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The analytical performance of *Saccharomyces cerevisiae* and *Bacillus megaterium* microbial consortium as recognition element in ethanol biosensor

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Abstract. *Nurdiani, Iswantini D, Nurhidayat N, Wahyuni WT, Kartono A. 2023. The analytical performance of* Saccharomyces cerevisiae *and* Bacillus megaterium *microbial consortium as recognition element in ethanol biosensor. Biodiversitas 24: 5928-5936.* Alcohol, particularly ethanol, is commonly found in human food and plays a significant role in degenerative diseases and disability. Accurate measurement of alcohol in food products is essential to ensure adherence to the Muslim halal rule. However, existing alcohol biosensor that relies on a single microbe has limitations in measuring a wide range of ethanol concentrations. To address this issue, a microbial consortium is needed to expand the measurable range. Therefore, this study aimed to develop an innovative biosensor to widen the range of measured ethanol concentrations based on the microbial consortium of *Saccharomyces cerevisiae* YSAPMI.2 and *Bacillus megaterium* BSAPMI.1. The performance of the biosensor was evaluated using the cyclic voltammetry method. The results showed that the linear range, linearity, coefficient of determination, sensitivity, and response time, were 0.02-6.0%, 0.9968, 0.9936, 83.157 µA (%)-1, and 11 seconds. The LoD and LoQ theoretical values of the method obtained in the ethanol oxidation reaction were 0.060% and 0.182%, respectively. The confirmatory test for the LoD value of 0.01% yielded a positive response, while the confirmed LoQ value of 0.02% showed good precision and accuracy. The biosensor had precise %RSD values of 0.568, 1.338, and 4.632% for the high, medium, and low ethanol concentrations, respectively. The accuracy reflected as the recovery percentage was in the range of 90.27-111.07%. The biosensor was relatively specific and had no interferences with common ethanol compounds including methanol, sodium chloride, formic acid, and glucose. The stability obtained with biofilm showed a better result reaching 88% in 70 days. Based on the result, this microbial consortium biosensor could widen the range of measured ethanol concentrations and should be further developed to create a prototype for an accurate and practical analysis.

Keywords: ADH, alcohol, biofilm, microbe, SPCE

Abbreviation: LoD: limit of detection, LoQ: limit of quantitation, HPLC: high-performance liquid chromatography, GC: gas chromatography, GC-MS: gas chromatography-mass spectrophotometry, DNA: deoxyribonucleic acid, ADH: alcohol dehydrogenase, AOX: alcohol oxidase, SPCE: screen printed carbon electrode, IUPAC: International Union of Pure and Applied Chemistry, KM: Michaelis-Menten constant, Vmax: reaction rate

INTRODUCTION

Alcohol, produced in various fermented food ingredients is widely used in the pharmaceutical, biotechnology, food, and beverage industries, as a solvent for coloring and flavoring agents. Various methods have been used in detecting alcohol including GC-MS (Park et al. 2016), fluorescent emission (Akamatsu 2015), and spectrophotometry UV Vis (Nahak et al. 2021). GC-MS has LoD, LoQ, %RSD, regression, and percent recovery values of 0.25 mg/kg, 1.16 mg/kg, <7%, 0.999, and 90.79-101.50%, respectively, while spectrophotometry UV Vis has LoD of 0.056 mg/L, LoQ of 0.187 mg/L and %RSD of 91.5% (Nahak et al. 2021). However, these methods have several weaknesses, such as HPLC and GC can accurately measure the concentration of alcohol in samples, but the equipment and measurement costs are relatively high, coupled with a complex sample preparation method (Park et al. 2016). The use of biosensor is an efficient alternative to measuring ethanol content.

A biosensor is a measurement method that uses biological components as elements to recognize changes in analytes, including enzymes, antibodies, DNA, cells, and microorganisms (Goradel et al. 2018; Ali et al. 2017). This method has advantages such as providing a fast response, simple operation, fairly good validity, miniaturization, and low cost of analysis (Meshram et al. 2018; Clementino et al. 2016). Based on a previous report, enzyme biosensor is the most widely used method (Istrate et al. 2021) due to its associated high selectivity, activity, and sensitivity.

However, pure enzymes have several drawbacks, namely expensive prices and low stability. These limitations can be overcome through the use of microbes as enzyme producers which have the advantages of longevity, low cost, tolerance to pH, and high stability compared to pure enzyme sensors. Biosensors from enzymes are a dependable alternative that can be applied to a variety of analytical processes in a wide range of fields. These tools have been used successfully for early disease detection, toxins, viruses, elevated blood levels, and other purposes (Alizadeh et al. 2020). Biosensors are used in the food industry to detect food allergens, contamination, and antioxidant power (de Oliveira Neto et al. 2017). In the environment, their applications detect pollution in the air or contaminants in water or soil (Othman and Wollenberger 2020). For alcohol biosensors, enzymes commonly used are ADH and AOX which can catalyze reactions of alcohols as substrate molecules, thereby increasing sensitivity, specificity, and accuracy (Park et al. 2013). ADH is a reversible oxidoreductase enzyme that oxidizes ethanol to acetaldehyde (Kuswandi et al. 2014). The ADH enzyme-based ethanol biosensor has better stability and specificity compared to the AOX but requires an extended response time (Castritius et al. 2010). Iswantini et al. (2017) also successfully developed an ethanol biosensor from *Bacillus* sp. as a producer of ADH enzymes. Nurdiani et al. (2023) screened 5 *Bacillus* isolates and found that based on the oxidation current data, *Bacillus megaterium* 23/6/22 produced the highest current compared to other isolates. Susparini et al. (2019) also examined an alcohol biosensor using *Acetobacter aceti*, producing the AOX enzyme with a sensitivity, LoD, and LoQ of 43.076 μA $\%^{-1}$, 2.32×10^{-5}, and 7.03×10^{-5} % respectively. In addition, microbes from pineapple juice, namely *Saccharomyces cerevisiae*, produce the ADH enzyme, which can be used as an alcohol biosensor (Iswantini et al. 2018). This tool was also developed using ADH microbes with a linear range of 0.05-2 mM (Istrate et al. 2021).

Alcohol biosensor relying on a single microbe has a limited range of measuring ethanol concentrations. This limitation can be addressed by using microbial consortium to widen the measurable range. The interaction between bacteria within a consortium exhibits a synergistic relationship provided the substrate is sufficient and the bacteria do not inhibit the growth of each other (Liu et al. 2017; Alvarado et al. 2021). The use of microbial consortium tends to yield better results compared to the use of single isolates. This is due to the expectation that enzymatic activities from different microbial strains complement each other, allowing survival through the use of available nutrient sources in the carrier media (Pantanella et al. 2013). The synergism among two or more inoculated bacteria plays an important role in their effective collaboration (Kardena et al. 2020; Deng and Wang 2016). The consortium produces a product that not only supports the growth of individual isolates but also fosters mutual growth (Pantanella et al. 2013; Djatmiko et al. 2023). Pringgenies et al. (2020) successfully determined the potential of consortium microbes as tofu preservatives. However, there is a lack of study on biosensor development using a consortium of *B. megaterium* and *S. cerevisiae* microbes as producers of the ADH enzyme. It is also necessary to effectively optimize the use of microbial biodiversity in Indonesia.

This study was conducted to strengthen the performance of the electrodes and expand the detection area for alcohol, specifically ethanol in food or drinks using a microbial consortium of *S. cerevisiae* and *B. megaterium*. A consortium was prepared on the surface of SPCE to develop a sensitive, valid, accurate, and highly stable ethanol biosensor model.

MATERIALS AND METHODS

Biofilm formation

Bacillus megaterium BSAPMI.1. and *Saccharomyces cerevisiae* YSAPMI.2 cultures in an equal density were mixed homogenously in a 0.1% agar solution, at a temperature of 40-50°C in a 1:1 (μL) ratio. An aliquot of 20 μL suspension by Mettler Toledo micropipette was applied on the surface of a SPCE refs. 110 (DropSens, Asturias, Spain), and airdried at room temperature for 5 minutes. The culture layering was repeated 5 times for a total of 100 μL suspension. The SPCE was subsequently stored in an airtight container at room temperature for 10 days to ensure optimal conditions for use.

Electrochemical measurement

The electrochemical measurements were carried out by the cyclic voltammetry method using eDAQ potentiostats (Ecorder 10 ADInstruments Australia) and Echem v2.1.0 software (Elgrishi et al. 2018). The working, reference, and counter electrode was 4 mm diameter carbon, silver (Ag/AgCl), and carbon respectively. The three electrodes were integrated as SPCE, and the current measurement used the following parameters: mode: cyclic; initial E: -1000 mV; final E: +1000 m; E Lower: -1000 mV; E Upper: +1000 mV; step W: 20 ms; rate: 100 mV/s; and range: 1 V.

The analytical parameter measurement

The linearity, limit of detection, limit of quantitation, sensitivity, accuracy, precision, response time, and selectivity of the analytical measurement were all evaluated (Sujarweni 2015; Harvey 2009).

Linearity

A calibration curve of the relationship between 96% ethanol concentrations (Sigma-Aldrich, Saint Louis, USA), and their current responses was used to determine linearity. Ethanol solution was prepared in the concentration series of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 1, 1.5, 2, 3, 4, 5, and 6% in 50 mM PBS with pH 7.5. The current response was measured with a scan speed of 100 mV/s and a potential interval of -1 Volt to +1 Volt. The values obtained were corrected against a 50 mM phosphate buffer solution with pH 7.5 by TOA DK HM-250 pH meter. Each ethanol concentration was measured by cyclic voltammetry and a linear equation was made ($y = a + bx$). Variable A represents the intercept of the calibration curve, and B denotes its slope. The linearity of the calibration curve was calculated using the coefficient of determination (R^2).

Response time

Response time refers to the duration needed for an electrode to reach a constant potential, attributed to the occurrence of equilibrium reactions at the electrodes. The assessment of response time was conducted using two distinct methods: firstly, by determining the time taken to achieve a constant potential, and secondly, by gauging the stability of the potential for 300 seconds. The second method is in line with the IUPAC standard, and the final calculated response time was derived as an average of the time required to reach a constant potential.

Stability

The stability of the SPCE-bearing microbial biofilm was evaluated based on the cyclic voltammetry of 1% ethanol in phosphate buffer with a pH of 7.50 mM. This analysis was conducted within a potential window range of -1 Volt to +1 Volt at a scanning speed of 100 mV/s. Measurements were made every 7 days for 10 weeks using the same SPCE and stored at room temperature (about 25°C). The stability value was expressed in percent and calculated using the equation:

Stability (%) = It/Io x 100%

Where:

I_t : oxidation current on day-x th (µA)

I_0 : oxidation current on day-0 th (µA)

Limit of Detection (LoD) and Limit of Quantitation (LoQ)

The LoD and LoQ were calculated from the standard curve linear regression equation obtained from 6 repeated measurements in each concentration series. The values were calculated from the average slope of the line and the standard deviation of the curve intercept, with the equation:

$$LoD = \frac{3 \times SD}{s} \quad ; LoQ = \frac{10 \times SD}{s}$$

Where:

LoD : limit of detection (%)

LoQ : limit of quantitation (%)

SD : standard deviation of the intercept of the standard curve (n = 6)

s : the slope of the line equation

Precision

The precision test was conducted using the most optimum biosensor performance. The relative standard deviation (%RSD) was used to measure accuracy according to the equation:

$$SD = \sqrt{\frac{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)^2}{n-1}}$$

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n}\left(x_i - \bar{x}\right)^2 \qquad \%RSD = \left|\frac{SD}{\bar{x}}\right| \times 100\%$$

Where:

RSD: relative standard deviation

SD : standard deviation

xi : ethanol content per replicate

$\bar{x}$: average ethanol content

N : amount of measurement data

Selectivity

The ability of the method to measure only certain substances carefully and thoroughly in the presence of other components in the sample matrix was referred to as its selectivity or specification. The selectivity was measured by mixed and separate methods. In each measurement variation, 3 repetitions were carried out (n = 3), and the influence of interfering ions was determined based on the selectivity coefficient. Electrode selectivity was determined by measuring the main ion E of the 4% ethanol and interfering ion E in the citric acid, sodium chloride, glucose, and methanol each at a concentration of 4.0%.

Selectivity measurements using separate methods were carried out on ethanol and interfering substances dissolved in PBS pH 7.5. Meanwhile, in the mixed methods, measurements were carried out on two types of mixture variations, namely the matrix (mixture of interfering substances) with and without the addition of ethanol.

Sensitivity

The linear regression equation of the calibration curve obtained from the LoD and LoQ tests was used to calculate the sensitivity.

Real sample

Real samples were measured three times (n = 3) in the -1 to +1 Volt range using an 11.7% alcohol beer beverage. A buffer phosphate solution pH 7.5 50 mM was used to dilute the beer samples.

Determination of alcohol dehydrogenase kinetics

The magnitude of the oxidation current generated by biosensor measurements from SPCE with a series of ethanol concentrations, as in the linearity test, was used to determine AOX kinetics. The Michaelis-Menten, or Lineweaver-Burk, equation was used to calculate ADH kinetics including KM and Vmax values. The Lineweaver-Burk plot was then created using the equation:

$$\frac{1}{V_0} = \frac{1}{V_{maks}} + \frac{K_M}{V_{maks}} \times \frac{1}{[S]}$$

Where:

V_0 : initial reaction rate

Vmax : maximum reaction rate which is analogous to Imax

KM : Michaelis-Menten constant

[S] : substrate concentration

Data analysis

The electrical current data obtained from the cyclic voltammetry potentiostat (Handayani et al. 2020; Kucherenko et al. 2020) was processed and analyzed using the Echem v 2.1.0, Origin Pro 7.0, and Microsoft Excel applications. The statistical Independent Sample T-test ($p>0.05$) (Sujarweni 2015) analysis was applied to determine the significance between the ethanol biosensor and the GC method.

RESULTS AND DISCUSSION

Validation is useful for proving that a parameter meets the requirements in an analysis process. In addition, it is an important element of quality control and assures that measurements are reliable. The analytical parameters of ethanol biosensor were reviewed based on linearity, sensitivity, selectivity, accuracy, precision, quantitation, and detection limit, as well as response time (Iswantini et al. 2014).

According to Harvey (2009), linearity is the capacity of the analytical method to respond proportionately to the analyte concentration in a sample. Under optimum conditions, the developed microbial consortium biosensor was tested against various ethanol concentrations. Based on the results, the biosensor showed a proportional relationship in the dynamic range of 0.1 to 6.0%. The linearity of the curve was determined as a parameter of quantitative analysis. Each concentration was measured by cyclic voltammetry, resulting in a linear equation (y = a + bx). Variable a represents the intercept and b denotes the slope of the calibration curve. The linearity of the calibration curve was obtained from the value of the coefficient of determination (R^2). A calibration curve was made to determine the linear response of ethanol biosensors to the measurement of oxidation currents using cyclic voltammetry. The concentration range used was 0.1-6.0% with a linearity of 99.68%, denoted by the equation y = 83.157x + 24.266, and the R^2 value was 0.9936 as shown in Figure 1. The R^2 value refers to the coefficient of determination used to measure the effect of the independent variable or current on the dependent (concentration). Sensitivity was expressed as the slope of the calibration curve, and the value obtained was 83.157 µA $(\%)^{-1}$. This means that a 1% change in the analyte concentration value resulted in a corresponding 83.157 µA alteration in the current response of the ethanol biosensor. The higher the value, the greater the sensitivity of the instrument.

Precision referred to the degree of agreement between individual test results and was reflected in the dispersion of data from the average when the procedure was repeated on homogeneously mixed samples (Harvey 2009). Precision is also known as repeatability or reproducibility. Repeatability is the precision of a method when it is repeated by the same analyst under the same conditions and in short time intervals, while repeatability refers to the precision obtained under different conditions (Sujarweni 2015; Harvey 2009). Precision or repeatability indicates the value of the accuracy of the measurement expressed as the relative standard deviation (%RSD). The smaller the %RSD value, the greater the precision of the method used. The precision in this study was determined by measuring ethanol content in 50 mM phosphate buffer pH 7.5 with concentration series, as in the cyclic voltammetric linearity test with 6 replications. The repeatability value was tested using 3 different ethanol concentrations, and each was measured 6 times. According to the (AOAC 2016), the %RSD value category is divided into 4, namely: (i) very thorough: %RSD < 1, (ii) thorough: %RSD 1-2, (ii) moderate: %RSD 2-5, and (iv) not precise: %RSD >5. This study obtained very precise (0.568%), precise (1.338%), and moderate (4.632%) RSD values for the high, moderate, and low ethanol concentrations respectively.

The true value of the analytical method was determined by adding quantitatively traceable reference standards to the sample. In this study, a standard ethanol solution was theoretically added to the sample, and the accuracy results reflected in the percentage of return ranged from 90.27 to 111.07%. This indicated that the recovery of standard ethanol at low, medium, and high ethanol concentrations was in the adequate range compared to the recovery value of NATA (NATA 2013) ranging from 80-120% (Table 1).

Table 1. Accuracy data of microbial consortium biofilm

Spike concentration (%)	Measured average concentration (%)	% Recovery
0.300	2.170 ± 0.013	90.27
0.500	2.441 ± 0.007	108.37
0.700	2.677 ± 0.004	111.07
Acceptance conditions NATA[20]		80-120%

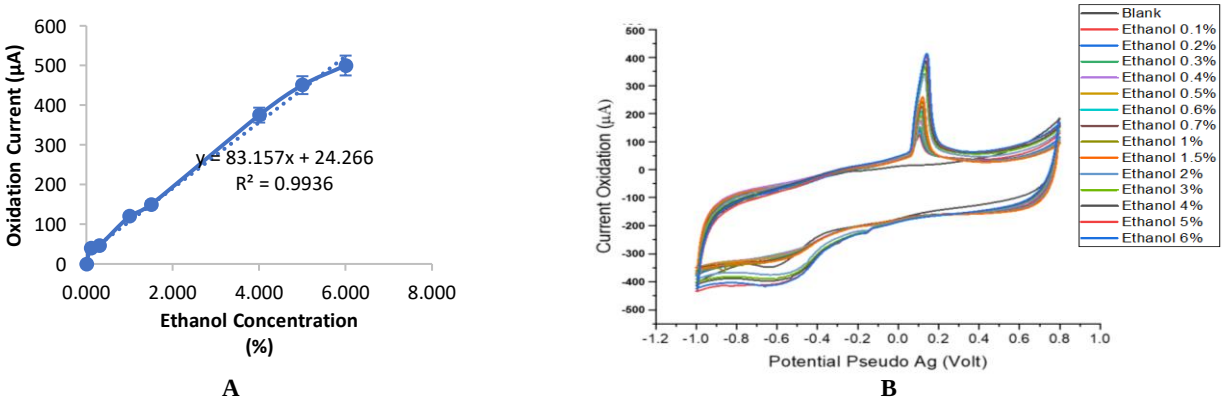


Figure 1. (A) Linearity curve of microbial consortium ethanol biosensor, (B) Standard series voltammogram of microbial consortium ethanol biosensor

The LoD indicates the minimal detectable analyte concentration that produces a significant response compared to a blank (Harvey 2009). On the other hand, the quantitation limit (LoQ) shows the smallest analyte concentration that still meets the criteria of being careful and thorough (Harvey 2009). The LoD and LoQ values of the instrument obtained in the ethanol oxidation reaction were 0.041 and 0.135%. Consequently, the minimum detectable ethanol concentration is 0.041% and the lowest concentration that can be detected accurately, and precisely is 0.135%. For the LoD and LoQ methods related to ethanol oxidation reaction, the values recorded were 0.060 and 0.182% (Table 2). This suggests that the minimum detectable ethanol concentration is 0.060% and the lowest concentration capable of being detected accurately and precisely is 0.182%. The confirmation test results for the LoD value of 0.01% yielded a positive response, while the confirmed LoQ value of 0.02% showed good precision and accuracy. The low LoD and LoQ values, specifically 1% can be used to detect ethanol in food and beverages (Mulyawan et al. 2022).

The primary challenge with ethanol biosensors based on ADH is their poor stability. Using the SPCE biofilm microbial consortium, the ethanol biosensor was evaluated to determine the biofilm formed to produce a relatively stable oxidation current. Stability was determined by comparing the current at a specific time with the initial current measurement. The results showed the microbial consortium biofilm-based biosensor was still stable after the 10 weeks or 70th day of measurement with an activity of 88% (Figure 2). These results indicated that the biosensor did not experience a significant decrease from the initial activity, measured repeatedly for 10 weeks or 70 days at room temperature.

In contrast, a study by Harvey (2009) reported a significant decrease in the detection capability of the alcohol dehydrogenase biosensor. This was shown by a 70-day stability test of the biosensor in 400 µm ethanol solution at 0.2 V. The results showed that the initial amperometric response of the biosensor for alcohol decreased, by 12.6, 54, and 72.2% on days 2, 3, and 7 respectively. In this study, the stability of the biosensor using biofilm showed a better result up to 88% within 70 days or 10 weeks with a high amount of current using the same electrode (Figure 2). These results indicated that the biosensor did not experience a significant decrease from the initial activity, measured repeatedly for 70 days or 10 weeks at room temperature. Biofilm formed by microbial consortium provided an excellent biological microenvironment in which cells survived and maintained their enzymatic activity.

Table 2. Data of method detection and quantitation limits of consortium biofilm

Repetition	Regression equation	Slope (b)	Intercept (a)	R^2
1	y = 82.178x + 32.809	82.178	32.809	0.9922
2	y = 82.115x + 33.161	82.115	33.161	0.9922
3	y = 82.489x + 30.736	82.489	30.736	0.9915
4	y = 81.794x + 35.454	81.794	35.454	0.9924
5	y = 81.988x + 33.031	81.988	33.031	0.9919
6	y = 82.165x + 32.914	82.165	32.914	0.9923
	Average	82.122	33.018	0.9921
	SD		1.498	
	LOD (%)		0.060	
	LOQ (%)		0.182	

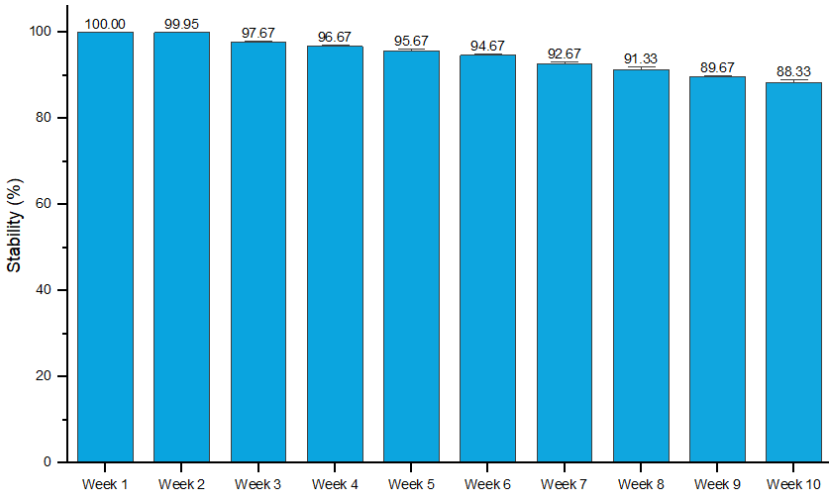
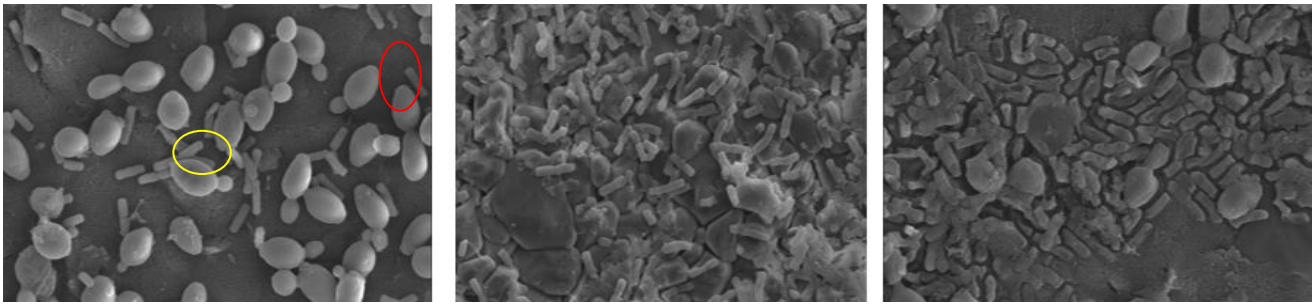


Figure 2. Microbial consortium biofilm stability



A **B** **C**

Figure 3. Surface morphology of microbial consortium biofilm at 10.000x magnification; (A) Morphology of microbial consortium before measurement stored for 10 days; (B) Morphology of microbial consortium before measurement stored for 70 days; and (C) Morphology of microbial consortium after measurements stored for 70 days

Based on the SEM analysis results, the morphology of the 10-day-old unused microbial consortium biofilm showed a thin layer. The intact microbial form of *B. megaterium* was rod-shaped (red circle in the picture) (Tiwari et al. 2019) and *S. cerevisiae* was spherical (yellow circle in the picture) (Andersen et al. 2014) (Figure 3A). For the 70-day-old unused microbial consortium biofilm, the morphology indicated a thick layer. The microbial form of *B. megaterium* which remained intact was in the form of a rod with some dead microbes (Figure 3B), while the SPCE surface of biofilm used for measurement showed a very thick layer (Muguruma 2018; Nguyen et al. 2019) (Figure 3C). According to Bilgi and Ayranci (2016), the *B. megaterium* and *S. cerevisiae* microbial consortium, when applied on the SPCE surface tend to aggregate due to gravity, forming a biofilm. This biofilm assumes the form of an extracellular polymer matrix made up of polysaccharides, proteins, nucleic acids, and lipids (Wu et al. 2015; Bilgi and Ayranci 2018). Based on its morphological characteristics, commercial *S. cerevisiae* cells have a spherical shape with a cell diameter reaching 5-10 µm, a smooth surface, embossed, and a yellowish color (Ramírez 2015).

Biofilm protects cells from extreme environmental conditions, enhancing their survival over an extended period even without nutrition. Microbial consortium within these biofilms exhibits greater resistance to antimicrobial substances compared to planktonic bacteria. Based on the results, the *B. megaterium* and *S. cerevisiae* microbial consortium biofilm despite being used repeatedly and stored for quite a long time (70 days) without being given nutrients and stored at room temperature, managed to survive and produce the ADH enzyme. After 70 days or 10 weeks, the microbial consortium developed a biofilm matrix that almost covered the entire SPCE surface. These results support the stability data obtained, indicating an 88% decrease in stability on the 70th day or 10 weeks. Although biofilm receives nutrients from dead bacteria in a living bacterial colony (Pantanella et al. 2013), the declining enzyme secretion from the dead bacteria leads to decreased activity within a biofilm.

T90, which represents the time needed for the signal to reach 90% of its concentration value, is a common reference for the biosensor response time. This signifies the duration required for the biosensor to stabilize its signal following exposure to a change in analyte concentration. Furthermore, the response time is essentially the period needed for an electrode to reach a constant potential due to the reaction equilibrium occurring at the electrode. The detection time required to reach a peak signal was approximately 11 seconds (Figure 4).

The ability of the biosensor to measure ethanol content accurately and thoroughly in the presence of other components was examined by determining the selectivity based on mixed method measurements. This method was carried out by measuring the potential of a solution containing a mixture of main and interfering compounds with constant concentration. Glucose 4%, NaCl 4%, methanol 4%, formic acid 4%, and ethanol 4%, were selected to study the effects of interfering compounds commonly found in alcoholic beverages. Consortium microbe biofilm was used to test these compound solutions in PBS at pH 7.5 and track the effects of the sample matrix. Based on the results, the 4% ethanol compound produced the highest oxidation current response of 400 µA, while the 4% glucose mixed with 4% ethanol yielded 316 µA oxidation current. Moreover, 4% NaCl, 4% formic acid, and 4% methanol mixed with 4% ethanol each produced 300 µA, 300 µA, and 313 µA current respectively. The results show that the mixture with 4% ethanol addition produced a current of 360 µA (Figure 5 A and B). This proved that microbial consortium biofilm used as an ethanol biosensor yielded the highest oxidation current response in the presence of ethanol in the sample matrix being measured.

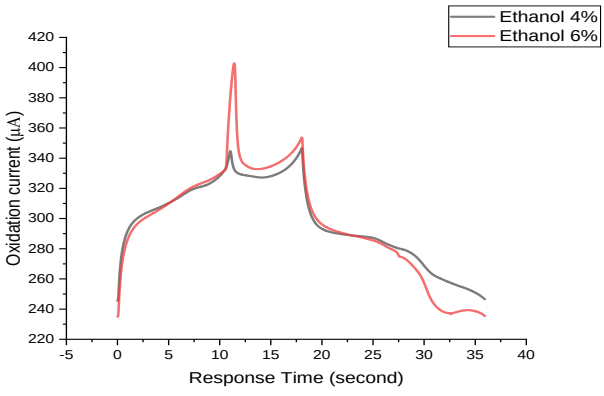


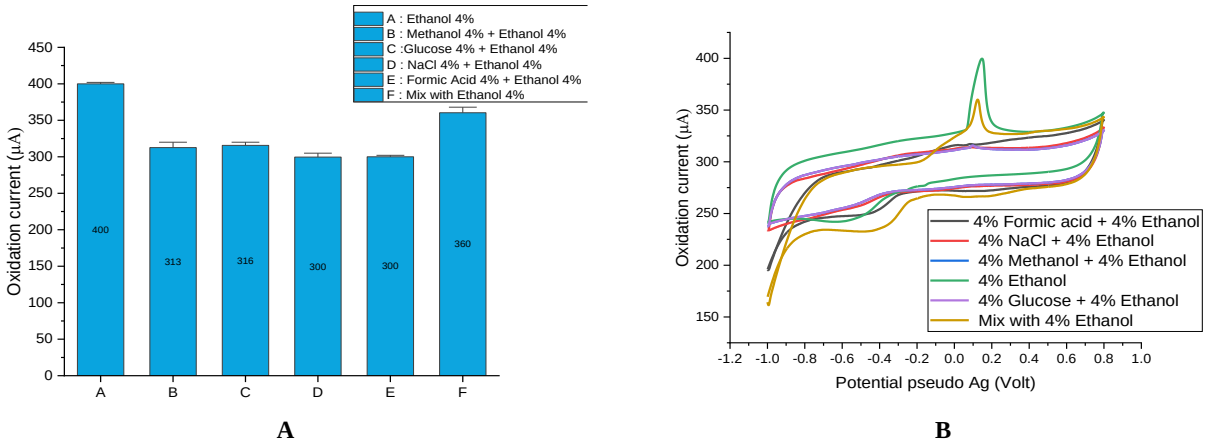
Figure 4. Response time curve of microbial consortium biofilm

Measurement results on samples of alcoholic beverages

The alcohol content in alcoholic beverage samples was determined by measuring the peak oxidation current using cyclic voltammetry. Samples of alcoholic beverages on the packaging have an alcohol concentration of 11.70%. This was determined using the line equation of the linearity test, namely y = 83.157x + 24.266 with x being the alcohol concentration and y being the peak oxidation current.

The real sample test was carried out to compare the results obtained from measurements using SPCE and GC. The alcohol concentration was determined using the line equation of the linearity test, namely y = 1000000x + 27453 where x is the alcohol concentration and y is the peak oxidation current. The real sample test results using microbial consortium-based biosensors were verified by a standard measurement method, namely GC. The two methods were then statistically tested using the T-independent test, and there was no significant difference in their results at the 95% confidence level. The ethanol content stated on the beer packaging showed a value of ± 11.70%. The measurement results obtained with the biosensor averaged 11.23% while those from GC amounted to 11.44% (Table 3).

The columnographic normality test indicated no significant differences between the values. Similar to the conventional difference test, when the significance is less than 0.05, it implies a significant difference, while values greater than 0.05, indicate no significant difference. The results obtained a significant value above 0.05, specifically a P value of 0.557, indicating that the test data and standard normal data did not differ significantly from one another. The comparison of the analytical performance of the alcohol biosensor from several bioreceptors is seen in Table 4.



A **B**

Figure 5. A. The selectivity bar graph of the interfering compound on ethanol substrate; B. The selectivity curve of the interfering compound on ethanol substrate

Table 3 Consortium biofilm and GC testing on alcoholic beverage samples

Parameter	Concentration measurement results with consortium biofilm (%)			Concentration measurement results with GC (%)		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Beer (11.70%)	11.30 ±0.05	11.60±0.05	10.80±0.06	11.71±0.04	11.00±0.05	11.60±0.04

Table 4. Biosensor performance based on various bioreceptors

Bioreceptor	Linearity (%)	Sensitivity (µA %$^{-1}$)	LoD (%)	RSD (%)	Stability (%)	Reference
ADH-NAD	0.1-2.0	0.02	70000	2.7	50 after 30 days	Samphao et al. (2015)
G. oxydans	na	117-121	2-6	<1	na	Šefčovičová et al. (2015)
Bacillus sp	0.1-5.0	na	na	na	na	Iswantini et al. (2017)
Saccharomyces ellipsoideus	0.02-0.2	43.6	0.008	8.22	50% after 9 days	Rotariu and Bala (2003)
Saccharomyces cerevisiae	0.01-3	87.61	0.006	<5%	87% after 49 days	Yusuf (2019)
A. aceti	5x10^{-5} - 3x10^{-4}	43,076	2.32 ×10^{-5}	1.08	98.99 after 49 days	Ninik et al. (2019)
A. aceti	0.99-1.96	57,29	0.003	1.95	100.34 after 55 days	Iswantini et al. (2020)
Consortium microbial *Bacillus megaterium* and *Saccharomyces cerevisiae*	0.02-6.0	83.157	0.010	0.568	88 after 70 days	This research

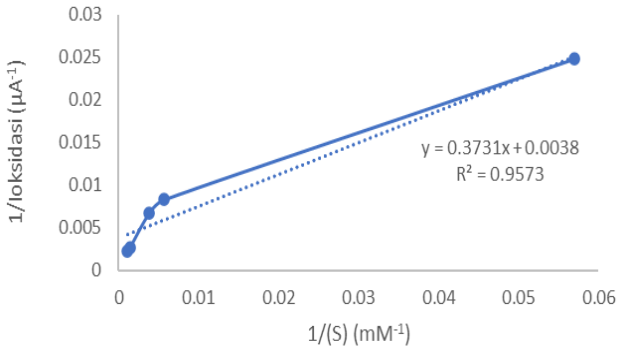


Figure 6. The relationship between 1/[S] and 1/Ioxidation as a Lineweaver-Burk curve

ADH kinetic parameters in microbial consortium

One of the characteristics of the enzyme examined in this study was the kinetics, in the form of KM and Vmax parameters. Kinetic measurements were carried out to determine the specificity, and the enzyme used was ADH present in the cell membrane of microbial consortium. The parameters of the enzyme kinetics were the KM and Vmax, equivalent to the maximum current (Imax) obtained from cyclic voltammetric measurements of ethanol solution. These parameters were determined based on the Lineweaver-Burk equation, and a graphical regression line was generated by plotting 1/[S] against 1/I with a series of ethanol concentrations, namely 0.1-5% (Figure 6). Based on the results, the KM value obtained was 0.56% and Vmax was 263.16 µA. The KM value can also be interpreted as a measure of the affinity or binding capacity of the enzyme to the substrate. The lower the value, the stronger the enzyme binding to its substrate, hence, even a low substrate concentration is sufficient to saturate the enzyme and achieve maximum catalytic efficiency. Vmax represents the rate at which the enzyme catalyzes the reaction.

In conclusion, *B. megaterium* and *S. cerevisiae* successfully formed biofilm on SPCE surfaces, enabling the use of microbial consortium in the development of ethanol biosensor. The results showed that the consortium microbial biofilm had the potential to be used in biosensor development, with significant sensitivity reaching 83.157 µA $(\%)^{-1}$ for the determination of ethanol, particularly at low concentrations. The biofilm showed ethanol oxidation activity and maintained stability at 88% for 70 days stability. Under optimal conditions, this tool could be used as an identifier for biological ethanol. This was evidenced by the analytical parameters, such as limit of detection and quantitation, linearity, sensitivity, precision, accuracy, response time, and selectivity, which were all improved. In the presence of matrix commonly found in food, the manufactured biosensor was quite selective toward ethanol. Based on the result, the consortium microbial biofilm of *B. megaterium* and *S. cerevisiae* could be developed further in the future.

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