Enhancing Activity and Stability of Uricase from *Lactobacillus plantarum* by Zeolite immobilization

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Abstract. *Lactobacillus plantarum* has been known be able to produce uricase for uric acid biosensor. Durability and stability of *L. plantarum* in generating uricase enzyme was low. Hence, we tried to enhance its durability and stability by immobilizing it onto activated 250 mg zeolite at room temperature using 100 μL *L. plantarum* suspension and 2.87 mM uric acid, while Michaelis–Menten constant (Kₘ) and Vₘₐₓ were obtained at 6.7431 mM and 0.9171 μA consecutively, and the linearity range was 0.1-3.3 mM (R² = 0.9667). Limit of detection (LOD) and limit of quantification (LOQ) value of the measurement were 0.4827 mM and 1.6092 mM respectively. Biosensor stability treatment was carried out in two different treatments, using the same electrode and using disposable electrode. The disposable electrode stability showed better result based on repeated measurements, but stability was still need improvement.

1. Introduction

Uric acid is the final metabolism product of purine and its derivatives from the body. Normally, uric concentration in blood is around 0.13-0.46 mM and 1.49-4.46 in the urine [1]. The increase of uric acid in blood above the normal level may lead to stimulate some disease, like gout, hyperuricemia, and Lesch-Nyhan syndrome. However, at low concentration it can lead to cause oxidative stress and multiple sclerosis [2]. Hence, a simple measurement method needs to be developed for monitoring the uric acid concentration in human body.

Some spectrophotometry methods have been developed for measuring uric acid concentration, like colorimetry [3], high performance liquid chromatography [4], chemiluminescence [5], and fluorescence [6]. This approach offers high precision and selectivity, however this method is highly influenced by light, needs trained operator, relatively high cost, complex sample preparation, and long time analysis [7]. Overcoming these difficulties, biosensor as an alternative method has been intruduced for further development [8].

Biosensor is an analytical device which applies bioreceptor as detector, to detect a signal that indicate biochemical response. Transducer then transfers the signal produced by bioreceptor, transmitting it into analyzer. Frequently biosensor uses enzyme as bioreceptor, because it has specific and seletive reaction. For detecting uric acid, the applied enzyme which can be used is uricase,
because it can catalyze uric acid, transforming it into allantonin facilitated by O$_2$ [9]. However, using pure enzyme is not economically applicable because of the difficulty in purification process, and in addition it only produces small portion of enzyme. The enzyme utilization can be substituted by a selective microbe, since the microbe can produce uricase enzyme and be regenerated easily so that the enzyme can be obtained easily [10]. Several former investigations have been conducted and one of the microbe candidate which easily produce uricase was *Lactobacillus plantarum* [11].

As analytical instrument for routine analysis, biosensor should have high stability and can be used for long time. To support this function, *L. plantarum* was immobilized with zeolite. In our previous research, we used base activated zeolite which was compatible with the enzyme range, but not with the optimum pH of *L. plantarum*. Furthermore, we used measurement at 40°C, hence it is unpractical for routine analysis. In addition, the averag produced enzyme by microbe was unknown. Hence, in this research mainly we would like to enhance the biosensor stability. *L. plantarum* was still immobilized into zeolite. The measurement was conducted at room temperature condition. Therefore, applying activated natural zeolite we studied the optimum condition (pH, zeolite mass, uric acid) of this biosensor.

2. Research Methods

2.1. Tools and Materials

The tools used here were eDAQ potensiostat-galvanostat with computer installed *Echem* v.2.0.1 (3 electrodes system) and Minitab 16, incubator (Himawari), microwave (Sanyo), autoclave (Hirayama), pH meter (TOA DK HM-250), spectrophotometer BioSpec-1601 (Shimadzu), micropipette 1000μL, analytical scale, laminar airflow, and other glassware. And the used material were: *L. plantarum* isolate from LIPI, activated natural zeolite, uric acid (Nacalaic tesque, Japan), K$_3$[Fe(CN)$_6$] (Merck, Germany), KCl (Merck, Germany), 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q$_0$, Merck, Germany), dialysis membrane, O-ring, calcium carbonate (Merck, Germany), NaAcO (Merck, Germany), NaCl (Merck, Germany), polysorbate 80, yeast extract, beef extract, agar, phosphate buffer (Merck, Germany), and deionized water.

2.2. Methods

2.2.1. *L. plantarum* preparation. Solid GYP media was made by mixing homogenously 5 g CaCO$_3$, 15 g agar, 10 g glucose, 5 g pepton, 1.4 sodium acetate, 5 mL NaCl solution, 10 mL Tween 80, 2 g beef extract, and 10 g yeast extract with 100 mL aquadest, autoclave at 121°C for 15 minutes. The liquid GYP media was made in the same procedure, but without adding agar and CaCO$_3$. The prepared GYP media then was poured into petri dishes and was let until became harden. Later, a loopful of *L. plantarum* was smeared onto its surface and incubated at 37°C for 1-3 days anaerobically. After the incubated microbe had been grown, it was relocated into liquid MRS media and reincubated aerobically at 37°C for 24 hours. Later, its liquid was measured for OD$_{600}$ value until it had range value of 0.5-0.6, then separated by centrifugation (10 000 rpm, 10 minutes, 4 °C), to produce pellet. The pellet was rewashed twice then diluted into phosphate buffer, mixed with activated zeolite for immobilization process later.

2.2.2. Biosensor Preparation Measurement. Carbon paste electrode was prepared by mixing graphite, paraffin oil (2:1 m/m) and Q$_0$ as mediator (3% of graphite mass). Q$_0$ was diluted first into DMSO before mixing it with graphite. After Q$_0$ and graphite mixed homogenously (DMSO should has been evaporated), paraffin was added and mixed again, transforming it into carbon paste. Next, the modified carbon paste was put into the electrode compartment, packed densely and polished. After 5 days from the first preparation, the electrode characterized by cyclic voltammetry method using KCl...
0.1 M and K$_3$[Fe(CN)$_6$] 0.01 M. The parameters were: mode cyclic, initial 500 mV, final 500 mV, rate 250 mV/s, step W 20 ms, upper E 900 mV, lower E -600 mV, range 5 V, and arus 100 μA.

After the electrode was tested by voltammetry cyclic, the harvested microbe at the previous step was immobilized onto the electrode surface. Firstly, 10 mL phosphate buffer was prepared and mixed with several mg of zeolite. From the mixture, 10 μL mixture was mixed suspended _L. plantarum_, then 10 μL of the mixture was immobilized as bioreceptor onto the electrode's surface. After immobilization process was done, the electrode was stored into buffer phosphate solution (pH 7, 4 °C) until the usage. Biosensor measurement was conducted by setting the parameters in the computer as like in the electrode characterization process. In this step, we measured the oxidation peak of the current from every treatment.

For measurement analysis the optimum condition was set by combining 3 factors, zeolite, _L. plantarum_, and uric acid used for further test. The combinations of 3 variables are listed in table 1 by using Central Composite Design. Before optimizing 3 variables given, we searched the optimum the acidity of _L. plantarum_ (pH 5, 6, 7). The measurement was conducted in room temperature, with blank sample (2 mL phosphate buffer). After adding some uric acid into the cell, the current was again measured.

| No. | Zeolite (mg) | [Uric Acid] (mM) | _L. plantarum_ (µL) |
|-----|-------------|-----------------|---------------------|
| 1   | 250         | 4               | 20                  |
| 2   | 50          | 0.1             | 100                 |
| 3   | 50          | 2.05            | 60                  |
| 4   | 250         | 0.1             | 100                 |
| 5   | 150         | 2.05            | 20                  |
| 6   | 150         | 2.05            | 60                  |
| 7   | 150         | 2.05            | 60                  |
| 8   | 150         | 2.05            | 60                  |
| 9   | 150         | 2.05            | 60                  |
| 10  | 150         | 2.05            | 100                 |
| 11  | 50          | 4               | 100                 |
| 12  | 50          | 4               | 20                  |
| 13  | 250         | 2.05            | 60                  |
| 14  | 150         | 2.05            | 60                  |
| 15  | 150         | 0.1             | 60                  |
| 16  | 150         | 2.05            | 60                  |
| 17  | 250         | 4               | 100                 |
| 18  | 250         | 0.1             | 20                  |
| 19  | 50          | 0.1             | 20                  |
| 20  | 150         | 4               | 60                  |

At the optimum point, we measure the kinetic parameters of uricase activity according to Michaelis-Menten equation, and transformed it into Lineweaver-Burk plot. Here is the formula:

\[
I = \frac{I_{\text{m}}^\text{opt} [\text{Uric acid}]}{K_M + [\text{Uric acid}]}
\]
There were 2 kinds of validation technique performed in this research: LOD (limit of detection) and LOQ (limit of quantization) value, and stability. LOD and LOQ value were counted according this equation:

\[ Q = \frac{k \cdot sb}{b} \]

Q : LOD or LOQ value
k : constant, LOD = 3, LOQ = 10
sb : standard deviation of linearity measurement
b : slope of linearity measurement

The parameter of stability was divided into 2 kinds of treatment. The first was by using the same electrode from the initial day, and the second was by using new-made electrode for each day. Both treatments were done for 20 days. From both treatments, current data were obtained and calculated using this formula:

\[ \text{Stability} (\%) = (100\% - \frac{[I_0 - I_t]}{I_0} \times 100\%) \]

I₀ = oxidation current at day-0
Iₜ = oxidation current at day-ₜ

3. Result and Discussion

In this research we used uricase from \textit{L. plantarum}. Uricase activity was measured electrochemically. The enzymatic reaction oxidizes uric acid to allantoin facilitated by oxygene. It produces \( \text{H}_2\text{O}_2 \) as the product of \( \text{O}_2 \) reduction. This peroxide is very reactive and rapidly change to \( \text{O}_2 \) and transferring electron. This electron was transmitted to electrode and detected as signal. hereby the following reactions [12]:

\[ \begin{align*}
\text{Uric acid} + \text{O}_2 + \text{H}_2\text{O} & \xrightarrow{\text{Uricase}} \text{Alantoin} + \text{CO}_2 + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 & \rightarrow \text{O}_2 + 2\text{H}^+ + 2\bar{e}
\end{align*} \]

By using modified carbon paste electrode (2:1 graphite/paraffine mass) we examined the characteristic of this biosensor. We used \( Q_0 \) (2,3-dimethoxy-5-methyl-1,4-benzoquinone) as the modifier for enhancing oxidation current detected by the potentiostat. Having the ready electrodes for further measurements, we tried to immobilized \textit{L. plantarum} directly to the electrodes at various pH (5, 6, and 7) then we measured the produced current (using 0.5 mM uric acid). Obviously we obtained at pH 5.0 the electrode produced higher current compared the others. It means that \textit{L. plantarum} was more stable at pH 5.0, like in the planktonic mode [13].
Based on this pH, the amount of *L. plantarum* and zeolite applied onto the electrode surface was examined. After analyzing the result, we got the optimal current would be produced by mixing 250 mg zeolite with 100 μL *L. plantarum* at 2.87 mM of uric acid. Therefore, at this optimum point, we studied the uricase activity of *L. plantarum* after being immobilized onto the zeolite by observing the current after introducing various uric acid concentration (0.1-4.0 mM). At room temperature, it only produced 5.73 μA, the maximum current, at 3.3 mM uric acid (figure 2). The result was lower compared to the similar research which have been conducted before, because the pH affected the enzyme stability. And also, it used a lower temperature compared to former study [14].

**Figure 1.** *L. plantarum* response to various pH (5, 6, and 7).

**Figure 2.** Voltammogram profile of immobilized *L. plantarum* onto zeolite.
Analytical properties of measurement method will describe how the proposed method works. In this research we examined LOD, LOQ, linearity, and range of measurement. Based on figure 3, this proposed method had best range between 0.1-3.3 mM uric acid. It is still inside the normal range of uric acid in blood. This method also had good linearity and had strong correlation between signal and uric acid concentration, obviously seen from $R^2$ value (96.97%). It had low LOD value, 0.4827 mM which means it can be useful for detecting uric acid at small concentration. However, it has a higher value of LOQ, up to 1.6092 mM, indicating this method is surely good for detecting uric acid above stated LOQ value. We conducted also a test about the stability of this biosensor for 16 days, however it didn't produce good stability. Along that period. Some further enhancement are needed for developing this alternative method for measuring uric acid.

![Figure 3. Linearity and measurement range of this uric acid biosensor.](image)

$y = 1.544x + 1.0291$

$R^2 = 0.9667$

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