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Superoxide Anion Radicals Biosensor Based on Protein Extract from *Deinococcus Radiodurans* Immobilization by Glutaraldehyde Cross-Linked

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Abstract

A common enzyme for superoxide anion radicals biosensors is superoxide dismutase (SOD). *Deinococcus radiodurans* protein extract can replace the use of pure SOD enzymes for superoxide anion radicals biosensors. The purpose of this research is to determine the analytical performance of the superoxide anion radicals biosensor with protein extract of *Deinococcus radiodurans* immobilized on carboxymethylcellulose-gelatin-zeolit (CMC-G-Z) that is crosslinked by glutaraldehyde. The response surface method showed that the optimum condition for the biosensor was pH 7, protein extract concentration of 1075 µg/ml, zeolite concentration of 5 mg/ml, and glutaraldehyde concentration of 0.0042 M. Stability of the biosensor retained 67% of its sensitivity after use for 24 hours. The biosensor exhibits good analytical performance with a linear range from 0.1 – 0.8 mM, a detection limit of 77.84 µM, and a limit of quantification of 259.5 µM with a correlation coefficient of 0.9905.

Keywords: Superoxide anion radicals; *Deinococcus radiodurans*; Biosensor; Superoxide dismutase (SOD); Glutaraldehyde.

INTRODUCTION

Reactive oxygen species (ROS) are chemical molecular species formed after the incomplete reduction of oxygen and are highly reactive. ROS consists of hydroxyl radicals (·OH), superoxide anions ($O_2^{\cdot-}$), and hydrogen peroxide (H_2O_2) (J. Liu et al., 2023). Mitochondria produce $O_2^{\cdot-}$ through oxidative phosphorylation in living aerobic organisms for ATP synthesis (Schröder, 2020). Superoxide anion can be converted into H_2O_2 and oxygen (O_2) by the enzyme superoxide dismutase (SOD), which is present in mitochondria. However, $O_2^{\cdot-}$ can be dangerous if the amount of superoxide anion is uncontrolled. (Andrés et al., 2023). An excessive increase of superoxide anion radicals in biological systems can cause cancer (Sahoo et al., 2021), Parkinson's (Jiang & Bai, 2022), autoimmune (Tavassolifar et al., 2020), and inflammation (Albano et al., 2022).

Up to date, there are several methods for measuring $O_2^{\cdot-}$ as well as for evaluating antioxidant activity, including spectrophotometry (Yang et al., 2020), fluorescence sensor (Song et al., 2017), liquid chromatography/mass spectrophotometry (LC/MS) (Wang et al., 2018), and electrochemical biosensor (Zouleh et al., 2025); (Crulhas et al., 2017). Among the above methods, electrochemical is considered an ideal superoxide anion detection technique due to it is simple, cost-effective and has a fast response. (F. Liu et al., 2022). One of the $O_2^{\cdot-}$ biosensor methods uses the superoxide dismutase (SOD) enzyme which has a good correlation with spectrophotometric methods to detect $O_2^{\cdot-}$ (Braik et al., 2016). However, the use of SOD enzyme in biosensors has a limitation due to its high cost. The use of microbes as an alternative solution to reduce biosensor production costs. One of the potential microbial sources of SOD in Indonesia is *Deinococcus radioduran* (Iswantini et al., 2019); (Sukma et al., 2023).This microbe contains manganese superoxide dismutase (Mn-SOD) within its cells (Gaidamakova et al., 2022). Enzim SOD dapat diperoleh dengan menghancurkan membran sel *D. radiodurans* yang berupa ekstrak protein *D. radiodurans.* Ekstrak protein SOD yang diimobilisasi pada elektroda pasta karbon menghasilkan aktivitas SOD lebih tinggi daripada enzim murni SOD. Sehingga protein *D. radiodurans* berpotensi untuk biosensor antioksidan (Iswantini et al., 2019); (Sukma et al., 2023)(Iswantini et al., 2019; Sukma et al., 2023).

Research on *D. radiodurans*-based superoxide anion biosensors is continuously being conducted to improve their performance, focusing on optimizing the immobilization method of the biorecognition element. Enzyme stability can be maintained by immobilizing it on nano-materials (Nemiwal et al., 2022). Zeolite has been widely used in enzyme biosensor immobilization matrices due to its advantageous properties, including size selectivity, charge, hydrophobic/hydrophilic interactions, and high thermal stability (Zhang et al., 2021). using zeolite in biosensors as a microbial immobilization matrix has been proven to enhance current response (μA) in electrochemical methods (Vu et al., 2020). Natural zeolite is abundant in Indonesia, including Bayah zeolite from Banten, Indonesia. Bayah zeolite as a natural zeolite still needs to be optimized for use in all areas such as immobilization matrix because its abundance, easy availability, and low cost of natural zeolite support this research for immobilization matrix development. However, the stability of biosensors using *D. radiodurans* protein extract needs to be improved by combining zeolite with other materials as immobilization agents for the *D. radiodurans* protein extract (Iswantini et al., 2019). The immobilization method for enzyme-based biosensors using glutaraldehyde as a cross-linking agent can increase the stability and performance of biosensor electrodes (Bounegru & Apetrei, 2023); (Kocabay et al., 2012); (Karakaya et al., 2020).

This research continues the previous research by Iswantini et al. (2019), where *D. radiodurans* protein extract was immobilized with zeolite on a carbon paste electrode (Iswantini et al., 2019). Previous research has shown that SOD from *D. radiodurans* protein extract exhibits higher affinity compared to purified SOD enzyme. Further research is needed to improve analytical parameters, especially the stability of biosensors. Improvement of immobilization through crosslinking between gelatin and glutaraldehyde dispersed on the surface of zeolite can optimize the performance of *D. radiodurans* protein. It is concluded that using this immobilization method can improve the stability and activity of the *D. radiodurans* protein extract for anion superoxide radicals biosensors.

MATERIALS AND METHODS

Materials

The materials used *D. radiodurans* bacterial cells grown from waste sauce isolation in Indonesia. Natural zeolite from Bayah, Indonesia. Lactose Broth medium from Miller. Gelatin, carboxymethylcellulose, glutaraldehyde, xanthine oxidase, and xanthine, were all sourced from Sigma Aldrich, and DMSO was from Merck.

Growth of *D. radiodurans* Cells and SOD Protein Extraction

D. radiodurans from culture were grown in 25 mL of LB media. The bacteria were incubated at 30°C and measured at a wavelength of 600 nm for an optical density (OD) value of 0.5–0.6. The cells were harvested by centrifugation at 7000 x g, 4°C for 10 minutes to separate the microbial cells from the media. The cells (pellet) were washed three times with phosphate buffer pH 7.0 and then resuspended in phosphate buffer solution pH 7.0. The cell suspension was sonicated at 80% pulse intensity to lyse the microbial cells for 2 minutes, with 1 x 2-minute intervals, and a 1-minute resting period between sonication cycles. During sonication, the cell suspension was cooled in an ice bath. The cells were centrifuged at 10,000 x g, 4°C for 30 minutes to separate the supernatant from the pellet. The crude protein extract was present in the supernatant. The cytoplasmic extract after dialysis was then measured for absorbance at wavelengths of 260 nm and 280 nm to determine the protein concentration and the protein-to-DNA ratio (Chou & Tan, 1991).

Preparation of Carbon Paste Electrode

The electrode was prepared by dissolving 3 mg of ferrocene in 1 mL of DMSO, and then adding 100 mg of graphite to the solution. The mixture was left to stand for 2 hours. After 2 hours, the solvent was evaporated in an oven at 105°C until dry. Graphite was mixed with liquid paraffin in a 2:1 ratio until a paste was formed. The carbon paste was inserted into the electrode body and compacted until it reached the glass surface. The glass surface of the electrode was polished and cleaned using sandpaper.

Immobilization of *D. radiodurans* Protein Extract on the Surface of the Carbon Paste Electrode

A polymer mixture (CMC-gelatin) was prepared in a 0.1 w/w ratio in 10 mL phosphate buffer. 50 mg of zeolite was dispersed into the polymer mixture to form the (CMC-G-Z) mixture. 60 μL *D. radiodurans* protein (in phosphate buffer) and 10 μL of 0.05 M glutaraldehyde were added to 30 μL of CMC-G-Z (Kocabay et al., 2012); (Karakaya et al., 2020). The immobilization system mixture (CMC-G-Z-Protein) was applied to the surface of the carbon paste electrode. The electrode was left to stand until the solvent evaporated at 25ºC. The electrode surface was coated with a dialysis membrane. The immobilization process of CMC-G-Z-Protein of *D. radiodurans* can be seen in Figure 1.

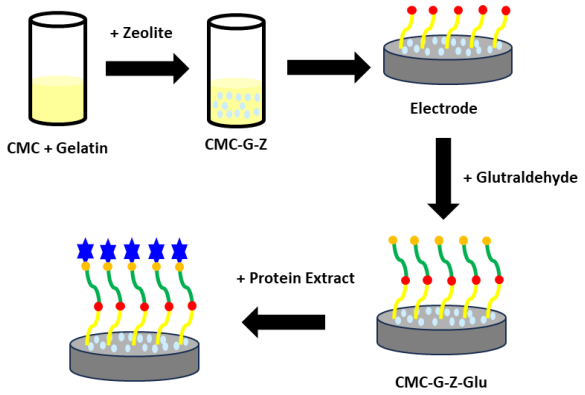

Figure 1. Schematic representation of the stepwise immobilization process of protein extract on CMC-G-Z by glutaraldehyde crosslinked.

Electrochemical Measurement

Electrochemical measurements were conducted using a potentiostat/galvanostat eDAQ system and a computer with the Echem v2.1.0 data processing software. The electrodes used were an Ag/AgCl as the reference electrode, platinum wire as a counter electrode, and a carbon paste electrode as the working electrode. 1.9 mL of 0.05 M phosphate buffer solution at pH nine was added to the electrochemical cell, and the anode current peak formed was observed as the blank. 100 µL of 0.1 U/mL Xanthine Oxidase solution and 1 mL of 2.1 mM xanthine substrate were added to the electrochemical cell. The change in current was monitored until a steady-state current was reached.

Optimization of Superoxide Anion Biosensor

Optimization was performed by combining variations in pH (7, 8, and 9), *D. radiodurans* protein extract concentration (500, 1000, and 1500 µg/ml), glutaraldehyde concentration (0, 0.005, and 0.01 M), and zeolite concentration (5, 7.5, and 10 mg/mL). The method used for optimizing was the *Response Surface Methodology* with *MINITAB 16* software.

Determination of Electrode Stability

Electrode stability was determined by measuring the activity of the protein extract after optimal conditions were achieved. The activity value obtained from the initial measurement was considered 100%. The activity was remeasured at specific time intervals, and the remaining activity was calculated.

Electrode Analytical Parameters

The performance of the biosensor electrode was tested for two electrodes, one with glutaraldehyde and the other without glutaraldehyde. The parameters determined were linearity, the limit of detection (LOD), and the limit of quantification (LOQ) for superoxide anion biosensors.

RESULTS AND DISCUSSION

Growth of *D. radiodurans* and Extraction of SOD Protein

The *D. radiodurans* cells were grown in LB medium for approximately 18 hours with a shaker (aerobic conditions) at room temperature. The *D. radiodurans* cells were lysed to obtain crude protein containing the SOD enzyme. The protein extraction results, shown in Figure 2, indicate that the optimal induction treatment was achieved with 3 minutes of exposure to UV light. UV light induction can generate free radicals that attack the DNA and membranes of microorganisms, leading to cell damage. However, *D. radiodurans* is a microbe that can survive damage by free radicals because of its ability to repair DNA damage (Chen et al., 2023). Proteins repair DNA damage within the cell. The MnSOD enzyme in *D. radiodurans* is capable of counteracting free radicals, one of which is caused by radiation (Gaidamakova et al., 2022). Therefore, UV light induction facilitates the expression of the SOD protein, leading to a greater protein extract yield compared to conditions without UV irradiation (Palmieri et al., 2019). *D. radiophilus* is also able to survive in extreme environments. The Mn-SOD produced by *D. radiophilus* increases with increased environmental stress, such as UV radiation. However, if the UV radiation is excessive, Mn-SOD production decreases (Yun & Lee, 2003). UV induction for 5 minutes can damage *D. radiodurans* cells, leading to a decreased in cell growth due to excessive UV exposure.

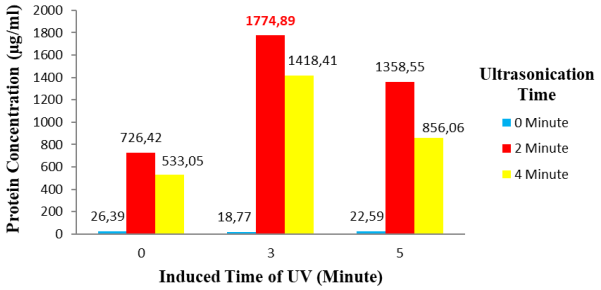

Figure 2. Protein Concentration of *D. radiodurans* to UV Radiation and Ultrasonication Time Treatment.

The optimal ultrasonication time for obtaining protein extract from the cells is 2 minutes, with a protein concentration of 1774.89 µg/ml. The SOD enzyme in *D. radiodurans* is located in the cytoplasm, requiring ultrasonication or cell membrane lysis for its extraction (Sadowska-Bartosz & Bartosz, 2023). Ultrasonication for 0 minutes resulted in very low yields, as the SOD protein extract remained within the cell membrane. Ultrasonication for 4 minutes resulted in a decrease in protein extract concentration, as some of the SOD proteins in the cytoplasm were denatured due to the ultrasonication process.

Immobilization of *D. radiodurans* Protein Extract

The immobilization of *D. radiodurans* protein extract using CMC-gelatin and glutaraldehyde aims to increase the stability and activity of the protein extract. The immobilization process is performed by crosslinking glutaraldehyde with protein extract on a solid phase of CMC-gelatin. The reactions in the biosensor system are:

$$\text{Xantine} + H_2O + O_2 \xrightarrow{\text{XOD}} \text{Uric Acid} + 2H^+ + O_2^{\cdot-}$$

$$2H^+ + O_2^{\cdot-} \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

$$H_2O_2 \longrightarrow 2H^+ + O_2 + 2e^-$$

The voltammogram of the immobilized *D. radiodurans* protein extract can be seen in Figure 3. The activity of the protein extract can be determined by the anodic peak at a voltage of 0.4V after adding xanthine and xanthine oxidase (XOD) to the electrochemical cell solution. The blank peak can be determined by measuring the buffer at pH seven before adding xanthine and XOD. Electrochemical measurements using the SOD enzyme crosslinked with glutaraldehyde produce an anodic peak at a voltage of 0.4–0.5 V, confirming that the anodic peak of *D. radiodurans* protein extract is comparable to that of the pure SOD enzyme (Vu et al., 2020).

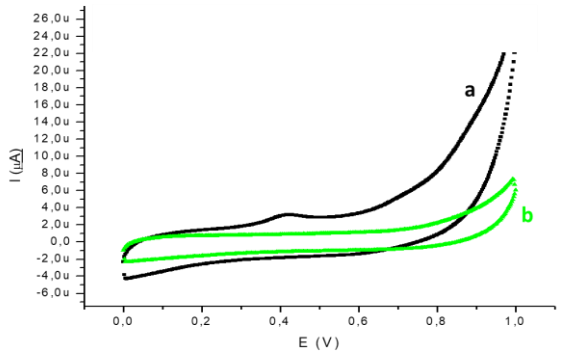

Figure 3. Voltamogram of *D. radiodurans* protein extract activity with xanthine and XOD (a), without xanthine and XOD (b)

The carbon paste electrode was coated with a dialysis membrane which functions as an intermediary for the diffusion of the substrate into the protein extract and to keep the protein extract out of the immobilization system. The dialysis membrane used is *Thermo Scientific SnakeSkin Dialysis Tubing*, 10K MWCO (Molecular Weight Cut Off), which ensures that the protein extract containing the SOD enzyme is retained, as it has a molecular weight greater than 10 kDa (Chen et al., 2023). However, the pores of the dialysis membrane can allow the superoxide radical substrate to pass through and interact with the *D. radiodurans* protein extract.

Figure 4 shows that higher current response is obtained from the immobilized protein extract with glutaraldehyde crosslinking while without glutaraldehyde the current response is lower. The increased current with glutaraldehyde crosslinking can explain that the crosslinking agent can bind the protein extract optimally, resulting in a higher activity of the protein extract to react with the substrate (Karakaya et al., 2020).

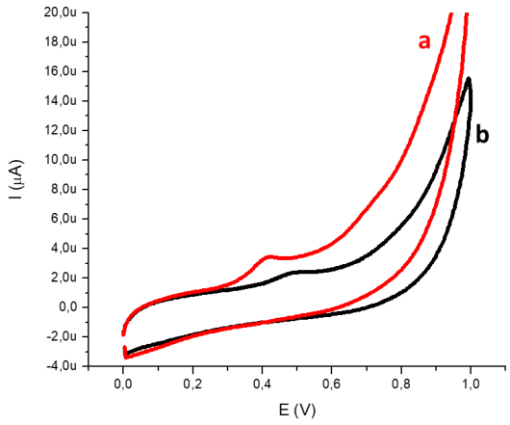

Figure 4. The voltammogram of the activity of *D. radiodurans* protein extract immobilized with crosslinking and without glutaraldehyde.

Optimization of Anion Superoxide Biosensor

Optimization was performed on a 0.7 mM xanthine substrate with several variations of protein concentration, glutaraldehyde concentration, zeolite concentration, and pH using the *Response Surface Method* in *MINITAB 16* software. Figure 5a shows the effect of glutaraldehyde concentration as a crosslinking agent, which produces the optimal condition at approximately 0.005 M. A glutaraldehyde concentration of 0.005 M results in the maximum current compared to concentrations below or above 0.005 M. The optimum concentration of glutaraldehyde obtained in this study shows results that are suitable when using pure SOD enzyme to detect superoxide (Karakaya et al., 2020). However, when the glutaraldehyde concentration is higher, there will be a reduction in current because it will bind to the active site in the protein extract, causing no reaction with the substrate to form, and the pores on the electrode surface will become tighter, which can make diffusion of the substrate more difficult (Kocabay et al., 2012); (Karakaya et al., 2020). Without glutaraldehyde in the immobilization system, the protein extract will only be adsorbed into the phase of the CMC-gelatin-zeolite polymer. This adsorption is physical, which causes the protein extract to result in a lower current (Berillo et al., 2024).

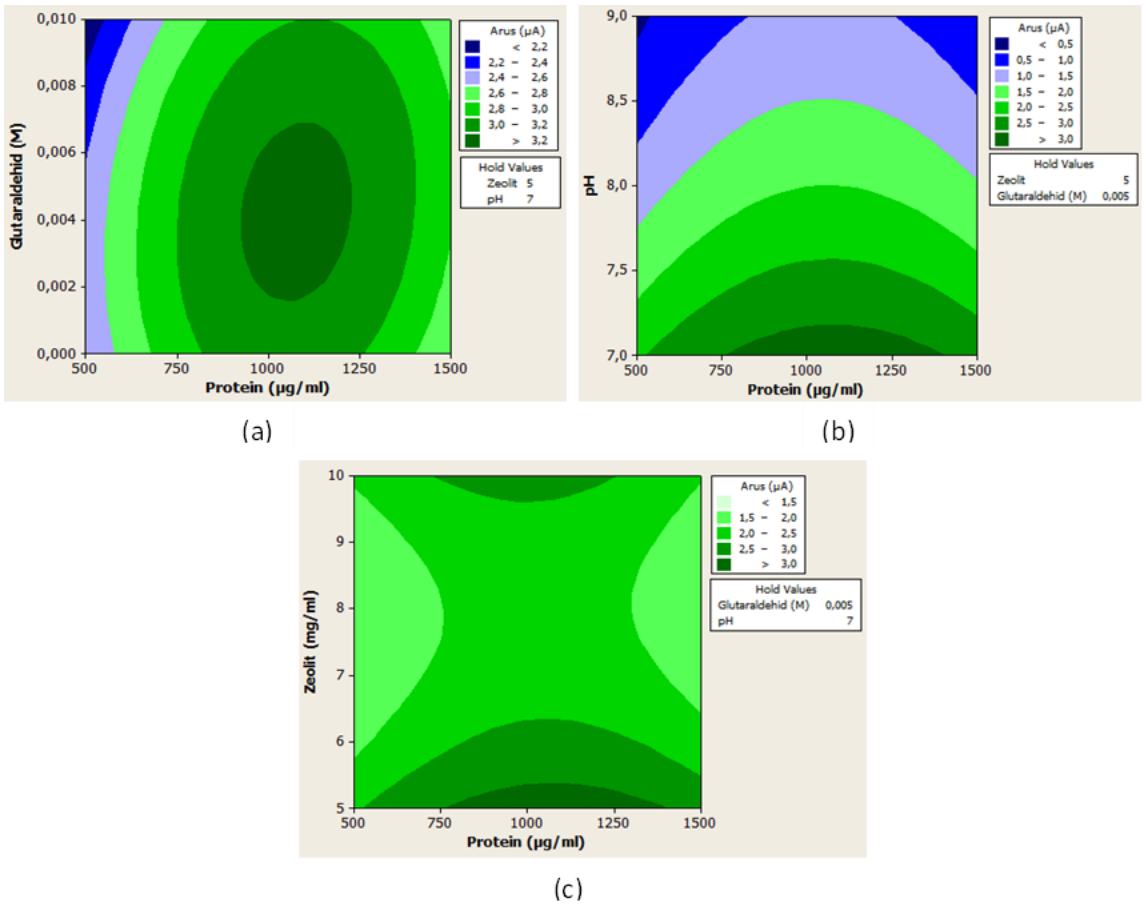

Figure 5. The effect of glutaraldehyde concentration on protein concentration (a); the effect of pH on protein extract concentration (b); and the effect of zeolite concentration on protein extract concentration (c) on the change in peak current.

Figure 5b shows that the optimal condition for pH is at pH 7, with a protein concentration of approximately 1000 µg/ml. The optimal pH condition is crucial because enzymes are susceptible to pH. The pure SOD enzyme immobilized with glutaraldehyde crosslinking shows an optimum pH condition of 7 (Kocabay et al., 2012); (Karakaya et al., 2020). Figure 5c shows the effect of zeolite concentration, which results in the optimum condition at a concentration of 5 mg/ml. Zeolite, as an immobilizer for *D. radiodurans* protein extract can increase the signal's current (Iswantini et al., 2019). Zeolite has been widely used as a matrix in enzyme immobilization systems because zeolite can improve the catalytic properties of enzymes based on hydrophobic or hydrophilic interactions, electrostatics, and hydrogen bonds. The ability of zeolite to increase the biosensor response is influenced by the Si/Al ratio. The Si/Al ratio affects the biosensor response, with a higher Si/Al ratio leading to a stronger biosensor response (Soldatkina et al., 2019). The zeolite concentration increased to 7.5 mg/ml and 10 mg/ml, the signal current became lower. This is probably due to the form of the zeolite covering the electrode surface and making the electrode surface rigid (Khasanah et al., 2018).

Based on Figures 5a, 5b, and 5c, it is shown that each contour shows the optimum concentration for the *D. radiodurans* protein extract, which is 1000 µg/ml. These results probably show the higher the concentration of protein extract, the more saturated the enzyme will be in the matrix pores, which limits the reaction between the substrate and the enzyme (McDonald & Tipton, 2022). The protein extract from *D. radiodurans* contains several proteins besides SOD. These other proteins in the extract may interfere, making it more difficult for the substrate to react with the SOD enzyme (Iswantini et al., 2019).

Electrode Analytical Parameters and Stability

The effect of xanthine concentration on protein extract activity was measured using xanthine concentrations ranging from 0.1 to 0.8 mM. Figure 7 shows the relationship between xanthine concentration and the activity of protein extract crosslinked with glutaraldehyde and without glutaraldehyde. The *D. radiodurans* protein extract biosensor immobilized on CMC-G-Z with glutaraldehyde crosslinking shows a linear measurement range of 0.1–0.8 mM, with an R^2 value of 0.9905, while without glutaraldehyde, the range is also 0.1–0.8 mM, with an R^2 value of 0.9861. Limit of Detection (LOD) and Limit of Quantification (LOQ) obtained with glutaraldehyde are 77.84 µM and 259.50 µM, respectively, while without glutaraldehyde, the LOD and LOQ are 94.39 µM and 283.16 µM, respectively.

The protein extract activity on CMC-G-Z-glutaraldehyde is higher at xanthine concentrations than CMC-G-Z. This is because, when the protein extract is crosslinked with glutaraldehyde, the active sites are preserved on the carbon paste electrode, allowing them to fully interact with the substrate. In contrast, the active sites of the protein extract without glutaraldehyde are less stable on the carbon paste electrode.

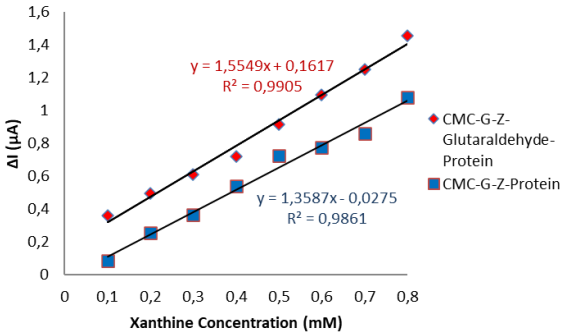

Figure 6. Linearity of the anion superoxide biosensor with glutaraldehyde crosslinking and without glutaraldehyde.

Linearity is another important factor, indicating the linear connection between the sample's concentration and the related analytical signal (Aldiansyah et al., 2025). The difference in linearity between the protein extract immobilized on CMC-gelatin-zeolite crosslinked with glutaraldehyde provides a wider linear range and higher current compared to the non-immobilized extract. This indicates that the immobilization of the protein extract can enhance its activity. In another research, *Clostridium difficile* used as a producer of Mn-SOD resulted in a measurement range of 0.1352–1.160 mM (Ye et al., 2014). Biosensors using pure SOD enzyme produce a wider measurement range and a lower detection limit compared to biosensors that use microbes. This is because the composition of microbes is more complex than pure enzymes, which results in slower diffusion between the desired protein and the substrate, making it more difficult to form the catalytic reaction (Ratautė & Ratautas, 2024). The difference in electrode types, depending on the material's electrical conductivity, can also affect the results of the measurement range and other analytical parameters (Thandavan et al., 2013).

In this study, the stability of the electrode was tested using the same electrode, which was evaluated at the initial time and subsequently over time. The stability of the electrode can be calculated by comparing the current at each hour (time) with the initial measurement current at hour 0. In Figure 6, the stability of the biosensor with protein extract immobilized using the crosslinking agent glutaraldehyde results in an activity of *D. radiodurans* protein extract of 67.74% for 24 hours. In contrast, without glutaraldehyde, the activity is 46.38% for 24 hours. Glutaraldehyde binds the protein extract, stabilizing the position of the active site, which leads to more optimal performance of the protein extract in reacting with the substrate (Karakaya et al., 2020); (Kocabay et al., 2012); (Ionescu, 2022); (Moya et al., 2020). Based on the graph results in Figure 6, it can be shown that there is a decrease in current between the samples with and without glutaraldehyde. This is due to the enzyme's nature, which reacts with the substrate based on the key-lock principle. When the active sites on the enzyme surface are utilized, enzyme activity is reduced (Ionescu, 2022); (Moya et al., 2020).

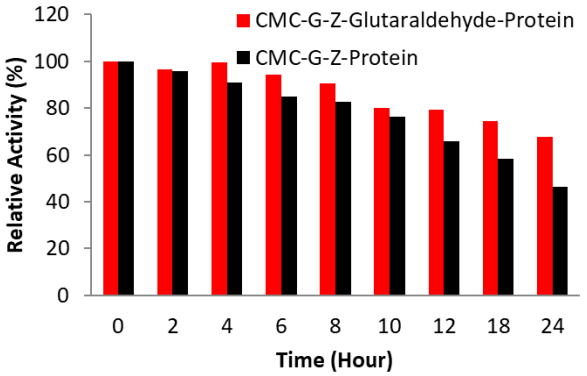

Figure 7. The stability of the anion superoxide biosensor with glutaraldehyde crosslinking and without glutaraldehyde.

The stability of the protein extract is not only influenced by the immobilization process but also by the presence of other proteins in the extract, such as peptidase. Peptidase is an enzyme that can break peptide bonds in proteins, and therefore, the presence of peptidase may potentially damage the superoxide dismutase enzyme within the crude protein extract (Dalmaso et al., 2015).

CONCLUSIONS

The superoxide anion radicals biosensor using *D. radiodurans* protein extract immobilized in a CMC-gelatin-zeolite matrix with glutaraldehyde crosslinking resulted in greater electrode stability over time than without glutaraldehyde. The technique of cross-linking glutaraldehyde immobilization with *D. radiodurans* protein extract increases the analytical performance of the biosensor by generating a higher current in the measurement against the xanthine substrate. Further research is needed to remove other proteins, especially peptidase in the *D. radiodurans* protein extract.

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Competing Interests: The authors declare that there are no competing interests.

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