Pineapple juice’s microbe as bioreceptor for detecting alcohol by electrochemical method

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Abstract. Alcohol is commonly found in food and beverage. This alcohol contents must be precisely measured to ensure the halalness of a product, considering the fatwa of Ulema's Council of Indonesia. Alcohol measurement can be carried out using alcohol biosensor based on microbe from pineapple juice, Saccharomyces cerevisiae yeast, which is known to produce alcohol dehydrogenase. This enzyme can reversibly convert ethanol to acetaldehyde. 1 % Ethanol measurement shows oxidation and reduction peak current at potential 0.736 V and 0.248 V, respectively. The result shows that immobilized electrode with OD600 value of 0.300 microbe cells generate the highest peak current. One of requirement sufficiently biosensor is high linearity. The best linearity measurement value is 0.9936 which is would be obtained at 0.01-2.50% concentration range. This linearity of ethanol measurement is similar with ethanol measurement using pure enzyme. The result would make this study potentially prospective.

1. Introduction

Recently alcohol can be commonly found in many kind of food and beverages. This raise awareness from consumers especially moslems to ensure the halalness of the product. Moreover, based on that fact, Ulema's Council of Indonesia (MUI) has issued a fatwa No. 4/2003 which stated that the minimum threshold of ethanol presents in the foods or beverages is 1%.

Measuring alcohol concentration may be conducted using several methods. Some of them are: redox titration, chromatography, and spectroscopy. Those methods offer excellent accuration and precision. However, those methods have many disadvantages such as relatively expensive, complicated in sample preparation, complex in technique, longer time in result analysing [1]. Based on those facts, some proposed methods have been developed, such as electrochemical measurement. One kind of electrochemical measurement is biosensor, a sensing method by applying enzymatic reaction for detecting the targeted compound. Biosensor provides high selectivity and sensitivity. Substrate would be selectively oxidized or reduced by enzymatic reaction [2]. Other advantages are fast detection time, simple, relatively cheap, sensitive, selective, and portable [3]. Thus, we conduct a research to detect alcohol by using biosensor.

Most of the alcohol biosensor designed today are applying alcohol oxidase (AOX) and alcohol dehydrogenase. Both enzymes are selective for detecting short aliphatic of alcohol, especially ethanol. Some research show that ADH works more stable and specific compared to AOX. This happens because AOX easily reacts to those short aliphatic alcohol [1]. However, using pure enzyme for
biosensor needs high cost, hence other enzyme source is needed. Thus, applying microbe as the whole cell bioreceptor can minimize the cost for fabricating the biosensor. Besides, it is more tolerable to pH change, obtained easily, and no need for further enzyme purification [4]. Previous research shows that Hansenula Polyphorma [5] and G. Oxydans [6,7] can be used as bioreceptor for alcohol biosensor. Those microbes have enzyme AOX and ADH respectively. Other potential microbes are S. cerevisiae dan A. Aceti [8]. Both microbes are easily isolated from fruit juice, such as: apple, grape, and pineapple. Therefore, we tried to make a biosensor for alcohol measurement by using isolated microbe from one of those fruit juices: apple, grape, and pineapple.

2. Materials
Materials used in this research were: juice from apple, grape, and pineapple, graphite paraffin oil, ferrocene, diethyl ether, phospat buffer, KCl, K3Fe(CN)6, ethanol 97%, dialysis membrane, o-ring rubber, and glassy tube.

3. Methods

3.1. Modified carbon electrode preparation
Modified carbon paste electrodes were made into 3 different graphite and paraffin compositions: 1:1(m/m), 3:1(m/m), and 7:3(m/m). Later on, ferrocene concentration was assorted depended on graphite mass: 1:100(m/m) dan 5:100(m/m). Ferrocene later was diluted into diethyl ether. All of the material, except paraffin, then mixed homogenously until diethyl ether evaporated. Later, paraffin poured into the mixture, transforming the mixture into a carbon paste. The carbon paste then was placed inside a tube glass compartment (Figure 1) 3 mm, being densed, and polished. The electrodes later was allowed to dry for 7-12 days before characterizing them using K3Fe(CN)6 solution.

![Figure 1 Biosensor construction](image)

3.2. Bacterial isolation from juice
Bacteria was isolated from the juice by letting the juice stay still for one night in room temperature. Later, 2 mL of juice from each fruit (apple, grape, pineapple) was added up by 1%, 2%, 5%, and 8% ethanol. After 2 hours, the sample then was smeared into solid agar media and incubated for one night at 37°C. The well-grown bacteria at the highest concentration was collected by a loopful and poured into phosphate buffer solution (pH 6.80) which had contained the corresponding alcohol concentration, then incubated once more for one night in room temperature. The most clouded solution later was smeared once more onto the solid agar media for one night at 37°C. Later, a loopful of bacteria was diluted into 2 mL phosphate buffer solution 50 mM pH 6.8 for its electrochemical response testing by using 1% and 5% of ethanol. The bacteria which showed the highest current response would be chosen for further test as the bioreceptor.
3.3. Microbe harvesting, counting, and immobilizing

As much as a loopful of pure isolate was used for rejuvenation, and diluted into liquid agar media and shaken in shaker water bath for one night (37°C, 0 rpm). Later, the microbe was rejuvenated once more with liquid agar media (1:50) for 2-3 hours before counting the microbe's population by using spectrophotometer at 600 nm. The used OD600 value must fall in range 0.5-1.0. The OD600 value of 0.1 equals to 1-2x106 cells/mL. After counting the microbe, it was later separated from the media by centrifugation (10,000 rpm, 4°C, 10 minutes) producing pellets. The pellets were washed by sterilized aquadest and recentrifugated two times with the same previous procedure. The pellet later was diluted into phosphate buffer 50 mM pH 6.8, mixed homogenously before immobilized onto the electrode. The harvested microbe then was assorted into 3 different concentrations, based on OD600 value: 0.300, 0.600, 0.900. As much as 7.5 µL of microbe was directly put by micropipette onto electrode surface, and let dried. The electrode was stored into phosphate buffer solution 50 mM pH 6.8 before usage.

3.4. Ethanol measurement using cyclic voltammetry

Electrochemical measurement by voltammetry cyclic was performed by using potentiostat (eDAQ, and computer installed with Echem v2.1.0). There are 3 kinds of electrode used here: Ag/AgCl (reference electrode), platinum (auxiliary electrode), and biosensor (working electrode). Hereby the parameters for cyclic voltammetry measurement. For the blank measurement, 7 mL phosphate buffer solution 50 mM pH 7.5 was poured into the compartment cell. Later, various ethanol concentrations (0.01%, 0.05%, 0.50%, 1.20%, 1.50%, 2.00%, 2.50%) were used for observing the peak of oxidation current. This was done for 3 immobilized microbe concentrations used as bioreceptor.

3.5. Comparison measurement using spectrophotometry method and biosensor

At the same ethanol concentration used in cyclic voltammetry, we conducted the measurement using UV-Vis spectrophotometry method. Before running the method, the maximum wavelength absorbance was observed. The linearity of this measurement, later was compared to the previous measurement using electrochemical method.

3.6. Stability

Biosensor stability was measured by comparing the current produced from the disposed electrode and reused electrode. Both electrode types were tested once for a day.

4. Results and discussion

4.1. Electrode fabrication and applied bioreceptor

Electrode is a part of the biosensor that will transduce the electric signal into the analyzer. The modification of the electrode will produce more electron detection, because the redox reaction is accelerated. In this research, ferrocene as mediator will enhance the electron transfer process, decreasing the over-potential, and enhancing enzyme selectivity [9]. The well-fabricated electrode was easily known by the presence of oxidation and reduction peak in the voltammogram. The characterization process used KCl and K3[Fe(CN)6] solution. By using only KCl (blank solution) there was no any oxidation peak (Figure 2). The redox peak appeared after adding K3[Fe(CN)6] diluted into KCl. After measuring the current produced by the electrodes, it showed that the electrode which had composition of 7:3:0.35 (graphite, paraffin, ferrocene) produced the highest oxidation peak current, 2.62×10-4 A (Figure 2). This happened because the composition was good for conducting the electron [10].
Figure 2 Voltammogram profile using: — KCl 0.1 M and K$_3$Fe(CN)$_6$ 0.1 M in KCl 0.1 M with graphite:paraffin:ferrocene composition — 1:1:0.01, — 1:1:0.05, — 3:1:0.03, — 3:1:0.15, — 7:3:0.07, dan — 7:3:0.35.

There were 3 kinds of juice as the microbe source: apple, grape, and pineapple. Those juices are highly potential to carry microbe which is able to metabolize alcohol. Microbe selection is needed to harvest the best microbe that produce the highest oxidation current. The chosen microbe was taken from the isolate of the juice which could cope the highest ethanol concentration applied into the juice. The result showed that all of the isolates from 3 juice were able to detect ethanol. This could be concluded after the presence of redox peak in the voltammogram (Figure. 3, Figure. 4, Figure. 5).

Figure 3 Voltammogram profile of 1% and 5% ethanol measurement using — unmodified electrode; unmodified electrode+apple juice microbe
Compared to all microbe sources, microbe from apple juice produced the highest oxidation peak (6.44×10^{-6} \text{ A}) at 1% ethanol. However, at 5% ethanol, only microbe from pineapple juice produced the highest peak while other microbe isolates could not produce high current. It means that, pineapple juice microbe could survive at higher level of ethanol concentration, whereas apple and grape juice microbe could not deal with higher concentration. Ethanol concentration can affect the cell's metabolism, especially the viability, cell growth, and structure of membrane. At low concentration ethanol can inhibit cell growth and replication. It also affects cell's volume and specific cell growth. Conversely, at higher concentration it affects cell membrane strength and its mortality [11]. For
bioreceptor usage, higher survival chance for detecting alcohol is needed, thus we choose the microbe from pineapple juice.

Later, microbe identification was conducted by observing its colony shapes and cell morphology. The colony shape that was seen were round, white milk coloured, while its morphology was oval and several cells were being duplicated (Figure. 6).

![Figure 6 Microbe harvested from pineapple juice using light microscope with 100 times magnification.](image)

Based on this data, we concluded the observed microbe was Saccharomyces sp. It has characteristics of having egg-shell shaped, and able to divide itself [12]. This microbe is able to oxidize alcohol, thanks to the presence of alcohol dehydrogenase, located in its cytosol (Adh1p dan Adh2p), and mitochondrial matrix (Adh3p). The enzyme was induced by the presence of ethanol. Thus, ethanol were catalyzed by this microbe for the sake of energy source reversibly to acetaldehyde, followed by NAD+ or NADP+ reduction [8] This redox reaction was observed indirectly in voltammogram as oxidation and reduction peak.

4.2. Alcohol Measurement by Biosensor and Comparison to Spectrophotometry

Alcohol measurement by cyclic voltammetry can reveal the oxidation and reduction process of the enzymatic reaction. When detecting the alcohol, oxidation peak current represents the oxidation of alcohol to acetaldehyde, and reduction peak current represents the reverse reaction, since alcohol dehydrogenase conducts reversible reaction. As showed at Figure. 7, the redox reaction happens as many redox chain reaction happens. The first system is the oxidation of ethanol to acetaldehyde by alcohol dehydrogenase. This reaction leads NAD+ converted to NADH. NADH later oxidizes ferrocene then transfers electron to the electrode.

![Figure 7 Redox reaction during alcohol detection](image)
By using biosensor for detecting 1% ethanol and OD_{600} value as bioreceptor, it shows a slow electron transfer. It can be seen from the difference between oxidation and reduction voltage, 0.248V and 0.736V respectively. By the comparison between oxidation and reduction peak current, it reveals that it has tendency having higher amount of reduction current (1.78×10^{-6} A) compared to the oxidation current (2.48×10^{-6} A). Thus, it is consistent with the enzyme characteristic, since converting to ethanol is easier than converting to acetaldehyde. Both compounds have similar property, an inhibitor to alcohol dehydrogenase, though acetaldehyde more competitively inhibits enzyme.

Different OD600 value has effect on the current produced during alcohol detection. As OD600 value gets higher, the current tends to decrease (Figure 8). This trend happens in both oxidation and reduction peaks. It happens because as OD600 get higher, the microbe concentration is increasing. It leads to the higher amount of microbe usage in the same electrode's surface area. Thus, it creates competition for consuming alcohol to replenish every single microbe. As the concentration of ethanol get lower for every cell, it lead to current decreasement. Therefore, we use microbe concentration with OD600 value of 0.300.

![Figure 8](image_url)

**Figure 8** Oxidation and reduction current for each measurement

We conduct this research in range between 0.01-3.00%. During the measurement, appearance of oxidation peak is the main source for calibration curve. We observe that oxidation peak appears between 0.532-0.724 V, while the reduction current occurs at range 0.168-0.344 V. As predicted, more alcohol will increase the oxidation peak. In this condition, 0.01-2.50% gives the best linearity (R2 = 0.9939, Figure. 9a).
Several research have been conducted using pure alcohol dehydrogenase. Combining the enzyme with blue toluidine gives linearity up to 0.9968 [13]. Another research using this enzyme directly immobilized onto modified electrode with ZrO$_2$, it gives 0.9984 for R$^2$ value. Comparing those result to our research result, it presents almost similar linearity using the pure enzyme [14].

Since the enzyme purification needs sophisticated steps, therefore only using microbe obviously gives chance for developing more stabilized biosensor [15]. Both methods offer precision for detecting alcohol. For the same linearity range (Figure. 9), it is clearly shown that biosensor has the advantage as alternative measurement method. It is reflected from R$^2$ value, since linearity could be affected by other interferences which may have the same wavelength absorbance. Eventhough biosensor offers easiness for detecting alcohol, biosensor needs more development as the alternative method of measuring alcohol, since using biological matters should deal with the uncertainty of its viability. Accordingly, this would affect much on the biosensor performance.

Other tests are also performed, like: stability and accuracy. At Figure 10, both electrodes indicate low stability. The electrode stability was rapidly reduced below 20% signal, meaning it only has two days to remain stable. Tkac et al. (2003) reported by using unimmobilized $S$. cerivisiae had given better stability compared to usage of $Gluconobacter$ immobilized to cellulose acetate membrane, which was only stable for 8.5 hours [7]. The result of this research still has lower stability, compares to biosensor using $Saccharomyces$ sellipsoideus which could remain stable for 9 days until it has only a half from the initial current [15]. The difference occurs based on the microbe species used and the amount of microbes immobilized. Hence, for further research an immobilization method should be considered for enhancing the lifetime of the biosensor.
Figure 10 Biosensor stability profile between disposed and undisposed electrode

It would be concluded that isolated microbe from pineapple juice, Saccharomyces cerevisiae, evidently could detect alcohol by using cyclic voltammetry with the best electrode composition 7:3:0.35 (graphite, paraffin:ferrocene). Microbe concentration of OD600 could affect the performance of biosensor, and the highest current achieved at OD600 as much as 0.300. Result showed that it had similar R2 value with the other research using pure enzyme. However, it still needs more effort in validation technique before fabricating the whole installation.

References
[1] Chen-An L and Yu-Chen T 2009 Sensors and Actuators B 138(2009) 518-523
[2] Iswantini D, Trivadila, Nurhidayat N and Nurcholis W 2013 WASET 7(6) 263-270
[3] Alferov V A et al 2011 J. of Anal. Chem. 66(12) 1205-1211
[4] Iswantini D, Nurhidayat N, Trivadila and Nurjayati A 2011 JIPI 16(2)112-118
[5] Kostyantyn V D et al 2007 BMC Biotechnology 7(2007) 33
[6] Milan V, Jaroslav K, Ernest S and Peter G 2009 Sensors and Actuators B 138(2009) 581-586
[7] Tkac J et al 2003 Biosen. Bioelect. 18(2003) 1125-1134
[8] Smidt O D, Preez J C D and Albertyn J 2008 FEM Yeast Res. 8(2008) 967-978
[9] Bean L S et al 2006 Malay J. of Anal. Sci. 10(2) 313-320
[10] Svancara I, Kalcher K, Walcarius A and Vytras K 2012 Electroanalysis with Carbon Paste Electrode (New York: CRC Press)
[11] Stanley D et al 2010 J. of Appl. Microbiol. 109(2010) 13-24
[12] Andayani P, Wardani A K and Murtini E S 2008 Jurnal Teknologi Pertanian 9(2) 95-105
[13] Alpat S and Telefoncu A 2010 Sensors 10(2010) 748-764
[14] Salimi F et al 2012 Int. J. Electrochem. Sci. 7(2012) 7225-7234
[15] Dhewa T 2015 Jour. Env. Res. 3(2) 212-218