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Optimization and Validation of Electrochemical Biosensor Based on *Pseudomonas* sp. Biofilm Immobilized on Screen-Printed Carbon Electrode in Detecting Benzene

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Abstract. Benzene is known as one of the hazardous compounds potentially interfering the health and polluting the environment. Generally, detecting benzene still requires long analysis time and expensive costs. Several methods have already reported of benzene detection including raman spectrometry, liquid chromatography, chemiluminescence, and electrochemical detection. Among electrochemical detection methods, electrochemical biosenseor is one of the most prospective in detecting benzene. Therefore, this study aimed to evaluate the analytical performance of a biosensor with microbes as the biodetection element. *Pseudomonas* sp. biofilms which produces benzene dioxygenase enzyme and then immobilized on a Screen-Printed Carbon Electrode (SPCE). The results of the optimization of the biosensor obtained a benzene concentration of 3 mM, a bacterial density of 1.4×10^{11} cells/mL and suspension pH of 7.5. The optimization results were used to measure the analytical performance of the biosensor. The value of analytical performance produced on linearity was in the measurement range of 0.1 - 3 mM, the equation y = 7.4118x + 80.048 with R^2 = 0.9744. The detection limit and quantity limit are 0.5630 mM and 1.8767 mM respectively, with a sensitivity of 7.4188 µA/mM. The precision obtained shows that the SPCE biofilm method has moderate accuracy with a %RSD value below 5%. The selectivity of this method still needs to increase, but the stability of the benzene biosensor increases up to 35 days with an activity of 100.36%. This indicates the immobilization of *Pseudomonas* sp. had potency as an alternative method for detecting benzene and it can be developed for a prototype.

Introduction

Benzene is an aromatic hydrocarbon compound with one aromatic ring component. Benzene does not only have negative impact on people's health, but also pollutes the environment. Benzene could contaminate soil, surface water, sediment, and groundwater. Conventionally, laboratory techniques use more sophisticated instruments such as HPLC, UPLC (Vinci *et al.*, 2013), and Raman spectrometry (Moreau and Rinnert, 2015) for hydrocarbon compounds analysis and determination. Those methods are able to provide good accuracy, yet they are costly, time-consuming, and generating potential unnecessary solvent waste. Therefore, a new approach is needed for *in situ* analysis, thus it promotes the green analytical chemistry application for immediate results. In the other hand this new technique should be user-friendly, accurate, faster, and sensitive. A promising developing method in this field is biosensor.

Biosensor is an analytical method using biological components as receptor-altering elements of analytical signals, such as: enzymes, antibodies, DNA, cells, and microorganisms (Kivirand *et al.*, 2013). The biosensor technology is currently growing rapidly in the fields of health, medication and environmental (Moretto & Kalcher, 2014). The electrochemical biosensor in detecting glucose with *E. coli* as its bioreceptor (Iswantini *et al.*, 2011) and

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potentiometric-based biosensor in detecting toxicity in water through oxidation of ammonia by bacteria (Zhang *et al.*, 2013) had been reported. Enzyme-based biosensors are also able to be used for detecting pollutants (Nigam & Shukla, 2015). Biosensors offer high specificity, however, the techniques used are still very complex and expensive.

Electrochemical biosensor is widely applied since its simplicity to miniaturize. The electrochemical biosensor is the analytical tool that converts biological responses into electrical responses (Bhalla *et al.,* 2016). The important component in the transmission of signals in this electrochemical biosensor is the electrode. There are various electrodes, one of which uses Screen-Printed Carbon Electrode (SPCE).

The biological component which can identify benzene is benzene dioxygenase (Obayori and Salam, 2010). However, using pure enzymes is expensive. In spite of that, microbes are able to produce extracellular enzymes, moreover the enzyme is still inside its native environment, minimizing the enzyme denaturation. Le Digabel (2015) had measured water quality from hydrocarbon pollutants using *Pseudomonas putida*. The microbes were immobilized on SPCE to form a biofilm. Benzene is able to be degraded through oxidative degradation, where some electrochemically active compounds; like cathecol, phenol, and many other similar compounds sharing similar structure (Liu et al., 2014). Considering the potency of biosensor as a *in situ* monitoring technology for the pollutant measurement, the authors were interested in developing the method of a biofilm-based electrochemical biosensor to determine benzene pollutants using Indonesian biodiversity.

Experimental

Materials and Method

Materials

Oil sample (LIPI collection sample from Balikpapan, Kalimantan), benzene 97.7% (Merck), phosphate buffer solution 50 mM pH 6.8, aquadest, yeast extract (Difco™), tryptone (Himedia, ref RM 014), NaCl (Himedia, ref RM 3949), agar (Himedia, ref RM 026), ethanol 70% and 96%, *Pseudomonas Isolation Agar* (PIA) (Difco™)**,** *plastic wrap*, and aluminium foil.

Cultivation of *Pseudomonas* sp.

Cultures of *Pseudomonas* sp. were taken and were entered in an Eppendorf tube containing 2 mL of 50 mM phosphate buffer solution with pH 6.8. The Eppendorf tube was shaken by vortex until the solution became homogenous. After that, 200 µL of suspension was taken and OD (Optical Density) was evaluated by microtiter reader 600 nm until OD achieved was 0.7. The buffer was used as the blank. Then, centrifugation was carried out by velocity 7000 rpm, 4 °C for 5 minutes. The pellet formed was separated from the supernatant and was washed using 1 mL of buffer. Centrifuged process was carried back out. The washing of phosphate buffer was worked triplicate. The pellet was diluted in 1 mL of phosphate buffer the it was shaken. The suspension resulted was used to form biofilm on the SPCE surface.

The Electrochemical Measurements

Pseudomonas sp. cells in the phosphate buffer were immobilized onto the surface of the working electrode of 100 µL, then incubated for 2 days at room temperature and protected from sunlight in a dry place. 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 of 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 -1 V, Final +1 V, Rate 250 mV/s, Step W 20 ms. The measurement was worked with 3 mM benzene solution in 50 mM phosphate buffer with the pH of 6.8 as the analyte, then phosphate buffer solution was used as blank.

Characterization of Biofilm *Pseudomonas* sp.

The characterization of biofim of the SPCE surface was carried out by Scanning Microscope Electron (SEM) JEOL JSM-5310LV. It aimed to check the biofilm and form of *Pseudomonas* sp. colonies

Optimization of Biosensor Measurements

Three parameters should be unraveled for the optimum value in immobilization technique were the amount of bacterial suspension volume (1.4×10^9, 1.4×10^{10}, 1.4×10^{11} cell/mL), benzene concentration (0.75, 1.5, 3 mM), and the pH (6, 7, 8). Response Surface Analysis method from software Minitab 17 was used to obtain the optimum value of enzyme activity.

Validation of Biosensor Measurements

The validation useful to know that the measurement through the several requirements. These were linearity, LOD, LOQ, precision, sensitivity, selectivity, and stability

Result and Discussion

Cultivation of *Pseudomonas* sp.

Based on the isolation result, there were four pure bacterial isolates that grew on solid Luria Bertani (LB) solid medium. Each isolate was mixed into a phosphate buffer 50 mM with the pH of 6.8 and selected through the electrochemical biosensor method. Measurement were carried out using phosphate 50 mM buffer solution with pH 6.8 as blank and 10 mM simulated benzene solution as a sample. Shown figure 1.

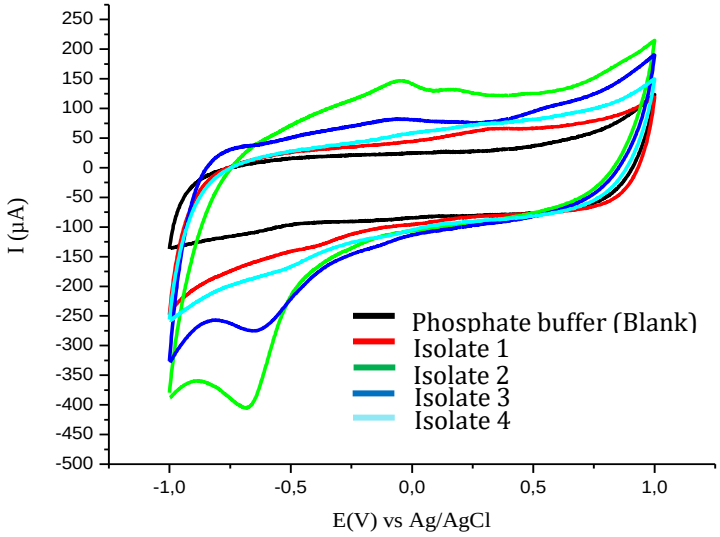

Figure 1. The cyclic voltammogram of four bacterial isolates of petroleum samples

The voltammogram results showed that all isolates produced oxidation peaks, yet some only produced both oxidation and reduction peaks. The presence of oxidation peaks indicates the occurrence of oxidation reactions on benzene by oxygenase-producing bacteria. Meanwhile, the reduction peak indicates the presence of reduction reaction due to dehydrogenase-producing bacteria. Selection results showed that the 4 isolates provided bacterial activity, however isolate 2 had the highest activity in detecting benzene. Isolate 2 produced oxidation peak as much as 120.1 μA.

Table 1. The value of peak current of oxidation and reduction of each isolate.

No.	Oxidation current (μA)			Reduction current (μA)		
	Blank	Sample	ΔI_a	Blank	Sample	ΔI_r
1	30.6	50.2	19.6	-110.3	-	-
2	30.6	150.7	120.1	-110.3	-490.8	-300.5
3	30.6	80.9	50.3	-110.3	-290.5	-180.2
4	30.6	70.4	39.8	-110.3	-	-

The selected isolate proved that the bacteria would grow in the media by using benzene as a carbon source and provided stronger resistance compared to the other three isolates. Furthermore, the selected isolate was characterized macroscopically and microscopically through gram staining method as shown at Figure 2.

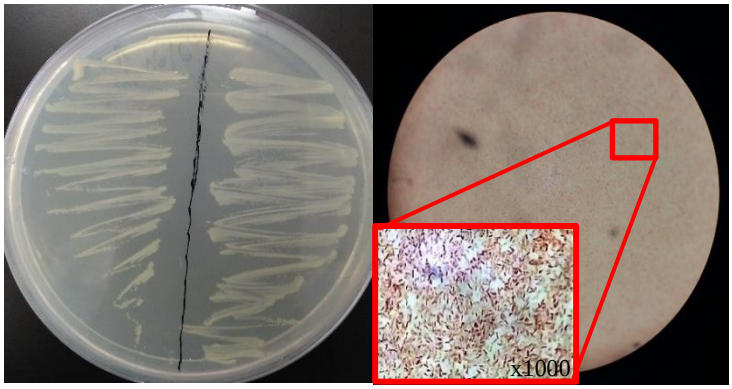

Figure 2. Bacterial colonies on the agar medium (left) and Micrograph of the morphology of *Pseudomonas* sp. at ×1000 magnification (right).

The purification results on Pseudomonas Isolation Agar (PIA) medium showed a large, smooth, flat surface and

greenish yellow colonies. Characterization of bacteria through Gram staining referred to Bergey's Manual Determinative Bacteriology seven edition. The selected microbe (isolate 2) had longitudinal form, short stature, Gram negative, aerobic, and rapidly well-grown on the surface with the temperature of 37 oC that resembled the family *Pseudomonadanceae*, genus *Pseudomonas*, species *Pseudmonas* sp. (Breed RS, et all. 1975). Pseudomonas species are known to be able to oxidize hydrocarbons aerobically.

The Electrochemical Measurements

The biofilm formation and growth test were used to find out how much time needed for Pseudomonas sp. to form biofilms. The average density reading of biofilm cell-forming through Microtiter Plate Biofilm Assays method was 0.211 for 2 days. Although with lesser OD, *Pseudomonas* sp still had the potential to form biofilms. Therefore, *Pseudomonas* sp. biofilm was formed on the SPCE surface to detect benzene compounds. The detection of benzene that signified the biofilm activity was carried out by measuring the oxidation current generated from the reaction between the substrate and the enzyme by cyclic voltammetry. The substrate used was the simulated solution of benzene and the enzyme used was benzene dioxygenase excreted by *Pseudomonas* sp. on the SPCE surface.

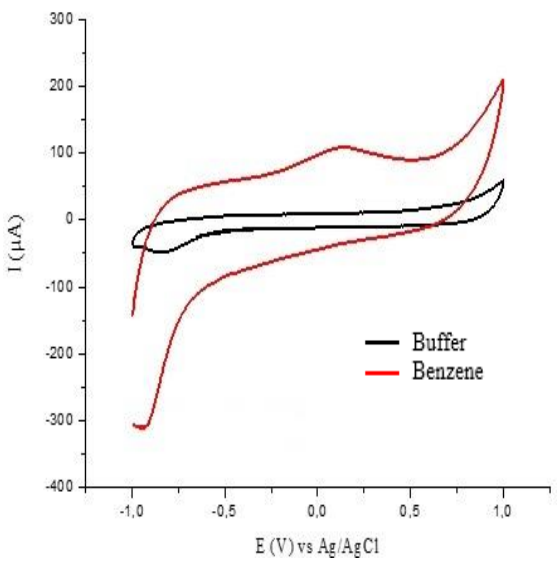

Figure 3. Cyclic voltamogram of buffer phosphat (black); benzene dioxidase (red).

The results of figure 3 showed that the oxidation current peak of 3 mM benzene in 50 mM phosphate buffer and 6.8 pH with SPCE biofilm was 108.7 μA and the peak was observed after scanning 11.40 seconds from the starting point. Lanyon (2006) had made a benzene biosensor by immobilizing 50 μL bacterial cultures with the 0.7 OD to the SPE surface and the results showed the highest measurement current of 25 μA. The results were much lower than the findings in this study. The success of *Pseudomonas* sp. in detecting benzene contamination required an understanding of the mechanisms of interaction between bacteria and benzene compounds as follows shown Figure 4 and 5.

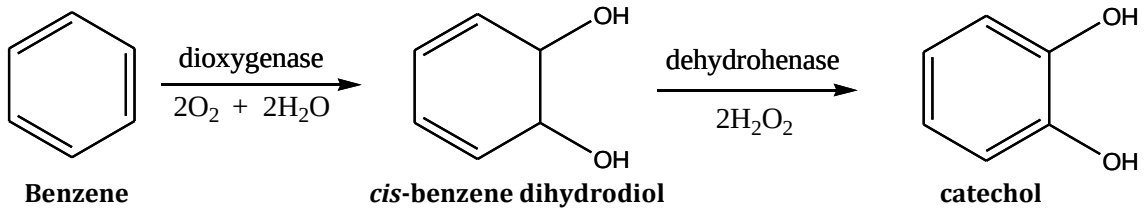

Figure 4. Oxidation and reduction reactions benzene (Xu *et al.,* 2003)

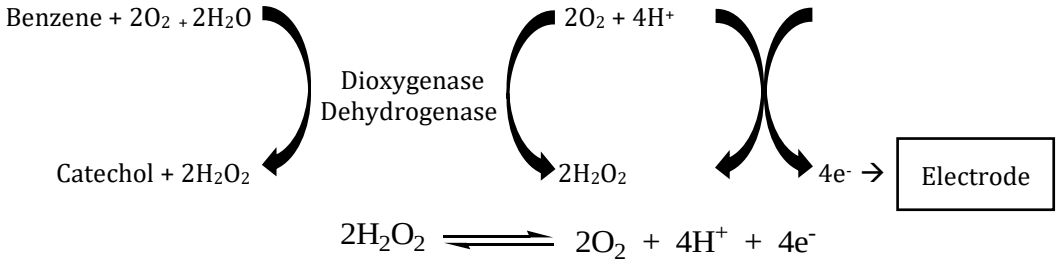

Figure 5. The mechanism of benzene oxidation through *Pseudomonas* sp. cells.

Catalysis was performed by phi bond breaking of benzene assisted by the oxygen existence as oxidizer to produce catechol and H_2O_2 (Figure 5). The reverse redox reaction between oxygen and hydrogen peroxide would produce electrons. The electron transfer created the current which then to be sent by the electrode to the transducer as signal wave. The transducer converted the signal into electromagnetic waves which can be read and recorded by the recorder as the benzene oxidation current peak.

Characterization of Biofilm *Pseudomonas* sp.

This characterization was done to investigate the biofilms formed by *Pseudomonas* sp. on the SPCE surface.

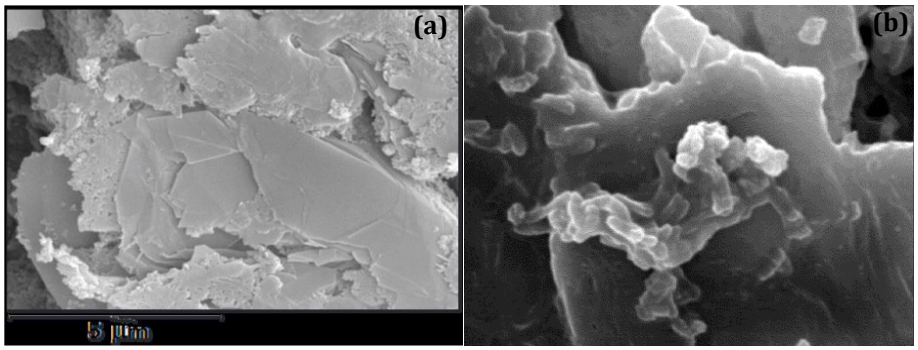

Figure 6. Morphology of the working electrode surface on the SPCE (Dropsens 2016) (a); the surface morphology of SPCE biofilms of *Pseudomonas* sp. after 15 days (b).

Figure 6 shows the comparison between the surface of the SPCE that has not been immobilized and the SPCE that has been immobilized. Figure 6a shows the blank electrode, without microbe immobilization. After 15 days of planting *Pseudomonas* sp. onto the surface of SPCE, the structure of the biofilm was clearly observed (Figure 6b). Even the shape of the bacteria forming the biofilm still was able to be seen well. It suggested that after the microbe's immobilization, the biofilm formed well on the SPCE surface. Based on SEM results in this study, the morphology of *Pseudomonas* sp. cell on the SPCE had a rod shape and colonized. This was in accordance with SEM results in the findings of Donlan *et al.* (2002) that *Pseudomonas* sp. biofilms had rod-shaped and which are immobilized on the SPCE surface will precipitate and form colonies following the gravitational force and then form the biofilm.

Biofilm is consisted by extracellular polymer matrix composed of polysaccharides, proteins, nucleic acids, and lipids (Flemming, et al., 2007). The biofilm protects the microbe cells from extreme environmental conditions, allowing the cells to remain alive for longer periods of time even without nutrients. Figure 6b still didn't show any obvious appearance of the extracellular matrices. Thus, we can safely assume that the biofilm is still on the building up stage. The biofilm hadn't achieved its maturation stage. A series of SEM analysis of different time frames are needed to observe the development of the biofilm from the microbe's colonies.

Optimization of Biosensor Measurements

Table 2 shows the optimization results for significant factors that influenced the current, namely bacterial density and benzene concentration with <0.05 p value. The positive coefficient meant the greater the value of the factor, the greater the value of the current generated.. The optimization was achieved at 3 mM benzene concentration, 1.4×10^{11} cells/mL and the 7.5 suspension pH. The results of biosensor optimization would be used in the next validation process.

Table 2. Analysis of factors influencing the current

Factor	Coefficient	*p* value
Constant	14.485	0.000
Bacterial density	18.180	0.000
Benzene concentration	19.250	0.000
pH suspention	4.180	0,074
R-Sq = 96.27%;	R-Sq(pred) = 75.71%;	R-Sq(adj) = 92.91%

Validation of Biosensor Measurements

There were 7 validation aspects studied in this research: linearity, detection limit, quantization limit, precision, sensitivity, stability, and selectivity.

The linearity was obtained around 0,1-3 mM with the linear equation y = 7.4118x + 80.048 (R^2 = 0.9744). Meanwhile, limit of detection and quantity were 0.5630 mM and 1.8767 mM. The low detection and quantity values showed the sensitivity of excellent method so that the biofilm SPCE used can be said to be sensitive for the benzene oxidation reaction. The sensitivity value obtained in this study amount of 7.4118 µA/mM which intended that any change in sample concentration of 1 mM would change the current response of 7.4118 µA. The sensitivity value indicates that this biosensor has good sensitivity. Whereas the precision showed that %SB value had a moderate accuracy value below 5% (RSD ≤ 5%) (Table 3)

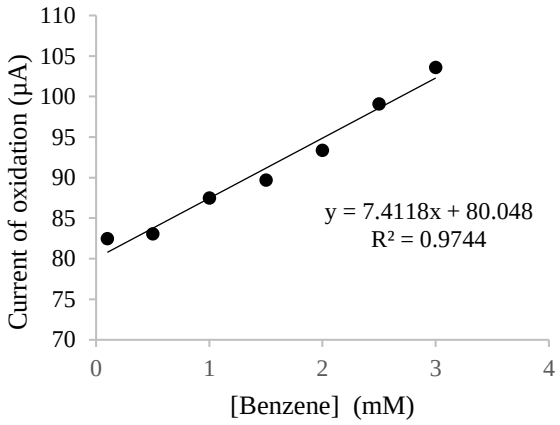

Figure 7. The linearity curve of benzene concentration to the current response.

Table 3. Analysis of precision.

[Benzene] (mM)	Replicate	I (µA)	Average (µA)	SD	RSD (%)
0.1	1	83.7	78.78	3.76	4.77
	2	82.5			
	3	78.7			
	4	78			
	5	75.9			
	6	73.9			
0.5	1	88.5	84.35	2.67	3.17
	2	83.1			
	3	80.4			
	4	83.9			
	5	84.9			
	6	85.3			
1	1	87.7	86.53	0.88	1.02
	2	87.5			
	3	85.4			
	4	86.3			
	5	86.1			
	6	86.2			
1.5	1	89.4	87.85	1.58	1.80
	2	89.7			
	3	86.5			
	4	87.5			
	5	88.3			
	6	85.7			
2	1	91.4	93.18	1.20	1.29
	2	94.3			
	3	94.3			
	4	93.8			
	5	92.1			
	6	93.2			
2.5	1	98.5	100.26	1.20	1.20
	2	99.1			
	3	101.5			
	4	101.2			

Table 3. Analysis of precision (continue).

[Benzene] (mM)	Replicate	I (µA)	Average (µA)	SD	RSD (%)
2.5	5	100.4	100.26	1.20	1.20
	6	100.9			
3	1	103	100.73	2.00	1.98
	2	103.6			
	3	99.5			
	4	99.5			
	5	99.5			
	6	99.6			
Average					2.18

Selectivity determination was done to investigate the response of bacterial to interferent ions. The results showed that the voltammogram of each ion did not show significant peak change (Figure. 8). This means that the interferences do not influence with the measurement. In other words, this bacteria-based biofilm of benzene biosensor has good selectivity. The selectivity coefficient showed the most influential contaminant compounds to the successive measurement respectively was NaCl> $CaCl_2$> KCl> NH_4Cl> $MgSO_4 \cdot 7H_2O$ = $CaCO_3$.

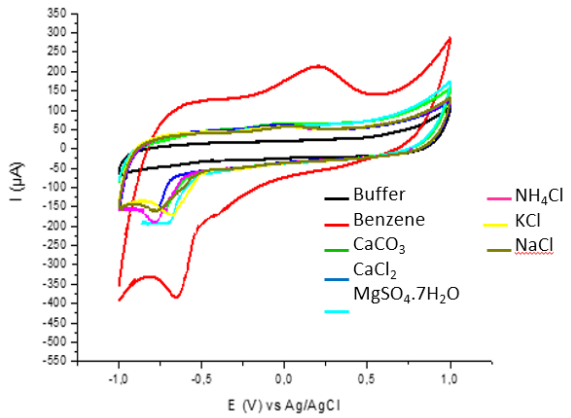

Figure 8. Voltammogram of selectivity measurements against interferent ions.

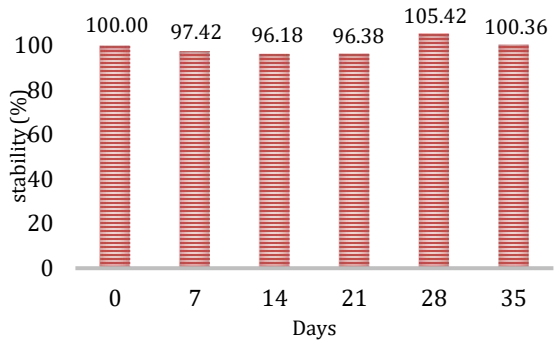

Figure 9. The bar chart of the stability of SPCE biofilm-based biosensor.

While the stability indicated that the biofilm of *Pseudomonas* sp. which was immobilized on the SPCE surface was still stable even stability increases at the 35th day after the first immobilization with relatively constant stability (Figure 9). The results showed that the immobilized *Pseudomonas sp*. biofilm on the SPCE surface remained stable after the 35 days of measurement with 99% residual activity (Figure 9). The optimum SPCE biofilm made has good stability with an RSD less than 5%.

Conclusion

In conclusion, benzene biosensor based on *Pseudomonas sp.* biofilm immobilized on SPCE surface showed the great analytical performance. Stability of electrode retained about 99% for 35 days. Accordingly, this method is possible to measure quantity of antioxidant products with lower cost and user-friendly.

Conflict of Interest

The authors declare that there is no conflict of interest.

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