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Cover Page Footnote

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Highly Sensitive Phenol Biosensor Utilizing Selected *Bacillus* Biofilm Through an Electrochemical Method

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Abstract

An eco-friendly phenol biosensor from *Bacillus* biofilm was prepared and investigated. The biofilm, which produced tyrosinase enzyme, was successfully immobilized on a screen-printed carbon electrode surface. A total of 72 *Bacillus* isolates were utilized because of their capability to produce tyrosinase enzyme in tyrosine media. Among them, *Bacillus* isolate code 100 was selected because it produced an adequate amount of tyrosinase enzyme and a high potentiostat current. The response surface methodology was also used to optimize the phenol sensing condition through an electrochemical method. Results showed that the optimum condition was achieved after 6 days on a phosphate buffer solution (pH of 8), with an optical density of 0.33. Furthermore, the limits of detection and quantification were 3.0 and 13 ng/L, respectively. The measurements of precision yielded a relative standard deviation of < 5%, which is remarkable. Although the biosensor material was used for 35 days, the current throughout was still maintained at 90%, indicating that the evaluated biosensor material has the potential to be used for phenol monitoring on environmental samples in the near future.

Keywords: *bacillus*, *biofilm*, *biosensor*, *electrochemical*, *phenol*

Introduction

Phenol is a harmful pollutant that is released to the aquatic environment from industrial activities and fuel processes. According to the regulation concerning the management of water pollution released in 2010 by the Indonesian Ministry for the Environment, the concentration of phenol compounds should be < 1 µg/L. Therefore, the researchers focused on phenol monitoring to maintain the environmental quality. Currently, phenol monitoring is conducted through various types of chemical and biological analyses. Although high-performance liquid chromatography is well known for its accuracy and is a sensitive protocol for phenol quantification, the technique has several disadvantages, such as time-consuming analysis, high-cost process and maintenance, and large-space apparatus. Thus, several simple phenol quantification methods are currently being optimized and developed [1].

Biosensors are analytical systems that use biological cells or tissues to identify and detect chemical signals.

These sensors are composed of biorecognition elements and different kinds of physicochemical transducers [2]. Biosensors based on tyrosinase activity have the potential to be used for phenol detection and quantification in technical applications [3]. The biological component of biosensors is generally composed of enzymes, and the catalytic activity induced by these molecules yields a highly sensitive and selective measurement. Thus far, tyrosinase [4], laccase [5-7], horseradish peroxidase, and their modifications [8-9] have been evaluated. Among them, the tyrosinase-based biosensor was considered for possible application to detect monophenol hydroxylase activity, which could not be achieved using laccase enzyme [10-11]. However, the direct use of an enzyme is quite expensive because the molecules require isolation. A solution for that problem is using selected bacteria as bioreceptors as they contain natural enzymatic pathways [12]. Several bacteria that produce tyrosinase, such as *Bacillus thuringiensis*, *Pseudomonas putida* [13-15], and *Ralstonia solanacearum* [16-17], have been reported. However, the *Bacillus* genus is resistant to extreme conditions while producing tyrosinase

enzyme [18]. Although rarely reported, selected *Bacillus* isolates are more suitable for phenol detection and quantification.

Therefore, in this study, we evaluated 72 *Bacillus* isolates for possible application as phenol biosensor on a screen-printed carbon electrode (SPCE) surface. The response surface methodology was utilized to determine the optimum condition for phenol sensing using a *Bacillus*-based biosensor. The limit of detection (LoD) and limit of quantification (LoQ) values were also determined to evaluate the sensitivity of the measurement method. Furthermore, the biosensor material was replicated several times and tested for up to 5 weeks to observe biosensor stability. The selectivity of the biosensor material for phenol detection and quantification was evaluated against benzene, toluene, resorcinol, and pyrogallol compounds.

Materials and Methods

Bacillus culture. *Bacillus* cultures (Health Microbiology Laboratory, Research Center for Biology, Indonesian Institute of Sciences, Cibinong, Indonesia) were grown and rejuvenated in heterotrophic medium and incubated (Sanyo MIR-162, Osaka, Japan) at 37 °C for 24 h. The heterotrophic medium contained 3.0 g of agar, 3.6 g of tryptone, 1.0 g of NaCl, and 0.5 g of K₂HPO₄ mixed in 200 mL of distilled water. The medium was sterilized in an autoclave (HVE 50 Hirayama, Japan) at 121 °C for 15 min. Cultures were incubated in the medium at 37 °C for ±48 h.

Selection of *Bacillus* isolates that produce tyrosinase. A total of 72 *Bacillus* isolates were incubated in the tyrosine agar at 30 °C for 2–3 days. The tyrosine agar was composed of 0.5% w/v tryptone, 0.3% w/v beef extract, 2% w/v agar, and 0.5% w/v L-tyrosine (Sigma-Aldrich) at pH 7. The production of a clear zone around the *Bacillus* isolates indicated the presence of tyrosinase [19]. Ten *Bacillus* isolates with the largest diameter of clear zone around them were selected for use in the bioreceptors.

Selection of *Bacillus* isolates that produce the highest current. Five doses of *Bacillus* isolates were transferred to 10 mL of liquid heterotrophic medium. The suspension was mixed vigorously on a vortex mixer and centrifuged (type 5415C) at 10,000 rpm for 5 min. This step was repeated three times with phosphate buffer solution (PBS). Approximately 100 µL of suspension was dropped on the working SPCE (DropSens, Oviedo, Spain). The electrode was set at room temperature for 5 days.

Electrochemical measurement. The electrochemical measurement of cyclic voltammetry was conducted using an eDAQ potentiostat (e-corder 410, Denistone East,

Table 1. Experimental Factors and Levels in Central Composite Design (CCD) of the Optimization

Factor	Units	Levels				
		-1.68	-1	0	1	+1.68
A: time	days	0	1	3	5	6.36
B: pH	–	5.32	6	7	8	8.68
C: OD	–	0.33	0.50	0.75	1	1.17

Australia) equipped with the Echem v2.1.0 software (Denistone East, Australia). The working electrode was composed of carbon with a diameter of 4 mm, the reference electrode was composed of silver (Ag/AgCl), and the auxiliary electrode was made of carbon. The measurement parameters were as follows: mode, cyclic; initial, -1,000 mV; final, -1,000 mV; rate, 100 mV/s; step W, 20 ms; upper, 1,000 mV; and lower, -1,000 mV. The measurement was set up with 10 mg/L phenol in PBS as analyte. Then, PBS was used as blank.

Optimization of the biosensor. The system was optimized using a variable combination of buffer, pH 6–8; time, 1–5 days; and optical density (OD), 0.5–1.0. The OD measurements were performed on a microplate reader (BioRad iMark, Kyoto, Japan) at the maximum wavelength of 595 nm. The response surface methodology was used to determine the optimal conditions from the aforementioned parameters. The variable combination was inputted into the statistical software program MINITAB 17. Then, the variable combination was explored and yielded several factors. The experiment was conducted on the basis of these factors, as shown in Table 1.

Characterization of *Bacillus* biofilm. Characterization of the biofilm of the SPCE surface was conducted by scanning electron microscopy (SEM, HITACHI TM3030, Oklahoma, Japan). The system was used to view the biofilm and the formation of the *Bacillus* colonies.

Determination of analytical performance. Analytical performance was determined in terms of linearity (phenol = 1×10^{-8} to 10 mg/L), LoD, LoQ, precision, selectivity (phenol, benzene, toluene, resorcinol, and pyrogallol), sensitivity, and stability for 5 weeks [20].

Results and Discussion

A total of 72 *Bacillus* isolates were successfully grown and rejuvenated on solid heterotrophic medium. Figure 1 shows the reaction between tyrosine and tyrosinase enzyme. *Bacillus* isolates that produced tyrosinase formed a circular clear zone in the media. The diameters of the clear zone of the 72 *Bacillus* isolates increased as they grew from 1 day to 5 days. Then, 10 *Bacillus* isolates, namely, 85, 81, 75, 99, 87, 142, 192, 100, and 190 (replicated three times, with the standard deviation of <5%) were selected as they produced the largest diameters of clear zone surrounding the colonies.

Among the 72 *Bacillus* isolates, 10 were used to make biofilms on SPCE. The current of each biofilm was measured using the eDAQ potentiostat. The voltammogram shown in Figure 2 illustrates the peak values of oxidation current. The presence of an oxidation current peak indicates the occurrence of phenol oxidation to quinone by tyrosinase from *Bacillus* [21]. This result shows that enzyme activity is able to detect phenol pollutants electrochemically. The voltammogram also shows that all *Bacillus* isolates respond to the current, except code 87. This isolate was not resistant to the 10 mg/L phenol solution. On the basis of enzyme activity during phenol detection, *Bacillus* isolate code 100 is the

candidate with the most potential in this study. The high current produced by 9 of the 10 isolates proved that *Bacillus* grew using phenol as a carbon source and was resistant in the long term.

Figure 3 shows the microscopic observations of bacterial isolates after Gram staining and illustrates the elliptical shape of the cells, which is suitable for the identification of *Bacillus* morphology [22]. This characterization was performed to investigate the formation of *Bacillus* cells. In addition, SEM was used to view the biofilms of *Bacillus* isolate formed by the bacteria on the SPCE surface after 7 days compared

Figure 1. Biotransformation of L-tyrosine into L-DOPA by Tyrosinase Enzyme [21]

Figure 2. Current Produced by Biofilms of 10 *Bacillus* Isolates used in the Phenol Biosensor

Figure 3. Micrograph of the Morphology of *Bacillus* Biofilm (Code 100)

with that at 0 day. Figure 4 shows the aggregation of bacteria immobilized on the surface after being kept for 0 and 7 days, and the shape of the cells is observed to be round. This finding is in accordance with the results of previous reports [23] that stated that the morphology of the cells and biofilms of *Bacillus* are round and clustered. Optimization was performed using the response surface methodology with MINITAB 17 (Table 2). The tested treatments were buffer pH, number of days that the biofilms were formed, and OD. The optimum biosensor measurements were obtained after 6 days on PBS (pH of 8), with an OD of 0.33. This result was used to determine analytical performance. An OD of 0.33 was used because it yielded a higher biofilm stability than an OD of 1.17. Bacterial biofilms with a high density of cells on the SPCE surface enabled cells to escape easily. Table 3 shows the results of the optimization that significantly influenced current, time, and pH at a confidence value of $p < 0.05$. The coefficient shows a positive value, which means that the greater the value of the factor, the greater the value of the current.

Analytical performance was simulated using samples of phenol and determined on the basis of linearity, precision, LoD, LoQ, sensitivity, selectivity, stability, and repeatability. Linearity was measured with phenol in a concentration range of 0.000001 mg/L to 0.001 mg/L; the linear regression line equation was expressed as $y = 2.047x + 18.651$, with an R^2 value of 0.9594. The R^2 value, which is close to 1, represents the anodic peak current generated linearly with the increase in analyte concentration. Therefore, phenol levels in the sample can be determined by measuring the current response.

The LoD and LoQ values obtained using the International Conference on Harmonisation (ICH) method in the oxidation reaction were 3.0 and 13 ng/L, respectively. The low LoD and LoQ values show that the sensitivity of the method is remarkable and that the biofilm SPCE used is sensitive to the phenol oxidation reaction. In addition, the voltammetry readings of the biosensor showed good precision, with a relative standard deviation (RSD) of $<5\%$. The selectivity of phenol detection and quantification was investigated to determine the response of bacteria to interference from similar phenol compounds. The obtained results showed that the voltammogram of each similar phenol compound (i.e., benzene, toluene, resorcinol, and pyrogallol) exhibits significant peak changes, as shown in Figure 5. Although the selectivity observed in this study is not satisfactory enough because of the similar chemical structure of the aforementioned compounds, it should be noted that phenol was often detected in wastewater, whereas benzene, resorcinol, and pyrogallol were hardly detected on the environmental samples [24,25]. Furthermore, the obtained LoD and LoQ values were in the ng/L level, indicating that it is still possible to use this biosensor material for phenol detection and quantification in real applications.

The stability of the biosensor synthesized was determined by measuring peak oxidation using the optimum biofilm-coated SPCE every 7 days for 5 weeks. The results showed that the immobilized *Bacillus* biofilm on the SPCE surface remained stable until 35 days of measurement, with 90% residual activity. Therefore, the optimum SPCE biofilm has good stability, with an RSD of $<5\%$.

Figure 4. SEM Morphology of Biosensor *Bacillus* Biofilm (code 100) at a Magnification of $\times 7,000$: (a) 0 Day and (b) 7 Days

Figure 5. Selectivity Voltammogram of the Phenol Biosensor

Table 2. Results of the Response Surface Methodology

Factor variable	OD	Time (days)	pH	Oxidation current (μA)	Predicted oxidation currents (μA)
1	0.33	3	7	43.45	44.05
2	0.5	1	6	24.15	23.45
3	0.5	1	8	28.79	28.54
4	0.5	5	6	42.79	44.17
5	0.5	5	8	47.63	47.64
6	0.75	0	7	23.04	23.21
7	0.75	3	5	30.33	28.54
8	0.75	3	7	35.9	35.45
9	0.75	3	7	35.66	35.45
10	0.75	3	7	35.62	35.45
11	0.75	3	7	34.19	35.45
12	0.75	3	7	35.44	35.45
13	0.75	3	7	35.51	35.45
14	0.75	3	9	35.79	35.03
15	0.75	6	7	47.71	45.11
16	1	1	6	30.48	32.25
17	1	1	8	35.73	36.13
18	1	5	6	39.12	41.15
19	1	5	8	40.93	43.41
20	1.17	3	7	51.01	47.89

Table 3. Analysis of the Effects of the Factors on the Current

Factor	Coefficient of variance	p value
Constant	36.049	0.000
OD	1.450	0.139
Time (days)	10.983	0.000
pH	3.182	0.005
$R^2 = 96.34\%$		$R^2(\text{adj}) = 93.04\%$
		$R^2(\text{pred}) = 71.48\%$

Conclusion

A phenol biosensor based on selected *Bacillus* biofilm immobilized on the SPCE surface has been successfully prepared, with the LoD and LoQ values of 3.0 and 13 ng/L, respectively. The stability of the current through the electrode was maintained at approximately 90% for 35 days. The optimum value was obtained after 6 days on PBS (pH of 8), with an OD of 0.33.

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References

- [1] Vinci, G., Antonelli, M.L., Preti, R. 2013. Rapid Determination of Polycyclic Aromatic Hydrocarbons in Rainwater by Liquid-Liquid Microextraction and LC with Core-shell Particles Column and Fluorescence Detection. *J. Separation. Sci.* 36(1): 461–468, <http://dx.doi.org/10.1002/jssc.201200854>.
- [2] Neethirajan, S., Ragavan, V., Weng, X., Chand, R. 2018. Biosensors for Sustainable Food Engineering: Challenges and Perspectives. *Biosensors*. 8(1): 23, <http://dx.doi.org/10.3390/bios8010023>.
- [3] Vigneshvar, S., Sudhakumari, C.C., Senthikumar, B., Prakash, H. 2016. Recent Advances in Biosensor Technology for Potential Applications: An Overview. *Bioeng. Biotechnol.* 4(11): 1–9, <http://dx.doi.org/10.3389/fbioe.2016.00011>.
- [4] Karim, N., Lee, H.J. 2013. Amperometric phenol biosensor based on covalent immobilization of tyrosinase on Au nanoparticle modified screen-printed carbon electrodes. *Talanta*. 116(1): 991–996, <http://dx.doi.org/10.1016/j.talanta.2013.08.003>.
- [5] Sekretaryova, A.N., Volkov, A.V., Zozoulenko., Turner, A.P., Vagin, M.Y., Ericksson, M. 2016. Total phenol analysis of weakly supported water using a laccase based microband biosensor. *Anal. Chem. Acta*. 11(907): 45–53, <http://dx.doi.org/10.1016/j.aca.2015.12.006>.
- [6] Bilir, K., Weil, M.T., Lothead, J., Kok, F.N., Werner, T. 2016. Construction of an oxygen detection-based optic laccase biosensor for polyphenolic compound detection. *Turk. J. Biol.* 40(1): 1303–1310, <http://dx.doi.org/10.3906/biy-1602-40>.
- [7] Delgado, M.R., Nava, G.A., Delgado, J.R., Assad, G.D., Chapa, S.M., Barcelo, D., Parra, R. 2015. Laccase-based biosensors for detection of phenolic compounds. *Trends. Anal. Chem.* 74(1): 21–45, <http://dx.doi.org/10.1016/j.trac.2015.05.008>.
- [8] Elkaoutit, M., Naranjo, R.I., Temsamani, K.R., Vega, M.D., de-Cisneros, J.L. 2007. Dual laccase-tyrosinase based sonogel-carbon biosensor for monitoring polyphenols in beers. *J. Agric. Food. Chem.* 55(20): 8011–8018, <http://dx.doi.org/10.1021/jf0711136>.
- [9] Dai, Z., Xu, X., Wu, L., Ju, H. 2005. Detection of trace phenol based on mesoporous silica derived tyrosinase-peroxidase biosensor. *Electroanal.* 17(17): 1571–1577, <http://dx.doi.org/10.1002/elan.200403256>.
- [10] Karim, F., Fakhruddin, A.N.M. 2012. Recent advances in the development of biosensor for phenol: A review. *Rev. Environ. Sci. Biotech.* 11(1): 261–274, <http://dx.doi.org/10.1007/s11157-012-9268-9>.
- [11] Pogni, R., Baratto, M.C., Sinicropi, A., Basosi, R. 2015. Spectroscopic and computational characterization of laccases and their substrate radical intermediates. *Cell. Mol. Life. Sci.* 72(5): 885–896, <http://dx.doi.org/10.1007/s00018-014-1825-7>.
- [12] Tecon, R., Van, D.M.J.R. 2008. Bacterial Biosensors for Measuring Availability of Environmental Pollutants. *Sensors*. 8(7): 4062–4080, <http://dx.doi.org/10.3390/s8074062>.
- [13] Liu, Y., Liu, H., Yang, H., Yang, Y., Shen, G., Yu, R. 2005. A phenol biosensor based on immobilizing tyrosinase to modified core-shell magnetic nanoparticles supported at a carbon paste electrode. *Anal. Chem. Acta*. 533(1): 3–9, <http://dx.doi.org/10.1016/j.aca.2004.10.077>.
- [14] Claus, H., Decker, H. 2006. Bacterial tyrosinases. *Syst. Appl. Microb.* 29(1): 3–14, <http://dx.doi.org/10.1016/j.syapm.2005.07.012>.
- [15] McMahon, A.M., Doyle, E.M., Brooks, S., O'Connor, K.E. 2007. Biochemical characterisation of the coexisting tyrosinase and laccase in the soil bacterium *Pseudomonas putida* F6. *Enzyme. Microb. Tech.* 40(5): 1435–1441, <http://dx.doi.org/10.1016/j.enzmictec.2006.10.020>.
- [16] Molloy, S., Nikodinovic, R.J., Martin, L.B., Hartmann, H., Solano, F., Decker, H., O'Connor, K.E. 2013. Engineering of a bacterial tyrosinase for improved catalytic efficiency towards D-tyrosine using random and site-directed mutagenesis approaches. *Biotech. Bioeng.* 110(7): 1849–1857, <http://dx.doi.org/10.1002/bit.24859>.
- [17] Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H., Sugiyama, M. 2006. Crystallographic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. *J. Bio. Chem.* 281(13): 8981–8990, <http://dx.doi.org/10.1074/jbc.M509785200>.
- [18] Sarwade, V.D., Gawai, K.R. 2014. Biodegradation of phenol by alkaliphilic *Bacillus* D1. *J. Environ. Sci.* 8(5): 28–35, <http://dx.doi.org/10.9790/2402-08522835>.
- [19] Raval, K.M., Vaswani, P.S., Majumder, D.R. 2012. Biotransformation of single amino acid L-tyrosine

- into a bioactive molecule L-DOPA. *Int. J. Sci. Res. Pub.* 2(5): 2250-3153.
- [20] International Conference on Harmonisation. 2005. Validation of Analytical Procedures: Text and Methodology Q2(R1). New York (US): International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Available at https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2R1/Step4/Q2R1Guideline.
- [21] Gennaro, D.P., Bruzzese, N., Anderlini, D., Aiossa, M., Papacchini, M., Campanella, L., Bestetti, G. 2011. Development of microbial engineered whole-cell systems for environmental benzene determination. *Ecotoxicol. Envir. Safety.* 74(3): 542-549, <http://dx.doi.org/10.1016/j.ecoenv>.
- [22] Zolock, R.A., Li, G., Bleckmann, C., Burggraf, L., Fuller, D.C. 2006. Atomic force microscopy of Bacillus spore surface morphology. *Micron.* 37(1): 363–369, <http://dx.doi.org/10.1016/j.micron.2005.11.006>.
- [23] Bridier, A., Meylheuc, T., Briandet, R. 2013. Realistic representation of *Bacillus subtilis* biofilms architecture using combined microscopy (CLSM, ESEM and FESEM). *Micron.* 48(1): 65–69, <http://dx.doi.org/10.1016/j.micron.2013.02.013>.
- [24] Raza, W., Lee, J., Raza, N., Luo, Y., Kim, K., Yang, J. 2018. Removal of phenolic compounds from industrial waste water based on membrane-based technologies. *J. Industrial. Eng. Chem.* 71(1): 1–18, <http://dx.doi.org/10.1016/j.jiec.2018.11.024>.
- [25] Anku, W.W., Mamo, M.A., Govender, P.P. 2017. Phenolic Compounds in Water: Sources, Reactivity, Toxicity and Treatment Methods. United Kingdom. InTechOpen. pp. 420–443.