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Antioxidant Biosensor based on Deinococcus radiodurans Biofilm immobilized on Screen-printed Carbon Electrode (SPCE) Surface

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ABSTRACT

Antioxidant biosensor based on *D. radiodurans* biofilm has been investigated in this research. The biofilm producing SOD enzymes were immobilized on SPCE surface. Optimization of experimental measurements were carried out by the response surface method. The optimum value obtained was at the buffer pH 7, suspension pH 6, and optical density (OD) 0.5. The morphology of SPCE surfaces was characterized by SEM. The optimum result was used to determinate analytical performance, including linearity, sensitivity, limit of detection (LOD), limit of quantity (LOQ), precision, selectivity, stability, and repeatability. Linearity was achieved in the xanthine concentration range of 0.1-0.6 mM with the equation y = 40.79x + 57.173 and R^2 = 0.99. The apparent Michaelis-Menten constant K_M was evaluated. It was found that the biosensor had a low K_M of 40 µM. LOD and LOQ respectively 40.8 µM and 123.7 µM with sensitivity 40.79 µA mM^{-1}. Precision showed that RSD was less than 5%. Stability was measured for 35 days and retained 90% of current for the period. Repeatability showed RSD ≤ 5%. The selectivity of this method still needs to increase. In conclusion, antioxidant biosensor based on *D. radiodurans* biofilm may be used to measure the capacity of antioxidant products practically.

Keywords-- antioxidant biosensor, biofilm, *D. radiodurans*, SOD, SPCE.

I. INTRODUCTION

Antioxidant products including foods, drinks, and any other products play an important role in decreasing free radicals in the human body. The performance of products can be known by their strength in removing free radicals. For that reason, quantitative control needs to be carried out so that we can identify the antioxidant quantity exactly, easily, and rapidly. The general methods have been used to measure such as spectrophotometry [1] and chromatography [2]. However the methods have several disadvantages such as high cost, time consuming, highly skilled worker, hard sample preparation and extraction in the many cases. A nowadays electrochemical method based on biosensor was developed. The measurement of antioxidant quantity using this method was more efficient than spectrophotometry since it has advantages as well as high sensitivity, response time, accuracy, low cost, and simple preparation [3]. Some researchers utilized electrochemical method based on biosensor for antioxidant quantity measurement. Modification of an electrode contained encapsulated tirosinase with a cellulose acetate dip-coating film. Sensitivity and LOD respectively, were 5.68 Ω and 200 µM [4]. Immobilization of tirosinase in a derivated copolymer of N-nonylcarbazole on Pt electrode enhanced electron transfer so that the active side of enzyme could increase. LOD was achieved 0.02 µM and electrode stability can be retained 4 months with current response almost 100% [5]. In conclusion, this method deserves to determine the antioxidant quantity [6].

One of the enzymes used in antioxidant biosensor is superoxide dismutase (SOD) because the enzyme has the good activity and sensitivity to analyze. Nevertheless, it has very expensive to routine test in order that microbes can be as an alternative choice in biosensor design where can be cultured [7-8]. D. radiodurans could be used as bioreseptor in this method as the bacterium has high stability [9].

Many developments of antioxidant biosensor, such as zeolite nanoparticels were made to immobilize the protein extract of D. radiodurans. LOD was obtained about 0.05 µM. However, electrode stability only retained 59% of its initial activity for 8 hours [10]. A matrix of carboxymethylcellulose-gelatin-zeolite cross-linked glutaraldehyde to immobilize protein extract of D. radiodurans and LOD was achieved about 67 µM. Nevertheless, electrode stability, less enhanced cause it could

be retained 67% of its initial activity for 24 hours [11]. As the result, we can know that the analytical performance and the stability of antioxidant biosensor have to increase. The reason is supported by bacteria lifetime, hence biosensor can be used to detect antioxidant quantity in a long time. Indeed the electrode used has limited surface area as the electron transfer process is not optimum. Thus, this research aims to form D. radiodurans biofilm on SPCE surface which can enhance the analytical performance and the stability of biosensor. Consequently, it can be used as a practical method to measure antioxidant quantity.

II. EXPERIMENTAL PROCEDURES

X0626 Sigma xanthine and X2252 sigma xanthine oxidase microbial (7 U/mg) were purchased from Sigma Aldrich. Electrode used was SPCE refs. 110 (DropSens Spanyol) and SPCE DRP-CAC71190 Metrohm connector. The electrochemical measurement was performed at room temperature utilizing potensiostat/galvanostat eDAQ with three-electrode system completing Echem v.2.1.0 and Origin pro 7.0 software. OD measurements were performed on the microplate reader BIORAD iMark at the maximum wavelength 595nm. Scanning electron microscopy (SEM) was investigated JEOL JSM-6360LA.

2.1 Isolation and Cultivation of D. radiodurans.

Tomato-sauce was added 100 µL distilled water. It was spread in GTY agar containing bactoagar 1% (b/v); glucose 0.5% (b/v); trypton 1% (b/v); yeast extract 0.5% (b/v) [12]; K_2HPO_4 0.5% (b/v); NaCl 0.5% (b/v) [13]. After that, the medium was given UV for 15 minutes. Furthermore the medium was incubated for ±48 hours at 37 ºC so that it resulted an isolate. Then the isolate obtained was grown on the medium plate and was incubated for 48 hours at 37 ºC [12]. It produced a colony which was observed at a magnification of 1000 times with the help of a microscope. After that, it was characterized by electrochemical method of cyclic voltammetry. D. radiodurans strains were grown in the GTY liquid. Next the strains were incubated ±20 hours at 37 ºC using a shaker incubator. Then, cultures were taken and were entered in an eppendorf containing 2 mL of 50 mM phosphate buffer solution (PBS) with pH 7. The eppendorf was shaken until the solution was homogenous. After that, 200 µL of suspension was measured OD by microtiter reader 595 nm until OD achieved was 0.6. The buffer was used as a blank. Then, centrifugation was carried out by velocity 7000 x G, 4 ^oC for 5 minutes. The pellet formed was separated from the supernatan and was washed using 1 ml of buffer. Centrifuged process was carried back out. The washing of PBS was worked triplicate. The pellet was diluted in 1 ml of PBS the it was shaken. The suspension resulted was used to form biofilm on the SPCE surface.

2.2 The Electrochemical Measurement

The suspense about 70 µL was dripped on the working electrode of SPCE. Then, the electrode was at the room temperature for 3 days. After that, the electrochemical measurement of cyclic voltammetry was carried out using eDAQ potentiostat (Ecorder 410) completed Echem v2.1.0 software. The electrode was formed carbon with diameter 4 mm as the working electrode, silver (Ag/AgCl) as reference electrode, and carbon as auxiliary electrode. The performance of measurement was following: Mode Cyclic, Initial -600 mV, Final -600 mV, Rate 250 mV/s, Step W 20 ms, Upper E 900 mV, Lower -600 mV. The measurement was worked with xanthine and xanthine oxidase in PBS as analyte, then PBS was used as blank.

2.3 The optimization of antioxidant biosensor

Optimization was worked by a variable combination at buffer pH (6-8), suspension pH (6-8), and OD (0.5-0.7). The method used to get the optimized condition was the response surface method. The variable combination was entered into a statistical software called as MINITAB 16. Then the combination would be explored which were yielded several factors of free variable combination. The experiment was carried out based on the factors.

2.4 Characterization of Biofilm D. radiodurans

The characterization of biofim of the SPCE surface was carried out by SEM. It aims to see biofilm and form of D. radiodurans colonies

2.5 The determination of kinetic performance

The determination of kinetical performance of SOD enzyme secreted by D. radiodurans based on the power of oxidation current yielded from the biosensor measurement with the xanthine concentration range such as linearity determination. The kinetical performance of D. radiodurans biofilm as K_M and V_{max} was determined by derivation of Michaelis-Menten Lineweaver-Burk equation [14].

2.6 The determination of analytical performance

The determination of analytical performance useful to know that the measurement through the several requirements. These were linearity, LOD, LOQ, precision, sensitivity, selectivity, stability, and repeatability [15].

III. RESULTS AND DISCUSSION

3.1 Isolation and cultivation of D. radiodurans

D. radiodurans was isolated by tomato-sauce because the sauce was be going process of gamma ray. The aim of the radiation was to kill all kinds of most microbes, especially bacteria that are resistant to radiation. In a research, the bacterium can survive gamma ray doses of about 14 kGy [16]. Medium of D. radiodurans growth is HTR as it contains many nutrients needed to the bacterium. The productivity growth of the bacterium needs some requirements, these are a carbon, nicotinamide acid, sulfur, nitrogen, and manganese source [12].

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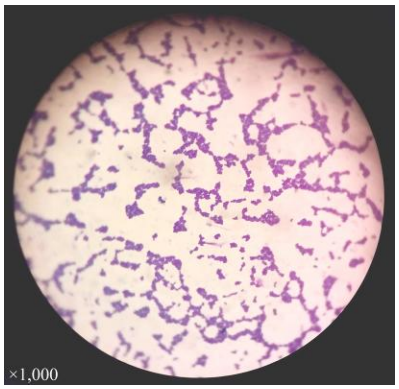


Figure 1. Micrograph of cell form of D. radiodurans with help a microscope. Coloration of cells using crystal violet 2%

Before the process of growth, the bacteria in the medium were radiated of UV for 15 minutes to prevent the possibility of contaminating the other microbes. The bacteria could resist to ionizing radiation, UV, drying, and several damage DNA agents [17]. The resistance could be caused possibly by the Mn^{2+} presence in the bacteria which would protect the proteins from the oxidative stress. Thus, these have high survival. D. radiodurans can produce SOD. The enzymes act as an antioxidant system which strike radical oxygen species damaging. The bacteria contain more manganese so the more SOD is Mn-SOD [18]. A study reported that DNA of D. radiodurans play an important role cause it can be protected of ROSs by Mn^{2+} [16]. The optimum temperature and pH for growth of D. radiodurans were 37 °C and 7 respectively.

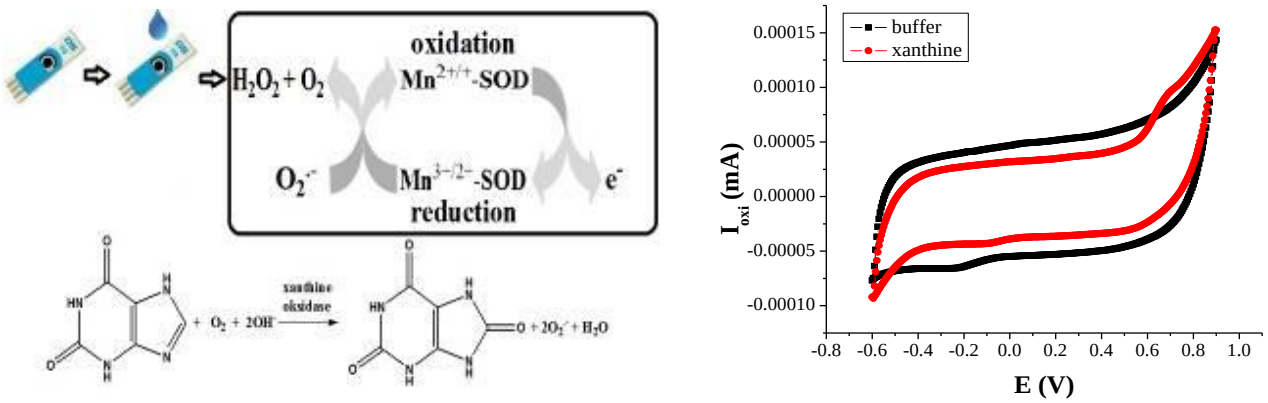


Figure 2. The construction of antioxidant biosensor measurement. (a) The reaction for formation and dismutasion of superoxide radical by D. radiodurans SOD. (b) Cyclic voltamogram of buffer phosphat (black); xanthine and xanthine oxidase (red).

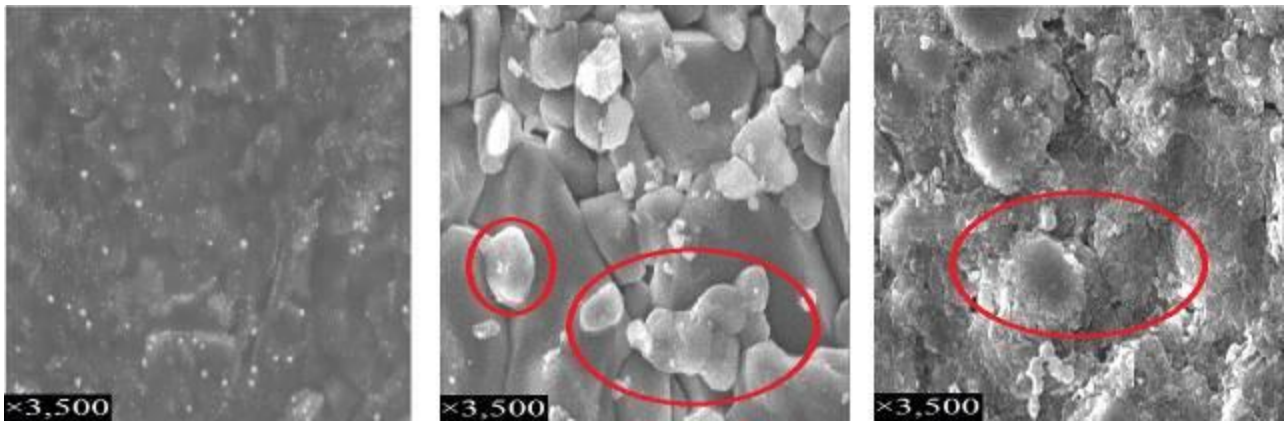
These could accelerate bacterial growth. Based on Fig.1, microscopic observations showed a cocus shape, grown as single or tetrad-shaped cells. This was suitable to the morphology of D. radiodurans in a study that this bacterium was coccus and single-celled or tetrad-shaped [19].

3.2 Electrochemical Measurement

Suspensions of D. radiodurans were immobilized on the SPCE surface. The suspensions were allowed for 3 days at the room temperature to form a bacterial biofilm. The room temperature is used to allow bacteria to form biofilms under extreme conditions. A report showed that D. radiodurans DSM 20539 could form biofilms [20]. It was also supported a review that D. radiodurans KanR could form biofilms [21]. The immobilized mechanism occurred involves physical adsorption because carbon electrodes have pores which possess functional groups.

It can interact with biofilm matrix called extracellular polymeric substances (EPS) containing proteins, polysaccharides, extracellular DNA (e-DNA), peptidoglycans, lipids and phospholipids [22]. Interaction utilizes a combination of Van der Waals and hydrophobic forces, hydrogen bonds, and ionic force [23]. The use of bacteria biofilm as molecule recognition is more efficient than enzyme. As we know that enzyme needs a supporting material to make immobilized on the working electrode. If the bacteria form biofilm, so it does not need supporting material. It was caused by their matrix can act as supporting material.

Measurements were carried out using 2.1 mM of xanthine solution and 0.1 U/ml of xanthine oxidase enzyme solution which were dissolved in PBS. Vast potential windows were set at -600 mV to -600 mV because the potential ranges the analytical oxidation peaks could be observed good.



(a) (b) (c)

Figure 4. SEM images of SPCE surface. (a) Clean surface of working electrode (Oviedo, spain). (b) Bacteria bound before measurement for 3 days. (c) Biofilm matrix of bacteria bound after measurement for 35 day

In addition, the ranges were not disturbed by redox peaks of electrolytes. The xanthine and xanthine oxidase reactions undergone enzymatic reactions and yielded superoxide radicals (Fig. 2a). Superoxide radicals were catalyzed by the SOD bacteria immobilized on the electrode surface and were resulted oxidation peak current of H_2O_2 in the cyclic voltammogram (Fig. 2b). Oxidation peak in this study showed more than +600 mV. The cyclic rate used was 250 mV/s. Each cyclic rate was made triplicate to get a stable voltammogram. Detection of antioxidant product is based on measuring the H_2O_2 quantity, the oxidation of H_2O_2 often requires higher working potential, usually over +600 mV (Monasik et al. 2012), 650 mV (Campanella et al. 2002), and over +0.5 V (Di et al. 2003).

3.3 Optimization of antioxidant biosensors

The optimization was performed using a response surface method using MINITAB 16. The tested treatments to the current were buffer pH, suspension pH, and OD. Based on Fig. 3, the optimum value of the optimization of biosensor measurements was obtained at a pH of buffer 7, pH of suspension 6, and OD 0.7. This result was used in determining analytical performance, but OD used was OD 0.5 because it has higher biofilm stability than OD 0.7. Bacterial biofilm cells that have high density on the SPCE surface make the cells escape easily.

3.4 Characterization of Biofilm D. radiodurans

This characterization was performed to investigate D. radiodurans cell form. In addition, this treatment was also intended to see biofilms formed by bacteria on the SPCE surface. The morphology of the surface of the working electrode for SPCE without immobilizing with bacteria (Fig. 4a) shows black lumps. Fig. 4b shows aggregation of bacteria immobilized on the surface after hushed for 3 days and its shape is round. This is in accordance with the results of [21] that the morphology of cell shape and biofilm D. radiodurans are round and clustered. After 35 of days (Fig. 4c), the bacteria through growth and development process so that they form the biofilm matrix covering with the entire surface of the working electrode.

3.5 Determination of SOD D. radiodurans Kinetic

The measurements of kinetic parameter were performed using the derivation of Michaelis-Menten equation, the Lineweaver-Burk equation. SOD Kinetics in this study were determined using xanthine concentration series as linearity test, ie 0.1 mM-0.6 mM with 0.1 mM interval. The equation curve is made by the relationship between 1/[xanthine] on the x-axis and 1/oxidation peak current on the y-axis (Fig. 5).

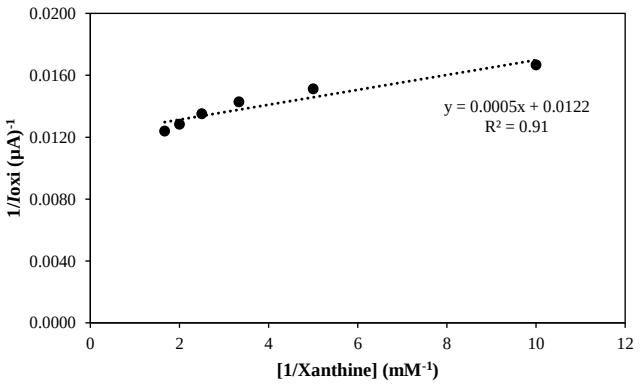


Figure 5. Lineweaver-Burk curve relationship between 1/[xanthine] and $1/I_{oxidation}$

Based on the equation (Fig. 5), the values of K_M and V_{max} obtained to the equation are 40 µM and 81.97 µA respectively. The SOD enzyme produced by D. radiodurans will reach half of its maximum reaction rate if the antioxidant substrate concentration is about 40 µM with a maximum oxidation current of 81.97 µA. If the K_M value is low so the enzyme has a high affinity to bind the substrate. The low

substrate concentration can make the enzyme to be saturated. Whereas if the value of large K_M so the enzyme has a less affinity to bind the substrate where it needs more substrate to saturate the enzyme.

Table 1. Comparison of kinetic parameter values

Kinetic factors	SOD of *D. radiodurans* biofilm (present study)	Pure enzyme [9]	Protein extract of *D. radiodurans* [9]
K_M (mM)	0.040	0.3694	0.1930
I_m (µA)	81.97	0.0803	0.1089

The K_M SOD value of bacterial biofilm obtained in this study is smaller than the pure enzyme K_M and SOD protein extract of D. radiodurans (Table 1). While the value of I_m in this study is greater than I_m pure SOD and protein extract of D. radiodurans. This suggests that the formation of D. radiodurans biofilms and the useful of SPCE as electrode can significantly increase the anodic current peak. Pure enzyme had great sensitive over so that K_M value was lower than SOD of the bacterium. It was because bacterium matrix would bother and block the presence of sample (Dhull et al. 2013). In fact of this study, SOD of the bacterium had lower K_M than pure SOD. That happened because electrode used was SPCE which had larger diameter of surface area than conventional electrode. As the result, volume of suspension loaded was plenty [24]. Besides, SPCEs had low background currents and vast potential windows [25-26].

3.6 Determination of analytical performance

The determination process of analytical performance, simulated samples of xanthine and xanthine oxidase were used to produce superoxide compounds. There are several analytical parameters determined, for example linearity, precision, LOQ, LOQ, sensitivity, selectivity, stability, and repeatability.

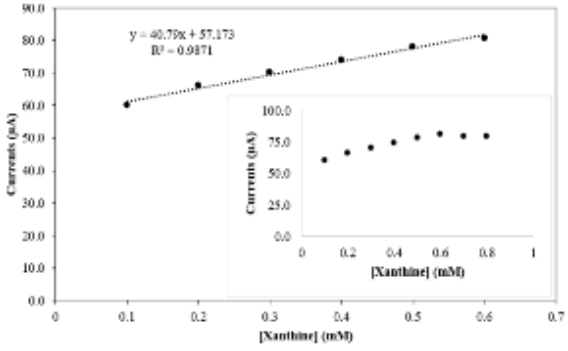


Figure 6. The linearity curve of xanthine concentration to the current response. Inset is when the substrate fulfills all parts of active site enzyme at 0.6-0.8 mM of xanthine concentration

Linearity measurement of xanthine concentration range 0.1 mM to 0.6 mM with 0.1 mM intervals obtained by equation y = 40.79x + 57.173 with R^2 = 0.9871. The value of R^2, which is close of 1 means the anodic peak current generated linearly to the increase of analytical concentration (Fig. 6). It meets the criteria for quantitative analysis. The antioxidant levels in a sample can be determined by measuring the current response. The response obtained is achieved into the linear equation so that the value of x as the antioxidant sample concentration can be known. The values of LD and LK obtained in the oxidation reaction were 40.8 µM and 123.7 µM respectively.The low LOD and LOQ values show the sensitivity of excellent method so that the biofilm SPCE used can be said to be sensitive for the xanthine oxidation reaction. The sensitivity value obtained in this study amount of 40.79 µA mM^{-1}, which intended that any change in sample concentration of 1 mM would change the current response of 40.79 µA. The sensitivity value indicates that this biosensor has good sensitivity. The measurement using biosensor with voltammetri cyclic has a good precision (RSD ≤ 5%).

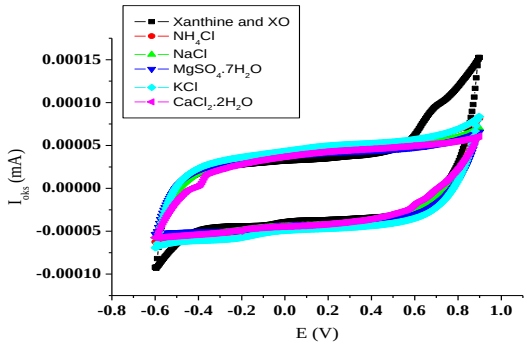


Figure 7. Voltammogram of selectivity measurements against interfferen ions

Selectivity determination was done to investigate the response of bacterial SOD to interferen ions. The results showed that the voltammogram of each ion did not show significant peak change (Fig. 7). This means that the ions of interference do not interfere with the measurement. In other words, this bacteria-based biofilm of antioxidant biosensor has good selectivity.

The stability of biosensor in this study was done by measurement of the SOD activity through the peak oxidation by superoxide antioxidants using the optimum SPCE biofilm every 7 days for 35 days.

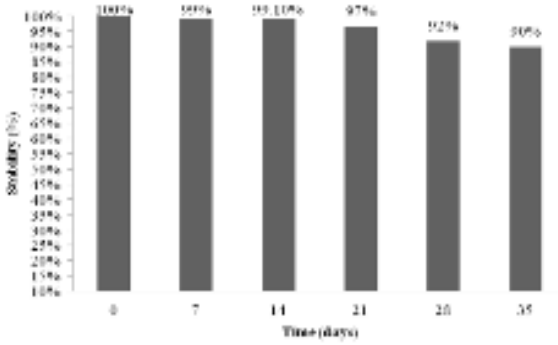


Figure 8. Stability of biosensor antioxidant since 35 of days

The results showed that the immobilized D. radiodurans biofilm on the SPCE surface remained stable after the 35 days of measurement with 90% residual activity (Fig.8). The optimum SPCE biofilm made has good stability with an RSD less than 5%, and 0.5% at the oxidation peak.

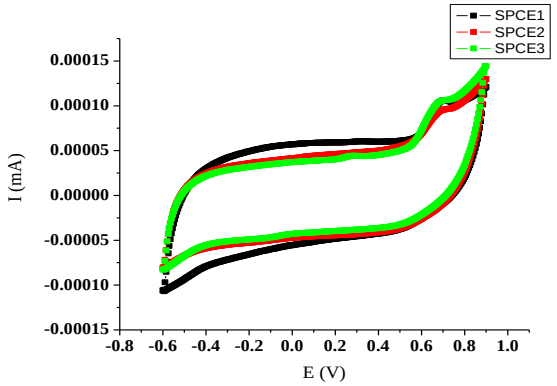


Figure 9. Voltamogram repeatability of optimum condition of the antioxidant biosensor

The repeatability measurements showed that the optimum biofilm SPCE has a good enough repeatability. The yielded voltammogram with 3 different biofilm SPCE was identical, which were having an oxidation peak at a potential of 0.750 V. The oxidation peaks resulting for 3 SPCE biofilms were at 106.73 µA, 106.27 µA, and 106.43 µA (Fig. 9). The difference of oxidation peak for 3 SPCE can be caused by the difference of cell biofilm density on the SPCE surface so that the amount of SOD produced is also different. Thus, the resulting oxidation peak will be different. The RSD value was less than 5%. This indicates that homogeneity of bacterial suspension in the SPCE biofilms is good and there is no significant change for measurement with different biofilm SPCE. In conclusion, antioxidant biosensor based on D. radiodurans biofilm immobilized on SPCE surface showed the great analytical performance. Stability of electrode retained about 90% for 35 days. Accordingly, this method is possible to measure quantity of antioxidant products practically and cheaply.

IV. CONCLUSION

In conclusion, antioxidant biosensor based on D. radiodurans biofilm immobilized on SPCE surface showed the great analytical performance. Stability of electrode retained about 90% for 35 days. Accordingly, this method is possible to measure quantity of antioxidant products practically.

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