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Activity and Stability of Uricase from *Lactobacillus plantarum* Immobilizated on Natural Zeolite for Uric Acid Biosensor

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Abstract: Determination of uric acid concentration in human urine and blood is needed to diagnose several diseases, especially the occurrence of kidney disease in gout patients. Therefore, it is needed to develop a simple and inexpensive method for uric acid detection. The purpose of the research was to observe the use of Indonesian microbe that was immobilized on natural zeolite as a source of uricase for uric acid biosensor. Selection of mediators and determination of optimum condition measurement, the stability and kinetic properties of *L. plantarum* uricase were performed using carbon paste electrode. Cyclic voltammetry was employed to investigate the catalytic behavior of the biosensor. The result indicated that the best mediator for measurement of *L. plantarum* uricase activity was Qo (2,3-dimethoxy-5-methyl-1,4 benzoquinone). Optimum conditions for immobilization of *L. plantarum* uricase on zeolite were obtained at pH 7.6, with temperature of 28°C, using uric acid concentration of 0.015 mM and zeolite mass at 135 mg K_M and V_{Max} of *L. plantarum* uricase obtained from Lineweaver-burk equation for the immobilization uricase on zeolite were 8.6728×10^{-4} mM and 6.3052 mM, respectively. K_M value of *L. plantarum* uricase directly immobilized onto the electrode surface was smaller than K_M value of *L. plantarum* uricase immobilized on zeolite. The smaller K_M value shows the higher affinity toward the substrate. The Electrode when kept at 10°C was stable until 6 days, however the immobilized electrode on zeolite was stable until 18 days. Therefore, Indonesian *L. plantarum* could be used as a uric acid biosensor.

Key words: Activity, stability, uricase, *Lactobacillus plantarum*, natural zeolite, uric acid biosensor

INTRODUCTION

Uric acid (2,6,8-trihydroxypurine) is the main final product of purine metabolism in humans. Abnormalities of uric acid level are symptoms of cardiovascular disease and hypertension (Johnson *et al*., 2003). Therefore, the uric acid level in the body need to be measured rapidly and accurately. One method that is commonly and widely used to determine uric acid level is spectrophotometry method. However, because of its low specificity, expensive cost and sensitivity to the light, spectrophotometric method is now becoming obsolete and began to change into biosensor. Biosensor is an analytical tool to determine the concentration of substances using biological system, usually an enzyme (Turner *et al*., 1987). One of the enzymes used as a biological component in uric acid biosensor development is enzyme urate oxidase (uricase). This enzyme plays an important role in catalyzing the uric acid oxidation reaction. Generally, uric acid can be isolated from vertebrate animals. However, due to the complicated isolation procedure, limited materials and enzyme sources availability and also high costs, then used alternatives sources such as molds, yeasts and bacterias (Atalla *et al*., 2009). One of the uricase-producing bacteria is *Lactobacillus plantarum*. Previous studies showed that the activity of uricase isolated from *L. plantarum* measured by spectrophotometric methods was still lower

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than the purely-commercial uricase activity from *Bacillus* sp. In addition to the low activity, stability was also low, until the second day. The low activity and stability of *L. plantarum* uricase was allegedly due to the lack of spectrophotometry method selectivity in measuring uricase activity (Iswantini *et al*., 2009).

One way to overcome these shortages is by immobilizing the enzyme in a matrix or material such as nanomaterial (Tian *et al*., 2005). The proper immobilization technique known to be able to increase the specificity and stability of immobilized enzyme (Campanella *et al*., 2004; Caramori and Fernandes, 2004). The right material selection for immobilization matrix can also determine the generation of high currents response in electrochemical biosensor. Nanocomposite, combination of two or more nano-scaled material substance, has a potency as a good immobilization matrix. In this research, natural zeolite nanocomposite from Indonesia were used. So far, there are no any reports about utilization of Indonesia natural zeolite nanocomposite as enzyme immobilization matrix. Therefore, the activity of *L. plantarum* uricase immobilized in natural zeolite nanocomposite using electrochemical biosensor method were studied.

MATERIALS AND METHODS

Microbial cells and reagents: Indonesian microbe: *Lactobacillus plantarum*, were grown on Glucose Yeast Peptone (GYP) medium for 24 h at 37°C and were incubated to reach the OD_{610} value of 0.5. The cells were harvested by centrifugation, were washed twice with a saline solution (0.85% NaCl) and were kept at 5°C.

Q_0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone) were purchased from Sigma Chemical Co. All other chemicals used were high purity, commercially available materials.

Zeolite activation: Zeolite (from Indonesia) was washed with aquadest until reached neutral pH, filtered and dried in oven at 105°C. Dried zeolite was activated by adding HCl and stirred for 1 h. Activated zeolite then was washed with aquadest until reached neutral pH and chlorine free. After that, zeolite was dried at 300°C for 3 h. Finally, it was mashed and sifted (100 mesh).

Preparations of the electrode modified with microbial cells: A carbon paste was constructed by packing a mixture of graphite powder and paraffin liquid with ratio 2:1 into one end of a glass tubing and the surface was smoothed using a piece of waxed paper. Zeolite nanocomposite matrix was suspended in 1 mL saline solution containing *L. plantarum*. The mixture then was stirred and suspended for 1 h. Onto the surface of carbon paste electrode, a 10 μL of aliquot of the cell suspension of *L. plantarum* was dropped, the solvent was allowed to evaporate, then the surface was covered with a dialysis membrane and fixed with nylon fibre. The electrode thus prepared, which is referred to as a whole cell electrode in the following, was used for enzyme-electrochemical measurements.

Electrochemical measurements: Electrochemical measurements were carried out with eDAQ potensiostat (Ecorder 410) under anaerobic conditions and Echem v 2.1.0 software. In which an Ag|AgCl| Sat. KCl electrode, a platinum disk and carbon paste electrode were used as the reference, counter electrodes and working electrode, respectively. The measurements were conducted at room temperature with an electrolysis cell containing 1 mL of basal solution of borate buffer at a certain pH, the optimum pH was determined previously. The test solution was stirred with a magnetic stirrer and was deaerated by passing over argon gas, unless stated otherwise. The change of currents response after each addition were monitored and recorded.

Kinetic properties: Kinetic properties of immobilized *L. plantarum* uricase were determined using Michaelis-menten equation. Lineweaver-Burk plot was also made by derived the Michaelis-menten.

RESULTS

Mediator selection: According to the result of three substance using as mediator, i.e $K_3[Fe(SCN)_6]$, 2,3-dimethoxy-5 methyl -1,4 benzoquinone (Q_O), dan ferrocene ($Fe(C_5H_5)_2$), the suitable substance for mediating the electron transfer generated from uric acid oxidation reaction catalyzed by immobilized uricase to electrode surface was Qo. As shown in Table 1, oxidation peak generated from Qo-mediated electron transfer was the highest amongst other mediators.

Immobilization of *L. plantarum*: Table 2 shows current response generated from two different methods. *L. plantarum* uricase immobilized in zeolite matrix produced higher oxidation current than uricase immobilized directly onto electrode surface.

Table 1: Oxidation current peak of $K_3[Fe(SCN)_6]$, 2,3-dimethoxy-5 methyl-1,4 benzoquinone (Q_O) and ferrocene ($Fe(C_5H_5)_2$) as mediators for measurement of uric acid concentration

Mediator	Oxidation current (μA)
Qo	28.9267
Ferrocene	5.3767
$K_3[Fe(SCN)_6]$	3.5700

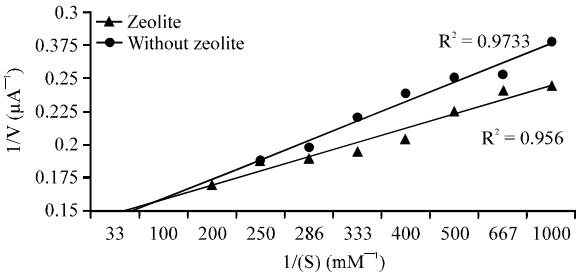

Fig. 1: Lineweaver-burk plot of uricase activity from *L. plantarum* immobilized with zeolite and without zeolite

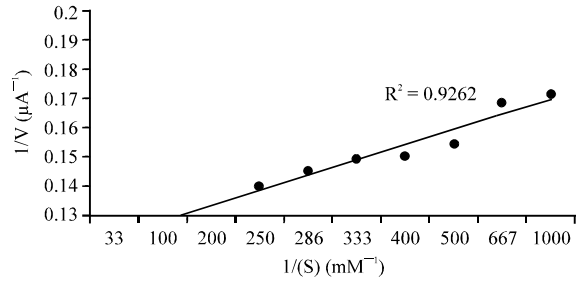

Fig. 2: Lineweaver-burk plot of pure uricase

Table 2: Oxidation current response of *L. plantarum* uricase that was immobilized without zeolite and with zeolite

Sample	Ip_{ox} (μA)
Buffer	1,3300
Qo	3,3000
Uric acid	5,5700
Uric acid, zeolite	12,8800

Kinetic properties of immobilized *L. plantarum* uricase: Figure 1 shows linearity between uric acid as substrate concentration and *L plantarum* uricase activity. Immobilized uricase with and without nanocomposite zeolite matrix had same regression value, i.e 97.74 and 97.37%, respectively. Lineweaver-Burk plot can be seen in Fig. 1 and 2. The figures indicate that K_M and V_{Max} values obtained from Lineweaver-Burk equation were 4.9038×10^{-3} mM and 5.8514 μA for the *L. plantarum* uricase directly immobilized onto electrode surface and 8.6728×10^{-4} mM and 6.3052 μA for the *L. plantarum* uricase immobilized in zeolite firstly.

Electrode stability: Electrode stability was determined from uricase activity measurement from initial time (t = 0) to some definite times. It was a relation between time and stability percentage where percentage of initial time considered as 100%. It is shown in Fig. 3. Immobilization of *L. plantarum* in zeolite could increase the stability up to sixth day at 28°C and 18th day at 10°C.

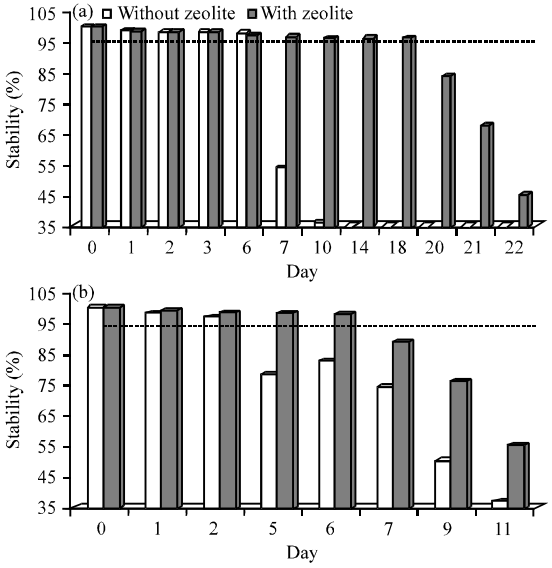

Fig. 3(a-b): The effect of zeolite on the stability of *L. plantarum* uricase that was immobilized on the surface of carbon paste electrode when kept at (a) 10°C and (b) 28°C

DISCUSSION

In this study, determination of the activity of uricase in *L. plantarum* immobilized in natural zeolite nanocomposite using electrochemical biosensor method as uric acid biosensor were performed. Firstly, the best mediator for uric acid concentration measurement was determined. Mediator is an electron transfer agent which can be easily involved in redox reaction with a particular biological component. Mediator can accelerate electron transfer process in enzymatic reaction. The best mediator is one that can produce the highest current as a response of oxidation process (Zhao and Jiang, 2010). Based on the result of the research that has been done to three kinds of mediator, i.e $K_3[Fe(SCN)_6]$, Q_O and ferrocene ($Fe(C_5H_5)_2$), the most suitable mediator for uric acid oxidation by *L. plantarum* uricase was Qo (Table 1).

Selectivity and stability of immobilized enzyme, beside be affected by the substrate, were also by immobilization technique and matrix material used (Yao *et al*., 2007). Therefore, it was necessary to select the suitable method so that the high current responses can be generated. There were two kinds of immobilization technique, first, *L. plantarum* was immobilized onto carbon paste electrode surface directly and second, *L. plantarum* was immobilized in zeolite before immobilized onto carbon paste electrode surface. The amount of *L. plantarum* cell for every immobilization on to

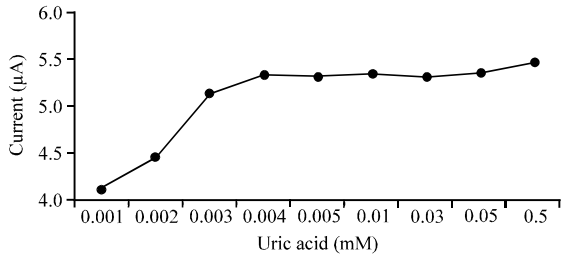

Fig. 4: Relationships between uric acid concentration and current responses

electrode surface was $7,5\times10^{5}$ CFU mL^{-1}. Current response produced by two immobilization methods can be seen in Table 2. It shows that *L. plantarum* firstly immobilized in zeolite was generated higher current response than directly immobilized onto electrode surface. This is agree with Varoyd *et al.* (2007) which modified carbon paste electrode with zeolite and methylene blue as mediator, the modified electrode could produced high electrocatalytic oxidation currents. Balal *et al.* (2009) also modified carbon paste electrode with zeolite nanocomposite and $FeCl_3$ as mediator that could increase the current responses. Mazloum-Ardakani *et al.* (2009) used zeolite as nanocomposite for carbon paste electrode modifier that increase not only the current response, but also the sensitivity in Cu^{2+} ion measurement. The ability of zeolite in increasing current response is because zeolit has a unique characteristic, i.e uniform pores and frame structure, so that resulted adsorbed enzymes with higher selectivity and reproducibility.

Optimalization of immobilized *L. plantarum* uricase activity was needed to be done to study the effects of zeolite addition to its activity and stability. The parameter were temperature (25-45°C), pH (7-10), uric acid concentration (0.001-0.05 mM) and zeolite weight (30-240 mg). Based on those contours, the optimal conditions for immobilized *L. plantarum* uricase activity were pH 7.6, temperature 28°C, uric acid concentration 0.0043 mM and zeolite weight 135 mg. The most affected conditions were pH and temperature. pH shift occurred because enzyme was immobilized within a matrix having different charge, while temperature shift occurred because of immobilized enzyme produced inhomogenity so that caused any deviation to the Arrhenius plot (Bisswanger, 2008).

Characterization of enzyme specificity to the substrate can be studied by determination of its kinetic properties, i.e Michealis-menten (K_M) constant and Maximum rate which analogous with maximum current (I_{Max} or V_{Max}). These properties were determined by measuring the activity of immobilized *L. plantarum* uricase activity against its substrate (uric acid) concentration. It is showed in Fig. 1 and 2, the curve shape is identical with Michealis-menten curve. It shows that enzyme-catalyzed reaction was occurred in two stages. First, in the substrate concentration range of 0.001-0.005 mM, the active sites of immobilized *L. plantarum* uricase did not fully bind the uric acid. It was agree with previous study by Iswantini *et al.* (2009). Second, when the uric acid concentration was higher than 0.005 mM, when the uricase was saturated by the uric acid so that it reached steady state condition where the advanced uric acid addition did not affect uricase activity. Immobilized uricase with and without nanocomposite zeolite matrix had same regression value, i.e 97.74 and 97.37%, respectively (Fig. 4).

Most of enzymatic reactions can be analyzed quantitavely using Michealis-menten theory, but the K_M and V_{max} value are difficult to be determined by Michealis-menten curve. Therefore, it needs another way to determine K_M and V_{Max} value using Lineweaver-Burk equation as shown in Fig. 1 and 2, based on Lineweaver-Burk equation, K_M and V_{Max} values of the *L. plantarum* uricase directly immobilized onto electrode surface obtained were 4.9038×10^{-3} mM and 5.8514 μA and for the *L. plantarum* uricase immobilized in zeolite firstly were 8.6728×10^{-4} mM and 6.3052 μA. K_M value of *L. plantarum* uricase directly immobilized onto electrode surface was smaller than K_M value of *L. plantarum* uricase immobilized in zeolite. The smaller K_M value shows the higher affinity to the substrate, so that lower substrate concentration can saturate the enzyme active site immediately. These values were different with pure uricase isolated from *Bacillus fastidious* (3.1384×10^{-3} mM) and *Candida* sp. (5.2×10^{-3} mM) (Yang *et al.*, 2011). This differences were due to different bacteria producing different amounts of uricase.

K_M and V_{Max} values obtained from this study were different with the values obtained using spectrophotometry method, i.e 0.1541 mM and 1.3635. This is probably caused by the sensitivity and selectivity differences of both methods. Spectrophotometer only detects based on absorbed and scattered light by the sample, while the electrochemical method only detects the electron transfer generated by redox reaction. Uric acid concentration could be detected *in vivo* using electrochemical method up to 0.0045 mM, this result was similar to previous result that was performed by spectrophotometer method (Iswantini *et al.*, 2009). The result indicated that the capability of *L. plantarum* uricase for uric acid biosensor still could not be increased by utilization of nanomaterial and electrochemical method.

Electrode stability: Electrode stability was determined by measuring immobilized uricase activity after obtaining the optimum condition. The stability described as the relationship between stability percentage and time, where the stability percentage at the initial time (t = 0) was considered as 100% (Fig. 4). Stability of electrode stored at 10°C was longer, i.e up to sixth day, while at room temperature (28°C) only up to second day. It caused by enzyme denaturation so the activity decreased. The stability at room temperature obtained was similar with the result obtained using spectrophotometry method. Immobilization of *L. plantarum* pada in zeolite could increase the stability (Fig. 4), i.e up to sixth day at 28°C and 18th day at 10°C. The increasing of electrode stability by *L. plantarum* immobilization in zeolite was probably caused by zeolite protection to the part of enzyme active site so that it slower the enzyme denaturation.

However, the results reported in this paper indicated that *L. plantarum* originated from Indonesia could be used as uric acid biosensor with high stability. Other Indonesian microbe, *E. coli* whole cells also had high capability as glucose biosensor (Iswantini *et al*., 2011).

CONCLUSION

L. plantarum uricase immobilized in zeolite had higher activity than *L. plantarum* uricase immobilized directly onto electrode surface. Enzyme activity was related to the current changing. K_M value of *L. plantarum* uricase directly immobilized onto electrode surface was smaller than K_M value of *L. plantarum* uricase immobilized in zeolite. The smaller K_M value shows the higher affinity to the substrate. Immobilization of *L. plantarum* uricase could increase the electrode stability and more increasing if it stored at 10°C.

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