Optical fiber mercury biosensor based on immobilized urease and bromothymol blue onto the alginate-chitosan membrane in the flow-system

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Abstract

An optical fiber biosensor has been developed for the detection of mercury ion based on inhibition of urease immobilized onto alginate–chitosan membrane, coupled with bromothymol blue (BTB) in the flow system. To get a good performance of the biosensor toward Hg(II) ion detection, the experimental parameters of the biosensor were optimized. Here, the maximum wavelength was detected at 580 nm, with the optimum response at pH of 6. The calibration curve had a dynamic working range at 10 to 500 µg/L of Hg(II) ion with a detection limit of 12.1 µg/L (equal to 10% inhibition) and high reproducibility (RSD= 0.86%). The regeneration of the biosensor has been performed by the addition of 1 mM ethylenediaminetetraacetic acid (EDTA) solution, in which five-time cycles have been achieved with the inhibition decrease to 9.94% from the original biosensor response. Applying the biosensor to the real samples showed conformity of results with the reference method, cold vapor atomic absorption spectrometry (CV-AAS). Therefore, this biosensor can be used as a method for routine analysis in the determination of Hg(II) concentration in an aqueous sample.

Keywords: Alginate–chitosan membrane; BTB; Hg(II) ion; optical fiber biosensor; urease.

1. Introduction

Mercury (Hg) as heavy metal, is known as a global pollutant (Driscoll *et al.*, 2013) that is released into the environment from geological and anthropogenic sources. Its compounds that are highly mobile and toxic at trace level (Belluzzi-muiños *et al.*, 2017) cause distress for human and environmental health (Sures, 2004; Urek *et al.*, 2013) and induce many illnesses even death (Nordberg *et al*., 2000). Hence, a simple and rapid method for mercury detection at trace level in environmental samples is needed (Apilux *et al.*, 2012; Samphao *et al.*, 2012).

The conventional methods for the determination of mercury are atomic absorption spectrometry (AAS) (Nakadi *et al.*, 2020; Szkoda *et al.*, 2006), inductively coupled plasma with mass spectrometry (ICP-MS) (Hofer *et al.*, 2017; Nixon *et al.*, 1999), etc. Another analytical method that has been developed for this purpose is an electrochemical method (Pujol *et al*., 2014). However, these standard methods require sample preparation, laborious procedure, and sophisticated instrumentation as well as high cost. Alternatively, the optical biosensor offers more benefits over the other existing methods, due to their simple, quick, and inexpensive analysis (Long *et al.*, 2013), particularly for the determination of heavy metal ions in the field. In

addition, an optical biosensor is a powerful alternative technique, by combining the specificity and sensitivity of biological systems, such as enzymes in small devices.

Enzyme inhibition method in the optical biosensor is commonly used for inhibitor detection, such as heavy metal ion. This method could use a wide range of enzymes that are specifically inhibited by trace concentration of certain metal ions (He *et al*., 2018; Ilangovan *et al*., 2016; Turdean, 2011, Shi & Jiang, 2002). Kuralay *et al.* (2007) developed an amperometric biosensor based on inhibition of immobilized urease in poly(vinylferrocenium) film for the Hg^{2+} ion determination and Kuswandi (2003) fabricated a simple optical fiber biosensor for Hg(II) determination. Here, the determination of Hg^{2+} by enzyme-based biosensor can be very sensitive but the inhibition of enzymatic reaction by Hg^{2+} leads to poor selectivity. Other heavy metal ions such as Fe^{3+}, Cu^{2+}, Cr^{3+} were also enzyme inhibitors. Improving the selectivity is important in Hg(II) determination since these interference ions possibly coexist in a real sample.

Immobilization of enzyme into suitable solid support material is one of the key successes in designing biosensors since this step plays an important role in the overall biosensor performance, including its selectivity. Zeng *et al.,* (2015) developed a sensitive potentiometric biosensor for Hg(II) determination by using a three-dimensional network structure as a matrix, chitosan–polyvinyl alcohol with glutaraldehyde crosslinker. In this study, a better and more simple preparation network matrix was used, polyelectrolyte complexes (PEC) of alginate-chitosan. Here, carboxylic groups of alginates interact with protonated amine groups of chitosan through ionic interactions to form complexes without the addition of crosslinkers at certain conditions and ratios. It was indicated by FTIR spectra of alginate-chitosan PEC at 1550 cm^{-1} (NH_3^+), 1577 $cm^{-1,}$ and 1398 cm^{-1} ($-COO^-$) (Hermanto *et al*., 2020; Ismillayli *et al*., 2020; Kulig *et al*., 2016). This strong ionic bonding was the main interaction inside the network. It has been reported that the immobilization of enzymes onto PEC, maintains higher enzyme activities than original enzymes and it was stable for long periods (Yabuki, 2011). Hence, PEC is a potentially attractive membrane for biosensor application. So far, alginate-chitosan PEC has not been used as an enzyme immobilization matrix in optical biosensor design. In previous studies (Hermanto *et al.*, 2019), this membrane was used as a support material for urease immobilization which showed good characteristics as a matrix. Hence, in this study urease coupled with BTB was immobilized onto the alginate–chitosan membrane. The membrane was used as a biosensor matrix for the determination of Hg(II) ions using the optical fiber flow injection analysis in aqueous solutions. The optimization for urease immobilization in alginate–chitosan membrane was carried out and the effects of pH and temperature in the biosensor response as well as stability of biosensor were studied along with biosensor analytical characteristics.

2. Experimental

2.1 Chemicals and instruments

The urease (E.C. 3.5.1.5.), sodium alginate, and chitosan used to prepare biosensors were purchased from Sigma (St. Lois, USA). Urease (272 u/g) was isolated from jack beans (Type III and U1500), sodium alginate was isolated from brown alga (300-400 cp) and chitosan was isolated from crab shell with 95% deacetylated. Hydrochloride acid (37%), glacial acetic acid (98%), sodium hydroxide, potassium dihydrogen phosphate (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA), and Bromothymol blue (BTB) were received from Merck (UK). Urea stock solution (1000 µg/mL) was prepared in an aqueous solution. The

standard metal solutions of Ag(I), Hg(II), Cu(II), Cd(II), and Pb(II) (1000 mg/L) (grade of analytical, Merck) were diluted with appropriate buffer solution. Deionized water was used for solutions preparation. An optical fiber USB 2000 spectrometer (Ocean Optic, USA) was used for the reflectance measurements. While all pH measurements were conducted by using A pH meter model IM-20E (TOA Electronics Ltd.).

2.2 Preparation of membrane

The membrane was prepared by mixing two polymer solutions consist of chitosan and alginate hydrosols, as described by previous work (Hermanto *et al.*, 2019). The produced membrane was used as solid support of urease and BTB immobilization.

2.3 Immobilization procedure

The 3 µL alginate-chitosan hydrosol was added to a 1 µL phosphate buffer (pH 6.5). The buffered mixture was added to 3 µL of urease (enzyme solution in phosphate buffer, pH 6.5) and 3 µL of BTB (1.5 mg/mL in ethanol) (Hermanto *et al.*, 2019). Here, maintaining enzyme activity was achieved by using phosphate buffer in the modified hydrosol process. Immediately, the mixture (10 µL) was formed in the designed circular mold (i.d.10 mm and 1 mm depth) thinned and flattened using a magnetic stirrer (300 rpm) for 10 seconds then stored for the aging process (5 days, 4 °C). The obtained film was released from the mold and stored in a closed container at 4 °C until used.

2.4 Optical biosensor design

For optical biosensor design, the alginate-chitosan/urease and BTB membrane were packed into a flow cell (i.d. 50 mm and 50 mm depth) at room temperature (Figure. 1), then was faced directly to the circular end of a fiber optic bundle probe. The flow-cell device integrated two needles assigned at 120° opposite each other (stainless-steel, i.d. 1 mm and 20 mm length) for the inlet/outlet solution flow with an internal flow cell volume of approximately 20 µL. The circular bottom end of the flow cell is provided with a PTFE disk for a reflective surface backing.

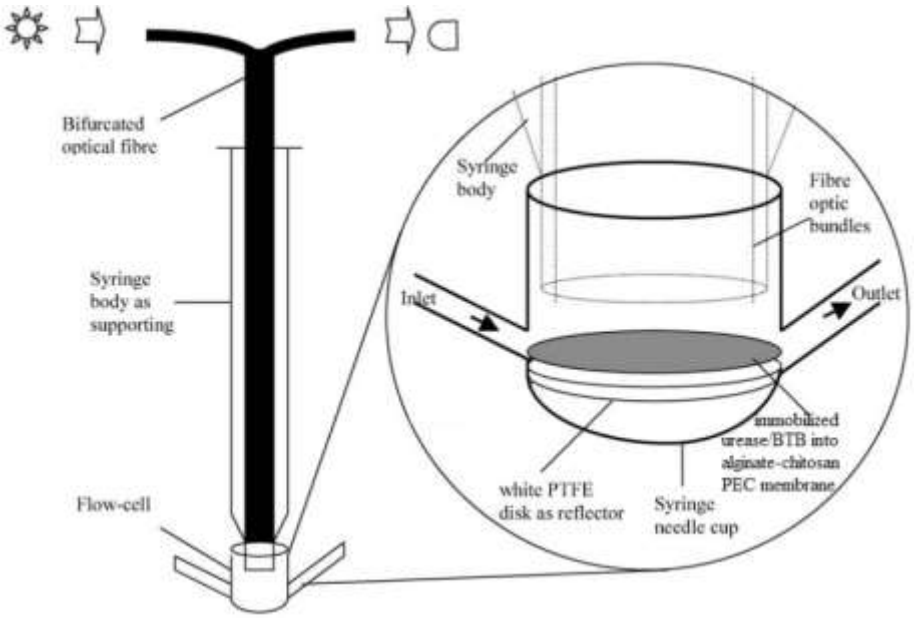

Fig. 1. The construction of optical fiber Hg(II) biosensor using spectrophotometry

2.5 Flow manifold and measurement procedure

Flow injection analysis (FIA) has a flow manifold configuration as shown in Figure 2. The FIA system consists of three injection valves (Omnifit 1106) and a four-channel peristaltic pump equipped with a Tygon tube (1.0 mm i.d.). Teflon® tubing was used between all components in the FIA system (FIA 5010, Tecator). In the beginning, the carrier solution of phosphate buffer was pumped and flowed through the FIA system producing a baseline signal. Then, the urea solution was injected manually at injection valve 2 to determine the initial activity of the enzyme without inhibition (Eo). While the Hg (II) ion in the sample solution was injected into valve 1. After the inhibition, residual enzyme activity was revealed by urea injection, and the inhibition percentage (Ec) was calculated based on the following equation:

$$Inhibition\ (\%I) = [(Eo - Ec) / Eo] \times 100 \quad (1)$$

Where Eo and Ec are substrate signal intensities without and with inhibitors. After inhibition determination, reactivation solution (1 mM EDTA) was injected through injection valve 3 to leach heavy metal ions from urease so that its activity was restored. The same cycle was carried out in other Hg(II) concentration measurements. The membrane must be washed carefully after the measurement to avoid blockage due to salt precipitation in the buffer solution.

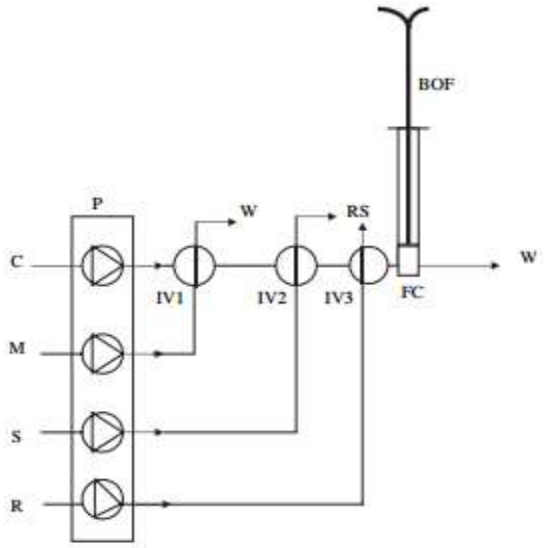

Fig. 2. The flow manifold is used for the measurement of urease-based biosensors for the determination of Hg(II) ions. C, carrier (pH 6.0, 0.1 N phosphate buffer solution); M, sample (metal ions solution); S, substrate (urea solution); R, regeneration solution (1 mM EDTA solution); P peristaltic pump; IV injection valve; RS reservoir; FC flow-cell; BOF bifurcated optical fibre; W waste.

2.6 Detection of Hg(II) ions in the real sample

Optical biosensor evaluation for the detection of Hg(II) in the real samples was carried out on tap water obtained from our lab, river, and seawater from Sekotong district, West Lombok regency, Indonesia. Firstly, water samples were filtered using a syringe filter (0.22 μm), then a standard solution of Hg(II) 20 ppb was added to the sample solution. The reflectance intensity was recorded on the optical fiber spectroscopy at 580.15 nm using the same measuring conditions. As a comparison, the CV-AAS (GBC HG 300) was also used as the reference method for the determination of Hg(II) ions in the real water sample.

3. Results and discussion

3.1 Biosensor scheme

The optical biosensor used the immobilized urease as a bio-catalytic element for the selective substrate. Urease broke carbon-nitrogen bond in urea and release CO_2, NH_3, and water as products, according to Equation.1.

$$(NH_2)_2CO_{(aq)} + 3H_2O_{(l)} \xrightarrow{\text{urease}} 2\,NH_3(aq) + CO_{2(g)} + 2H_2O_{(l)} \quad (1)$$

$$NH_{3(aq)} + H_2O_{(l)} \rightleftharpoons NH_4^+{}_{(aq)} + OH^-_{(aq)} \quad (2)$$

Ammonia was protonated by water to form NH_4^+ and OH^- (Equation 2) and produced an increase in pH. This process was detected by BTB indicator (pKa 7.2) that was showed by

membrane color change from yellow to green. This process also caused a change in spectra and was used as the optical response. Furthermore, the inhibition measurements were carried out by passing the Hg(II) ion into the biosensor. The Hg(II) ion has a strong affinity to sulfhydryl (-SH) group of urease to form Hg(II)-mercaptide as given Equation. 3.

$$E—SH + Hg^{2+} \rightleftharpoons E—S—Hg \quad (3)$$

The inhibition of Hg(II) ions in urease activity caused the very low rate of urea hydrolysis reaction and even caused loss activity of urease. Consequently, less ammonia was formed and lower pH change was detected when urease was reacted with urea. It was indicated quantitatively by the decrease in signal intensity at the presence of Hg(II), as shown in Figure 3. Since the amount of Hg(II) ions was proportional to the detected pH change before and after inhibition, Hg(II) concentration in the sample can be determined.

As it is illustrated in Figure 3, the maximum wavelength for biosensors was obtained when the signal intensity difference before and after reaction with Hg(II) ions was the greatest. The signal intensity of the biosensor changed due to the pH increase, as increasing of ammonium ion (product of urea hydrolysis by the urease). Then, a pH increase was detected by the BTB indicator.

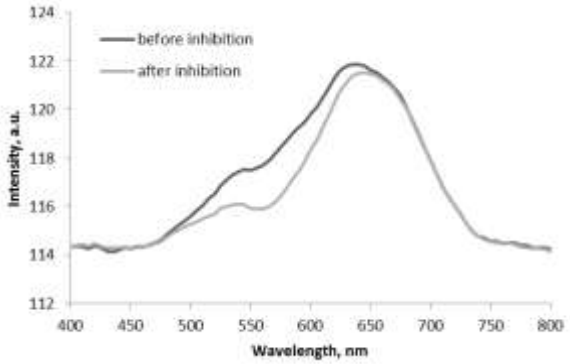

Fig. 3. The BTB spectra (i) before and (ii) after inhibition by Hg(II) ions.

Here, the color changed from yellow to green leading to a decrease in the reflected signal intensity at 580.15 nm. This aspect is important since the pH working range of the pH indicator fits very well with the pH change associated with the enzyme-substrate reaction. On the other hand, the plain shape of the pH indicator allowed easy access for the analyte, which in turn, resulted in high sensitivity of pH indicator to the net of pH change. Furthermore, the matrix effect on pH indicators had been minimized by using a buffer to maintain the stability of the biosensor response. It means that pH change only correlated to the Hg(II) ion concentration and not to other parameters (Kuswandi & Suwandari, 2007).

3.2 Optimum parameters

The optimization of the experimental parameters is an essential step in the analytical characteristics of the mercury biosensor performance. Table 1 shows some of the investigated experimental parameters and their optimum value. It was found that pH 6 of the buffer solution was optimum and was used for further measurement.

The enzymatic reaction will occur effectively when the substrate concentration is proportional to enzyme activity. Therefore, optimization of urea concentration as the substrate is needed to obtain good reproducibility results. The range of urea concentration was 10 to 100 mM. Here, the concentration of 75 mM was optimum for the urea concentration and used for further measurements.

The rate of enzyme catalysis can be affected significantly even with a small change in their chemical environment. In this study, both urease and BTB were immobilized into the alginate chitosan membrane. To interact with the enzyme, the substrate must diffuse and penetrate the membrane. A certain period is needed, hence maximum change is obtained. Substrate flow at a controlled rate to ensure urea hydrolysis produces ammonium and hydroxy ions which induce a color change of the membrane. In this case, the flow rate of 10 mL/min produced a relatively constant response of biosensors toward the substrate. Then, this flow rate was used for further measurements.

The inhibition time was also optimized to provide optimum time for binding of Hg(II) ions with the active site of urease. It has been performed bypassing of Hg(II) solution toward the biosensor, and it was found that 7 min was the optimum time for inhibition. Reaction temperature also affected on blank and inhibition signal of the biosensor. Here, the temperature at 25°C was the moderate temperature in this enzymatic inhibition reaction. Since at higher temperatures, immobilized enzyme activity decreased. Temperature affects the alginate–chitosan membrane as solid support for the immobilized urease and BTB, which cause small leakage of urease and BTB.

Table 1. Optimization of biosensor parameters

Parameter	Range	Optimum
pH	5–8	6
Substrate/Urea (mM)	10–100	75
Flow Rate (mL/min)	5-15	10
Inhibition Time (min)	4–8	7
Temperature (^{o}C)	25–35	25

3.3 Analytical characteristics

By applying optimized experimental parameters, dynamic response for Hg(II) ions was observed as the change of signal intensity reflected before and after inhibition at 580 nm. The calibration curve was made by measuring biosensor response to urease activity due to the presence of Hg(II) ions. For example, series of Hg(II) solutions at 10–500 µg/L interval concentration were passed into a biosensor system with three replications (Figure 4). First, the biosensor was injected buffer to give the baseline signal, followed by urea to give the blank signal. Then, a sample contained Hg(II) ions was injected, followed by urea to give the inhibited signal. To regenerate the inhibited signal, the regeneration solution (EDTA) was injected to recover the immobilized enzyme activity. However, the inhibited urease cannot be regenerated fully (100% of its enzyme activity) due to strong metal-urease binding that was considered an irreversible reaction. This might be the reason for increasing response at EDTA regeneration. From Figure 4, the relationship between Hg^{2+} ion concentration and % urease inhibition was described in Table 2.

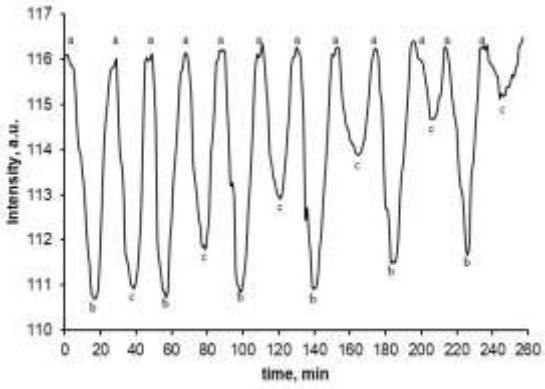

Fig. 4. The average response curve of inhibited urease biosensor by Hg(II) in triplicate mode (in this case toward 10, 20, 50, 100, 200, 500 µg/L Hg(II)) and its regeneration using EDTA; (a) substrate; (b) Hg(II) ions (c) regeneration.

Table 2. Evaluation of the urease inhibition by Hg(II) in biosensor

$[Hg^{2+}]$ (µg/L)	Log $[Hg^{2+}]$	% Inhibition			Average	STDEV	RSD
		x1	x2	x3			
10	1.000	3.235	3.459	3.430	3.375	0.122	3.617
20	1.301	19.335	19.195	19.118	19.216	0.110	0.573
50	1.699	36.289	36.308	36.186	36.261	0.065	0.180
100	2.000	55.406	55.332	55.643	55.460	0.162	0.293
200	2.301	63.162	63.431	63.238	63.277	0.139	0.220
500	2.699	73.529	73.205	73.580	73.438	0.203	0.277
						Average	0.860

By evaluating the inhibition degree against the concentration of Hg(II) ions, calibration curves were plotted. For Hg(II) concentration intervals from 10 to 500 µg/L, the logarithmic curve was obtained as given in Figure 5. Based on this Figure, a linear correlation was generated by plotting % inhibition of urease versus log Hg(II) concentration to obtain a correlation coefficient (r) of 0.9778 as seen in Figure 6. The detection limit determined by inhibition of Hg(II) to the enzyme at 10% ($I_{10\%}$) was 12.1 µg/L of Hg(II) ions. Shi & Jiang (2002) have developed a dip and read test strip for mercury determination based on urease inhibition with cellulose acetate membrane as a matrix. It was observed that 0.2 µg/L mercury can be detected with a detection range of 0.2-200 µg/L. This value indicated lower LOD than this proposed biosensor. However, this biosensor has the advantage over a wider linear range of 10 to 500 µg/L.

Biosensor reproducibility is one of the influential analytical performances. The reproducibility of the proposed biosensor was presented as the coefficient of variation (CV) or relative standard deviation (RSD) toward the determination of standard solutions of Hg(II) ions (n=3) using the same alginate–chitosan membrane in the concentration range 10 to 500 µg/L. The obtained reproducibility biosensor (RSD) was 0.86% (Table 2), meaning that the reproducibility was good.

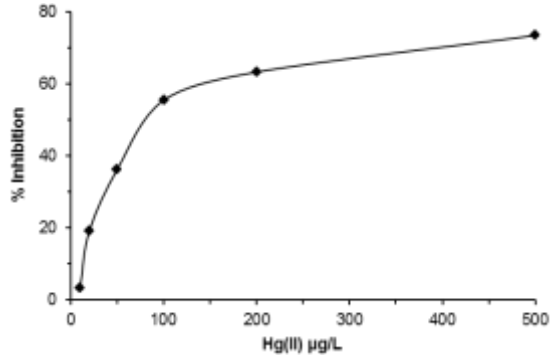

Fig. 5. The calibration curve of urease inhibition vs concentration of Hg(II).

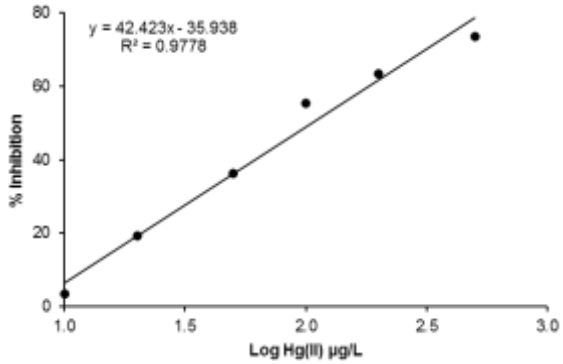

Fig. 6. The calibration curve of urease inhibition vs log concentration of Hg(II).

The enzyme-based biosensor that has been inhibited by Hg(II) ions could be regenerated by EDTA as a reactivation agent. Here, it was found that 1 mM of EDTA solution was sufficient to reactivate the enzyme again. The stability of the biosensor is determined by measuring the decreasing enzyme activity during the period of investigation within one week. Besides inhibition caused by the metal ions, the immobilized enzyme activity also decreased during the storage at room temperature (25 °C). While the biosensor was not used, the immobilized urease and BTB into alginate–chitosan membrane was stored in the refrigerator (4 °C). However, the reactivation or regeneration of the proposed biosensor was achieved up to five time cycles, since the data showed that enzyme activity decreased < 10% after five times used. This might be due to the reactivation effect, such as hysteresis, which implies the biosensor stability during the measurements of Hg(II) ions. The biosensor selectivity was determined by adding several potentially interfering ions commonly found in water samples with varying concentrations, such as Pb(II), Cu(II), Cd(II), and Ag(I) at 100 µg/L. Figure 7 illustrates that the inhibitory value of interference ions (Pb(II), Cu(II), and Cd(II)) compared to a blank (without inhibition) was very small (< 5%). This indicates that these heavy metal ions at this concentration (100 µg/L) did not significantly cause interference. However, only Ag(I) ions caused a relatively large interference at this concentration compared with other heavy metal ions tested. This might be because Ag(I) ions have a similar inhibition effect to Hg(II) ions, where it will also affect the inhibition of urease activity, particularly at the high concentrations (Azmi *et al*., 2012).

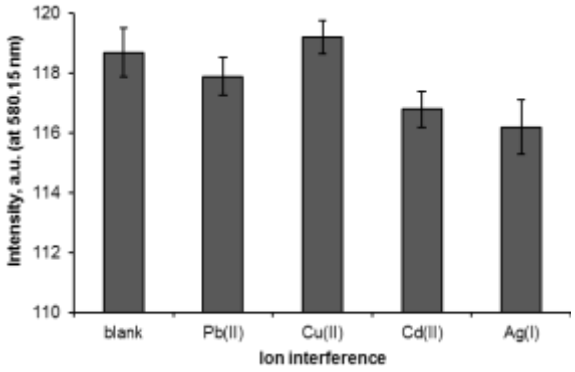

Fig. 7. The biosensor responses toward other heavy metals ions

Determination of Hg(II) ions in real water samples were carried out using the certified reference method. Here, the sample was spiked with a concentration of Hg(II) ions of 20 ppb. The results of the Hg(II) ions determination are summarized in Table 3. Table 3 shows the measured Hg(II) sample concentration values corrected with the added standard Hg(II) solution. The high concentration of Hg(II) in the Sekotong seawater sample is related to the high activity of gold processing waste disposal using mercury as gold extractor (amalgamation) into rivers that flow into the sea. The developed optical biosensor has similar results for the determination of Hg(II) in the real water samples with the reference method using the CV-AAS. AAS is an AOAS official method for determining the metal concentration and is commonly used (Arshad *et al.*, 2020). By using statistical analysis (t-test), it was obtained that there was no significant difference for both methods. Thus, It can be stated that the result of the proposed biosensor measurement has good conformity with the reference method (AAS).

Table 3. Measurement of Hg (II) concentration in aquatic samples

Sample	Various Water Samples	*The biosensor /ppb	*AAS (std Hg(II)) /ppb
1	Buffer	0.00	0.00
2	Tap water	0.00	0.00
3	River water	0.003±0.003	0.000±0.000
4	Seawater	44.95±0.163	40.23±0.002

*average of triplicate measurements.

5. Conclusions

In the present work, the optical biosensor based on immobilized urease and BTB onto alginate-chitosan membran had good analytical performance and rapid response towards Hg(II) ions in the sample solution. In addition, biosensors could be reused up to five-time by treatment of 1 mM EDTA solution. The result of Hg(II) measurement in the real water samples showed a good agreement with the reference method (CV-AAS) based on statistical analysis. Hence, the proposed optical biosensor is a reliable alternative method for the simple and rapid determination of Hg(II) ions in the aqueous samples.

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