures (Suo et al., 2018). In the present research, magnetite chitosan has been used as a support for cholesterol oxidase.

Magnetite chitosan has also been used for non-enzymatic reactions, but non-enzymatic reactions are slower than enzymatic methods. The aim of the present study was to explore the use of modified magnetite chitosan for the enzymatic reaction of cholesterol oxidation. A further aim was to investigate the effect of enzyme immobilization on the oxidation reaction between immobilized cholesterol oxidase and its substrate. The enzyme was tested for its ability to conduct the oxidation reaction by varying the enzyme concentration, substrate concentration, and reaction time. The immobilized enzyme material was characterized by Fourier transform infrared (FTIR) spectroscopy and the oxidation reaction was quantified by high performance liquid chromatography (HPLC).

2. Methods

All chemicals used for synthesis, immobilization, and oxidation were commercially available and were analytical grade from Sigma-Aldrich (St. Louis, USA). The chemicals were chitosan, Fe_3O_4 powder, glutaraldehyde, chloroacetic acid, cholesterol, and Triton-X100. Methanol and propanol were purchased from Merck (Darmstadt, Germany). The cholesterol oxidase enzyme was produced from *Streptomyces* sp. with a submerged fermentation method. The *Streptomyces* sp. culture was purchased from the Indonesian Culture Collection (InCC) LIPI (Bogor, Indonesia).

2.1. Synthesis of Chitosan-Fe_3O_4

The chitosan-Fe_3O_4 was synthesized using the process described by Suo et al. (2018), with some modifications. Commercial Fe_3O_4 and chitosan were used in this process at a ratio of 1:0.2 (w/w). The chitosan used in this study was commercial material purchased from Sigma-Aldrich. Both materials were dissolved in 50 mL 1% chloroacetic acid. The mixture was homogenized by ultrasonic mixing for 5 min at 60°C. The homogenous mixture

was then separated from the solvent using a vacuum filter and dried in an oven. The dry synthesized material was activated with the addition of glutaraldehyde and sonicated for 90 min at 60°C. The mixture was stored for 12 h and then the material was washed with buffer (0.01 M phosphate buffered saline, pH 7.0) to remove the excess glutaraldehyde. The final chitosan-Fe_3O_4 was divided for immobilization and characterization studies. FTIR was used to characterize the functional groups in the material.

2.2. Immobilization of Cholesterol Oxidase on Chitosan-Fe_3O_4

The cholesterol oxidase enzyme was attached to the synthesized chitosan-Fe_3O_4 by a crosslinking method. The ratio between enzyme and material was 1:2 (w/w). The prepared support material was allowed to react for 4 h with glutaraldehyde (2% v/v), which served as the immobilizing agent. A known enzyme concentration was then added to the mixture and allowed to react, with occasional shaking, at 4°C for 12 h. The immobilized enzyme was then used for the oxidation reaction with cholesterol as the substrate (Xu et al., 2013).

2.3. Oxidation of Cholesterol

Phosphate buffer (0.1 M), which was used as the solvent to dissolve the cholesterol oxidase, was prepared by combining 8.66 g Na_2HPO_4 and 5.31 g KH_2PO_4 in 250 mL distilled water and continuously stirred until the mixture became homogenous. Triton X-100 (0.05%) and distilled water were added until the mixture reached a 1 L volume. Cholesterol oxidase from *Streptomyces* sp. was weighed and dissolved in phosphate buffer. The concentration of enzyme solution used in this study was 0.5 mg/mL. Cholesterol was dissolved in 2-propanol at 2.5 mg/mL and sonicated to create a homogenous solution.

The cholesterol oxidation reaction was initiated by addition of 3 mL buffer solution to the reaction tube, followed by adding the catalyst containing 100 µL cholesterol oxidase. The mixture was incubated in a shaking water bath at 37°C for 5 min. After 5 min, the cholesterol solution was added to the mixture and incubated in the shaking water bath at 37°C for 5, 30, 60, 120, and 180 min.

The reaction products were quantified by HPLC with C-18 column using methanol:propanol (70:30, v/v) as the mobile phase at a 25°C and 1 mL flow rate. The cholesterol peak eluted at a retention time of 7.25 to 7.5 min.

3. Results and Discussion

3.1. Chitosan-Fe_3O_4 Synthesis and Characterization

Fe_3O_4 nanoparticles are commonly used as immobilization supports because of the nanoparticles have high surface area and magnetic ability that make them useful in separation methods. Fe_3O_4-chitosan material was synthesized well using the method of Suo et al. (2018) with some modifications. The chitosan-Fe_3O_4 particles were prepared at a low pH using 1% (v/v) chloroacetic acid.

Magnetite is a metal oxide hybrid that consists of organic and inorganic constituents and crosslinkers that promote ion transport at the material interface (Ramachandran et al., 2019). Magnetite chitosan was synthesized well by a precipitation method using iron chloride as a precursor and the addition of chitosan. The obtained particles had a lumpy shape and were formed from a hard aggregation of bulky particles. The magnetic characteristics were exploited to allow easy separation of the nanoparticles from the reaction solution. The ferromagnetic properties of magnetite are antiparallel (Bezdorozhevvet al., 2017). In this study, the magnetite chitosan was prepared by precipitation and a chemical method. The superparamagnetic properties of magnetite were investigated in the immobilization reaction. Addition of a strong dispersing agent to the solution promoted the formation of a covalent bond between the particles and the enzyme.

The crosslinking method is an easy method for immobilization, and this process does not require a matrix. In this method, the crosslinking reagent is glutaraldehyde, which has aldehyde groups in two end chains that can react with the amino acids in enzymes. The advantage of the crosslinking method is that it is more stable than the adsorption method and it prevents enzyme leakage. However, the limitation of this method is that it causes temporary changes in the active site of the enzyme, which leads to a loss of enzyme activity (Ghosh et al., 2017). Ahmad and Goswami (2014) used chitosan beads without modification as an immobilization support and glutaraldehyde as the activation agent and found that the optimum temperature increased to 50°C with immobilization, with an optimum pH of 7.5, but the enzyme began to be denatured at 55–70°C. Repetition of up to 12 cycles resulted in only a 33% loss of the initial enzyme activity. A previous study that examined the fabrication of chitosan nanoparticles also reported that an activation of the crosslinking agent with persulfate anion is reached at 70°C. Chitosan was assumed to form a dense network structure by sulfate bridges. The effect of a monocarboxylic acid on the reaction was a degradation and shortening of the chitosan chain (Kusrini et al., 2015). These conditions were similar to those in the present study; however, we used chloroacetic acid to shorten the chitosan chain and crosslink with other amino groups.

The chitosan-magnetite material that was successfully synthesized was used for the immobilization of the cholesterol oxidase enzyme (20 mg). Before the immobilization step, the chitosan-magnetite material is added to the glutaraldehyde solution. The addition of glutaraldehyde enlarges the pores of the chitosan-magnetite material and coats the outside of the material (Wang et al., 2015). The addition of 2% v/v glutaraldehyde can form particles with a diameter of 50–200 nm (Xu et al., 2013). Following the protocol of Xu et al (2013), the minimum time reaction between glutaraldehyde and materials was set at 2 h for the crosslinking step. We added a further 2 h for the immobilization step to account for the difference in the enzyme used in the present study. As reported previously (Wang et al., 2015), the immobilization of cholesterol oxidase requires at least 4 h at 4°C and was continued to 12 h with occasional shaking.

A crosslinker is an intermediate agent that binds two or more materials. The functional groups of specific materials and the crosslinker agent react with each other to form covalent bonds. Here, glutaraldehyde acted as the crosslinker in the reaction, and chemical processing is needed for immobilization between the enzyme and support material. The -NH_2 or amino acid functional groups interact with the metal oxide (M-O) group from the materials. By this reaction, the enzyme is covalently bonded to the metal in a reaction between the amino acid functional groups and the metal oxide. A dispersing agent helps to build the covalent bond space on the magnetite surface (Ramachandran et al., 2019). Upon addition of glutaraldehyde, the mixture is then washed several times with a buffer, followed by reaction with enzymes to form covalent bonds between the enzyme and the support material. In order to form a homogenous composition, the mixture of enzymes and material is allowed to stand for 12 h with periodic stirring.

The behavior of the immobilized enzyme was further analyzed. The success of the immobilization process was evident by the oxidation reaction of the cholesterol oxidase enzyme, as indicated by the more rapid decrease in the substrate. The strong chemical bonding between the enzyme and chitosan magnetite reinforced the enzyme robustness and chemical stability.

The crosslinker agent acted as a stabilizer between enzyme and metal. Chitosan has hydrophobic characteristic so it only soluble in an acidic reagent. Ultrasound was used to produce a homogenous mixture of chitosan-Fe_3O_4. Magnetic stirring method is not compatible with this procedure because of the magnetic characteristics of the Fe_3O_4.

According to Tang et al. (2003), chitosan should not be exposed to ultrasonic mixing for longer than 10 min, as the chitosan particles will start to fragment and lose activity. Therefore, the ultrasonication process was done for only 5 min. After the ultrasonication, a shaking water bath was used for 90 min to allow complete mixing of the chitosan-Fe_3O_4 (Tang et al., 2003). The synthesized chitosan-Fe_3O_4 was then separated from the solvent using a magnetic separation method, and the excess solvent was removed by evaporation. The dried chitosan-Fe_3O_4 was characterized by FTIR, as shown in Figure 1.

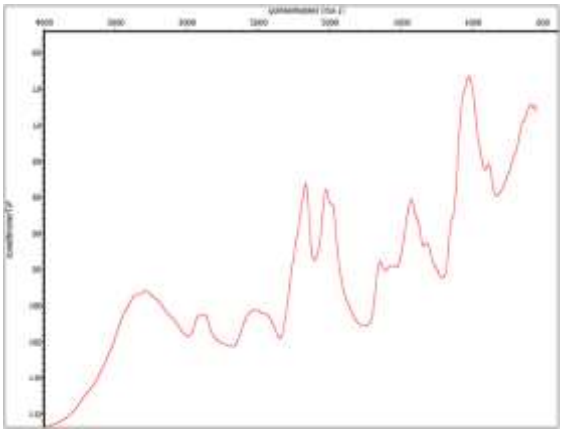

Figure 1 FTIR spectrum of chitosan-Fe_3O_4

Each spectrum peak in Figure 1 represents functional groups in the chitosan-Fe_3O_4. According to references, some important peaks indicate the presence of chitosan and Fe_3O_4. The wavenumber 589.68 cm^{-1} refers to the Fe-O group, 1022.67 cm^{-1} refers to the C-O group, 1643.98 cm^{-1} refers to the N-H group, 2874.02 cm^{-1} refers to the C-H group, and 3286 cm^{-1} refers to the O-H group. The FTIR spectrum shown in Figure 1 agrees with the spectrum previously published by Freire et al. (2016), who showed that magnetite material (M-O functional group) will appear at wavenumber of 580 cm^{-1}. The obtained peak also agreed with the results of Suo et al. (2018), who indicated that the important peaks of chitosan will show the functional groups of C-O at 1091 cm^{-1}, N-H at 1600 cm^{-1}, C-H at 2881 cm^{-1}, and vibration O-H at 3432 cm^{-1}. The band at 2874.02 cm^{-1} found in the present study indicated the C-H stretching vibration in the polymeric backbone, as confirmed in a previous study which showed the C-H band at 2890 cm^{-1} (Kusrini et al., 2014).

Figure 1 shows that the N-H group from chitosan appeared as a small peak at wavenumber 1643.98 cm^{-1}. This peak provides evidence that chitosan has been coated onto the support. However, a previous study showed that the N-H group also appeared at the wavenumbers of 1600 cm^{-1} (Suo et al., 2018) and 1594-1597 cm^{-1} (Kusrini et al., 2014). The shifted wavenumber in FTIR indicates the different coordination of anions or cations in the amine group.

3.2. Oxidation of Cholesterol

As shown in Figure 2, the immobilized enzyme was better at oxidizing cholesterol when compared with the free enzyme. The immobilized enzyme activity fell to 60% of the initial activity. The free enzyme, regardless of initial concentration of enzyme, showed a much slower reduction of the substrate.

The immobilized enzyme is more active than the free enzyme at reducing the cholesterol substrate in the oxidation reaction. The oxidation reaction confirmed that the cholesterol oxidase enzyme had attached to the chitosan-Fe_3O_4 and that the immobilization process had been successful.

The increase in enzyme concentration also affected the oxidation reaction. For the oxidation reaction, a 100 µL volume of enzyme solution was added to 3 mL phosphate buffer and incubated for 5 min, followed by addition of cholesterol and a further 5 min reaction. The reaction was initiated when the substrate was added to the enzyme solution.

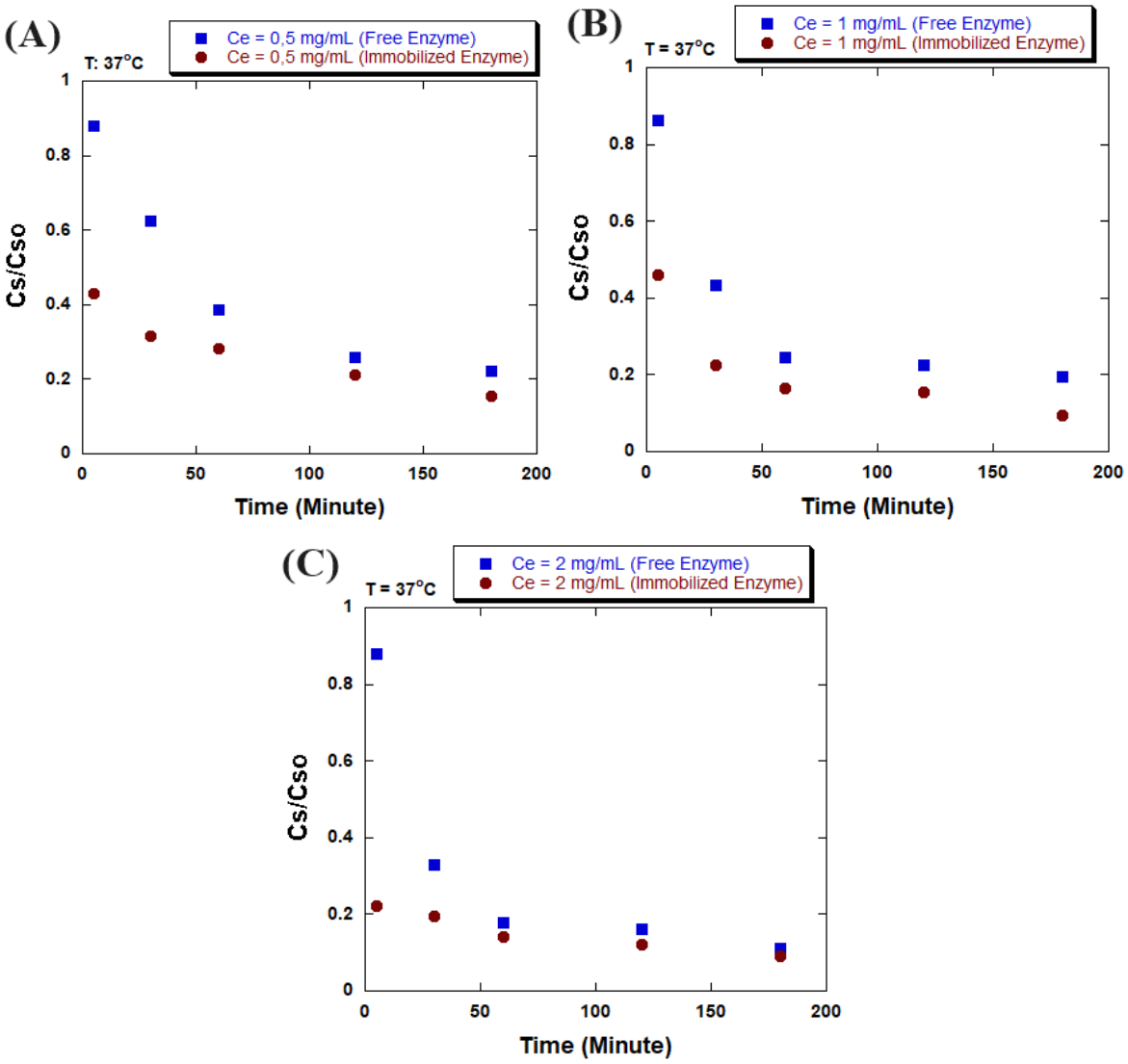

Figure 2 Substrate reduction with free and immobilized enzyme with different concentrations of enzyme. Concentration of enzyme: (a) 0.5 mg/mL; (b) 1 mg/mL; and (c) 2 mg/mL

Figure 2c shows that the immobilized and free enzyme at a concentration of 2 mg/mL showed a significant difference in the reaction. The immobilized enzyme was able to oxidize up to 93% of the substrate within 180 min, whereas the free enzyme only oxidized up to 90% of the cholesterol. Therefore, the immobilized enzyme retained the ability to oxidize and degrade the substrate in the oxidation reaction; however, the bulky aggregation of the chitosan magnetite became a barrier to obtaining optimal enzyme activity. This problem was also described by Freire et al. (2016), who noted a weight loss of magnetite during the reaction and separating stage that may have affected the enzyme properties. The bulky forms of magnetite chitosan exhibited a breakdown of the main chitosan chains that covered the magnetite. The loss of chitosan chains could lead to low binding with the enzyme, so the enzyme would be unable to interact perfectly with the materials. However, the use of glutaraldehyde for crosslinking did not show any disruption of the enzyme. The

immobilization of cholesterol oxidase with chitosan was successful, and the support was also able to maintain the enzyme performance (Ahmad and Goswami, 2014). Chitosan was chosen because it has a polymer chain that can bind with the enzyme and form a strong linkage between the support and the enzyme. In addition, the immobilization techniques also allowed the enzyme to maintain its biocharacteristics (Mohamad, 2015).

A previous study also noted that modified magnetite silicon dioxide can act as a supporting agent for the immobilization of cholesterol oxidase and the immobilized enzyme showed a higher retention of activity than was observed for the free enzyme. The peak behavior of enzymatic oxidation occurred at the initial reaction time and was slightly different from the activity of the free enzyme. Controlling the stability of the enzyme appeared difficult when it reacted with the specific substrate in different temperatures (Perdani et al., 2020).

4. Conclusions

Cholesterol oxidase immobilized with chitosan magnetite was able to oxidize up to 90% of a cholesterol substrate at different reaction times. The enzyme interacted with magnetite nanoparticles covered with aminated chitosan. The NH_2 functional group was recorded during the immobilization step. Cholesterol oxidation with the immobilized enzyme showed that the use of a support material can significantly change the behavior of the enzyme to oxidize the substrate. However, the concentration of enzyme also affected the behavior of the oxidation reaction. Chitosan-magnetite could be a candidate for a cholesterol biosensor due to the sensitivity of the oxidation reaction for the substrate. The chemical properties are better for the immobilized enzyme than for the free enzyme. The best concentration of the immobilized enzyme for substrate oxidation was 2 mg/mL with the maximum reaction time.

Acknowledgements

The authors acknowledge with thanks the research support provided by Universitas Indonesia and Ministry of Research, Technology and Higher Education Republic of Indonesia through PMDSU-PKPI grant number T/2093/D3.2/KD.02.00/2019 and World Class University program managed by Institut Teknologi Bandung.

References

Ahmad, S., Goswami, P., 2014. Application of Chitosan Beads Immobilized *Rhodococcus* sp. NCIM 2891 Cholesterol Oxidase for Cholestenone Production. *Process Biochemistry*, Volume 49(12), pp. 2149–2157

Aliyah, A. N., Edelweiss, E. D., Sahlan, M., Wijanarko, A., Hermansyah, H., 2016. Solid State Fermentation Using Agroindustrial Wastes to Produce Aspergillus Niger Lipase as a Biocatalyst Immobilized by an Adsorption-Crosslinking Method for Biodiesel Synthesis. *International Journal of Technology*, Volume *7*(8), pp. 1393–1404

Bezdorozhev, O., Kolodiazhnyi, T., Vasylkiv, O., 2017. Precipitation, Synthesis and Magnetite Properties of Self-assembled Magnetite-Chitosan Nanostructures. *Journal of Magnetism and Magnetic Materials*, Volume 428, pp. 406–411

Devi, S., Kanwar, S.S., 2017. Cholesterol Oxidase: Source, Properties, and Application. *Insight in Enzyme Research*, Volume 1(1), pp. 1–5

Freire, T.M., Dutra, L.M.U., Queiroz, D.C., Ricardo, N.M.P.S., Barreto, K., 2016. Fast Ultrasound Assisted Synthesis of Chitosan-based Magnetite Nanocomposite as a Modified Electrode Sensor. *Carbohydrate Polymers Journal*, Volume 151, pp. 760–769

Ghosh, S., Ahmad, R., Khare, S.K., 2017. Immobilization of Cholesterol Oxidase: An Overview. *The Open Biotechnology Journal*, Volume 12, pp. 176–188

Hamzah, A., Ainiyah, S., Ramadhani, D., Parwita, G.E.K., Rahmawati, Y., Soeprijanto, Ogino, H., Widjaja, A., 2019. Cellulase and Xylanase Immobilized on Chitosan Magnetic Particles for Application in Coconut Husk Hydrolysis. *International Journal of Technology*, Volume 10(3), pp. 613–623

Handayani, D., Amalia, R., Yulianto, M.E., Hartati, I., Murni, M., 2018. Determination of Influential Factor during Enzymatic Extraction of Ginger Oil using Immobile Isolated Cow Rumen Enzymes. *International Journal of Technology*, Volume 9(3), pp. 455–463

Hermansyah, H., Maresya, A., Putri, D.N., Sahlan, M., 2018. Production of Dry Extract Lipase from *Pseudomonas aeruginosa* by the Submerged Fermentation Method in Palm Oil Mill Effluent. *International Journal of Technology*, Volume 9(2), pp. 325–334

Kusrini, E., Arbianti, R., Sofyan, N., Abdullah, M.A.A., Andriani, F., 2014. Modification of Chitosan by using Samarium for Potential Use in Drug Delivery System. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, Volume 120, pp. 77–83

Kusrini, E., Shiong, N.S., Harahap, Y., Yulizar, Y., Arbianti, R., Pudjiastuti, A.R., 2015. Effects of Monocarboxylic Acids and Potassium Persulfate on Preparation of Chitosan Nanoparticles. *International Journal of Technology*, Volume 6(1), pp. 11–21

Kusrini, E., Wu, S., Susanto, B.H., Lukita, M., Gozan, M., Hans, M.D., Rahman, A., Degirmenci, V., Usman, A., 2019. Simultaneous Absorption and Adsorption Processes for Biogas Purification using $Ca(OH)_2$ Solution and Activated Clinoptilolite Zeolite/Chitosan Composites. *International Journal of Technology,* Volume 10(6), pp. 1243–1250

Mackay, J.G., Mensah, S., Mendis, K., Greenland, 2004. *The Atlas of Heart Disease and Stroke*. World Health Organization, USA

Mohamad, N.R., 2015. An Overview of Technologies for Immobilization of Enzymes and Surface Analysis Technique for Immobilized Enzymes. *Biotechnology & Biotechnological Equipment Journal*, Volume 29, pp. 205–220

Perdani, M.S., Faturrohman, M., Putri, D.N., Hermansyah, H., 2019a. Oxidation of Cholesterol from Fatty Food by using Crude Cholesterol Oxidase *Streptomyces* sp. *In*: AIP Conference Proceedings, Volume 2193(1), pp. 1–6

Perdani, M.S., Sahlan, M., Farida, S., Putri, D.N., Soekanto, S.A., Hermansyah, H., 2019b. Kinetic Study of Cholesterol Oxidation by Cholesterol Oxidase Enzyme as Application for Cholesterol Biosensor. *In*: AIP Conference Proceedings, Volume 2092(1), 030027

Perdani, M.S., Sahlan, M., Yohda, M., Hermanysah, H., 2020. Immobilization of Cholesterol Oxidase from *Streptomyces* sp. on Magnetite Silicon Dioxide by Crosslinking Method for Cholesterol Oxidation. *Applied Biochemistry and Biotechnology*, Volume 191(8), pp. 968–980

Peter, M., 1995. Applications and Environmental Aspects of Chitin and Chitosan. *Journal of Macromolecular Science,* Volume 32(4), pp. 629–640

Piovesan, J.V., Haddad, V.F., Pereira, D.F., Spinelli, A., 2018. Magnetite Nanoparticles/Chitosan-modified Glassy Carbon Electrode for Non-enzymatic Detection of the Endocrine Disruptor Parathion by Cathodic Square-wave Voltammetry. *Journal of Electroanalytical Chemistry*, Volume 823, pp. 617–623

Ramachandran, R., Jung, D., Spokoyny, A.M., 2019. Cross-linking Dots on Metal Oxides. *NPG Asia Materials*, Volume 11(1), pp. 1–4

Suo, H., Xu, L., Xu, C., Chen, H., Yu, D., Gao, Z., Huang, H., Hu, Y., 2018. Enhancement of Catalytic Performance of Porcine Pancreatic Lipase Immobilized on Functional Ionic Liquid Modified Fe_3O_4-Chitosan Nanocomposites. *International Journal of Biological Macromolecules*, Volume 119, pp. 624–632

Tang, E.S., Huang, M., Lim, L.Y., 2003. Ultrasonication of Chitosan and Chitosan Nanoparticles. *Journal of Pharmaceutics*, Volume 265(1-2), pp. 103–114

Wang, X-Y, Jiang, X-P., Li, Y., Zeng, S., Zhang, Y-W., 2015. Preparation Fe_3O_4@chitosan Magnetic Particles for Covalent Immobilization of Lipase from *Thermomyces lanuginosus*. *International Journal of Biological Macromolecules*, Volume 75, pp. 44–50

Xu, R., Zhou, Q., Li, F., Zhang, B., 2013. Laccase Immobilization on Chitosan/Poly (vinyl alcohol) Composite Nanofibrous Membranes for 2, 4-Dichlorophenol Removal. *Chemical Engineering Journal*, Volume 222, pp. 321–329

Zdarta, J., Meyer, A.S., Jenionowski, T., Pinelo, M., 2018. A General Overview of Support Materials for Enzyme Immobilization: Characteristics, Properties, Practical Utility. *Catalysts*, Volume 8(3), p. 1–27