

Article

Electrochemical Biosensor Using *Acetobacter* Enzyme for Detecting Alcohol

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Abstract: Determining halal product of fermented foods and beverages becomes important for muslim consumers due to religious codification of halal. In order to help on site analysis, an alcohol biosensor was under development. The biosensor was constructed using bacteria producing alcohol oxidase (AOX). Bacteria from apple juice was isolated in advance to obtain pure bacteria. The bacteria was cultured in two solid media. Bacteria on the *Acetobacter* media had a resistance in a solution containing alcohol with a higher concentration. *Acetobacter* was cultured on solid and liquid media for measuring at various ethanol concentrations (0.01%-3%) with different absorbance value. Bacteria with absorbance value 0.500 had the highest oxidation current peak. Later, it was used as bioreceptor for measuring ethanol by cyclic voltammetry method. The linearity of measurement later was compared with spectrophotometric measurement. By using cyclic voltammetry the linearity had higher R² value than spectrophotometric method. However, it only had stability for two days, therefore it needs more improvement for lasting its lifetime. Basically, electrochemical method has potency for further being improved further as an alternative method of ethanol on-site measurement.

Keywords: alcohol oxidase (AOX); biosensor; halal products; cyclic voltammetry

1. Introduction

Alcohol, commercially sold as ethyl alcohol, has been applied to many kinds of goods because of its beneficial properties, such as volatile, good solvent, and less toxic. It also has been applied to many kind of foods and beverages production [1]. However, its usage on foods and beverages industry should be put into consideration when dealing with islamic halal codification. As fatwa of Ulema Council of Indonesia (*Majelis Ulama Indonesia*; MUI) No. 4/2003, all the fermented foods or beverages which have more than 1 % of ethanol are considered as haram [2]. So, for protecting the consumers, especially muslims, from being exposed of haram products there should be a method to detect alcohol in foods or beverages easily on site.

Several methods have been developed, like colorimetry [3]. However, this method could be interfered by a similar analyte which has the same wavelength absorption of light. Hence, Electrogenated chemiluminescence (ECL), a sophisticated method for measuring ethanol by applying a simple optical adjustment, was made. Therefore, it provides a high flexibility and able to detect the analyte at the trace concentration with wide range of linearity and low background noise effect [4]. However, this method should use the pure enzyme for conducting the measurement. So, it deals with cost and also time consuming.

Biosensors have become a research interest due to its high sensitivity and selectivity which is very important for applying it in fermented foods and beverage industry quality control. Consequently, the bioreceptor, a crucial component of biosensor for detecting the analyte, should be explored to produce a more sensitive and selective biosensor. In this case, an enzymatic process can be a tool for it. Two kinds of enzyme which are involved into an enzymatic reaction with ethanol have been proposed: alcohol dehydrogenase (ADH) and alcohol oxidase (AOX). These enzymes could be easily produced by the microbes. So, rather than extracting the enzymes out from the microbes, it would be cheaper for immobilizing the microbes directly onto the electrode. Thus, it produces a microbe biosensor which has at least two advantages: durable and cheap. In addition, microbe has an ability to reproduce itself in higher rate via cell culture. It is also able to be manipulated easily and has better stability [5].

Previously, we had a research about alcoholic biosensor by using *Bacillus* sp. as bioreceptor and measured by cyclic voltammetry. However, the result was not optimum because the bacteria's amount was unknown and different for each measurement (unpublished). So, it produced a big measurement inconsistency when measuring alcohol above 5%. Based on this result, we decided to produce AOX enzyme from *Acetobacter* sp. in apple juice and conducting research by voltammetry cyclic. Carbon paste electrode was used as the working electrode and also for immobilizing bacteria. We also compared the ethanol measurement using UV-VIS and this developed biosensor.

2. Materials and Methods

Tools used in this research were micropipet (Gilson), pH meter (TOA DK HM-250), microwave, autoclave (HVE 50 Hariyama, Tokyo, Japan), centrifuge, computer which had been installed with electrical equipments from E-Daq potensioestat and Echem v2.1.0 as software, shaker water bath (VWR GEN 2370), incubator (Sanyo, Osaka, Japan), UV-Vis spectrophotometer Biospec 1601 (Shimadzu, Kyoto, Japan), micropipette P10 and P1000 (Gilson, Middleton USA), laminar (Airtech, Tokyo, Japan), pH-meter HM-250 (TOA DKK, Yamagata, Japan), centrifuge, microwave, and glasswares. And for materials we used apple juice (from local market), *Acetobacter* and Heterotroph media for growing bacteria (Sigma-Aldrich, St Louis, USA), ferrocene, KCl solution, $K_3[Fe(CN)_6]$ solution, phosphate buffer 50 mM (pH 6.), ethanol 97% (Merck, Darmstadt, Germany), graphite, diethyl ether, and liquid paraffine..

2.1 Media Preparation for Growing Bacteria

There were two type of media, the solid and liquid form. The solid form of media was made in two types: *Acetobacter* and Heterotrof. *Acetobacter* media was made by mixing 0.5 g yeast extract, 0.3 g peptone, 2.5 g mannitol, and 1.5 g *Bacto Agar* and put into 300 mL Erlenmeyer 300 flask, then dilluted with 100 mL aquadest. Heterotroph media was made by mixing 1.5 g *Bacto Agar*, 1.5 peptone, 0.3 g triptone, 0.5 g NaCl, and 0.25 g K_2HPO_4 into 300 mL Erlenmeyer flask and dilluted with 100 mL aquadest. Those media were put into microwave until dilluted homogenously. After the solutions were ready, the mouth of Erlenmeyer flask were closed by cotton covered by aluminium, later covered again by plastic wrap. Those media than were put into autoclave for 90 minutes then poured into several warmed sterilized petri dishes. Liquid media for growing bacteria was made from *Acetobacter* media. with the same ingredients and procedure as making solid *Acetobacter* media before but without addition of *Bacto Agar*.

2.2 Bacteria Preparation

Bacteria were isolated from 2 mL apple juice, which is containing 1%, 2%, 5%, and 8% ethanol, into different eppendorf tubes, and let it fermented for one night. Later the solution was taken and scratched onto solid media with sterilized loopful, then incubated at 37 °C for one night.

The colony which was grown on the media later was moved into phosphate buffer containing 8% for one night. Later, the solution was scratched again onto the other solid agar media. After having the isolated bacteria, later it is moved onto another petri dish containing solid agar media by scratching it. The dishes were wrapped for preventing contamination and stored at 37 °C for one night. The colony on the solid agar media then was moved into liquid agar media and incubated for one night at 37 °C and shaken at 90 rpm inside shaker water bath. The incubated bacteria later was moved again into the new liquid agar media with the same procedure as stated before for 3-4 hours only. All the pocedures above were performed inside a steril laminar column.

The bacteria later was measured for its OD₆₀₀ absorbance. The harvested bacteria were dilluted into 3 types of concentrations which have absorbance values: 0.250, 0.500, and 0.750. Later, those solutions were stored into different eppendorf tubes and separated by centrifugation for 10 minutes (10,000 rpm, 4 °C). The formed pellets later was separated and washed with aquadest using vortex and centrifugated again. The washing process was repeated twice, then suspended into 0.7 mL phosphate buffer (0.05 M pH 6.8). For counting its density, we used spectrophotometer UV-Vis at the wavelength of 600 nm. The given absorbance value was 0.1 equal to $1-2 \times 10^6$ cell mL⁻¹, and it should be measured before its usage.

2.3 Electrodes Preparation

Into the mortar ferrocene and graphite were mixed homogenously and added with 1 mL of diethyl ether. Those mixture later was mixed again with paraffine until it formed carbon paste. The paste then was put into the small tube compartment, pressed, and cleaned with tissue. The electrodes then were incubated for 7-12 days. After that, all the electrodes were characterized by using K₃Fe(CN)₆ inside KCl solution. The carbon paste electrodes were made into 6 different compositions of graphite (mg) : paraffin (mg) : ferrocene (mg): (a) 70:30:0.7;(b) 70:30:3.5; (c) 30:10:0.3; (d) 30:10:1.5; (e) 100:100:1; and (f) 100:100:5.

2.4 Ethanol Measurement

For electrochemical measurements we used a computer installed with eDAQ potentiostat and *Echem v2.1.0* as the software. The applied electrodes were: (a) carbon paste electrode as working electrode; (b) Ag/AgCl as refference electrode; and (c) platinum as auxiliary electrode. The measurement parameters were set as follows:

Mode	: <i>Cyclic</i>	Step W	: 20 ms
E _{Initial}	: 0 mV	E _{Upper}	: 1000 mV
E _{Final}	: 0 mV	E _{Lower}	: -200 mV
Rate	: 200 mV/s		

Blank current response was observed by putting 5 mL phosphate buffer solution (0.05 M pH 6.8) in the vial. The selected bacteria were proliferated for further analyses with the same former steps. Different ethanol concentrations were used in this analyses: 0.01%, 0.1%, 0.5%, 1%, 1.5%, 2%, and 3%. The electrochemical measurement on the selected bacteria and in the whole ethanol concentration range.

Spectrophotometry analyses was begun by searching the maximum absorbance wavelength of ethanol. In this analyses we used 2 cuvettes, the first one was for phosphate buffer solution, and the other one is for the samples. The measurement used the same concentration as the electrochemical measurement method. Stability measurement was conducted in by comparing two kind of electrodes, a disposal type and undisposal one. Each type of electrode is measured for current produced.

3. Results

3.1 Bacteria and electrode preparation

Isolated bacteria were obtained from apple juice. This action was performed for harvesting AOX enzyme from *Acetobacter* which is living in the extract. Thanks to the ambient condition in the apple juice, it provides *Acetobacter* life's needs [6]. The isolate was reserved with 8% alcohol for make i adaptable to the higher ethanol concentration. The obtained bacteria via scratching method showed that the bacteria survived (Figure 1a). By the result, there was a trend that the more ethanol presence in the solution, it inhibited bacterial growth. From the bacterial screening profile, it was shown that the colony was separated well in Ap4. These bacteria colonies, which were marked on red circle, later would be planted on different solid media, *Acetobacter* and Heterotroph media.

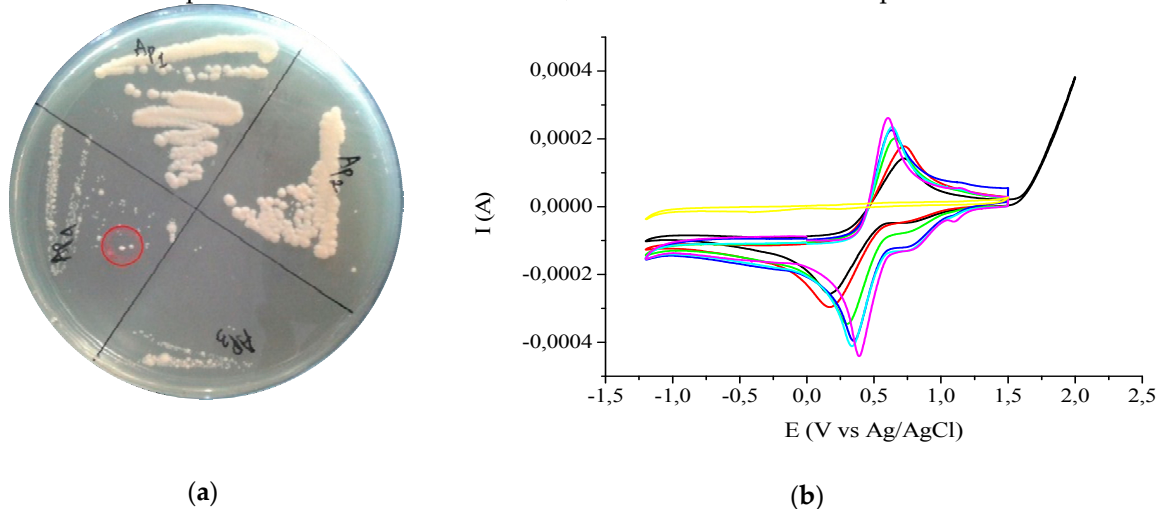


Figure 1. (a) Profile of bacteria on different alcohol concentration: 1% (Ap1), 2% (Ap2), 5% (Ap3), 8% (Ap4), the red circle (on Ap4) is the colony which was taken as the progenitor for further bacterial source; (b) Cyclic voltammogram profile of: — KCl 0.1 M (blank) and $K_3Fe(CN)_6$ solution; modified electrode —100:100:1 —100:100:5 —30:10:0.3 —30:10:1.5 —70:30:0.7 —70:30:3.5

Different composition of electrodes had different peak current (Figure 1b). By using KCl and $K_3[Fe(CN)_6]$ we screened the electrode to determine the best composition of modified electrode. The best electrode has a characteristic having a high oxidation peak, because it can provide a wide range of linearity. Based on the result, the electrode composition of 70:30:3.5 had the highest produced current (0.26 x mA for oxidation and -0.44 mA for reduction current respectively). Thus, we chosen this composition for further analyses.

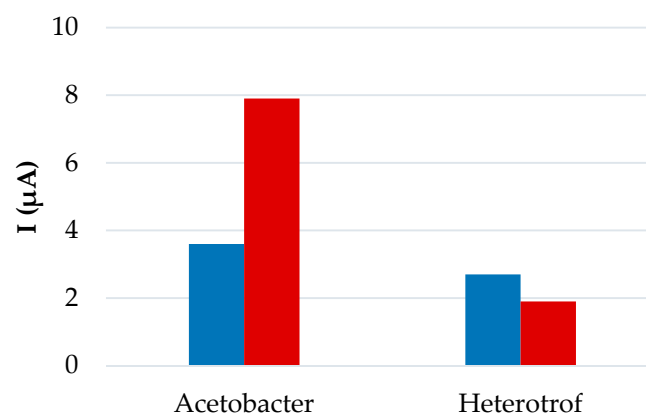


Figure 2. Oxidation peak current of bacteria from apple juice at ethanol concentration

■ 1% dan ■ 5%

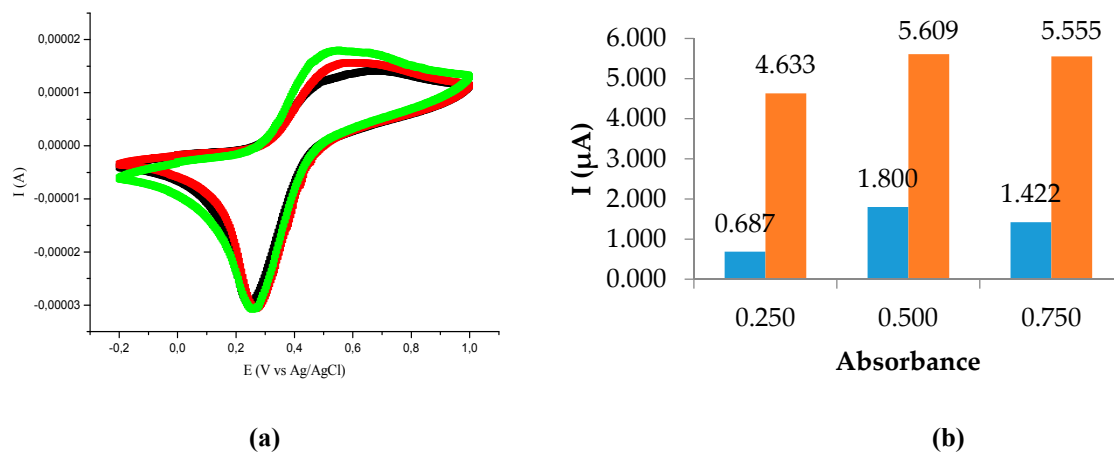


Figure 3. (a) Voltammogram of *Acetobacter* sp. with absorbance value 0.250 with — blank solution, ethanol 0.01% —, and 3.00% —; (b) The comparison between produced current which were produced from highest (3.00% —) and lowest (0.01% —) ethanol concentration.

Those two isolates later being measured using voltammetry method to determine which kind of media would produce the higher current. For this aim, we need to immobilize the bacteria onto the modified electrode. The immobilization process was simply by absorbing the bacteria onto the electrode surface. On Figure 2 it is shown that *Acetobacter* media had a higher oxidation current compared to Heterotroph media in both cases. The bacteria in *Acetobacter* media gave an increasement of oxidation current parallel to the increasement of the ethanol concentration. In other hand, the bacteria from Heterotroph media could not give higher current after ethanol concentration increasement. This indicates *Acetobacter* media is a better media for harvesting bacteria from apple juice. Hence, we chosen to use *Acetobacter* media for growing the bacteria from apple juice.

3.2 Bacteria concentration and oxidation current

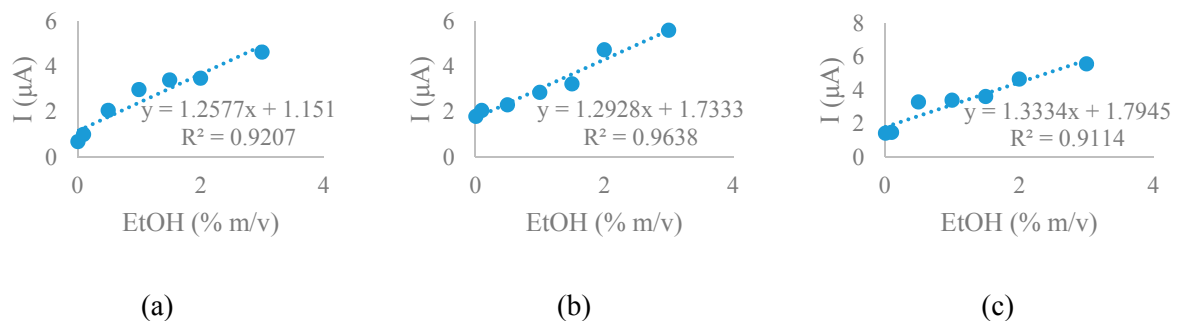


Figure 4. Linearity of three different *Acetobacter* sp. : (a) 0.25 (R^2 : 92.07%), (b) 0.50 (R^2 : 96.38%), (c) 0.75 (R^2 : 91.14%)

Different absorbance were set to 0.250, 0.500, and 0.750 creating 3 levels of cells concentration. These levels were made for differing the amount of bioreceptor on biosensor. Each absorbance value corresponded to 2.5-7.5 million of cells. However, not all cells were immobilized, for each cells concentration the immobilized cells were around 19, 36, and 56 thousand bacteria cells. It means that the more concentrated solution would give more thick immobilized cells on electrode. With increasing ethanol concentration, it was shown that the current getting higher as well (Figure 3a) as the the solution which had absorbance value 0.500 was the highest for both extreme points of linearity test. At the lowest ethanol concentration (0.01%), it gave current as much as 1.8 μ A, while the highest ethanol concentration (3.00%) produced 5.609 μ A (Figure 3b).

Biosensor is preferred for having high linearity between change of analyte concentration and the response signal. By comparing the R^2 value it was obtained that 0.500 absorbance value gave

higher R^2 value (Figure 4). It means, by using this concentration at absorbance value 0.500, it would produce more linear model. Thus, we can easily determine alcohol concentration without any big influence of residual error.

Thus, this result would be compared with the measurement result from spectrophotometry method (Figure 5). By comparing R^2 value it could be concluded that the proposed biosensor method had lower error compared to the spectrophotometer method, 96.38% compared to 94.07% respectively. However, the error difference was slight, so it needs more technique to enhancing biosensor for obtaining better measurement result.

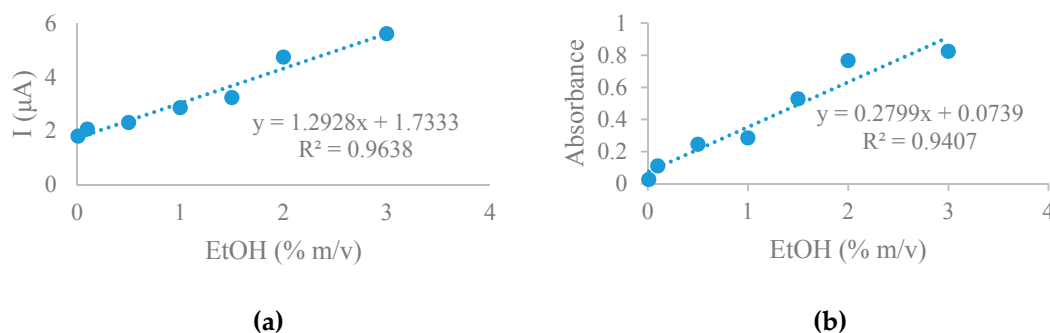


Figure 5. (a) Linear equation of biosensor with absorbance value 0.500 between the analyte's concentration and oxidation current peak ($R^2 = 0.957$); (b) Linear equation of UV-VIS spectrophotometer ($R^2 = 0.931$)

However, it only has low stability. A sharp decrease current produced happened at the second days after the fabrication (Figure 6, day-1 to day-3). It implies the stability of immobilization needs to be improved, or also the way for preserving it.

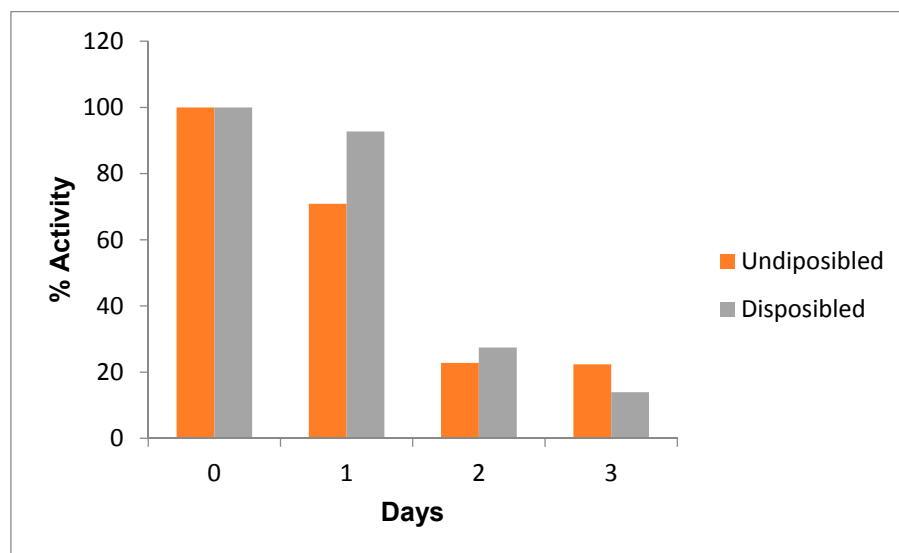
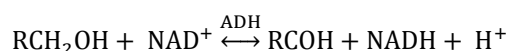


Figure 6. Stability profile of each type of biosensor measurement.

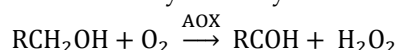
Discussion

There are several kinds of enzyme which are able to be used for detecting the presence of alcohol. Two kinds of the enzymes are alcohol dehydrogenase (ADH) and alcohol oxidase (AOX). Both of these enzymes utilize alcohol as the energy source for transforming it into other compounds. Both enzymes are grouped into oxidoreductase enzyme. However, ADH takes the reversible reaction comparing to AOX. In addition, ADH needs coenzyme nicotinamide adenine dinucleotide (NAD^+) for catalyzing primary alcohol to aldehyde reversibly as this reaction follows⁷:



Using ADH as the main base system for detecting short C-chain alcohol offers high specificity. However, it must be close to the cofactor, without any entrapment or linked. Several researches have been conducted by using ADH as the main bioreceptor. Alpat and his teams made an alcohol biosensor by combining ADH to the surface of modified electrode, covered by two kinds of layer: cellulose acetate-toluidine blue O and bovin serum albumin. Measuring using voltammetry cyclic, the fabricated biosensor had linear response between 10^{-5} - $4 \cdot 10^{-4}$ M selectively. It also provided high ratio of signal-to-noise up to three times and was stable of to 20 days which still had 50% activity of initial point⁸. Another research used polypyrrole{polyvinylsulphonate(PPy-PVS) with Meldola's blue as the mediator, facilitating electron transfer between the electrode and NADH. Interestingly, it had two linear range, at 1-10 μM and 0.01-0.1 mM. It also provided 17 days for stable measurement and 24 days when storing it in the phosphate buffer at 4 °C⁹.

Other researches using AOX as the bioreceptor also have been conducted. Compared to ADH, by using AOX measuring alcohol via enzymatic reaction is simpler, because it only uses O_2 as enzyme recover agent. Because of the O_2 presence in the air, it is easy to use, however it also conducted cross-reaction. It means, this enzyme also can react with other short chain alcohol. The measured current is obtained from the second reduction between H_2O_2 with the electrode's surface, transferring the electron to the electrode. Hereby the enzymatic reaction of AOX as follows⁷:



One of the research is by using colour change and detecting it visually are able to easen the alcohol detection. Kuswandi and his team have fabricated a optical biosensor for detecting ethanol visually, by using AOX binded to polyaniline (PANI), for halal verification. His works showed very good correlation at concentration range 0.01%-0.8%, and providing LOD up to 0.001%¹⁰. It means this biosensor can be used in daily application, compared to the electrochemical measurement method. It also provided a close range to the halalness threshold issued by *Majelis Ulama Indonesia* (MUI), 1 %. However, because of the nature of AOX, it also had slight cross-reaction with methanol and 1-propanol. It also has narrow linear range, only 0.01-0.8%. So, the linear range must be widened for ensuring the alcohol concentration has exceeded the threshold line.

Our reseach tried to use AOX produced *in situ* from *Acetobacter* sp. Based on our result, by using whole cell biosensor, we can widen the detection range, 0.01-3.00%. We also found that when ethanol concentration exceeded 5%, it gave low linearity. Thus, this proposed biosensor can easily track the alcohol concentration below and up of the threshold concentration. However, there were 2 crucial things that we have to concern: LOQ value and stability. LOQ value will give us information about the certainty of measurement. Whenever having a measurement value below the LOQ value, we have uncertain prediction of our analyt. And also stability, it is directly connected to the stability of bioreceptor in a biosensor. Since the system is adsorbtion only, deabsorption phenomenon could happen, influencing the biosensor's performance when measuring analyt. Therefore, a better immobilization method for combining *Acetobacter* sp. onto the bioreceptor layer and maintaining its presence and viability inside the immobilizer layer should be considered highly for the next future research. Since, without using any enzyme extraction from a microbe we can use it directly from the microbe and preserve its function as the bioreceptor longer. The stabibility of biosensor is 3 days with activity 22,34% for undisposibled electrode and 13,91% for disposibled electrode.

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