Activity and Stability of Biofilm Uricase of *Lactobacillus plantarum* for Uric Acid Biosensor

Dyah Iswantini*¹, Rescy Rachmatia ¹, Novita Rose Diana ¹, Novik Nurhidayat², Akhiruddin³ and Deden Saprudin¹

¹Department of Chemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor, 16680, Indonesia.
²Division of Microbiology R&D for Biology, The Indonesian Institute of Sciences, Bogor, Indonesia
³Department of Physics, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor, 16680, Indonesia.

E-mail: dyahprado@gmail.com

**Abstract.** Research of uric acid biosensor used a *Lactobacillus plantarum* was successfully conducted. *Lactobacillus plantarum* could produce uricase that could be used as uric acid biosensor. Therefore, lifetime of bacteria were quite short that caused the bacteria could not detect uric acid for a long time. To avoid this problem, development of biofilm for uric acid biosensor is important. Biofilms is a structured community of bacterial cells, stick together and are able to maintain a bacteria in an extreme environments. The purpose of present study was to determine and compare the activity of uricase produced by *L. plantarum*, deposited with biofilm and planktonic bacteria on glassy carbon electrode (GCEb & GCE), also to determine the stability of biofilm. The optimization process was conducted by using temperature, pH, and substrate concentration as the parameters. It showed that the activity of uricase within biofilm was able to increase the oxidation current. GCEb and GCE yielded the oxidation current in the amount of 47.24 µA and 23.04 µA, respectively, under the same condition. Results indicated that the optimum condition for uric acid biosensor using biofilm were pH 10, temperature of 40 °C, and uric acid concentration of 5 mM. The stability of GCEb decreased after 10 hours used, with decreasing percentage over 86.33%. This low stability probably caused by the unprotected active site of the enzyme that the enzyme is easier to experience the denaturation.
1. Introduction

Uric acid (2,6,8-trihidroxypurine) is the final product of purine metabolism in human body. The high concentration of uric acid in the blood caused hypertension and cardiovascular deseases [1]. Therefore, uric acid detection method is very important to develop. One method that is commonly and widely used to determine uric acid level is spectrophotometry method, but Spectrophotometry method has disadvantage such as: expensive, low sensitivity, and unstable to the light. Biosensor is an analytical tool to detect specific analyte, combining a physicochemical detector component and biological components [2].

Biosensor is a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, termal or optical signal [3]. One of the enzymes used as a biological component in uric acid biosensor development is uricase. Uricase (urate oxidase) is an enzyme that participates in the purine breakdown pathway, catalyzing the oxidation of uric acid to allantoin and hydorgen peroxide in the presence of oxygen [4]. Generally, uric acid can be isolated from vertebrate animals. However, due to the complicated isolation procedure, limited materials and also high costs, then used alternatives sources such as molds, yeasts, and bacterias.[5].

To overcome the problem, research related to uric acid biosensor used Lactobacillus plantarum as uricase source was successfully conducted. Lactobacillus plantarum were known can produce enzymes, among of them are uricase for uric acid biosensor, pyruvate oxidase for pyruvate biosensor and lactic oxidase for lactic acid biosensor [6]. Lifetime of bacteria is quite short that makes uricase activity is not long enough to detect an uric acid. Biofilms are defined as communities of bacteria encased in a self-synthesized extracellular polymeric matrix that attaches to a biotic or abiotic surface [7]. This formation of biofilm can maintain the bacteria to survive in extreme environments, able to survive against an antibiotics and disinfectants. Jalilsood et al (2015) had researched that L. plantarum PA21 is a very potent biofilm produces provided specific local micro-enviroments which were favorable to some pathogen or spoilage microorganisms [8]. The aim of present study was to determine and compare the activity of uricase produced by L.plantarum, deposited within biofilm and planktonic bacteria on glassy carbon electrode (GCEb & GCE), also to determine the stability of biofilm. The ability of biofilm to detect the uric acid concentration also be measured.

2. Materials and Methods

Reagents

The uric acid used in this work is from nacalaiq tesque, Japan, K₃Fe(CN)₆ is from Merck, German, and the 3-dymetoxy-5-methyl-1,4-benzoquinnon (Q₀) is from Sigma, German.

Microorganism

The Lactobacillus plantarum strain used in this study was previously isolated and collected in microbiology laboratorium of LIPI Cibinong, Bogor, Indonesia.

Preparation of Cultures

The L. plantarum cells were cultured on Glucose Yeast Peptone (GYP) agar plates and were incubated in 37°C for 24 hours. Then L. plantarum cells were subcultured in 5 ml liquid GYP and were incubated in 37°C for 24 hours and stored as the starter. The starter was then mixed with the liquid GYP and incubated for 4 hours to reach the Optical Density 600 (OD₆₀₀) of 0,5-0,6. They were harvested by centrifugation at 10.000 rpm for 10 minutes at 4°C. The pellets were washed with physiological salt and were centrifugated in the same condition. Supernatant was removed and the boric buffer was added into the pellets and separated with the vortex.
The Formation of Biofilm

The biofilm was formed by using microtiter plate method with several modifications. The *L. plantarum* was inoculated onto GYP and was incubated in the shaker incubator at 37°C over night. The adsorbent measurement was conducted by using spectrophotometer to observe the amount of bacteria used. Then, the bacteria suspension was diluted in the GYP until reached the concentration of $10^6$. The diluted culture was inoculated into the microtiter plate with the liquid GYP as the negative control, then they were incubated for 72 hours. After that, they were washed with aquadest to remove the planktonic bacteria and were dried and colored. The coloration used violet crystal 1% then was incubated in the room temperature for 15 minutes. After being washed and dried, the boric buffer was added. Then the absorbance was read by using microplate reader in the wavelength of 595 nm.

The Construction of Carbon Paste Electrode

Carbon paste electrode was constructed by mixing paraffin oil and graphit (1:2). Then the mixture was mixed with Q0 which has dissolved into DMSO. The mixture was deposited in the glass electrode and the surface was leveled. The electrode was stored within 2 days before being used.

Electrochemical Measurement

Electrochemical measurement was conducted with cyclic voltametric by using eDAQ potensistate (Ecorder 410) which was equipped with Echem software v 2.1.0. The electrodes used in the measurement were Ag/AgCl as the reference electrode, Pt as auxiliary electrode, and carbon paste as working electrode. Boric buffer solution was added into the measuring cell, and the current peak yielded was observed as the reference peak. Then the uric acid was added to observe the alteration of the current peak.

The Optimization of *L. plantarum* Uricase Activity

The optimization was conducted by varying the temperature (25-40) °C, pH (7.10-10.00), and uric acid concentration (1-5) mM by using Response Surface Method. After combining the factors of variables, then the study was conducted to gain the optimum activity.

The Determination of Electrode Stability

Electrode stability was determined from the uricase activity measurement after gaining the optimum condition. The electrode stability can be observed by measuring the activity time to time. The decreasing of stability was confirmed by the decreasing of the current yielded in the measurement. The stability percentage was calculated with the equation (1).

$$% \text{ stability} = \left( 100\% - \frac{I_0 - I_t}{I_0} \times 100\% \right) \ldots (1)$$

where $I_0$ is the initial oxidation current and $I_t$ is the oxidation current at certain time.

3. Results and Discussions

Characterization of Electrode

Before being used, the electrode was characterized with K3Fe(CN)6 to confirm that the electrode was proper to use. The proper electrode will result the voltamogram as showed in Figure 1.
The confirmation of biofilm formation was conducted by using microplate reader Bio-Rad 680. Figure 2 showed the average OD at pH 7.10, 8.55, and 10.00. The average OD of pH 10.00 was lower than the average OD of pH 8.55, however the oxidation current of pH 10.00 was higher than both pH 8.55 and 7.10. This may caused by the amount of enzyme excreted by the biofilm at pH 10.00 was much higher than at other pH. The cultures were inoculated onto wells 1, 2, 4, 5, 7, 8, and 10, whereas the liquid GYP as negative control was inoculated onto well 12. Table 1 indicated that the average of OD of column 12 smaller than others column, it means that *Lactobacillus plantarum* is biofilm formation agent.

![Voltamogram of carbon paste electrode characterization with K₃Fe(CN)₆](image1.png)

**Figure 1.** Voltamogram of carbon paste electrode characterization with K₃Fe(CN)₆

**Formation of Biofilm of *L. plantarum* Cells**

The confirmation of biofilm formation was conducted by using microplate reader Bio-Rad 680. Figure 2 showed the average OD at pH 7.10, 8.55, and 10.00. The average OD of pH 10.00 was lower than the average OD of pH 8.55, however the oxidation current of pH 10.00 was higher than both pH 8.55 and 7.10. This may caused by the amount of enzyme excreted by the biofilm at pH 10.00 was much higher than at other pH. The cultures were inoculated onto wells 1, 2, 4, 5, 7, 8, and 10, whereas the liquid GYP as negative control was inoculated onto well 12. Table 1 indicated that the average of OD of column 12 smaller than others column, it means that *Lactobacillus plantarum* is biofilm formation agent.

![Comparison of average OD from biofilm extraction](image2.png)

**Figure 2.** The comparison of average OD from biofilm extraction
Table 1. Result of biofilm of Optical Density (OD) measurement to confirm formation of *L. plantarum* cells biofilm

|     | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A   | 0.160 | 0.059 | 0.045 | 0.093 | 0.13 | 0.046 |
| B   | 0.138 | 0.084 | 0.045 | 0.116 | 0.07 | 0.048 |
| C   | 0.154 | 0.125 | 0.046 | 0.141 | 0.09 | 0.049 |
| D   | 0.111 | 0.056 | 0.042 | 0.086 | 0.13 | 0.056 |
| E   | 0.139 | 0.205 | 0.045 | 0.092 | 0.08 | 0.051 |
| F   | 0.128 | 0.097 | 0.039 | 0.104 | 0.10 | 0.055 |
| G   | 0.172 | 0.076 | 0.044 | 0.146 | 0.10 | 0.051 |
| H   | 0.138 | 0.107 | 0.042 | 0.084 | 0.10 | 0.049 |

For the purpose of doing a comparative analysis of test results, the adherence capabilities of the test strains were classified into four categories (Table 2) [9].
Table 2 Classification of bacterial adherence by microtitre plate method

| Mean OD value | Adherence | Biofilm formation |
|---------------|-----------|-------------------|
| <0.062        | Non       | Non               |
| 0.062-0.124   | Weak      | Weak              |
| 0.124-0.248   | Moderate  | Moderate          |
| >0.248        | Strong    | Strong            |

Result of formation of biofilm could detected uric acid and produced oxidation current higher than planktonic bacteria (Fig. 3). The oxidation current of *L. plantarum* biofilm and planktonic bacteria were of 47.24 and 23.04 µA, respectively, at the same pH, temperature, and uric acid concentration.

![Voltammogram resulted by planktonic bacteria ( - ) and biofilm of *L. plantarum* ( - )](image)

**Figure 3.** Voltammogram resulted by planktonic bacteria ( - ) and biofilm of *L. plantarum* ( - )

Detection of uric acid using *Lactobacillus plantarum* biofilm could be performed at optimum condition of pH 10.00, temperature 40 °C, and uric acid concentration 5 mM. The stability of biofilm decreased after 10 hours used, with decreasing percentage over 86.33%. This low stability probably caused by the unprotected active site of the enzyme that the enzyme is easier to experience the denaturation.

**The Optimization of Uricase Activity of Planktonic Bacteria**

The combination of variables were varied by using Response Surface Method to result the variation such as showed in table 2.
Table 2. The combination of optimization of *L. plantarum* planktonic bacteria uricase

| pH  | temp (°C) | uric acid (mM) | Oxidation Current (μA) |
|-----|-----------|----------------|------------------------|
| 8.55| 32.5      | 1              | 2.69                   |
| 8.55| 32.5      | 3              | 3.81                   |
| 7.10| 40.0      | 5              | 3.15                   |
| 10.00| 25.0    | 1              | 5.21                   |
| 10.00| 32.5    | 3              | 5.76                   |
| 8.55| 32.5      | 3              | 4.77                   |
| 7.10| 32.5      | 3              | 0.04                   |
| 7.10| 25.0      | 1              | 5.34                   |
| 8.55| 32.5      | 3              | 4.60                   |
| 7.10| 40.0      | 1              | 5.28                   |
| 8.55| 32.5      | 3              | 4.50                   |
| 10.00| 40.0   | 5              | 23.04                  |
| 10.00| 40.0    | 1              | 6.43                   |
| 7.10| 25.0      | 5              | 2.58                   |
| 8.55| 32.5      | 3              | 14.55                  |
| 8.55| 32.5      | 5              | 20.65                  |
| 8.55| 25.0      | 3              | 4.72                   |
| 8.55| 40.0      | 3              | 10.40                  |
| 10.00| 25.0   | 5              | 20.27                  |
| 8.55| 32.5      | 3              | 3.32                   |
Figure 4 shows the voltamogram of uric acid measurement. There is the difference between the oxidation peaks of boric buffer as the reference and uric acid. The increasing of current peak was caused by the oxidation process of the uric acid into the allantoin, CO\textsubscript{2}, and H\textsubscript{2}O\textsubscript{2}. The highest oxidation peaks of pH 7.10, 8.55, and 10.00 were 5.34 µA at 25°C, 20.65 µA at 32.5°C, and 23.04 µA at 40°C respectively.

![Voltamogram](image)

**Figure 4** Voltamogram of the result of optimization of *L. plantarum* planktonic bacteria uricase activity at pH 7.10, temperature 40°C, uric acid concentration 1 mM (a), pH 8.5, temperature 40°C, uric acid concentration 3 mM (b), pH 10.00, temperature 40°C, uric acid concentration 5 mM (c)
To observe the interaction contour path of temperature vs pH, pH vs uric acid concentration, and temperature vs uric acid concentration, the plot contour analysis was performed. Optimum area was showed by the darker contour path and higher oxidation current. In figure 5, the interactions of pH, temperature, and uric acid concentration were showed. Figure 5 (a) showed the optimum area was at pH 10 and 40°C. Figure 5 (b) showed the optimum area was at pH 10 and the uric acid concentration was of 5 mM. Figure 5 (c) showed the optimum area was at 40°C and uric acid concentration was of 5 mM.

\[ \text{(a)} \]

\[ \text{(b)} \]

\[ \text{(c)} \]

**Figure 5.** interaction contour: pH vs temperature (a), pH vs uric acid concentration (b), temperature vs uric acid concentration (c)

**The Optimization of Uricase Activity of L. plantarum Biofilm**

Figure 6 showed the voltamogram of optimization of biofilm uricase activity in various condition. Figure 6 (a) showed the voltamogram under the condition were of pH 7.10, 40°, and 1 mM of uric acid concentration, yielded the highest oxidation current of 1.74 µA. Figure 6 (b) showed the voltamogram under the condition were of pH 8.55, 32.5°C, and 3 mM of uric acid concentration, yielded the highest oxidation current of 23.26 µA. Figure 6 (c) showed the voltamogram under the condition were of pH 10.00, 40°C, and 5 mM of uric acid concentration, yielded the oxidation current of 47.24 µA.

The oxidation current of biofilm uricase was higher than plantonic bacteria in pH 10.00. This
indicates that biofilm uricase has far better activity than planktonic bacteria at that condition. The ability of biofilm which is able to survive in an extreme condition might caused the high of uricase’s activity. However, in the neutral condition (pH 7.10) the biofilm uricase didn’t show the good activity even no signal peaks yielded. This might caused by the biofilm bacteria couldn’t optimized the condition over pH 7.

![Voltamogram](a)

**Figure 6.** Voltamogram of biofilm uricase activity at pH 7.10, 32.5°C, 3 mM of uric acid concentration (a), pH 8.55, 32.5°C, 3 mM of uric acid concentration, pH 10, 40°C, 5 mM of uric acid concentration.

In figure 7, interactions between temperature vs pH, pH vs uric acid concentration, and temperature vs uric acid concentration were observed by using contour plot. The darker area and higher oxidation current indicate the optimum area. Figure 7 (a) confirmed the highest oxidation current was at pH 10 and 40°C. At 32.5°C, the oxidation current yielded was very low, i.e. < 5µA. Figure 7 (b) confirmed the highest oxidation current was at pH 10 and 5 mM of uric acid concentration. Uricase’s activity wasn’t optimum at pH < 7.5 which was showed by the low oxidation current. Figure 7 (c) confirmed the highest was at 40°C and uric acid concentration was 5 mM.
Table 4 showed the stability of GCE and GCEb. The stability of GCE decreased over 28% after 4 hours. The GCEb uricase were more stable than another.

**Determination of the Stability of Electrode**

Figure 7. Interaction contour of pH vs temperature (a), pH vs uric acid concentration (b), temperature vs uric acid concentration (c)
Table 4 The stability of GCE and GCEb

| Time | Current (μA) | Planktonic | Biofilm |
|------|--------------|------------|---------|
| 0    | 1.25         | 19.70      |
| 6    | 2.95         | 23.35      |
| 8    | 1.51         | 22.34      |
| 10   | 4.05         | 18.06      |

Figure 8 showed stability curve of two kinds of electrodes. The stability of biofilm uricase contained electrodes increased after 6 hours, then significantly decreased after ten hours. The decreasing was over 86.33%. This low stability might be caused by the unprotected active site that the enzyme could be denatured easily. The low stability could be addressed by immobilization method.

4. Conclusions

*Lactobacillus plantarum* could be functioned to form biofilm by incubating for 72 hours at 37°C. The uricase of *L. plantarum* biofilm is able to detect uric acid and yielded higher oxidation current than the uricase of planktonic bacteria. The optimum condition for biofilm uricase was at pH 10, temperature of 40°C, and uric acid concentration of 5 mM. The stability of *L. plantarum* biofilm uricase decreased after ten hours over 86.33%. This low stability might be caused by the unprotected enzyme’s active site that the enzyme could be denatured easily.

5. Acknowledgements

The authors would like to thank the Indonesian Government (IPTEK-DP2M DIKTI) who provided funding for this research.
References

[1] Johnson, R.J, Kang, D.H, Feig D, Kivlighn, S Kanellis, J, Watanabe, S, Tuttle, K.R, Rodriguez-Iturbe, B, Herrera-Acosta J and Mazzali, M. 2003. Is there a pathogenetic role for uric acid in hypertension and cardiovascular and renal diseases. Hypertension; 41: 1183-1190.

[2] Arslan, F., 2008. An amperometric biosensor for uric acid determination prepared from uricase immobilized in polyaniline-polypyrrole film. Sensors Articles; 8:492-500.

[3] Monosik R, Stredansky M, Sturdik E. 2012. Biosensor – classification, characterization and new trends. Acta Chimica Slovaca. 5(1): 109-120. Doi: 10.2478/v10188-012-0017-z.

[4] Madga Aly, Sanaa Tork, Saleh Al-Garni, Reda Allam. 2013. Production and characterization of uricase from Streptomyces exfoliates UR10 isolated from farm wastes. Turkish Journal of Biology. 37: 520-529. doi: 10.3906/bty-1206-3.

[5] Atalla, M.M., Farag, R.H.; Eman, M.S., Abd-El-Lataif and E.A. Nehad, 2009. Optimum conditions uricase enzyme production by Gliomastix gueg. J Microbiology; 5:45-50.

[6] Gamella M, Campuzano S, Conzuelo F, Curiel J.A, Munoz R, Reviejo, Pingarron JM. 2010. Integrated multienzyme electrochemical biosensors for monitoring malolactic fermentation in wines. Talanta. 81: 925-933.

[7] Cha JO, Yoo JI, Yoo JS, Chung HS, Park SH, Kim HS, Lee YS, Chung GT. 2013. Investigation of biofilm formation and its association with the molecular and clinical characteristics of methicillin-resistant Staphylococcus aureus. Osong Public Health Res Perspect. 4(5): 225-232. doi.org/10.1016/j.phrp.2013.09.001.

[8] Jalilsood T, Baradaran A, Song AAL, Foo HL, Mustafa S, Saad WZ, Yusoff K, Rahim RA. 2015. Inhibition of pathogenic and spoilage bacteria by a novel biofilm-forming Lactobacillus isolate: a potential host for the expression of heterologous proteins. Microb Cell Fact. 14(96): 1-14. doi 10.1186/s12934-015-0283-8.

[9] Saxena S, Banerjee G, Garg R, Singh M. 2014. Comparative study of biofilm formation in Pseudomonas aeruginosa isolates from patients of lower respiratory tract infection. Journal of Clinical and Diagnostic Research. 8(5): 9-11. doi: 10.7860/JCDR/2014/7808.4330