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Sensitive and stable ethanol biosensor development based on *Acetobacter aceti* biofilm for halal detection of food and beverages

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ABSTRACT

A highly sensitive ethanol biosensor based on *Acetobacter aceti* biofilm, which contains pyrroloquinoline quinone-alcohol dehydrogenase enzyme for halal detection in food and beverages, has been developed. In this study, *A. aceti* cells were purified by determining the conditions of growth time, incubation temperature, and pH to suppress the yeast growth. Selection of optimum conditions for biofilm formation was also carried out to obtain a stable sensor operation. The optimum biofilm formation was 14 days. The performance of biosensor was investigated by the cyclic voltammetry technique. Linear range, sensitivity, limit of detection (LOD), and limit of quantification (LOQ) were found to be 5×10^{-5}–3×10^{-4} %, 43,076 µA %$^{-1}$, 2.32×10^{-5}, and 7.03×10^{-5} %, respectively. The repeatability of biosensor was tested in $1 \times 10^{-5} - 1\%$ ethanol concentration and the relative standard deviation was 1.08% (for $n = 7$). The stability studies have shown that the cyclic voltammetry response of biosensor to ethanol leave the act of 98.99% at the 7 week. Thus, the developed biosensor is promising to be used for simple analysis of halal products and has been tested on real sample.

1. INTRODUCTION

The use of ethanol as a solvent in various industrial sectors, especially food and beverages, has become a major controversy in halal product analysis for the countries with the majority of muslim consumers. Every ethanol produced by anaerobic fermentation with concentration more than 1% is considered as Haram (prohibited), while ethanol produced naturally and less than 1% of its halal status is tolerable [1,2]. The presence of high levels of ethanol compounds in foods can cause poisoning, depression, mutation in tumor suppressant genes, hence the main trigger of the risk of colon and esophageal cancer [2].

Therefore, a proper technique is needed to detect its halal. The technique commonly used to measure ethanol content in food and beverages includes infrared spectroscopy [3], gas chromatography-mass spectroscopy [4], and fluorometry [5]. Although quantitatively those methods are quite accurate for practical reasons, they are less supportive in samples preparation and tools operation. Biosensor application is one of the solutions for developing ethanol detection devices that requires easily sample preparation, to use, sensitive, fast, and inexpensive.

The selection of bioreceptor components are a very crucial in making biosensors. Biosensor is a device that uses biological components as their bioreceptors [6]. Microbial cells are promising bioreceptors because there is no need to isolate enzymes, coenzyme, and cofactors already in the system, so enzymes are more stable in their natural environment within the cell [7]. Some microbes used as ethanol bioreceptors have been reported, including *Pichia methanolica* [8], *Candida tropicalis* [9], and *Pichia angusta* [10] as the producer of alcohol oxidase (AOX) enzymes. In addition, *Saccharomyces cerevisiae* [11], *Saccharomyces ellipsoideus* [12], and *Gluconobacter oxydans* [13,14] as a producer of alcohol dehydrogenase (ADH) enzymes. *Acetobacter aceti* is one of the microbes that has the potential to be used as an ethanol bioreceptor, because it has an ADH enzyme bound to pyrroloquinoline quinone (PQQ) which can convert ethanol to acetaldehyde [15]. In this study, used bacterial culture with K5 code obtained from the Indonesian Institute of Sciences (LIPI) collection. The culture has been isolated

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from the coconut from Indonesia. However, based on the results of preliminary tests with microscope observations, *A. aceti* has a susceptible risk of growing together with yeast as a contaminant. So, the purity of isolated bacteria becomes an important focus to make sure that the specific enzymatic reaction produced only comes from *A. aceti*. The PQQ-ADH enzyme of *A. aceti* has a higher ability to convert ethanol to acetaldehyde compared to the PQQ-ADH enzyme in *G. Suboxydans* [16]. The position of the enzymes that are on the periplasm [17] makes *A. aceti* cells react quickly to ethanol so that they are predicted to produce sensitive measurements. In order to keep live for a long time, *A. aceti* is formed into a biofilm so that the activity of the enzyme inside is relatively stable.

The biofilm is a community of bacterial cells encapsulated in an extracellular polymeric substance synthesized by these bacteria and attached to biotic and abiotic surfaces [18]. In previously study, simple visual detection biosensor based on AOX enzyme immobilized onto polyaniline film had been successfully created. It could last up to 7 weeks at storage temperature of 4°C, with 90.5% activity [19], whereas in this study, we wanted to maximize the ability of the bacteria themselves have forming polymers naturally. Planktonic bacteria can form biofilms in five stages, including attachment or colonization (I), growth of biofilm (II) maturation I (III), maturation II (IV), and erosion (V) [20]. Among the five stages, the maturation stage is the most stable stage where biofilms are well formed without the release of planktonic bacteria which is deposited on the abiotic surface. Well, the biofilms formed can protect cells from various external influences, such as changes in temperature and pH, so that enzyme activity is more stable. *Acetobacter aceti* bacteria can form a strong biofilm [21], but the formation time until the maturation stage is unknown. In this study, the optimum time and conditions for biofilm formation are important parameters that need to be known. The purpose of this study is to develop sensitive and stable biosensors based on biofilm *A. aceti* for halal analysis of food and beverages containing ethanol, especially at the levels below 1%. Screen printed carbon electrode (SPCE) is chosen as the basic electrode because of its simple use, low reagent consumption, fast response due to a specific surface area, good reliability, and reproducibility [22]. In addition, a flat SPCE surface allows *A. aceti* biofilm to form.

2. MATERIALS AND METHODS

2.1. Material

A culture with the K5 code containing *A. aceti* bacteria isolated from coconut water was obtained from LIPI, Bogor. Mannitol, agar powder, yeast extract, and peptone was obtained from Himedia (India). NaH_2PO_4, Na_2HPO_4, glucose, sucrose, fructose, sodium benzoate, ascorbic acid, 96% ethanol, methanol, 0.5 M HCl, 3% H_2O_2 was obtained from Merck (Germany). Crystal violet, lugol, and safranin was obtained from Sigma-Aldrich (USA). All solutions in the experiment were prepared just before their use. Commercial disposable electrodes SPCE obtained from DropSens (Spain) are used as standard electrodes.

2.2. Identification and Purification of *A. aceti*

The purification process was carried out in several stages, including breeding of bacterial culture, selection of the optimum incubation time, selection of incubation temperature, and selection of the growth pH. One loop of K5 culture containing *A. aceti* bacteria and yeast were suspended to a concentration of 10^7 in 50 mM sterile phosphate buffer solution (PBS) pH 6.8 (neutral) and pH 3 (adjust HCl), then vortex until homogeneous. One hundred microliter of bacterial suspension was spread over a solid medium of acetobacteria (0.5 g of yeast extract, 0.3 g of peptone, 2.5 g of mannitol, 1.5 g of agar powder in 100 ml of distilled water) as a bacterial selective medium of *Acetobacter* sp. [23]. Plates are incubated for 24, 48, and 72 hours. The incubation temperature was varied at 30°C and 37°C. Purification was carried out repeatedly until a single colony with milky white, smelled of acetic acid growth. Isolates were identified based on biochemical tests and cell morphological characteristics. Biochemical tests were carried out by glucose fermentation (1% and 5%) [24,25], ethanol fermentation (1% and 3%) [24–26], and catalase tests (3% H_2O_2) [24,27], while cell morphology was observed based on the appearance of cells under a microscope with 1,000× magnification (Nikon YS100) after gram staining.

2.3. Selection the Optimum Conditions for Biofilm Formation

The selection parameter was the concentration of bacteria measured by its optical density (OD) and the duration of biofilm formation before the measurement. Bacterial isolates that had been grown in acetobacteria liquid medium and washed with PBS pH 6.8, then their OD was measured with a microplate reader (BIO-RAD iMark) at λ = 595 nm with a variation range 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 Each variation of OD was measured by electrochemical activity, 3 days after dropping, then the average value and standard deviation of current were calculated. The highest current with the lowest deviation was chosen as OD that presented the best response. Selection of the duration of biofilm formation was carried out to determine the time needed for planktonic *A.aceti* forming biofilms This selection was led based on variations in storage time, namely, 3, 5, 8, 10, and 14 days and variations in the addition of nutrients both of distilled water and liquid acetobacteria media. The suspension of *A. aceti* that had been measured for its OD value was deposited on the SPCE surface. Nutrients such as distilled water and liquid acetobateria media were added separately on different SPCE as the last drop. Without the addition of nutrients used as the negative control, SPCE was deposited with bacteria and then stored in an airtight container at the room temperature during a varied storage time period.

2.4. Biosensor Making

A total of 10 loops of bacterial isolates grown in solid medium were re-grown in 50 ml of acetobacteria liquid medium [23]. Incubation was carried out aerobically, for 18 hours at the room temperature[28]. The growing suspension, then was washed using PBS pH 6.8 50 mM by stirring using vortex and centrifugation at 8,000 × g for 5 minutes, three times. The collected suspension was being measured to find its OD value using a microplate reader (BIO-RAD iMark). Ten microliter of *A. aceti* suspension was dripped on the surface of SPCE (Dropsen, Spain) periodically up to 10 times dripping [23]. The suspension that had been deposited

above the SPCE surface was then left in an impermeable container for 14 days at the room temperature before the measurement.

2.5. Electrochemical Measurement

Electrochemical measurements were taken by the cyclic voltammetry method using eDAQ potentiostat (Ecorder 410) equipped with Echem v2.1.0 software. SPCE used, consists of a work electrode is carbon, a reference electrode is silver, and an auxiliary electrode is carbon, were printed on the ceramic material. The overall dimensions of the electrode were 3.4 × 1.0 × 0.5 cm with a working electrode 4 mm in diameter (DropSens, Oviedo, Spain). Measurements were made in the range of −0.9 to + 1.2 V with a scan rate 200 mV/s. Initial measurements were made for 1% ethanol sample, 1% methanol, and 1% H_2O_2 to characterize the enzymatic catalysis reaction produced. The analytic biosensor performance was validated in the range of +0.1–1.2 V with same scan rate. Analytical performance was evaluated based on linearity, limits of detection, limits of quantization, repeatability, sensitivity, selectivity, and stability of biosensors.

2.6. Real Sample

Measurements in real samples were made by three times repetition (n = 3), in the range of +0.1–1.2 V using beer pilsener with a content of ±4.7%. Beer samples were diluted using PBS pH 6.8 50 mM to ±1%.

3. RESULTS AND DISCUSSION

3.1. Characteristic of *A. aceti*

The bacterial purification process aims to get a single colony of *A. aceti* which has potential to be used as bioreceptor for ethanol biosensor. The growth of acetic acid bacteria during the logarithmic phase might be affected by yeast species, such as *Kloeckera apiculata*, *Candida stellata*, and *Saccharomyces cerevisiae* [29]. For purification process, the used of breeding method is poured plate method. The principle of this technique is to reduce the concentration of bacteria, so that formed single colony is resulting of one cell division. From several variations of applied time incubation, 48 hours is the optimum time for *A. aceti* cells to divide until the stagnant phase (stationary phase), while more than that (72 hours) the growth of a single colony is no longer formed and is vulnerable to fungal contamination. In this case, even a single colony was formed, but, after observing its appearance with the microscope, it was still mixed with yeast, so the next step is to implement ideal growing conditions that produce *A. aceti* only.

The selection of incubation temperature was done to suppress the growth of yeast, especially in *S. cerevisiae*. At a temperature of 30℃, yeast was still susceptible to grow, while at a temperature of 37℃, yeast growth was relatively inhibited. These results are consistent with the results of a study proposed by Salvado *et al*. [30] which explains that most *Saccharomyces* sp. grow at temperatures below 30℃, while the *S. cerevisiae* have an optimum growth temperature at 32.5℃, nevertheless, some other yeast can still grow at 37℃ even in small amount. *Acetobacter aceti* is acetic acid bacteria which can grow in acidic conditions. pH 3 was chosen because based on research explained before, these bacteria can still grow at pH 3–3.5 [31,32], while the average growth of yeast, especially which isolated from coconut water, can grow at pH above 4 [30,33]. In this study, cultures that are added to acidic conditions can effectively inhibit the growth of yeast and other contaminants, such as fungi. The formed colonies have fresh milky white color and acetic acid aroma. Isolates that have been purified then characterized using biochemical tests to make sure bacterial identity based on their physiological properties.

Bacterial identification was done to make sure that the growth bacteria were *A. aceti.* Based on morphological and gram staining characteristics of bacteria have an ellipsoidal form, straight, or slightly curved, single and red color (gram negative). Similar results were also obtained from Lisdiyanti *et al.* [31]. *Acetobacter aceti* is a group of Gram-negative bacteria that will maintain the red color of safranin when gram staining was done. The cell wall structure of Gram-negative bacteria is dominated by lipids as the outer membrane, while the peptidoglycan layer is located between the inner and outer membranes. When washing with ethanol, the outer layer of the cell wall, lipopolysaccharide, may dissolve with ethanol, therefore it cannot support the purple color of crystal violet. Addition of dye agent (safranin) makes bacteria become red (See Fig. 1).

Biochemical tests were done to strengthen the results of bacterial identification. *Acetobacter aceti* from purification showed positive results on glucose fermentation which anaerobically lasted for 24 hours at 37°C , ethanol fermentation which lasted for 120 hours at 30°C and catalase test using 3% H_2O_2. The results of the biochemical test are presented in Table 1. *Acetobacter aceti* used glucose as a carbon source for its growth process, so both 1% and 5% glucose bacteria can grow well forming white suspension. These results are consistent with the reported that the *A. aceti* reaction to glucose is positive [24,25]. Glucose fermentation can change the initial pH of the liquid medium from 6 to 4. In sugar fermentation, the decrease in pH is caused by the formation of lactic acid and CO_2 as a result of a side reaction to the respiratory chain of *A. aceti* bacteria [16]. The effect of ethanol concentration is also observed in bacterial growth as well as its ability to produce

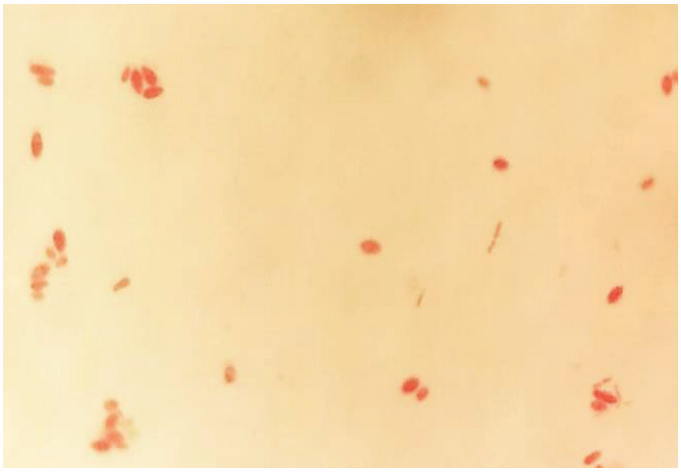

Figure 1: Visualization of gram-stained bacteria.

Table 1: Biochemical characteristics of purified *A. aceti*.

Biochemical test	Result	Conclusion
Glucose 1%	Bacteria have grown, CO_2 bubbles appeared, decreased pH of the media	+
Glucose 5%	Bacteria have grown, CO_2 bubbles appeared, decreased pH of the media	+
Ethanol 1%	Bacteria have grown, decreased pH of the media	+
Ethanol 3%	Bacteria have grown, decreased pH of the media	+
H_2O_2 3%	bubbles appeared	+

acetic acid. The growth of bacteria in liquid media, which is added with 1% ethanol is relatively more than 3% ethanol. Nevertheless, *A. aceti* is relatively tolerant for 3% ethanol. Similar results were also reported [25,26]. Higher levels of ethanol provided poor growth effects. In this study, the process of fermenting ethanol to acetic acid which lasted for 5 days at 30°C can change the initial pH of liquid medium from 6 to 3. In ethanol fermentation, the decrease in pH is caused by the conversion of ethanol to acetic acid [34]. *Acetobacter aceti* reaction with 3% H_2O_2 produced bubbles indicating of positive tests [27]. H_2O_2 is an organic chemical compound which has strong oxidizing properties. This compound is catalyzed by the enzyme catalase at neutral pH. The bubbles are produced by decomposition of H_2O_2 into H_2O and O_2.

3.2. *Acetobacter aceti* Biofilm Activity

The process of the biofilm formation above the surface of SPCE was executed with six suspensions of bacterial OD variations in 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0. Based on the results of measurements using the cyclic voltammetry technique, showed that the bacterial suspension with OD 3 had the best current response and consistency of enzymes secretion. This is related to the expression of enzymes produced by *A. aceti* biofilm above SPCE. The number of bacteria deposited on the SPCE surface, the more excreted enzymes will produce higher anodic currents. Furthermore, the results also showed that formed biofilm from OD 3 bacterial suspensions had relatively lowered random error values compared to the other five data. More bacterial cell numbers can produce more extracellular polymers so that the binding between bacterial cells by extracellular polymers get stronger. Extracellular polymers function as binding layers will protecting the bacterial colonies in them to be resistant to environmental conditions, so that the stability of the activity is maintained. Judging from the random error, % deviation standard obtained in the biosensor with OD 3 was quite low but has not been able to fulfill great sensor standards. A good repeatability of sensor was obtained when the standard deviation is less than 2.7% for analyzing measurements of at least seven times in the concentration of 1% [35]. The measurement results are presented in Table 2.

A consistent measurement value is obtained only if the formed biofilm perfectly above the SPCE surface to avoid ablation. Ablation is causing a large random errors value. In this study, 14 days is the optimum time of *A. aceti* biofilm formation, because the formed biofilm has reached the maturation stage in which the bacterial suspension is no longer in planktonic conditions, but it has formed a certain thickness of microcolony layer (maturation stage). It was shown by the absence of ablation from biofilm when it was enforced as much as 10 times or for 2 minutes. This layer is tightly bound by extracellular polymeric substances released by bacteria as a result of secondary metabolites. Variations in the addition of nutrients were also tested to see the effect on speed of biofilm growth. The addition of acetobacteria media over the suspension which deposited on SPCE surface can support the survival of bacteria. According to the theory, living colony in biofilms will get nutrients from the dead bacteria [36], but the died bacteria will not secrete enzymes, so even though the biofilm is physically deposited on top of SPCE, its activity will continue decline. Added nutrients at the last dropping before storage also serves to slow down the dispersion phase, so that the stability of biofilm-based sensors can last for a long time.

3.3. Biosensor Characterization

Characterization of biosensors was performed electrochemically using the cyclic voltammetry techniques. The cyclic voltammetry technique was chosen, because it has several advantages among others. The advantage of the cyclic voltammetry technique be able to detect peak currents for qualitative and quantitative analysis, and can be used to study the reversibility of electron transfer [37]. Characterization of biosensors was carried out to ensure that the produced current only came from the specific reaction between ethanol and the PQQ-ADH enzyme in *A. aceti*. Characterization was done by measuring 1% ethanol used an electrode (SPCE) without adding bacteria to ensure that produced current is only by a catalytic reaction. These results indicate that the pure SPCE electrode cannot oxidize ethanol which acts as an analyte, so the reaction is truly enzimatic reaction. Furthermore, biosensor characterization was taken by testing 1% ethanol, 1% methanol, and 1% H_2O_2. In some cases of biosensors, H_2O_2 is an intermediate product which compared with the amount of reagent. The current

Table 2: Mean currents and random errors based on the variation of OD.

OD	Means current ± % Deviation
0.5	66.13 ± 87.02
1	88.93 ± 88.04
1.5	130.33 ± 14.29
2	154.27 ± 11.91
2.5	165.13 ± 13.87
3	214.03 ± 5.63

absence in 1% H_2O_2 indicates, the intermediate product is not formed. Checking for 1% methanol was done to find out the type of enzyme in *A. aceti* biofilm that reacted to ethanol. Methanol is also chosen, because these compounds usually appear in alcoholic fermentation processes. *Acetobacter aceti* has specific activity on short chain of alcohols except methanol [15]. Bilgi *et al*. [38] also compared the performance of ADH and AOX enzyme-based biosensors to ethanol, methanol, and their mixture. The result is shown that the AOX enzyme produces higher oxidation current toward methanol than ethanol at the same concentration, while ADH is more specific to ethanol. In this research, the methanol response of biosensors in the voltage range of −0.9 to +1.2 V was almost undetectable. The resulting peak was not clearly visible, with the shape almost equaling the blank (Fig. 2). The result confirms that the enzymatic reaction truely produced by PQQ-ADH enzymes. This clearly makes *A. aceti* more potential to be used as a bioreceptor, because it produces a specific reaction.

Detection of ethanol is carried out by measuring the oxidation current resulting from the reaction between the substrate and the PQQ-ADH enzyme contained in *A. aceti* biofilm as bioreceptor. *Acetobacter aceti* does not require mediators for the transfer of electrons, so it can be effective direct electron transfer [39,40]. The direct bioelectrocatalysis of *A. aceti* cells, because it has two cofactors that work simultaneously [41]. The enzyme consists of subunits I (adhA), II (adHB), and III (adHS) [17]. Redox reactions occur in subunits I. Subunit I consists of a ADH enzyme in the periplasmic which depends on 1 molecule PQQ as coenzyme and 1 molecule of heme c as a prosthetic group. PQQ functions is to increase catalytic activity, while heme c functions as mediator for electrons transfer to electrode [41,42]. Ethanol is oxidized by the enzyme to produce acetaldehyde. Reduction of PQQ to $PQQH_2$ was produced two electrons. Then, electrons were transferred directly out of the membrane through heme c moiety. Heme c in *A. aceti* has an iron ion coordinated to porphyrin which responsible for electron transfer in some biological systems. So, this biosensor reaction is based on direct bioelectrocatalysis reaction. The substrate recognition reaction scheme is represented in Figure 3.

3.4. Analytical Performance of the Biosensor

The biosensor that had been designed under optimum conditions was tested for analytic performance using the cyclic voltammetry techniques. The experimental conditions in analytical performance testing are similar to the testing of biosensor characteristics, but the used of potential range is shortened to +0.1–1.2 V because anodic current only appeared around 0.83–0.92 V. It aims to accelerate the reaction process and reduce the occurrence of fatigue of bacteria in producing enzymes. The longer scanning time might cause death to bacteria. The analytical performance of biosensor was summarized as follows. The linearity range used for measuring sensitivity, LOD and LOQ was from 0.00005% to −0.0003%. The determination coefficient (R^2) value obtained was 0.9969, which means that as much as 99.69% of current changes are affected by increasing in ethanol concentration (Fig. 4). The obtained sensitivity was quite large, namely 43,076 μA%$^{-1}$ or

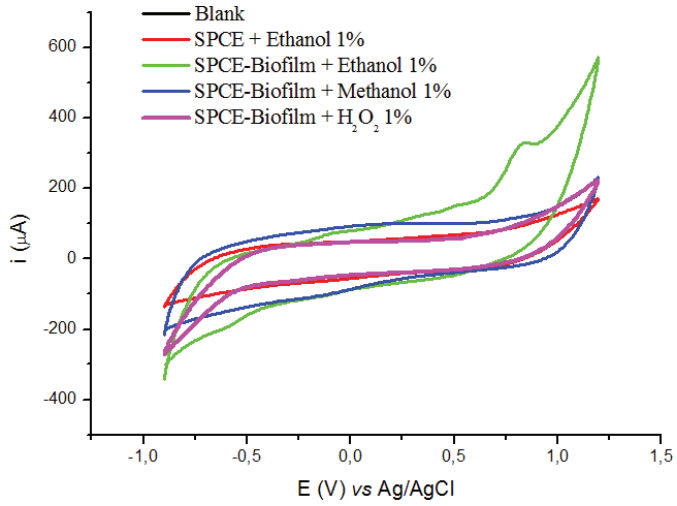

Figure 2: The reaction of A. aceti biofilm-based biosensors towards ethanol which is interpreted via cyclic voltammogram with υ = 200 mV s−1.

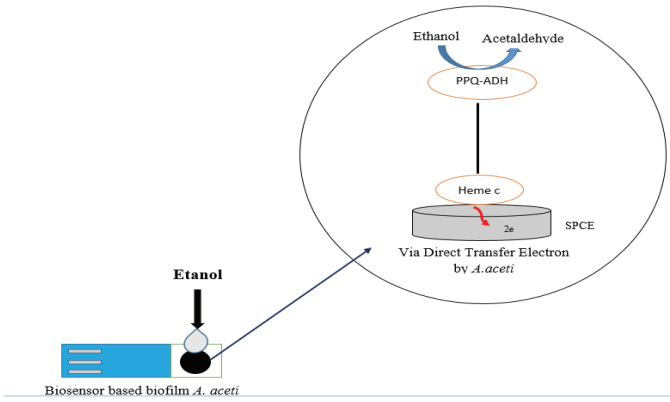

Figure 3: Schematic diagram of analyte recognition by bioreceptor. Ethanol oxidation reaction by PQQ-ADH enzyme.

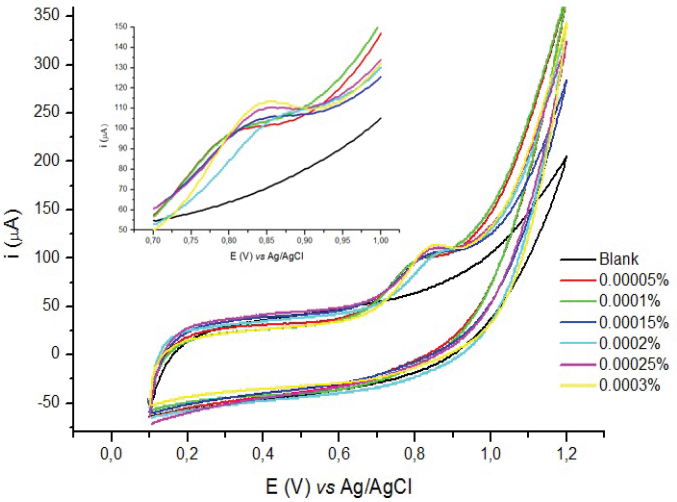

Figure 4: Cyclic voltamogram of dependent concentration and current with υ = 200 mV s−1.

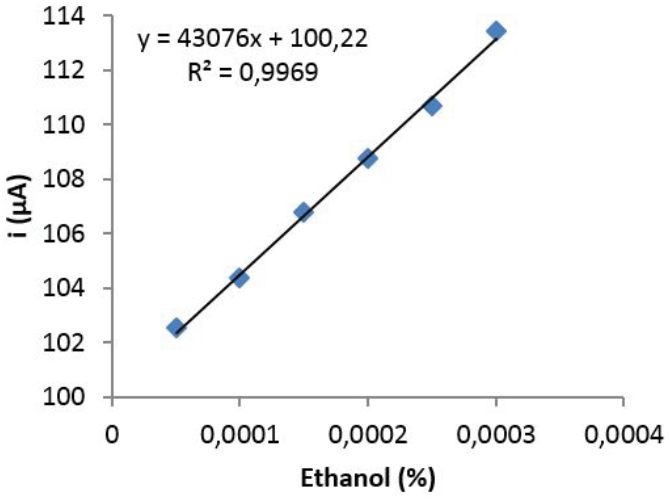

Figure 5: Linear regression curves relate ethanol concentration to oxidation currents with υ = 200 mV s−1.

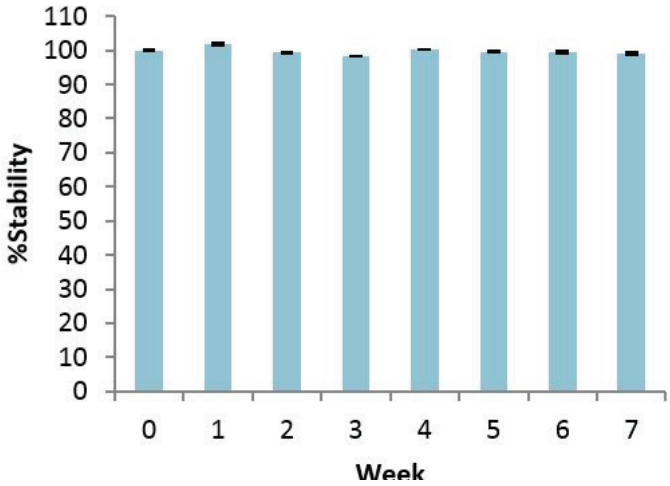

Figure 6: Bar diagram of ethanol biosensor stability.

equal to 251.42 µA mM^{-1} (Fig. 5). The obtained detection and quantization limits were 2.32×10^{-5} and 7.03×10^{-5}%, respectively. The existence of heme c moiety as quinohemoprotein predicted can be a link for the direct electron transfer between bacteria with electrodes. Thus, even without the addition of modifiers from other materials, *A. aceti* naturally has the potential to be used as a biosensor receptor because of the speed of the reaction.

Measurement repeatability was tested at seven times of ethanol solution concentration, i.e., 0.00001%, 0.0001%, 0.001%, 0.01, 0.1%, and 1% produced relative standard deviation (RSD) average value of 1.08%. The operational stability of biosensors was tested periodically for 7 weeks using 1% ethanol, three times repeated measurements (n = 3). The obtained voltammetric response up to 7 weeks left an activity of 98.99% (Fig. 6). *Acetobacter aceti*'s ability forming biofilm is able to support enzyme activity in bacteria both in short and long time intervals. In short time intervals, proven by repeatability measurements that produce RSD values 2.50 times smaller than the maximum limit set. It is known for measurements with 1% of ethanol, the RSD limit is 2.70%. In a long time interval, this biosensor is able to maintain its activity for up to 7 weeks with a decrease in activity is only 1.01%. Biosensor selectivity was tested on several matrices commonly found in foods, namely, glucose, fructose, sucrose, sodium benzoate, and ascorbic acid. For interference matrix, such as glucose, fructose, sucrose, and sodium benzoate, the biosensor performance was only selective toward ethanol, but less selective in the presence of ascorbic acid. Overall, biosensor based on *A. aceti* biofilm has good performance simple analysis. The results of several studies on ethanol biosensors can be summarized in Table 3.

3.5. Application in Real Sample

The measurements were done with the same conditions as analytical performance testing. The concentration of ethanol in the sample was determined using a calibration chart with the standard ethanol solution. Three measurement s used the same biosensor and analyte resulted in an average ethanol concentration of 1.42% (v/v) with an RSD value of 0.31% (n = 3).

Quantitatively, the ethanol content obtained should be ±1% but based on the measurement of the recovery value obtained was greater. This is possible because of the effects of fermentation that occur during the beverage storage period. In pilsener beer,

Table 3: Biosensors performace based on various bioreseptors.

Bioreceptor	Sensitivity (µA mM^{-1})	LOD (µM)	RSD (%)	Stability (%)	Refference
P. methanolica	Nd	50	nd	50 after 8 days	[8]
S. ellipsoideus	1.94	6	5.8	50 after 10 days	[12]
C. Tropicalis	0.037	nd	0.41	52 after 27 days	[9]
P. angusta	Nd	12	nd	82.6 after 5 days	[10]
G. oxydans	74	5	1.5	98.3 after 48 hours	[7]
G. oxydans	117-121	2-6	<1	nd	[14]
ADH-NAD^+	0.02	70000	2.7	50 after 30 days	[43]
ADH-NAD^+	0.49	96.1	1.57	72.7 after 7 days	[37]
ADH-NAD^+	1.36	7.1	1.65	38.88 after 30 days	[44]
ADH-NAD^+	Nd	5	<10	78 after 14 days	[45]
Biofilm *A.aceti*	251.42	3.9	1.08	98.99 after 50 days	This research

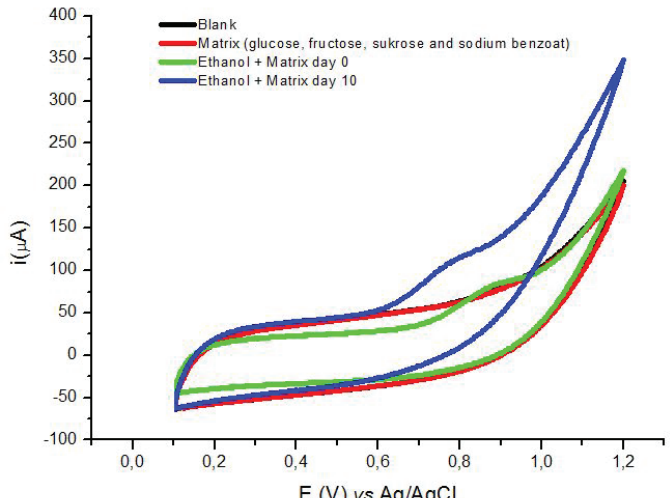

Figure 7: The effect of sugar fermentation in increasing the current of ethanol oxidation with υ = 200 mV s−1.

there is a sugar matrix which anaerobically can increase the ethanol concentration if is stored for a certain period of time. This phenomenon was also studied by researchers, by analyzing the effect of adding simple sugars (glucose, fructose, and sucrose) to 1% ethanol standard solution. After they had been stored anaerobically for 10 days, the existence of a sugars matrix are proven to increase the oxidation current from biosensor measurements. The graph can be seen in Figure 7.

4. CONCLUSSION

In conclusion, the results from this study have confirmed that *A. aceti* cells have the potential for design biosensors for the sensitive determination of ethanol until 251.42 μA mM^{-1}, especially at low concentrations. The enzymes position (in periplasm) and presence of heme two cofactors (PQQ and heme c) are a good synergy on the direct electron transfer process, so that little change in the analyte can be oxidized quickly. Electrochemically indicated by increasing current. In the form of biofilm, enzyme activity in cells only decreased by 1.01% for 50 days. So, naturally biofilm is able to maintain the stability of enzyme activity in cells. The manufactured biosensors were quite selective toward ethanol, in the presence of matrix which is commonly found in food, except ascorbic acid. So, there is needing to improve selectivity for eliminating ascorbic acid interferences. Overall, the manufactured biosensors have good performance.

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