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Immobilized bacterial biosensor for rapid and effective monitoring of acute toxicity in water

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ABSTRACT

The use of biosensors by using microorganisms such as bacteria have short life cycles and provide other advantages. One colorimetric biosensor technique that has been developed is the use of a biosensor utilizing the incorporation of Prussian blue formation reactions mediated by *E. coli* bioreactors with ferricyanide. Immobilization is a method that allows the bacteria can be used for long-term without reducing its ability as bioreceptor. This study aimed to develop a novel and rapid immobilized bacterial biosensor for the detection of toxic compound in water and to evaluate their analytical performances. Immobilization of *E. coli* performed by trapping method using alginate material support. The bacterial suspension was mixed with sodium alginate (1:1 v/v), and the mixture was continuously dropped in $CaCl_2$ solution to be a form of beads. The beads were used as bioreceptor to detect toxicants regarding cadmium, arsenic, mercury, chromium and lead solutions with Prussian blue as a colorimetric indicator. The linearity and sensitivity of detection of beads to the toxicants were tested, the stability of repeated use and storage were evaluated as well. The results showed that *E. coli* could be immobilized using alginate with response value was correlated with toxic concentration. The developed biosensor was more stable when used repeatedly and could be stored in a long time. The immobilization of *E. coli* in calcium alginate bead was successfully performed as a biosensor system for monitoring acute toxicity in water.

1. Introduction

Research and publications related to the development and manufacture of biosensors that are simple, fast, easy to make and low cost to monitor pollution and detect the contaminants in water are growing rapidly due to increasing the amount of pollution and industrial activity (Cao et al., 2018; Neufeld et al., 2006; Rodriguez-Mozaz et al., 2006). Several studies used biosensor for toxicant sensing in water with various kind of biosensing and detectors (Axelrod et al., 2016; Homaei, 2017; Mohseni et al., 2018; Rensing and Maier, 2003; Tsai et al., 2015). Each of them had advantages and limitations related to their application.

The use of organisms such as fish, shrimp, or animals living in the water to monitor water quality take a long time and need a sophisticated procedure and high cost. Thus, the use of biosensors by using microorganisms such as bacteria, become a solution to solve these problems, because the microorganisms have short life cycles and can provide an excellent response to pollution or toxins that can be used within the scope of health research that related ecosystem or environment (Bentley et al., 2001; Di Gennaro et al., 2011). Detection technique using a change of color, colorimetric, is widely used in the detection of biomolecules, metal ions, and the presence of other compounds because the response can be seen directly with the naked eye without the need for specialized instruments. Detection system by using colorimetric more simple and without using sophisticated instruments, furthermore this could reduce the costs significantly (Ali et al., 2008; Duke et al., 2008; Kim et al., 2012; Yuan et al., 2012).

Colorimetric biosensor for detection of toxicant in water can be used an early warning sign to monitor water toxicity in the environment. One colorimetric biosensor technique that has been developed was the use of a microbial biosensor utilizing the incorporation of Prussian blue formation reactions mediated by bioreactors with ferricyanide (Zhai et al., 2013). That study used *E. coli* as a model organism and the formation of Prussian blue with degradation of distinctive blue color as an indicator of the presence of contaminants or toxicant in water. The biosensor developed method has also been successfully used to detect the presence of toxicant in waters with the indicator of color change that can be observed visually with the naked eye. This study was still

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not satisfactory for practical application because the method was using fresh microbial cell suspensions that could only be used once time and the time of microbial preparation was quite long. Therefore, there is considerable interest in using immobilized microbes to solve the problem.

Immobilization is a technique that allows the bacteria can be used for long-term without reducing its ability as bioreceptor (D'souza, 2001). Among the immobilization techniques, trapping method is one method which can be used for cell immobilization. In this method, the bacteria will be in a trap in the form of a matrix polymer, for example in the form of a bead. Polymers commonly used are agarose, acrylamide, chitosan and alginate (Dong et al., 2014a). Alginate has the advantage among others because it has a good biocompatibility and low toxicity (Barin et al., 2009; Ruan et al., 2018). In this study, we developed immobilized *E. coli* bacteria as a biosensor for monitoring and detecting toxicity in waters. *E. coli* was entrapped in the form of beads using calcium alginate and evaluated their analytical performances regarding the sensing ability as a toxicant biosensor, reuse cycles of beads and storage stability. Finally, the immobilized bacterial biosensor was applied for monitoring toxicants in groundwater samples.

2. Material and methods

2.1. Chemical

Nutrient Broth (Merck, KgaG) was obtained from Darmstadt, German and used as a medium growth for *E. coli*. Sodium alginate (Sigma Aldrich, USA) and calcium chloride (Merck, Darmstadt, Germany) were used for the preparation of immobilized microbial beads. Potassium ferricyanide ($K_3(FeCN_6)$), sodium chloride, ferric chloride ($FeCl_3$), sulfuric acid, hydrochloric acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, were prepared as a reagent for sensing toxicant in the sample. Standard of cadmium ($Cd(NO_3)_2$), arsenic (H_3AsO_4), mercury ($Hg(NO_3)_2$), chromium ($Cr(NO_3)_3$) and lead ($Pb(NO_3)_2$) in HNO_3 solution were purchased from Merck (Darmstadt, Germany). The groundwater from our laboratory was chosen for the real sample analysis without any treatment. All other chemicals were of analytical grade.

2.2. Instruments

Autoclave YXQ-SG 41–280 (Ningbo, China), shaker incubator Memmert EN 60529 (Schwabach, Germany), and incubator IN-601 (Taipei, Taiwan) were used for beads preparation. The UV–Vis spectrophotometer for sensing measurement of OD_{600} was performed with a Cecil CE 3021 UV–visible Spectrophotometer (Cambridge, England). Scanning electron microscopes (SEM) TM-3000 (Hitachi, Japan) was used for bead characterization. All sensing measurements were carried out at room temperature.

2.3. Microbial culture

E. coli strain ATCC 25922 that subcultured from Laboratory of Microbiology, Faculty of Medicine, Jenderal Soedirman University, Indonesia was used as a microbial biosensor. It was added to 100 mL of Nutrient broth, pH 7.2. Bacterial medium subsequently incubated in a rotary shaker with a speed of 20 rcf for 24 h at 37 °C and transferred into 1.5 mL tubes for centrifuged at 50,000 rcf for 10 min. Part of pellets and the bacterial medium was separated by pipetting supernatant then the pellets washed with 9.0 mg/mL NaCl (w/v) sterile solution. Obtained pellets then resuspended in 10 mL of sterile saline, followed by stored at 4 °C. The incubation time for culturing bacteria found to be 25 h and used to obtain the desired bacterial concentration. The calculation of the concentration of bacteria was performed using the *McFarland* standard (Sood et al., 2011). Absorbance value is measured using spectrophotometer at a wavelength of 600 nm.

2.4. Microbial immobilization in calcium alginate

Immobilization of *E. coli* performed by trapping method using alginate material support. Immobilization of bacterial cells adopting from Rehn et al. (2012) The amount of 7.38×10^{11} cells/mL ($OD_{600} = 2.5$) suspension of *E.coli* added to a solution of sodium alginate with a ratio of 1:1 (v/v) in beaker glass. During the addition, stirring was performed by shaking slowly. The mixture was dripped continuously using a micropipette into 0.4 mol/L $CaCl_2$ solution to form immobilized bacterial beads. Bacterial Beads consecutively washed with a solution of 0.1 mol/L phosphate buffer, pH 7 and stored at 4 °C in 0.04 mol/L $CaCl_2$ solution. Surface topography of beads was examined by scanning electron microscope (SEM).

2.5. Sensing performance

Determination of the ability of immobilized bacteria as a biosensor was adopted from Zhai et al. (2013). The method was performed by 30 beads added 250 µL of 450 mmol/L $K_3(FeCN_6)$, 500 µL of deionized water and 250 µL of toxicants standard solution with concentrations of 10–500 µg/mL followed by incubating for 30 min at 37 °C. A 400 µL of solution was taken and mixed with 400 µL of 5 mmol/L $FeCl_3$ in 3 mol/L HCl and 1200 µL of deionized water. The absorbance was measured using spectrophotometers at 700 nm wavelengths and using deionized water as a blank. Measurement performed in three replicated and shown as relative activity between absorption intensity beads with toxicant and absorption intensity beads without toxicant with deionized water as a control. The stability of reuse cycles of beads was performed by measure the relative activity of beads that repeatedly used for toxicant detection. Beads washed with phosphate buffer solution three times and incubated for 15 min before subsequent use. The stability of storage of beads assayed by the evaluated relative activity of beads that stored in 0.04 mol/L $CaCl_2$ solution at 4 °C for 20 days. Application of biosensor for assays of groundwater samples was conducted using groundwater sample, and spiked groundwater that added Cd and As standard solutions.

3. Results and discussion

3.1. Immobilization of E.coli in calcium alginate

The bacteria of *E. coli* was harvested at the end of the log phase period and subsequently immobilized using calcium alginate matrixes in the form of beads. Bacteria cell was harvested and determined as the optimum incubation time because, in this phase, the bacteria achieved maximum growth (Odaci et al., 2009). The concentration result of *E. coli* suspension that calculated based on the McFarland approaches using spectrophotometer at OD_{600} 2.5 was 7.38×10^{11} cells/mL. The light emitted in the spectrophotometer will hit the bacterial cell resulted in some of the light will be absorbed and partly passed on. The amount of light absorbed was proportional to the concentration of bacterial cells (Domínguez et al., 2001; Koch, 2007; Rescott et al., 1988).

E.coli trapped in alginate beads when a solution of 5% alginate mixed with bacteria was dropped into the $CaCl_2$ solution. Beads were formed due to ionic interactions between Ca^{2+} bivalent cations with carboxylate groups of alginates (Bajpai and Sharma, 2004). The bead of immobilized *E. coli* was a round shape (Fig. 1). The results of this study were accordance with other research (Dong et al., 2014b) which states that the use of alginate with a concentration of 50 mg/mL can produce hard and round shape beads. The higher concentration of alginate would make the smaller pore size of the beads followed by in reduced efficiency of immobilization. The occurrence of diffusion detention whereas low alginate concentrations will result in fragile beads and large porous this will lead to the release of bacterial cells from beads (Bibi et al., 2015; Bilal and Asgher, 2015). Increased $CaCl_2$

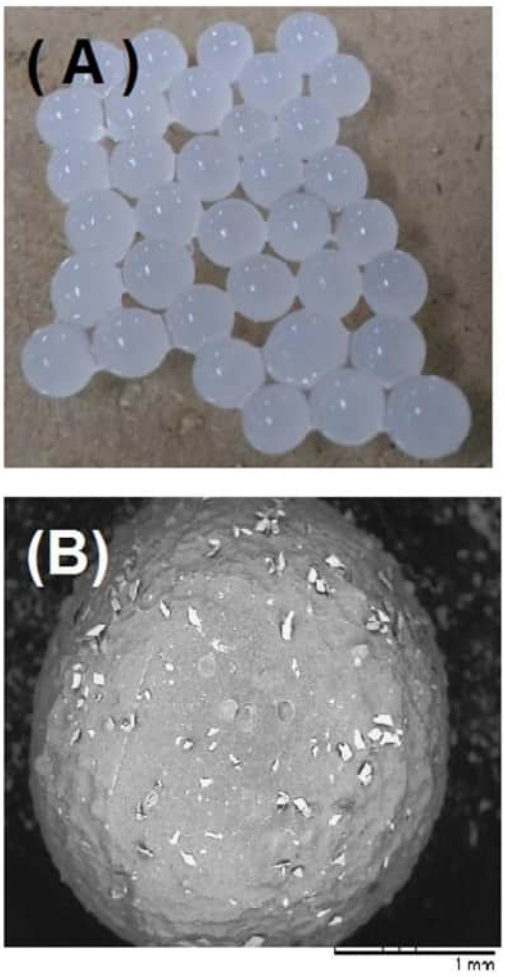

Fig. 1. Calcium alginate beads containing immobilized cells of *E. coli*. (A) The visual picture of beads (B) Scanning electron microscopy image of the bead with a diameter of 3–4 mm.

concentrations strengthen Ca-bonds with alginate chains thus the strength of the beads will also increase, and 0.4 mol/L $CaCl_2$ would produce stronger beads (Chai et al., 2004; Donati and Paoletti, 2009). In this study, 1000 µL of alginate-bacterial suspensions roughly formed 30 beads with a diameter of 3–4 mm.

3.2. Sensing performance of the immobilized microbial biosensor

Prussian blue was a colorimetric indicator of the process of reduction of ferricyanide to ferrocyanide during bacterial respiration with the addition of $FeCl_3$ and can be measured using spectrophotometer (Fig. 2). Consequently, selecting the wavelength that gave the optimum absorption in spectrophotometry was essential to prove appropriate measurement results (Wasito et al., 2018). The maximum wavelength of Prussian blue was 700 nm, this result was in accordance with related work that shows similar UV–Vis spectra (Zhai et al., 2013). This wavelength then used to measure the response of biosensors for this study. The feasibility of the novel immobilized bacterial biosensor in detecting toxicant was performed with the toxic metal standard solution of Cd, As, Hg, Cr, and Pb as the model toxicants. The absorbance data obtained than converted into relative activity. Calibration curves between

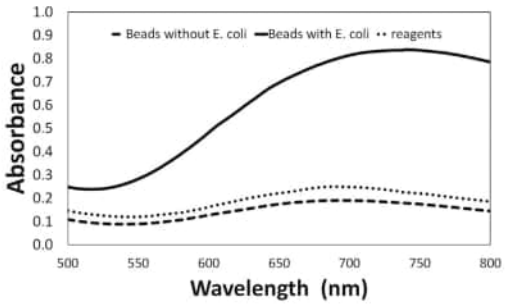

Fig. 2. UV–vis absorption spectra of Prussian blue formed by reagents that incubated using beads. Beads with *E.coli* could produce Prussian blue that has maximum absorption spectra of 700 nm, on the other hand, beads without *E. coli* that incubated with reagents, not interference the absorption spectra of Prussian blue formed.

Table 1
Values of regression coefficient between concentration of toxicant and relative activity for bacterial beads after incubated with various toxicants.

Toxicant	Regression equation	R^2	LOD (µg/mL)
Cd	y = −0.099x + 84.197	0.9349	61
As	y = −0.077x + 88.871	0.8265	8
Hg	y = −0.101x + 80.997	0.8915	82
Cr	y = −0.062x + 74.045	0.9562	51
Pb	y = −0.066x + 91.268	0.9058	76

the concentration of toxicant and relative activity (Table 1) shown that there was a linear relationship with the square of correlation coefficient of 0.8265–0.9562 over the concentration range of 10–500 µg/mL. Based on the standard deviation of the response and the slope of the calibration curve, limit of detection (LOD) was different among toxicants from 8 to 82 µg/mL.

The relative activity of bacterial beads for monitoring various toxicants with different concentration levels (Fig. 3) proved that the higher the toxicant concentration, the smaller the response value. The higher the toxicant concentration, the less the amount of $K_3[Fe(CN)_6]$ converted to $K_4[Fe(CN)_6]$ during bacterial respiration process resulted in the amount of Prussian blue produced was also decreased because toxicant inhibits bacterial respiration (Nwachukwu and Pulford, 2011; Zhai et al., 2013). Hg was seemed to be more toxic than other toxins. On the other hand, Pb appeared less toxic for *E. coli*. Each toxicant had different response depended on their characteristics. For instance, toxicants of Cd and As have different binding sites on *E.coli*; Cd has two binding sites whereas As has three binding sites. The difference between binding sites affects the toxicity and selectivity of toxicant (Chen and Rosen, 2014). This result was in accordance with the previous

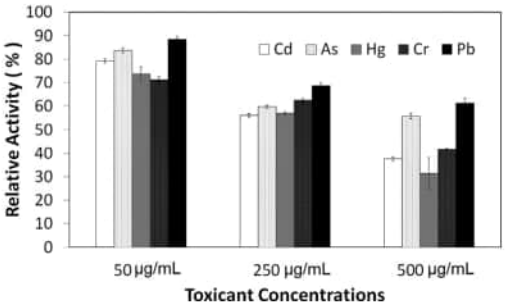

Fig. 3. The effect of Cd, As, Hg, Cr, and Pb with various concentrations on the relative activity of bacterial biosensor beads for monitoring toxicants in water.

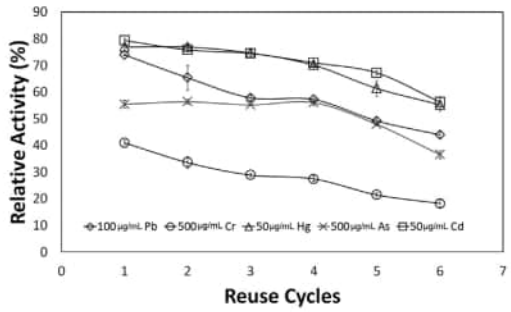

Fig. 4. Profile of reuse cycles of bacterial biosensor beads for monitoring of toxicants in water.

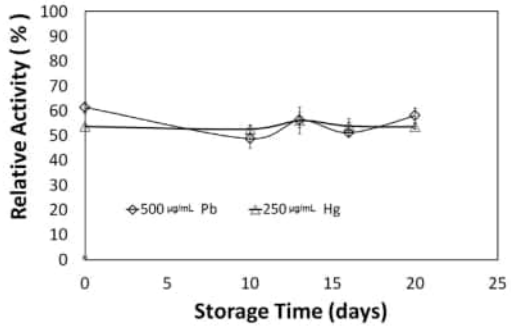

Fig. 5. The stability of bacterial biosensor beads after different storage times at 4 ℃ for monitoring toxicants in water.

study which reported that As not inhibit bacterial respiration significantly (Zhai et al., 2013).

The reuse cycles assay of bacterial beads was performed to determine the stability of bacterial beads biosensor when used more than one time for toxicant detection (Fig. 4). In general, it could be seen that immobilized of *E.coli* in beads after used four times could retain its response ability up to 85% and decreased gradually. The stability of beads on reuse cycles assay showed that immobilization of *E. coli* with calcium alginate proved the stability of beads form and bacterial entrapment. This result was in accordance with a previous study using other microorganisms of immobilized *Aspergillus* in calcium alginate which reported the ability to maintain the biosensor response of 91% after three times of use (El-Ghonemy, 2015). The gradual decrease in response activity could be caused by a loss of microbial cells during the washing process after each recycle used (Bibi et al., 2015).

Storage beads stability tests were performed to evaluate the stability of beads during storage in 4 ℃ for various periods (Fig. 5). Biosensor microbial beads seemed to be stable when it was stored up to 20 days. This stability of beads simplified the assay process for biosensing toxicant compared using free cells microbial biosensor. Microbial biosensor using free cells should be used on the day of harvest, so it is not possible to be stored (Zhai et al., 2013). This research found that the microbial biosensor with immobilized *E.coli* has higher stability than the free cell. According to the previous study with other microorganisms which reported that the immobilization of *A. fischeri* in alginate could be stored up to 10 weeks of storage and a longer storage time of 200 days immobilization of *E. coli* in alginate still shows stability response (Futra et al., 2014; Rehn et al., 2012).

The next step in this study was to apply the immobilized microbial biosensor developed to monitor some toxicants in groundwater samples. *E.coli* can be applied for toxic detection in groundwater and the response of relative activity correlated with increasing of toxicant

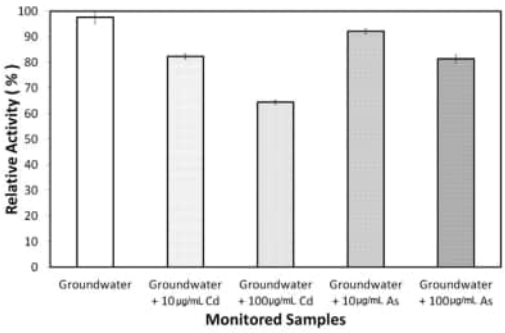

Fig. 6. Application of bacterial biosensor beads for monitoring toxicants in groundwater with and without added of toxicants.

concentrations (Fig. 6). In the assay using groundwater samples showed that no toxicant detected indicated by the relative activity value found to be 100% or the same as the blank sample. The assay was also performed with the toxicant of Cd, and As at the concentrations of 10 µg/mL and 100 µg/mL respectively, that added to the groundwater sample. On the addition of Cd or As, the relative activity was lower than the sample without toxicant; this result indicated that the biosensor beads was able to monitor the toxicant in groundwater samples where the higher the added toxicant concentration, the lower the relative activity biosensor response. This result proved that biosensors with immobilized bacterial of *E. coli in* calcium alginate proved a promising approach for early risk warning of acute water toxicity in groundwater.

4. Conclusion

The immobilized microbial biosensor of *E. coli* in calcium alginate beads was successfully developed and have appropriate sensing performances. The proposed microbial biosensor was novel, rapid and convenient for detection of acute toxicity and water monitoring effectively.

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Conflict of interest

Authors declare no conflict of interest.

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