Alcohol Dehydrogenase of *Bacillus* strain for Measuring Alcohol Electrochemically

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Abstract. Alcohol dehydrogenase (ADH) was applied to produce alcohol biosensor. The enzyme was collected from cultured *Bacillus sp.* in solid media. From 6 tested isolates, bacteria from fermented rice grain (TST.A) showed the highest oxidation current which was further applied as the bioreceptor. Various ethanol concentrations was measured based on the increase of maximum oxidation current value. However, a reduction value was happened when the ethanol concentration was higher than 5%. Comparing the result of spectrophotometry measurement, R^2 value obtained from the biosensor measurement method was higher. The new proposed method resulted a wider detection range, from 0.1-5% of ethanol concentration. The result showed that biosensor method has big potency to be used as alcohol detector in foods or bevearages.

1. Introduction

Alcohol is one of chemical substance which may be present in many kinds of foods and beverages. Introducing alcohol to biological system of human body exceeds the tolerable concentration can harm the biological function like nervous, transportation, and digestive system in human body [1], which can lead to many metabolism disfuctions and physiological deseases. Therefore, controlling the alcohol concentration in a processed food or beverage product becomes a major concerned. In addition, muslims consumers are also require assurance of halal products, since the Muslim people do not consume food or drinks that contain alcohol. Considering the people's need, Ulama's Council of Indonesia (MUI) released fatwa MUI No. 4/2003 stipulating that alcohol content inside foods or beverages should be below 1%. Therefore, there is necesity to have a new method that is able to measure alcohol content inside food materials sensitively and selectively [2].

For daily usage ethanol is frequently applied as part of food or beverage ingredient. Recently, several methods have been used in ethanol measurement, including gas chromatography [3], high performance of liquid chromatography [4], infrared spectroscopy [5] etc. These methods are very helpful for determining ethanol concentration based on its precision. However, it has some disadvantages because this method needs trained operator, has long time analysis, relatively high cost, and can be interferred with any substance which has same wavelength absorption. To solve this problem, we tried to develop other technique based on electrochemical reaction. One of the electrochemical measurement method is electrochemical biosensor. Biosensor has bioreceptor which

can detect substance selectively since it works is based on a selective enzymatic reaction. Biosensor has an opportunity being developed as selective, applicative, and fast time analysis, as well as a portable kit analyser [6].

In this method, choosing the right bioreceptor is a challenging since it determines the further process of detecting the targetted analyt. In this research microbe was used as the main bioreceptor because it is readily and easily to be regenerated. Many reports have written that *Bacillus sp*., a positive-Gram bacteria, has potency to be used for detecting alcohol, because this microbe produces alcohol dehydrogenase (ADH). Previous research has been conducted using *B. stearothermophillus* which was able to produce a stable ADH from heat of reversible oxidation reaction. In this reaction ADH required nicotinamide adenine dinucleotide (NAD^+) as cofactor. As the microbe becomes bioreceptor, during reaction, all biochemical reaction involving ADH would influence the charge of electrode. When proton is generated by turning NADH to NAD^+, it would be transfered into electrode directly or indirectly, by which the current changes then can detected [7]. Some experiments have been conducted in this basic concept and shown good result [8,9,10]. However, it still required more time for detection process and higher cost was needed for enzyme extraction. Therefore, using whole microbe cell as bioreceptor directly we expect that the technology may be able to reduce the fabrication cost of biosensor in the future.

This preliminary research aimed to develope an alcoholic biosensor using alcohol dehydrogenase (ADH) enzyme produced from *Bacillus sp.* for detecting ethanol content.

2. Research Methods

2.1. Tools and Materials

In this research we used a computer installed with software Echem v. 2.0.1 connected to E-Daq potentiostat E-Coder 410, spectrophotometer UV-Vis (Shimadzu 1700), centrifuge (Kokusan H-1500 F), autoclave (Hariyama), pH meter (TOA DK HM-250), micropipette (Gilson), oven, andglassware. The materials used in this research were *Bacillus* sp. isolate, solid agar media, graphite, ferrocene, liquid paraffin, simethyl sulfoxide (DMSO), buffer phosphate solution 0.05M pH 7.00, and ethanol (Merck).

2.2. Methods

2.2.1. Isolate preparation. There were 2 types of bacteria media: Heterotroph (HTR) and Yeast Mol (YM). HTR's preparation was carried out by pouring 1.5 g Bacto Agar, 1.5 g Peptone, 0.3 g Tripton, 0.5 g NaCl and 0.25 g K2HPO4 into 300 mL^3 erlenmeyer flask, dilluted with aquadest, while YM's preparation was prepared using 1.5 g Bacto Agar, 0.5 g Pepton, 0.3 g Yeast Extract, 1 g Glukosa, 0.05 g $MgSO_4.H_2O$, and 0.1 g K_2HPO_4 with the same aquadest volume. Both media were put into microwave and mixed homogenously. After mixing process the flasks were corked with aluminium foil. Later, it was put into autoclave 90 minutes and poured into several sterilized petri dishes in warm condition. There were 6 isolated bacteria of Bacillus sp., coded as follows: ET.OH, TST.A, BS 1, BS 2, BS 4, and BS 5. Those isolates were prepared by dethawing the isolate from freezer, taken a loopful and planted onto by smearing the solid agar media prepared before, and were stored into incubator for 24 hours (30°C). The first generation was replanted again with the same method, incubated for 24 hours. All the treatment was done inside steril laminar air flow. After 24 hours the second plantation, 10 loopfuls was put into eppendorf, diluted with 1 mL phosphate buffer 0.05 M pH 7.0, and washed by centrifugation. Later, the microbes was separated by 10 minutes centrifugation (10 000 rpm, 4°C). The pellet then was suspended into 10 minutes phosphate buffer 0.05 M pH 7.00.

2.2.2. Electrode preparation and microbe screening. Carbon paste electrode was made by mixing 3 mg ferrocene (diluted by 1 mL DMSO before) and 100 mg graphite, allowed to stand for 2 hours, and dried at 105 °C. The modified carbon paste then mixed with 30 μL liquid paraffin homogenously. The

paste was put into cyllindrical glass compartment, desed, and polished. The electrode was ready for further used after 7-12 days. All of the electrodes shoudl be characterized by $K_3[Fe(CN)_6]$ (diluted in 0.1 M of KCl), observing redox peak by cyclic voltammetry.The well-tested electrode later being used for further measurement. Ag/AgCl was used as refference electrode and Pt as auxiliary electrode. These following parameters was input into computer installed with Echem v.2.0.1 and connected to eDAQ potensiostat:

- Mode : Cyclic
- $E_{Initial}$: 0 mV
- E_{Final} : 0 mV
- Rate : 250 mV/s
- Step W : 20 ms
- E_{Upper} : 1000 mV
- E_{Lower} : 0 mV

For measurement, 5 mL buffer phosphate solution 0.05 M pH 7 was put into a vial, and the blank voltammogram profile was obtained. Later, 10 μL bacteria isolate, 1 mL ethanol 1% were also poured into blank solution, then the current observed. Difference between the sample current and blank current was considered as net current. The applied isolate bacteria was taken from aforesaid bacteria (ET.OH, TST.A, BS 1, BS 2, BS 4, and BS 5). Those six bacteria samples were measured electrochemically and screened for obtaining the highest oxidation current produced. The selected bacteria later was used for forward experiments.

2.2.3. Electrochemical and UV-Vis Measurement of Alcohol. Selected bacteria was rejuvenated and reproduced massively for linearity test with the same procedure listed before. The electrochemical measurement of enzymatic reaction was performed as written before, but with various ethanol concentration (0.1%, 0.3%, 0.5%, 0.7%, 1%, 3%, and 5%). The net current was calculated after observing the blank and sample current. With the same ethanol concentration, we applied it to spectrophotometry measurement method, using UV-Vis spectrophotometer (2 beams). The first cuvette was filled with phosphate buffer 0.05 M pH 7, and the second cuvette filled by pure ethanol 98%. After searching maximum wavelength absorbance, the first cuvette was added by ethanol increasingly (0.1%, 0.3%, 0.5%, 0.7%, 1%, 3%, and 5%). The absorbance value then was recorded.

3. Result and Discussion

Bacteria were collected from 6 different sources, the two first were isolated from fermented alcohol (ET.OH) and fermented rice grain (TST.A), while the rest bacteria sources were taken from laboratory isolate (B1, B2, B3, and B4). Only ET.OH isolate was planted in the HTR media, while the rest was planted on YM. By smearing method, the bacteria were planted into each proper media, and the result was presented in figure 1. These bacteria were chosen electrochemically (using hardware from EDAQ potentiostat and E-Coder 410: cyclic voltammetry method, data analyzed with software *Echem* v. 2.0.1) by using mixture of 1% ethanol and buffer (pH 7.00) read as blank current. The oxidation peak current was measured after 10 μL of suspended bacteria poured into the blank solution. The measurement showed that TST.A produced the highest amount of targeted enzyme indicated the highest amount of oxidation current (figure 2). The current was produced as much as 2.34μA at 0.545V. Therefore, we used TST.A as bacterial source for detecting ethanol.

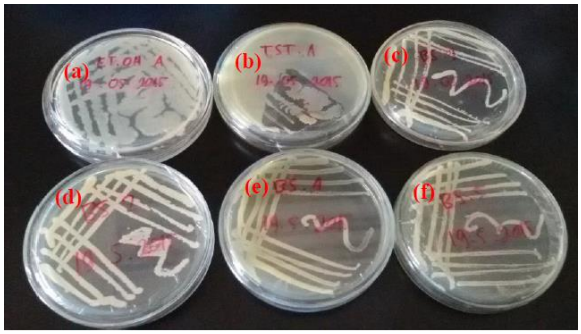
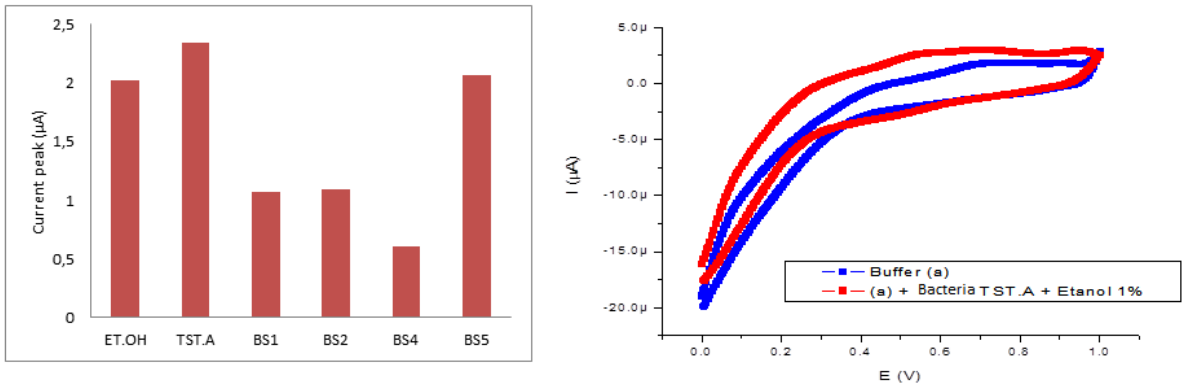


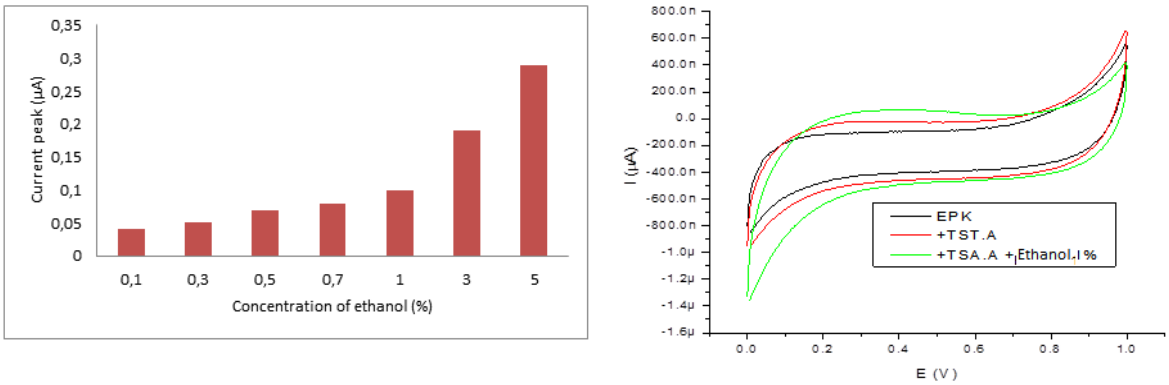
Figure 1 Isolated Bacteria: (a) ET.OH, (b) TST.A, (c) BS.1, (d) BS.2, (e) BS.3, (f) BS.4.



(a) (b)

Figure 2. (a) Oxidation peak current profiles of 6 bacteria; (b) voltammogram current profile of TST.A: blue line is blank solution, red line is after addition of TST.A isolate and ethanol.

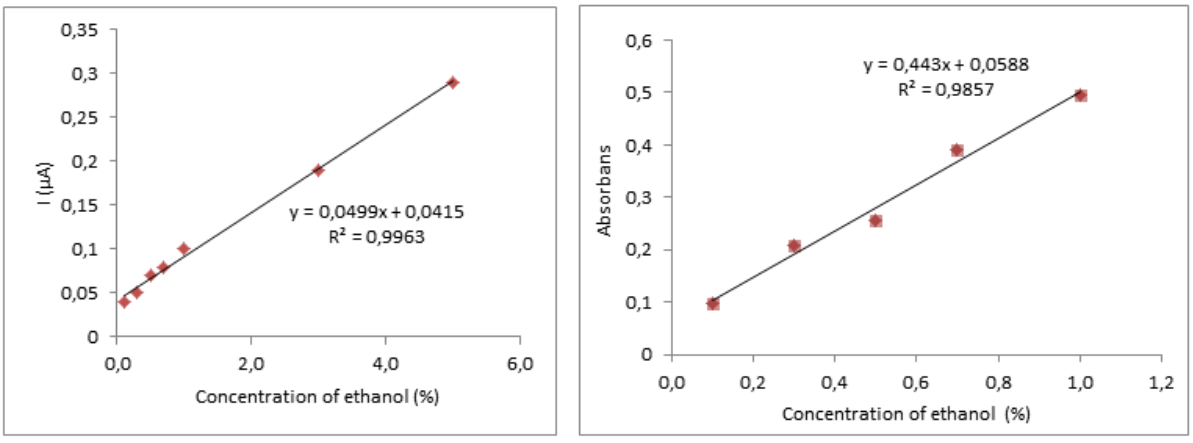
For the next experiment, we measured the various concentration of ethanol (0.1%, 0.3%, 0.5%, 0.7%, 1.0%, 3.0%, and 5.0%) by using biosensor, so that we can measure the measurement linearity. For this measurement, we got the average of oxidation current for each concentration (figure 3.a). The voltammogram profile also exhibited an increasement of current after adding ethanol to the mixture of bacteria and blank solution (figure 3.b). The result showed that the more alcohol concentration presented into the solution, the more oxidation current produced. However, at the concentration of 7.0% or 10.0% ethanol the reaction did not give linear result. So, using the measurement range of 0.1-5.0%, the graph produced R^2 value up to 99.63%. The result showed that using more etanol into sample solution decreased the current that was produced. It probably because the higher ethanol concentration lead to several chemical and physical changes inside the microbe's cell. Thus, the metabolism become ruptured because alcohol has potency as bactericide [10].



(a) (b)

Figure 3. (a) Average of thrice measurement on the same ethanol level concentration; (b) Voltammogram profile of ethanol measurement.

We also compared the linearity of the measurement with the electrochemical analysis using UV-VIS (Shimadzu 1700) at the same ethanol concentration. Than the R^2 value of both methods was evaluated. The analyzed results showed that both methods had good linearity range, however measurement using ethanol electrochemical method produced slightly higher value of R^2 (99.63%) compared to biosensor (98.57%, Figure 4). In addition, the measurement using biosensor only gave best result if the concentration of ethanol was below 1%. Above that concentration level, the graph was not linear anymore. Considering this, the proposed method had a good chance for being developed as alternative method for measuring ethanol even thogh some modification may need to be carried out.



(a) (b)

Figure 4. Measuring ethanol: (a) biosensor; (b) UV-VIS.

4. Conclusion

Alcohol biosensor can be constructed using bacteria which produced alcohol dehydrogenase enzyme (ADH). From 6 tested *Bacillus* sp., the bacteria isolated from fermented rice grain (TST.A) produced the best result (2.34 µA at 0.545 V) as compared to other bacteria. At linearity test, there was current decreasement above 5% of ethanol. The used measurement range was 0.1-5.0%. Therefore, measuring alcohol using ADH producer microbe potentially can be developed more in the future.

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