MICROBIOLOGY INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol 5, No 1, March 2011, p 9-14

Available online at:
http://www.permi.or.id/journal/index.php/mionline
DOI: 10.5454/mi.5.1.2

Glucose Biosensor Using Selected Indonesian Bacteria

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Microbial glucose sensors have been developed using *Escherichia coli* bacterial strains from Japan. However, there is interest in developing local bacteria as glucose sensors in Indonesia. In this research, the stability and the potential of a selected number of Indonesian bacteria as glucose biosensors was explored. Results of this study indicate that three of them, *E. coli*, *Bacillus subtilis,* and *Thermus filiformis* exhibit properties of high viability and stability at high temperature (30-60 ºC). Spectrophotometrical and electrochemical measurements showed significant absorbance values and highly stable current features for *E. coli* as indicated by its high capacity to produce glucose dehydrogenase. *E. coli, B. subtilis,* and *T. filiformis* produced currents of 3.25 µA, 0.2 µA, and 0.02 µA respectively, and *E. coli* also produced a much higher activity of glucose dehydrogenase. Electrochemical measurement using *E. coli*-modified carbon paste electrode allowed the determination of glucose concentration of up to 20 mM. Therefore, Indonesian *E. coli* has a high stability and can be used as a glucose biosensor.

Key words: glucose biosensor, electrochemistry, bacteria, *Escherichia coli*, *Bacillus subtilis*, *Thermus filiformis*

Escherichia coli asal Jepang telah digunakan sebagai sensor glukosa. Pengembangan menggunakan bakteri asal Indonesia yang berpotensi sebagai biosensor glukosa perlu dieksplorasi. Hasil seleksi menunjukkan ada tiga bakteri, yaitu *E. coli*, *Bacillus subtilis,* dan *Thermus filiformis* yang stabil pada suhu tinggi (30-60 ºC). Uji enzim glukosa dehidrogenase menggunakan metode spektrofotometri dan elektrokimia menetapkan *E. coli* sebagai bakteri yang berpotensi paling baik dalam menghasilkan serapan cahaya dan stabilitas arus dibandingkan dengan *B. subtilis* dan *T. filiformis. E. coli* dapat menghasilkan arus sebesar 3.25 µA, sedangkan *B. subtilis* dan *T. filiformis* berturut-turut hanya 0.2 µA dan 0.02 µA, maka *E. coli* dapat menghasilkan aktivitas glukosa dehidrogenase yang lebih besar daripada bakteri lain. Secara elektrokimia, *E. coli* yang diimobilisasikan di atas permukaan elektroda pasta karbon dapat mendeteksi konsentrasi glukosa sampai 20 mM. Oleh karena itu, *E. coli* asal Indonesia ini mempunyai peluang untuk dikembangkan sebagai biosensor glukosa.

Kata kunci: biosensor glukosa, elektrokimia, bakteri, *Escherichia coli*, *Bacillus subtilis*, *Thermus filiformis*

The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030, and the top four countries are India, China, United States and Indonesia for both 2000 and 2030 (Wild *et al.* 2004). Hence, the need for sensors that can quickly measure blood glucose level is high. Currently, a disposable glucose sensor is available in the market. However, the enzyme sensor is expensive and has a low stability. Recent developments show that microorganisms can also be used to produce glucose sensors (Ohfuji *et al*. 2004). The sensor is called a microbial sensor. Compared to enzyme sensors, microbial sensors have the advantages of increased length of sensor lifetime, and lower cost because the active enzyme does not need to be isolated and purified (Lobanov *et al.* 2001).

Microbial biosensors have been developed for the measurements of glucose (Richardson *et al*. 1991), succinate (Richardson *et al.* 1991), lactate (Smutok *et al*. 2007), and nicotinic acid (Takayama *et al.* 1995). Other related studies to glucose biosensor using microbe as biocatalyst had been reported by Ikeda *et al*. (1998) and Iswantini *et al*. (1998), while electrochemical method had been successfully applied to study the kinetics and thermodynamics of the holoenzyme formation *in vivo* and to the measurements of the enzymatic activity by *E. coli* K12 (IFO3301) cells. Ikeda *et al*. (2001) reported that freeze-dried *E. coli* cells had been prepared and utilized for the construction of the whole cell-based glucose sensor, meanwhile Yu and Liu (2003) used GOD enzyme with flow injection analysis. Recent research indicates that glucose dehydrogenase (GDH) derived from *E. coli* can function as an enzymatic biofuel cell, which can operate as a glucose biosensor (Chau *et al*. 2009).

Previous research has explored non-pathogenic Indonesian microbes in their ability to produce GDH enzyme. Nevertheless, this research in Indonesia has focused on GDH enzyme farming. The aim of this research was to obtain Indonesian bacteria to be used as glucose biosensors using the electrochemical method.

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MATERIALS AND METHODS

Microbial Cells and Reagents. *E. coli*, *B. subtilis*, and *T. filiformis* were isolated from Semurup Hotspring Area, Kerinci-Jambi, Sumatera. The bacteria were selected based on glucose utilization and Gram staining (Gram negative for *E. coli*, Gram positive spore forming for *B. subtilis,* and temperature resistance of the thermophile *T. filiformis*. The bacteria were grown on heterotropic liquid medium (1.5% tryptone, 0.5% yeast extract, 0.5% NaCl) for 2 h at 37 °C for *E. coli* and *B. subtilis*, and 50 °C for *T. filiformis* to a late logarithmic phase. They were then incubated to reach the OD_{610} of 0.5. The cells were harvested by centrifugation washed twice with a saline solution (0.85% NaCl) and were kept refrigerated at 5 °C.

PQQ (2,7,9-tricarboxyl-1H-pyrrolo[2,3-f]quinoline-4,5-dione) and Q_0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone) were purchased from Sigma Chemical Co. All other chemicals used were of high purity.

Spectrophotometrical Measurements. 75 µL of each cell suspension was added to phosphate buffer containing PQQ (0.3 M and 0.6 M) and $MgSO_4$ as prosthetic group and activator, respectively. 175 µL of 1 mM glucose was added as a substrate. Absorbance was measured at 340 nm every 15 sec for 3 min. Enzyme activity is equal to the concentration of NADPH produced per mL per min.

Preparations of the Bacterial Cell Modified Electrode. Carbon paste electrodes were constructed by packing a 2:1 ratio of graphite powder and paraffin liquid into one end of glass tubing, and the surface was smoothed using a piece of waxed paper. A 10 µL of aliquot of the cell suspensions was applied onto the surface of each carbon paste electrode. The solution was allowed to evaporate. Next, the surface was covered with a dialysis membrane, and fixed with nylon fibre. The finished electrodes, referred to as whole cell electrodes in the following text, were used for enzyme-electrochemical measurements.

Electrochemical Measurements. Electrochemical measurements were carried out with BASi EC Epsillon voltammetry analyzer under anaerobic conditions, in which an Ag|AgCl|Sat KCl electrode, a platinum disk, and a carbon paste electrode were used as a reference electrode, a counter electrode, and a working electrode, respectively. The measurements were conducted at room temperature (25 °C) with an electrolysis cell containing 1 mL of basal solution of phosphate buffer at a previously determined optimum of pH 6.5. The test solution was stirred with a magnetic stirrer and was deaerated by passing over argon gas, unless stated otherwise.

RESULTS

Selection of a High Lifetime Stability Indonesian Microbe. From a number of microbial species, *E. coli*, *P. fluoresens, Enterobacter, Bacillus, Bactotermo,* and *S. stearothermophyllus* were screened for temperature resistance from 25 °C to 50 °C over a 1-3 month period. Microbes showed temperature resistance and long stability (Tables 1 and 2).

***In Vivo* Spectrophotometrical GDH Activity Measurement.** *In vivo* bacterial GDH enzyme activity was determined spectrophotometrically at 340 nm. The results showed that GDH activity increased up to 200 times when in the presence of PQQ and $MgSO_4$. The activity of GDH in the presence of Na_2EDTA was much higher than that without Na_2EDTA (Table 3).

***In Vivo* Electrochemical Measurement of GDH Activity.** Using the reactivation of the *E. coli*-modified carbon paste electrode the capability of microbes as glucose biosensor was electrochemically measured by examining the effects of PQQ (Table 4) and Mg^{2+} ions and determination of the GDH activity. The current-time curve measured at 213 mV with the *E. coli*-modified electrode is shown in Fig 1, while Fig 2 shows the relation between the current and the pH of the solution, Fig 3 and 4 show the current-time curve with *B. subtilis*- and *T. filiformis*-modified electrodes.

Table 1 The life span of several Indonesian microbes

Microbes	Life span
Escherici coli	2 weeks
Bacillus subtilis	5 years
Thermus filiformis	2 weeks
Pseudomonas. fluorescens	2 days
Enterobacter	2 days
Streptococcus	1 week

Table 2 Temperature resistance of several Indonesian microbes

Microbes	Maximum temperature resistance (ºC)
Eschericia coli	37
Bacillus subtilis	42
Thermus filiformis	60
Pseudomonas fluorescens	37
Enterobacter	37
Streptococcus	30

Table 3 Activity of GDH enzyme in *Eschericia coli* (spectrophotometrically)

Buffer	GDH activity (µmol NADPH mL^{-1} min^{-1})		
	Without PQQ and $MgSO_4$	+PQQ	+PQQ+$MgSO_4$
Containing Na_2EDTA	6.7524×10^{-4}	4.0514×10^{-2}	3.4727×10^{-2}
Without Na_2EDTA	9.6463×10^{-4}	9.6463×10^{-4}	1.9293×10^{-2}

Table 4 Dependence of PQQ concentration on the current

[PQQ] (μM)	Current (μA)	Time (min)
0.1	1.5	30.0
0.5	1.0	30.0
1.2	1.6	14.5
2.3	3.0	6.0
4.6	5.5	6.0
6.0	4.5	5.5

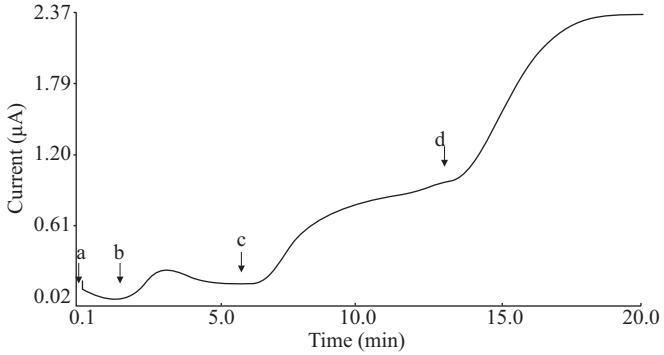

Fig 1 Current-time curve measured at 213 mV with the *Eschericia coli*-modified electrode. a, Starting point of measurement, addition; b, of 5 mM Q_0 and 10 mM glucose; c, 4.6 μM PQQ; and d, 10 mM $MgSO_4$ in the phosphate buffer pH 6.0.

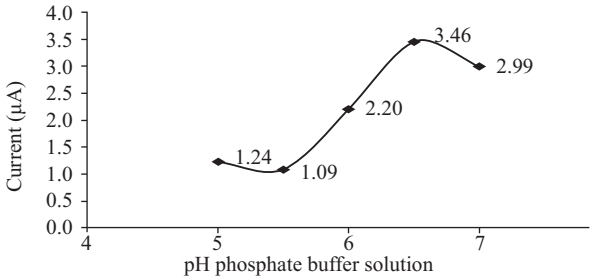

Fig 2 Curve of pH dependence of the current magnitude. —●—, glucose dehydrogenase activity.

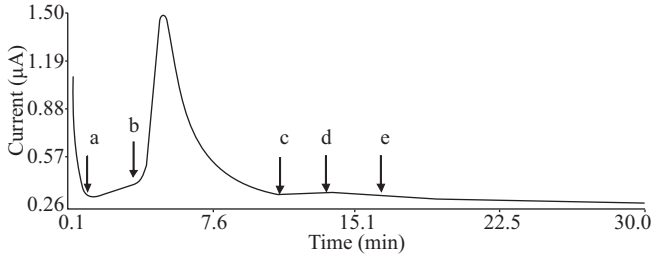

Fig 3 Current-time curve measured at 213 mV with the *Bacillus subtilis*-modified electrode. a, 5 mM Q_0; b, 10 mM glucose; c, 4.6 μM PQQ; and d, 10 mM $MgSO_4$ were added to the phosphate buffer pH 6.5.

Measurement of GDH activity using *E. coli*-modified carbon paste electrode at 213 mV showed significant current in the presence of PQQ and $MgSO_4$, the maximum current attained for 60 sec, the cyclic voltammetry of *E. coli*-modified carbon paste electrode as shown in Fig 6. Fig 7 shows the comparison of GDH activity in *E. coli*, *B. subtilis,* and *T. filiformis*. And Fig 8 shows the glucose concentration dependence of the GDH activity in *E. coli*.

Stability of GDH Activity. The stability of GDH activity was determined on immobilized *E. coli, B. subtilis,* and *T. filiformis* cells on the surface of the carbon paste electrode. The treated carbon paste electrodes were stored in NaCl 0.85% at room temperature. After 6 h the activity of GDH was measured. The results indicated that *B. subtilis* had the greatest stability with 74.04% of the GDH activity remaining after 6 h, followed by *E. coli* at 58% and *Bactotermo* at 27.61%.

DISCUSSION

Of the microbes tested, the isolated *E. coli* , *B. subtilis*, and *T. filiformis* had glucose utilization

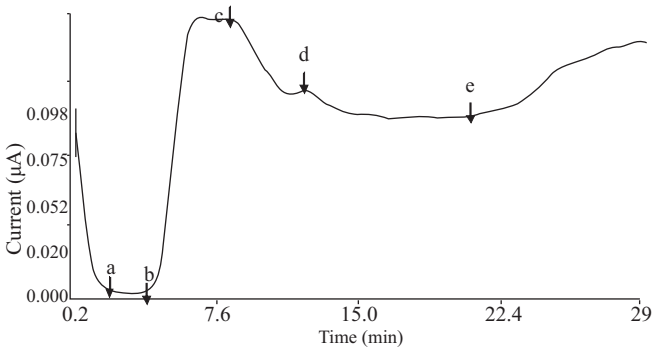

Fig 4 Current-time curve measured at 213 mV with the *Thermus filiformis*–modified electrode. a, Starting point of experiment; b, 5 mM Q_0; c, 10 mM glucose; d, 4.6 μM PQQ; and (e) 10 mM $MgSO_4$ on the phosphate buffer pH 6.

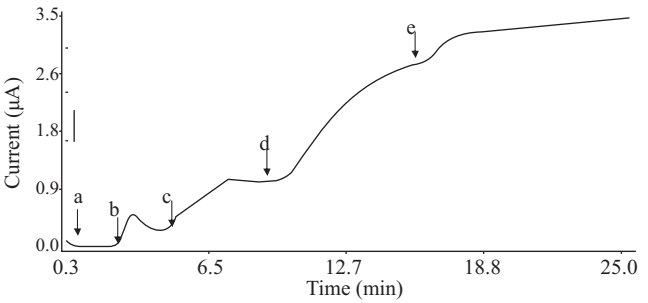

Fig 5 Current-time curve measured at 213 mV with the *E. coli*–modified electrode. a, Starting point of experiment; b, 5 mM Q_0; c, 10 mM glucose; d, 4.6 μM PQQ; and e, 10 mM $MgSO_4$ were added to the phosphate buffer pH 6.5.

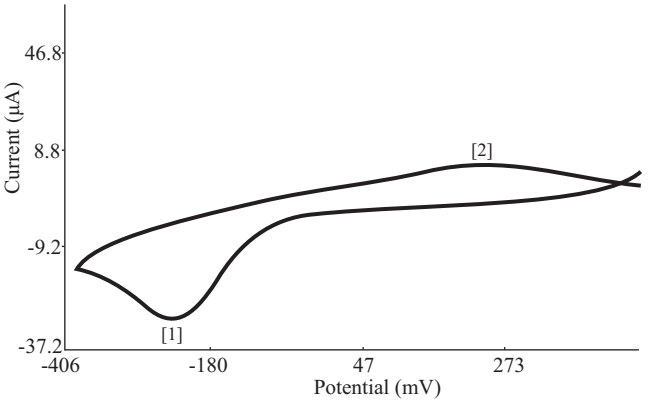

Fig 6 Cyclic voltammetry of *Escherchia coli*-modified carbon paste electrode.

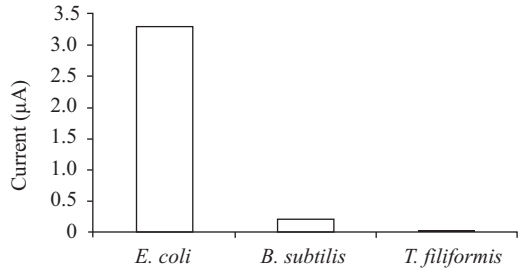

Fig 7 The activity of glucose dehydrogenase in *Escherichia coli*, *Bacillus subtilis*, and *Thermus filiformis* which measured electrochemically.

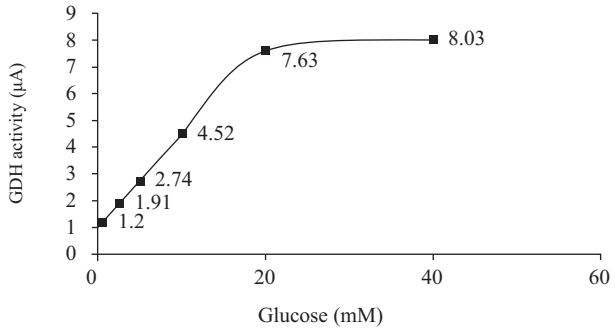

Fig 8 The glucose concentration dependence of the activity of GDH in *Eschericia coli.*

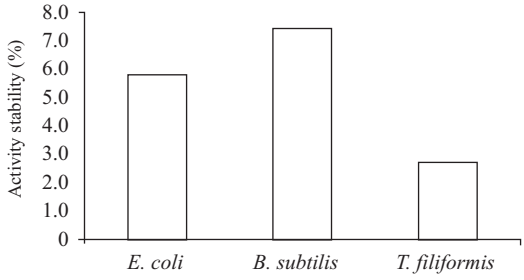

Fig 9 Stability of bacteria glucose dehydrogenase activity for 6 h time period.

characters essential for this glucose biosensor experiment. Their nature of cell wall and temperature respect would then give more on physiological characters on the biosensor output and higher stability with respect to temperature resistance and life span at room temperature than other microbes. *B. subtilis* and *T. filiformis* had the highest life span and stability on temperature resistance, respectively. Although *E. coli* had a lower life span and temperature resistance than that of *B. subtilis* and *T. filiformis*, but only *E. coli* to be potentially used in biosensor in this research supported by previous research (Ikeda *et al*. 2001) proved that *E. coli* could be used as a glucose biosensor. Therefore all of them were used in the experiment. The biosensor capacity is based on oxidation of glucose by GDH enzyme produces gluconate and NADPH with $NADP^+$ as an electron acceptor.

$$\text{Glucose} + NADP^+ \xrightarrow{\text{GDH}} \text{Gluconate} + NADPH$$

Absorbance values resulted from spectrophotometrically measurement indicated that the GDH activity was produced by *E. coli*, *B. subtilis*, and *T. filiformis*. It was known that the *in vivo* oxidation of glucose by GDH depended on the concentration of PQQ as a prosthetic group in the presence of Mg^{2+} ions as an activator of GDH. Meanwhile EDTA could decrease the activity of GDH in *E. coli* by chelating Mg^{2+} ions (Ikeda *et al* 1998; Iswantini *et al*. 1998). This prompted study on the effect of the addition of PQQ and $MgSO_4$ on the activity of GDH in the presence or absence, of Na_2EDTA. *E. coli* cells had the highest GDH activity compared to *B. subtilis* and *T. filiformis* (Table 3). *B. subtilis* had low GDH activity because this is a Gram positive bacterial having cell wall higher in containing peptidoglican much higher than that of Gram negative (*E. coli*) (Demchik *et al*. 1996). The presence and absence of Na_2EDTA in the buffer affected the activity of GDH.

From electrochemical measurements, a current of the fixed potential at 213 mV was applied to follow the appearance of GDH activity (Fig 1). A 5 mM Q_0, 10 mM glucose, 4.6 μM PQQ, and 10mM $MgSO_4$ were added to the buffer solution in that order. A small current started to appear when Q_0 and glucose were added. It started to increase only after the addition of PQQ, because apo-GDH (inactive GDH) in the *E. coli* cells was converted to holo-GDH (active GDH) by incorporating PQQ added to the solution, and the holo-GDH catalyzed the oxidation of glucose with Q_0 as an electron acceptor to produce a catalytic anodic current. When $MgSO_4$ was added to the solution, the current started to increase again and approached a significant magnitude, then continued to increase gradually and reached the steady state until at least 7 min. This result indicated that *E. coli* could produce GDH enzyme that could oxidize glucose to be gluconate, and the *in vivo* GDH activity could be determined easily spectrophometrically and electrochemically, at optimum pH of 6.5 (Fig 2) and 4.6 M of PQQ (Table 4). Electrochemically, GDH activity in *B. subtilis* could be detected, but unstable current had been produced after the addition of Q_0 and glucose (Fig 3). It may be due to the peptidoglican content of the cell wall of *B. subtilis* positive bacteria that could not excrete GDH enzyme from its periplasm easily (Demchik *et al*. 1996).

Measurement of GDH activity in *E. coli* modified carbon paste electrode had been carried out at optimum condition (pH 6.5 of phosphate buffer and 4.6 μM PQQ) as shown in Fig 5. Addition of Q_0 and glucose to the buffer solution had little observable effect on the current. It increased significantly about 1.8 A after the addition of PQQ and attained a steady-state. When

$MgSO_4$ was added to the solution, the current started to increase again and approached 0.8 A and attained a steady state for 60 sec while the current was stable for 8 min. This result indicated that measurement of GDH activity in *E. coli*-modified carbon paste electrode at optimum condition gave the highest activity of GDH compared to that of the other microbes. The result indicated that among several Indonesian microbes, *E. coli*, *B. subtilis*, and *T. filiformis* had high stability with respect to temperature resistance and life span.

Measurement of GDH activity using *E. coli*-modified carbon paste electrode at 213 mV gave significant current in the presence of PQQ and $MgSO_4$, the maximum current attained for 60 sec, the cyclic voltammetry of *E. coli*-modified carbon paste electrode as shown in Fig 6. Constant current could be maintained for 8 min. The capability of *E. coli* for glucose biosensor were good when it was done at pH 6.5 of phosphate buffer and 4.6 M of PQQ concentration. Electrochemically, GDH in *E. coli* gave a highest activity among three bacteria tested (*E. coli*, *B. subtilis,* and *T. filiformis*) as shown in Fig 7. The activity of GDH and the number of GDH in bacteria were supposed depend on the structure of their cell wall.

Dependence of glucose concentration on the current produced by *E. coli*-modified carbon paste electrode indicated that this method allowed the detection of glucose concentration up to 20 mM (Fig 8). This result similar to amperometric biosensor for continuous glucose monitoring based on the MWCNTs/graphite/Gox packed needle-type electrode (Jia *et al*. 2008). This indicated that *E. coli* can be developed as glucose biosensor, because the detection limit was comparable to carbon nanotube glucose biosensor. Also, this result showed that using microbe cell as glucose biosensor has similar capability compared to purified enzyme. This method provide less costly because the active enzyme does not need to be isolated and purified. This result was supported by recent research that GDH derived from *E. coli* could be used as the enzymatic biofuel cell, which could operate as a glucose biosensor (Chau *et al*. 2009). The glucose biosensor sensitivity activity of *E. coli* was observed to reach the steady state for as long as 120 sec at 58% of maximum activity after the addition of glucose. This stability is lower than that of GDH activity resulted by *E. coli* K-12 (IFO3301) which shown to reach 70% of maximum activity (Iswantini *et al*. 2000). Fig 9 showed the comparison of the stability of GDH activity resulted that among the 3 bacteria, the activity of GDH in *B. subtilis* was the highest, while the lowest was that of *T. filiformis*. This might be due to the ability of this bacteria to form endospore. Bacteria that have a high ability to form endospore have longer life spans than others. Therefore, a longer life span would result in higher GDH stability. The low GDH stability of *T. filiformis* might be due to the storing temperature: it is a thermophilic bacteria with an optimum temperature of 50-70 °C. Storing at less than these temperatures may cause a degradation of GDH activity. Because, GDH in *E. coli* gave a highest activity among three bacteria tested, electrochemically (Fig 7), however, Indonesian *E. coli* can be used as a glucose biosensor with high stability, even the stability still less than the purified enzyme biosensor that has excellent stability with almost 90% of its bioactivity maintained after storage at 4 °C in phosphate buffer solution for ten days (Wang *et al*. 2008).

ACKNOWLEDGEMENTS

This work was funded by the Indonesia Toray Foundation Research Grant 2005. We would like to thank Tokuji Ikeda from Laboratory of Bioanalytical Physical Chemistry, Department of Agricultural Chemistry, Kyoto University for providing the chemicals.

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