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Electrochemical *Bacillus licheniformis* Whole-Cell-Based Sensor and its Potential Application in Detecting Urea Concentration in Urine

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ABSTRACT

Electrochemical biosensor based on *Bacillus licheniformis* whole cell can detect urea level in a synthetic urine. We employed a synthetic urine solution as the urea source, and the level was indirectly monitored through the ammonia oxidation reaction using *B. licheniformis* microorganism on an electrode surface. The working electrode was a screen-printed carbon electrode (SPCE). *B. licheniformis* pellet was placed on the operated electrode carbon and was added with various urea concentrations in the synthetic urine, then kept for 30 minutes and tested using a cyclic voltammetry technique. The biosensor system results in a limit of detection of 0.01 M urea, 1.278 µA/M sensitivity, and linearity in the range of 0.01–0.2 M urea concentration with R^2 0.990. We conclude that the developed system performs better limit detection as compared to the previous work, and the range of linearity concentration range is within the urea level in the urine. Therefore, this biosensor system can be applied for urea level determination that is an indicator of the kidney health problems.

Keywords: Biosensor, urea, *Bacillus licheniformis,* cyclic voltammetry.

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INTRODUCTION

Urea sensor is required to monitor its concentration in some applied fields, including clinical, environmental, agricultural, and food industrial analysis[1,2,3]. The urea level in human urine is an indicator of kidney and liver health problems. A high level of urea may cause kidney failure and gastrointestinal bleeding, while the low-level causes liver failure, nephritic syndrome, and cachexia[4]. The urea concentration in the normal blood serum and urine are 2.7–7.5 mM[5] and 0.1–0.4 M, respectively[6]. Urea commonly exists in the environment as the result of nitrogen excretion from the organisms and fertilizers from the agricultural sector[7]. The urea would be a serious problem when it is converted into ammonia, which then increases the water pH level and algal bloom, thus decreases the aquatic life[8].

Although some techniques are available to determine the urea content, such as spectrophotometric[9], colorimetric[2], gas chromatography[10], high-performance liquid chromatography[11], optical[12], and fluorimetric[13], these methods are high cost, time-consuming, and require skillful persons to operate[14]. The enzymatic-based biosensor has been created to detect urea by providing a specific signal for a particular molecule[15,16,17]. However, this specific sensor system has limited replications, durability, and cost. Another constraint is the limited working condition based on temperature and pH ranges[18]. Therefore, a microbes-based biosensor is to be developed to overcome the limitations of the enzymatic biosensor[19]. The electrochemical method was also used due to more economical, more sensitive, and more accessible applications[20,21,22,23].

The whole-cell biosensor is proposed as having the potential to increase the enzyme stability in the natural environment, as well as reducing the extraction and the enzyme purification cost[24,25]. The whole-cell biosensor to detect urea had also been performed elsewhere[24,26]; this polymeric resistance changes as the pH increases due to the ammonia production. Another whole-cell urea sensor is using an enzyme with a nitrification bacterium and the total oxygen consumption of the bacteria is assessed through an amperometric technique[27]. The whole-cell biosensor urea is also performed using *Proteus vulgaris* as a biomolecule to detect the urea[28]. However, our proposed sensor will be simpler by using a carbon electrode, especially a screen-printed type.

We developed the screen-print carbon electrode from nanotube carbon ink using a carbon electrode work, where the *Bacillus licheniformis* pellet was placed. Our study aimed to preserve the system as a simple sensor device, where the whole cell of *B. licheniformis* can be potentially utilized to detect the urea concentration in urine. The primary method of urea detection is identified electrochemically through microbial urea conversion into ammonia on the working carbon electrode surface. The test comprised the electrode surface modification using the bacteria pellet, which indirectly monitored the urea concentration in a synthetic urine solution through the alteration of ammonia oxidation current peak from a cyclic voltammetry. We hypothesize that the bacteria placed on the carbon working electrode surface are capable of providing the signal; thus, the ammonia concentration should be proportional to the urea concentration in the synthetic urine.

MATERIAL AND METHODS

The materials used in this work: *B. licheniformis* were collected from the Laboratory of Microbiological Health, Biological Research Center, Indonesian Institute of Sciences. SPCE (DropSens, Oviedo, Spain), heterotrophic solid medium containing agar, tryptone, sodium chloride (NaCl), and dipotassium phosphate (K_2HPO_4). Synthetic urine composition: 8.001 g/L NaCl, 1.641 g/L KCl, 2.632 g/L K_2SO_4, 0.783 g/L $MgSO_4$, 0.661 g/L $KHCO_3$, 0.234 g/L K_3PO_4. The medium for qualitative detection for the presence of urease in the bacteria consisted of 0.20 g peptone, 1.00 g NaCl, 0.20 g glucose, 0.40 g K_2HPO_4, 2.40 mg phenol red, 3.00 g agar in 180 mL distilled water, and 4 g urea in 20 mL distilled water.

Bacterial culture

B. *licheniformis* was grown and rejuvenated on a heterotrophic solid medium in a petri dish, then incubated at 37 °C for 24 hours. The heterotrophic solid medium containing 3.70 g agar, 4.50 g Tripton, 1.25 g NaCl, and 0.63 g K_2HPO_4, was mixed with 250 mL distilled water and stirred until homogeneous in a microwave. The medium was sterilized using an autoclave for 15 minutes at 121 °C. The warm medium was poured into a petri dish, cooled and hardened in a laminar airflow. The solid medium was ready to be used for further bacterial cell growth and rejuvenation.

The qualitative test for *B. licheniformis* producing urease

An isolate of *B. licheniformis* was streaked on a urea medium surface, incubated at 30 °C for seven days, and observed the medium discoloration each day up to seven days. The medium discoloration of yellow to pink, red characterized the positive result of bacteria containing the urease. The composition of the urea medium comprising 0.20 g peptone, 1.00 g NaCl, 0.20 g glucose, 0.40 g K_2HPO_4, 2.40 mg phenol red, 3.00 g agar in 180 mL distilled water, was checked at pH 6.8 and sterilized in an autoclave for 15 minutes at 121 °C. Furthermore, 2% urea was added (4 g urea in 20 mL distilled water) and poured in a petri dish[29].

Whole-cell bacterial production

The bacteria grown on the solid media were transferred to 10 mL liquid heterotrophic medium. The bacterial suspension from the isolate was homogenized using a vortex and centrifuged at 3900 rpm for 10 minutes. This process was repeated twice and washed using a physiological saline solution (0.85% sterile NaCl). The various bacterial cell optical densities were 0.38, 0.42, 0.51, 0.65, 0.75, and 0.87. Each bacterial cell density was measured electrochemically by taking 20 µL bacterial pellets and placed them on the working electrode, added with 40 µL of 0.20 M urea solution, and kept for 30 minutes at the room temperature, then measured by cyclic voltammetry. The remaining bacterial pellets were stored at 4 °C to be used for the subsequent tests without dilution.

Electrochemical current profile measurement

Electrochemical measurements were performed using the cyclic voltammetry method with *eDAQ potentiostat* (Ecorder 410) equipped with *Echem v 2.1.0* software. SPCE used contained the working electrode carbon with a diameter of 4 mm, silver (Ag/AgCl) reference electrode, and carbon electrode counter. Based on ref. [23] with a slight modification, the current used was as the following: *Mode Cyclic, Initial E -0.15 V, Final E 0.15 V, Rate 50 mV/s, Step W 20 ms, Upper E 1.2 V, Lower E -0.15 V, Range 0.2 V*. The blank was the synthetic urine solution. The biosensor performance was evaluated based on the analytical parameters, such as sensitivity, detection limit, and linearity ([urea]: 0.2 M, 0.1 M, 0.05 M, 0.025 M, 0.010 M, 0.001 M).

RESULTS AND DISCUSSION

***B. licheniformis* producing urease**

The isolates of *B. licheniformis* grown on the solid heterotrophic media were tested for their ability to produce urease. The ability in providing urease can be seen by growing them on Christensen's agar medium[30]. The presence of urease causes a change of the change from yellow to pink or red (Fig. 1) as the medium contains phenol red as a pH change indicator. When the bacteria produce urease, they can hydrolyze urea in the medium, turning the yellow medium into a deep red color. Urease hydrolyzes the urea into ammonia and CO_2, thereby increasing the pH and carbonate concentration in the bacterial environments. Fig. 2 illustrates the morphology of *B. licheniformis* during seven days of incubation period at 30 °C in the Christensen's agar medium.

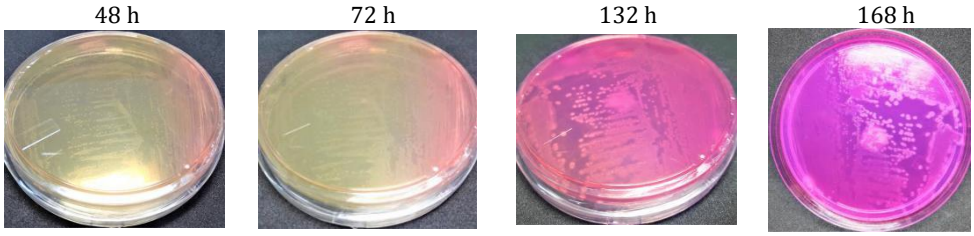

Fig. 1: The qualitative test of *B. licheniformis* bacteria in generating the urease during 48, 72, 132, and 168 hours of the incubation period

Fig.-2: The morphology of *B. licheniformis* during seven days of incubation period at 30 °C in Christensen's agar media

Electrochemical current profile measurement

B. licheniformis were immobilized on the working electrode surface, subsequently added with 40 µL 0.2 M urea in the synthetic urine and kept for 30 min before performing the voltammetry. Fig. 3 displays the electrochemical settings in this study whilst Fig. 4 displays the voltammogram and a hysteretic phenomenon related to the electrochemical reaction with redox. We also tested the blank solution to ensure that the current peak effect originated from the urea. There is a difference in the cyclic voltammogram between the system and that without the urea, meaning a successful urea detecting in the synthetic urine solution using *B. licheniformis* on the carbon electrodes. This study assumes that the current produced in the current derived from the electrons generated from the ammonia oxidation reaction.

The cyclic voltammogram optimization was also performed to give the maximum ammonia oxidation current, including the optimization of the column speed and cell density of the bacteria. The optimization scanning rate was in the range of 10 to 200 mV/s. Meanwhile, the optical density of *B. licheniformis* was optimized from 0.38 to and 0.87.

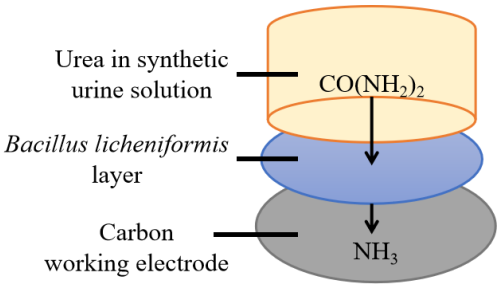

Fig. 3: Urea biosensors detection mechanism

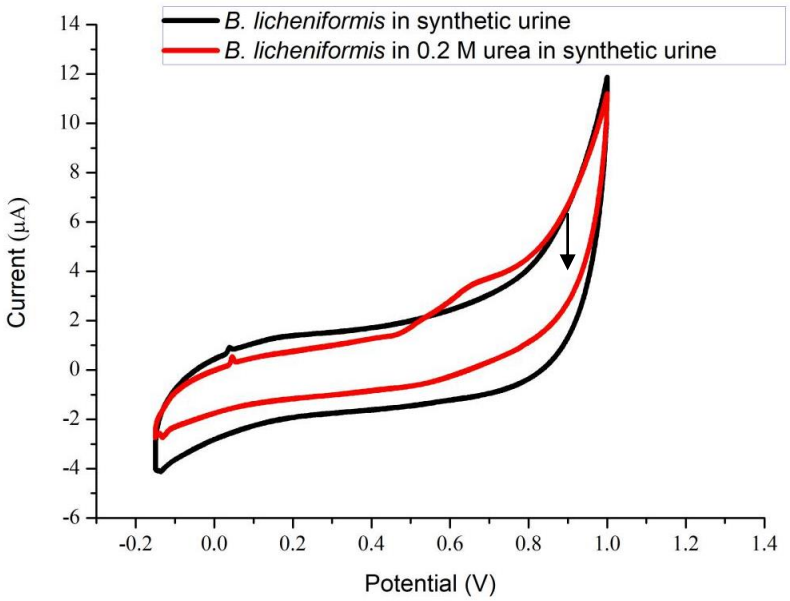

Fig. 4: Cyclic voltammograms representing indirect urea detection through ammonia oxidation in the presence of 0.2 M urea in synthetic urine solutions and in the absence of urea using SPCE *B. licheniformis* and scanning rate 20 mV/s. The arrows show ammonia oxidation peak current

The scan rate would affect the analyte response time. The larger the scanning rate, the faster the analyte response generated. Table 1 demonstrates the current response of the maximum oxidation bioreceptor suspension of the bacteria OD 0.87 in the urine samples containing 0.2 M urea against the various scanning rates. The maximum column speed occurred at 20 mV/s giving 0.30 µA oxidation under 0.650 V voltage in 30.8–45.0 s. Cyclic voltammogram under various scanning rates using 20 µL bacteria suspension of 0.87 optical density in 40 µL of 0.2 M urea solution on the matrix of synthetic urine solution was placed on the working electrode and incubated for 30 minutes before the voltammetric analysis.

Figure 5 illustrates the various bacterial optical densities (OD) with the maximum oxidation current result on the cyclic voltammogram. The voltammogram of various ODs using 20 mV/s scanning rate and 20 µL bacteria in 40 µL urea solution on the matrix of synthetic urine solution was placed on the working electrode and incubated for 30 minutes before voltammetric analysis. The maximum oxidation current was generated at 0.51 µA when the optimum OD of *B. licheniformis* was 0.75. The OD of 0.38–0.75 produces an improved oxidation current, indicating the exponential phase. Nevertheless, when the cell density was 0.87, the oxidation current decreased as predicted that the bacteria cells were in the death phase. The death

phase demonstrates the period of death, reducing the production of urease and oxidation current.

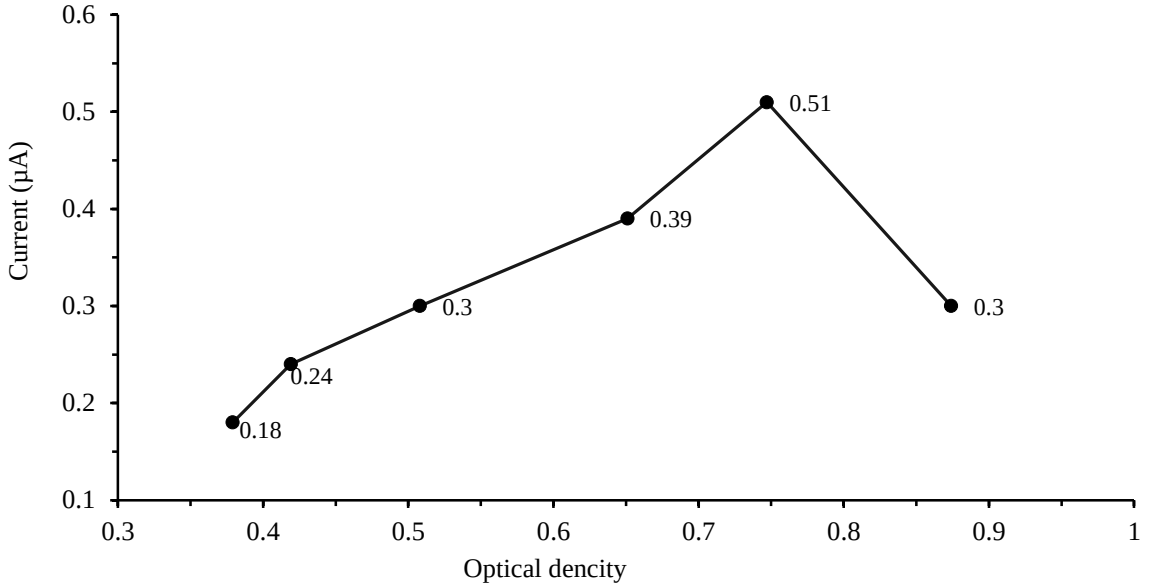

Fig. 5: Various *B. licheniformis* optical densities with the maximum oxidation current result on the cyclic voltammogram

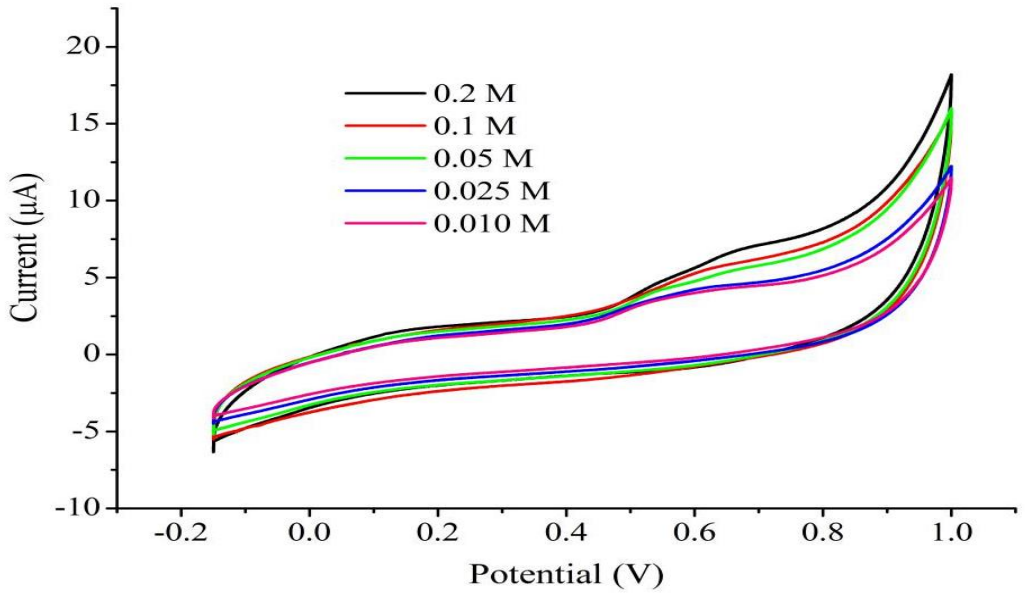

Fig.-6: Cyclic voltammograms indicating the effect of urea concentration at a scan rate of 20 mV/s, 20 μL of a *B. licheniformis* pellet OD 0.75 in 40 μL of urea in synthetic urine solution that was added to the working electrode and incubated for 30 minutes before the voltammetry analysis

The calibration curve (Figure 7) gives a linear range in 0.01–0.20 M urea. The calibration curve was plotted from 40 μL sample volume, 0.75 OD, and 20 mV/s scanning rate, which gives R^2 = 0.990. The detection limit in this study used the lowest concentration as the biosensor performed the detection starting from 0.01 M. The LOD indicates the lowest amount of analyte concentrations that can be detected and responded significantly as compared to the blank. The LOD biosensor in this study indicated better than that of a urea biosensor using the whole-cell of *Proteus vulgaris*, which is reported 0.027 M [28].

The sensitivity of detection can be observed based on the slope of the line equation, which is 1.278 μA/M, meaning that any changes in 1 M analyte concentration will produce the response current of 1.278 μA. The biosensor sensitivity in this study confirmed better production as compared to the urea biosensor sensitivity using nonenzymatic, which is 1.085 μA/M [31]. Therefore, *B. licheniformis* can be potentially utilized as the urea bioreceptor. Table 2 indicates the analytical performance sensors and compared with others.

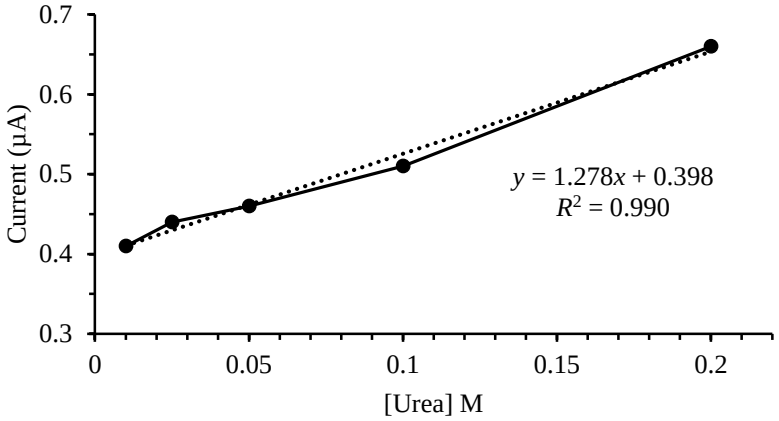

Fig. 7: The linearity relationship of between urea concentration and oxidation current using 20 µL *B. licheniformis* pellet of OD 0.75 in 40 µL synthetic urine solution that was added to the working electrode, the scan rate of 20 mV/s, and incubated for 30 minutes before the voltammetry analysis

Table 1: Maximum oxidation current response of the suspension bioreceptor *B. licheniformis* OD 0.87 in urine samples containing 0.2 M urea under various scanning rates

No	Scan rate (mV/s)	Oxidation current (µA)	Voltage (V)	Oxidation time (s)
1	10	0.18	0.527	58.2–79.8
2	20	0.30	0.650	30.8–45.0
3	40	0.22	0.635	16.4–21.9
4	100	0.12	0.672	7.1–8.6
5	125	0.03	0.683	5.5–6.9
6	200	0.01	0.699	3.5–4.3

Table 2: Comparison of urea sensor with similar sensors using amperometric detection method

Sensing material	Type	Analytical characteristics			Ref
		LOD	LR[a]	S[b]	
Carbon	Whole cell-*Bacillus licheniformis*	10 mM	0.01–0.20 M	1.278 µA/M	This work
Platinum	Whole cell-*Proteus vulgaris*	27 mM	0.01–0.05 M	17.890 µA/M	[28]
ITO/PDPA/PTA/Gra-ME	Nonenzymatic	100 mM	1–13 µM	1.085 µA/µM	[31]
Polyamidoamineccarbon nanotube	Urease	0.4 mM	1–20 mM	6.6 nA/mM	[32]

[a]Linearity Range, [b]Sensitivity

CONCLUSION

We successfully prepared biosensor using the whole-cell of *B. licheniformis* to measure the urea concentration in synthetic urine samples. This particular biosensor can perform a simple, low-cost method, and improves the sensitivity of the entire cell sensor that should be observed further for better performance of the sensor. This biosensor system has LOD of 0.01 M urea, 1.278 µA/M sensitivity, and from 0.01 M to 0.2 M urea linearity concentration range with an R^2 of 0.990.

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