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Screening and Optimization Consortium Technique of the *Bacillus megatherium* and *Saccharomyces cerevisiae* Microbial Consortium for Ethanol Detection

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Abstract

Determination of alcohol content is very important in the food and beverage industry. Biosensor is an alternative to measuring ethanol content. Alcohol biosensors with a single microbe still have a narrow measurement area at ethanol concentrations, so a microbial consortium is needed to widen the range of measured ethanol concentrations. Therefore, it is necessary to screen the microbes from *Bacillus sp* and *S. cerevisiae* which have the potential to produce alcohol dehydrogenase (ADH) enzymes and optimize the consortium technique that can provide the best response to oxidation currents. In the yeast microbial screening of 14 *S. cerevisiae* isolates and 5 *Bacillus sp* isolates, it can be concluded that for the 14 yeast S. cerevisiae isolates that have the potential to produce ADH enzymes, there is one isolate with the SCRF code. For the 5 bacterial isolates of *Bacillus sp* that have the potential to produce ADH enzymes, all *Bacillus* isolates with the code *Bacillus megatherium 29/9/14, Bacillus megatherium 23/6/22*, *Bacillus 6*, *Bacillus 53*, and *Bacillus 55*. Based on the oxidation current data, *Bacillus megatherium 23 /6/22* produces the highest current compared to other *Bacillus* isolates. The consortium technique that provides the highest current is the method of mixing 1:1 (µL) microbial suspension in an Eppendorf container. Optimization of the consortium's biofilms using the Response Surface Method was produced at 10 days of age, pH 7.5, and 75 µL of microbial suspension dripping volume.

Keywords: Alcohol biosensor; *S. cerevisiae; Bacillus*; consortium; biofilm.

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1. INTRODUCTION

Alcohol, especially ethanol, is often found in the food consumed by humans. According to WHO, alcohol consumption is the third largest risk factor in the world for degenerative diseases and disability. In addition, Indonesia especially for Muslims, really needs information on halal products, one of the indicators of which is the alcohol content in food and beverages and other products. Therefore, a sensitive and selective alcohol determination method is needed. Various methods have been developed to measure alcohol content, such as redox titration methods, chromatography, spectroscopy, and so on (Park et al, 2016; Boyaci et al. 2012). However, these methods have drawbacks including being expensive, time- consuming, and constrained by the turbidity of the sample being measured due to the high concentration of the sample and low sensitivity (Park et al. 2016; Castritius et al. 2010), so an alternative method is needed that is more precise, easy, fast, and sensitive for measuring ethanol content (Nuzulul et al, 2015). The electrochemical biosensor is an alternative method developed that is not constrained by the high concentration of the sample being detected (Mulyawan et al, 2022; Zusfahair et al, 2019; Iswantini, et al, 2014; Iswantini et al, 2014). However, the use of alcohol dehydrogenase (ADH) enzymes is expensive, so it is very important to use

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microbes that produce these enzymes, so the cost is low because they do not require enzyme purification. Several researchers have conducted previous studies using the formation of microbial biofilms to increase the stability of ethanol biosensors, but the concentration range is still narrow. Iswantini et al (2017) made an alcohol biosensor using Bacillus sp with a detection area of 0.1-5% ethanol. In addition, Iswantini et al (2018) also made an alcohol biosensor using Saccharomyces cerevisiae with a detection area of 0.01-2.5% ethanol. So, it is necessary to increase the ethanol concentration range. The use of a microbial consortium can be used to solve the problem. The aim of this study was to produce an alcohol biosensor model using a microbial consortium that is Bacillus sp and Saccharomyces cerevisiae which produces enzymes alcohol dehydrogenase (ADH). ADH is a group of reversible oxidoreductase enzymes and is the final enzyme in the formation of ethanol which oxidizes ethanol to acetaldehyde. This enzyme is widely distributed in bacteria, eukaryotes, and archaea (Kuswandi et al. 2014). ADH enzyme-based ethanol biosensor has better stability and specificity than biosensors using the enzyme alcohol oxidase (AOX). However, the response time is longer than the AOX enzyme-based ethanol biosensor (Castritius et al. 2010). Therefore, it is necessary to screen microbes from Bacillus sp and Saccharomyces cerevisiae which have the potential to produce ADH enzymes and optimize the consortium technique that can provide the best response to oxidation currents.

2. MATERIALS AND METHODS

Tools and Materials

The tools used was a Sanyo MIR-162 incubator, Hirayama HVE 50 autoclave, CVB 1300M laminar airflow, Mettler Toledo 100 µL micropipette, TOA DK HM-250 pH meter, Eppendorf tube, SPCE DRPCAC71190 Metrohm connector (DropSens, Asturias, Spain), potentiostat/galvanostat EDAQ (ADInstruments Pty Ltd, New South Wales, Australia) with a 3-electrode system from eDAQ (New Zealand, Australia), microplate absorbance reader BIORAD iMark (BIO-RAD, California. United States), Dino-Lite digital microscope, microwave, and other laboratory glassware.

The materials used, are Screen Printed Carbon Electrode (SPCE) refs. 110 (DropSens, Asturias, Spain), distilled water, ethanol 96% (Sigma-Aldrich, Saint Louis, USA), phosphate buffer solution, heterotrophic (HTR) and yeast maltose (YM) solid media, HTR and YM liquid media, and isolates of *Saccharomyces cerevisiae* and *Bacillus sp.* comes from a microbial collection of Health Microbiology Laboratory, National Research, and Innovation Agency in Cibinong.

Bacillus sp and *Saccharomyces cerevisiae* Cultures

A total of 1 pure colony of each isolate of 5 *Bacillus sp* isolates was carried out using the streaking technique for isolation purification on solid media for heterotrophic agar in a petri dish, then incubated at 37°C for 48 hours. Heterotrophic solid media was prepared from a mixture of 3.0 g bacto agar, 3.0 g peptone, 0.6 g tryptone, 1.0 g NaCl, and 0.5 g K_2HPO_4 mixed in 200 mL distilled water and then stirred until homogeneous. For 14 isolates of *Saccharomyces cerevisiae*, 1 pure colony was used for each isolate using the streaking technique for isolation purification in YM media in a petri dish, then incubated at 37°C for 48 hours. YM media was prepared from a mixture of 3.0 g of bacto agar, 1.0 g of peptone, 0.6 g of yeast extract, 2.0 g of glucose, 0.1 g of $MgSO_4.7H_2O$, and 0.2 g of K_2HPO_4 mixed in 200 mL of distilled water. then stirred until homogeneous. Then, the media was sterilized by autoclaving for 15 minutes at 121°C 15 lbs. After that, as much as 15 to 20 mL of warm heterotrophic media was poured into a petri dish and then allowed to cool and solidify in laminar air flow using UV light. The media that has solidified is ready to be inoculated and grown bacterial cells.

Harvesting Microbial Cultures Producing Alcohol Dehydrogenase Enzymes

Bacillus sp bacteria were incubated in a test tube containing 10 mL of liquid heterotrophic media (24 hours, 30 °C), while the *S. cerevisiae* microbe was incubated in a test tube containing 10 mL of liquid YM medium (24 hours, 30 °C) using an incubator shaker. Bacteria are harvested by centrifugation. A total of 1 ose of bacteria was transferred into 1 mL of distilled water, then vortexed until homogeneous. Then, 100 µL was transferred to liquid heterotrophic media for *Bacillus sp* and liquid YM media for *S. cerevisiae* which had been incubated and homogenized. Bacterial pellets were rinsed 3 times using a phosphate

buffer solution (PBS pH 7.0). The harvest is then resuspended with PBS solution, then the optical density (OD) value is periodically measured by means of 200 µL of bacteria from the media transferred to a microplate reader which is first checked for cleanliness with a value <0.05 until it reaches a value of 0.3 (bacterial exponential phase). The bacterial suspension that had grown was measured for its optical density (OD) at a wavelength of 595 nm. The optical density (OD) value was measured and the value of the number of bacterial colonies per milliliter was sought.

Microbial Consortium Biofilm Production and Measurement

Biofilms are made by mixing the harvested bacteria with 0.1% agar solution (temperature ± 40-50 °C) until it is homogeneous to prevent it from shifting the top of the working electrode. Biofilms were selected using an electrochemical approach immobilized on the surface of the working electrode on a screen-printed carbon electrode (SPCE). The production of the biofilm consortium was carried out in two ways, the first way was to drip 50 µL of *Bacillus sp* gradually onto the surface of the SPCE, then proceed with 50 µL of *Saccharomyces cerevisiae* gradually and wait for it to dry at room temperature, and do the same thing the other way around, with a variation of dripping 1: 1; 1:2; and 2: 1. After drying, drip warm agarose solution and let it stand until it forms a biofilm. The second method, namely the bacteria *Bacillus sp* and *Saccharomyces cerevisiae* are mixed in the Eppendorf tube with a mixing variation of 1: 1; 1: 2; and 2:1 and homogenized, then dripped on the SPCE surface as much as 20 µL, left to dry at room temperature and again dripped gradually up to 100 µL, then dripped with warm agarose solution and allowed to stand until it forms a biofilm. The suspension that has been deposited on the SPCE surface is then allowed to stand and stored in an airtight container for several days at room temperature until it dries. The selection of the best consortium biofilm was seen from the peak of the oxidation current formed when measured using mode cyclic voltammetry using a potentiostat/galvanostat EDAQ (ADInstruments Pty Ltd, New South Wales, Australia) with a 3-electrode system from eDAQ (New Zealand, Australia) (Ecorder 410) equipped with Echem v2.1.0 software with a scan rate of 100 mV/s. The SPCE used consists of a working electrode in the form of carbon, a reference electrode in the form of silver, and a supporting electrode in the form of carbon made of ceramic. The concentration of ethanol used ranges from 0.1% -1.5% (the concentration of ethanol below and above the halal limit of a beverage and food product by the MUI fatwa, namely 1%). The tested biofilms were distinguished by the following factors: (1) biofilm age (5-15 days), (2) PBS buffer pH (pH 6-9), and (3) microbial suspension drop volume (50-100 µL). The combination of these three factors is carried out using the Box Behnken design.

3. RESULTS AND DISCUSSION

Isolation and Selection of Selected *S cerevisiae* and *Bacillus sp* Microbes as Alcohol Biosensors

The optimization of the growth of microbe isolates process is carried out in several stages, including culturing bacterial cultures, selecting the optimum incubation time, selecting the growth incubation temperature, and selecting the pH of bacterial growth. Prior to planting bacteria (inoculation), all tools must be sterilized first using an autoclave at 121ºC to avoid contamination. Two of them that are often used are the quadrant stroke method and the pour cup method (Madigan et al., 2006). The principle of the two techniques is basically the same, namely by reducing the concentration of bacteria so that each colony formed is the result of one cell division. In this study, the culture method used was the pour plate method by spreading the culture which had been diluted in stages with a concentration of 10-7 on the surface of the agar media. The pour cup method aims to separate mixed populations of bacteria into single colonies with the assumption that a growing single colony consists of a population of the same type. The pour plate method was chosen because it has the advantage that the growing microorganisms can be completely separated over the entire surface of the agar.

The optimum incubation time is carried out to determine the time interval required for cells to divide into two folds (called the generation time). Each microbial species has a different generation time depending on whether physical conditions are sufficient. The choice of incubation time was carried out for 24 and 48 hours. From several variations of the incubation time applied, 24 hours is the optimum time for

S. cerevisiae cells to divide until they reach the stagnant phase (stationary phase), whereas after that time (24 hours) single colony growth is no longer formed.

The selection of incubation temperature is also done to suppress yeast growth. In this study, the growth temperatures tested were 30 ºC and 37 ºC. Based on observations using a microscope, at 30 ºC yeast is still prone to grow even in small amounts, while at 37 ºC yeast growth is inhibited. These results are in accordance with the results of a study put forward by Salvado et al. (2011) which explained that most of the yeast *Saccharomyces cerevisiae* grew at temperatures below 30 ºC, while for the species *S. cerevisiae*, the optimum growth temperature was at 32.5 ºC, although some other yeasts could still grow at 37 ºC although in small amounts.

Isolation of the yeast *S. cerevisiae* was carried out using a sample from the BRIN LIPI collection of 14 isolates, namely isolates with code R7.8 BS1; R7.8 BS2; R7.8 BS3; R7.8 BS4; R7.8 BS5; R10.5SCRF; Y2S1; Y3S1; Y5.S1; Y7S1; Y9S1; Y7D; Y9D; Y5R. Isolation of *S. cerevisiae* yeast was carried out using maltose yeast media which is a selective medium with high nutrition for the growth of *S. cerevisiae* yeast. The media contains yeast as a source of amino acids, peptone as a nitrogen source, and glucose as a nutrient for yeast. In the yeast growth medium for *S. cerevisiae*, sugar needs to be added because this fungus will thrive in sugar-containing habitats (Othman et al., 2014). The optimum temperature is 30°C, and pH is 7 (Hermansyah, 2018). This can accelerate the growth of the yeast *S. cerevisiae*.

Of the 14 isolates of *Saccharomyces cerevisiae* with code R7.8 BS1; R7.8 BS2; R7.8 BS3; R7.8 BS4; R7.8 BS5; R10.5SCRF; Y2S1; Y3S1; Y5.S1; Y7S1; Y9S1; Y7D; Y9D; The Y5R; can grow only one SCRF code. The other thirteen isolates still did not grow even though they had been grown in YM liquid media and spread from liquid to solid YM media and incubated again for 24 hours. As a result, 13 candidate isolates of *S. cerevisiae* still did not grow, only the SCRF code could grow. For the microbial *Bacillus sp*, 5 isolates from the LIPI BRIN collection were used with the code *Bacillus megatherium 29/9/14*, *Bacillus megatherium 23/6/22*, *Bacillus 6*, *Bacillus 53*, and *Bacillus 55*. After 24 hours of incubation in the incubator, 5 isolates of *Bacillus sp* with *Bacillus megatherium code 29/9/14*, *Bacillus megatherium 23/6/22*, *Bacillus 6*, *Bacillus 53* and *Bacillus 55* can grow as shown in **Figure 1**.

Isolates that have been pure then calculated the value of the Total Plate Count (TPC). The observation of the number of bacterial colonies is intended to determine the number of bacterial colonies that can grow on agar media. Observations using counting on agar media containing cultures are also called live bacteria counting methods or colony counting methods. The colony count is calculated using the Bacteria Colony Counter. The principle of the cup count method is to grow living microbial cells on the agar method; so that the microbial cells will multiply and form colonies that can be seen directly with the eye without using a microscope.

The agar plate containing the culture is selected and should have 30-300 colonies. Therefore, it is done by dilution. The number of microbes in the sample was determined by multiplying the number of colonies by the dilution factor on the agar medium. The unit used to express the number of bacterial colonies is CFU/mL (CFU = Colony Forming Units). Bacteria were taken as much as 1 mL to be put into the first diluent test tube (10^{-1}) containing 9 mL of distilled water. Then 1 mL of 10^{-1} was taken into diluent 10^{-2} and the tube was shaken until homogeneous. The experiment was repeated until the 10^{-7} dilution. Observations took dilutions 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. The solution was taken with a pipette and leveled with a glass rod as much as 0.1 mL of the solution to be cultured on Nutrient Agar (NA) media. The glass rod used must be sterile, by soaking it in alcohol and heating it over a fire. All Petri dishes were incubated for 24 hours in the incubator. Initially, the pure culture of *S. cerevisiae* was diluted 6x and 7x.

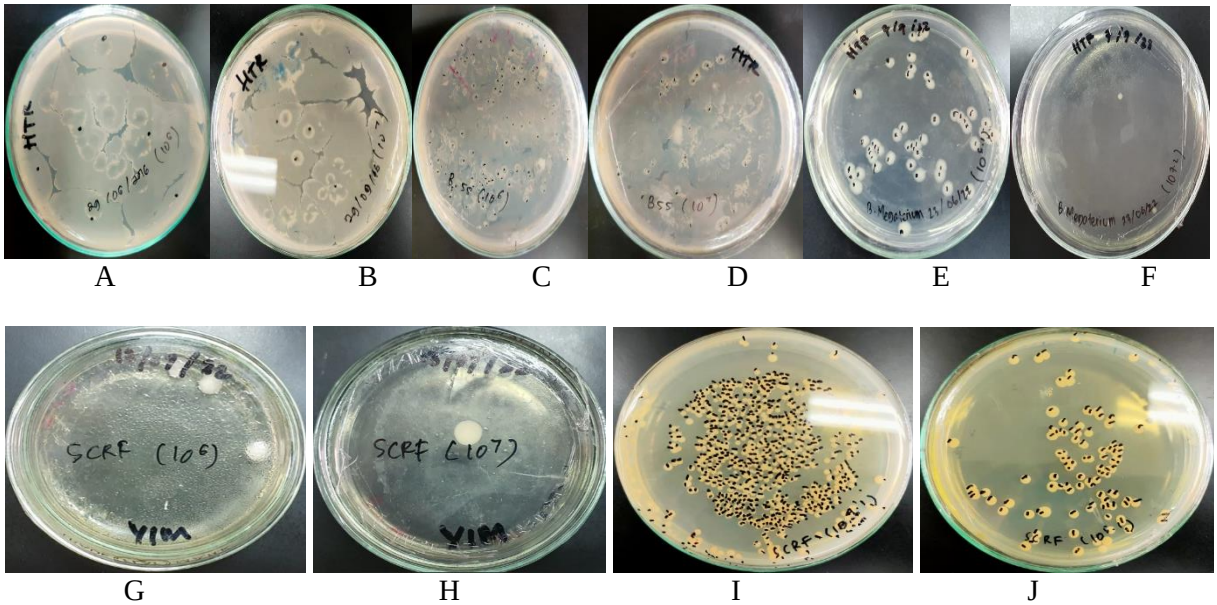
However, the pure culture of S. cerevisiae only grew on the second day with a TPC value of $3x10^{-6}$ CFU/mL for 6x dilution and a TPC value of 1 x 10^{-7} CFU/mL for 7x dilution, so 4x and 5x dilutions were made. The TPC value for the 4x dilution was 459 x 10^{-4} CFU/mL and for the 5x dilution, it was 105 x 10^{-5} CFU/mL. Bacillus 29/9/14 TPC value, 6x dilution = 7 x 10^{-6} CFU/mL, and 7x dilution = 4 x 10-7 CFU/mL Bacillus 55 TPC value, 6x dilution = 100 x 10^{-6} CFU/m, and 7x dilution = 28 x 10^{-7}, Bacillus 23/6/22 TPC value, 6x dilution = 54 $x10^{-6}$ and TPC Bacillus 23/6/22, 7x dilution = 3 x 10^{-7} (**Table 1**).

A B C D E F

Figure 1. Bacterial culture of *Bacillus sp* growing on agar media: (A) *Bacillus megatherium 29/9/14*, (B) *Bacillus megatherium 23/6/22*, (C) *Bacillus 6*, (D) *Bacillus 53*, (E) *Bacillus 55* (F) SCRF

Table 1. Total plate count value for several microbes

Microbe	Dilution	Total Plate Count Value (CFU/mL)
S. cerevisiae	7x	1×10^{-7}
	6x	3×10^{-6}
	5x	105×10^{-5}
	4x	459×10^{-4}
Bacillus megatherium 29/9/14	7x	4×10^{-7}
	6x	7×10^{-6}
Bacillus 55	7x	28×10^{-7}
	6x	100×10^{-6}
Bacillus megatherium 23/6/22	7x	3×10^{-7}
	6x	54×10^{-6}



A B C D E F

G H I J

Figure 2. The appearance of a single colony that grows after the purification process on (A) *Bacillus megatherium 29/9/14* 6x dilution, (B) *Bacillus megatherium 29/9/14* 7x dilution, (C) *Bacillus 55* 6x dilution, (D) *Bacillus 55* 7x dilution, (E) *Bacillus megatherium 23/6/22* 6x dilution, (F) *Bacillus megatherium 23/6/22* 7x dilution, (G) SCRF 6x dilution, (H) SCRF 7x dilution, (I) SCRF 4x dilution, (J) SCRF 5x dilution.

Not forming colonies or at least forming bacterial colonies can be caused by several things, including the cup containing the bacteria being too close to the fire so that the bacteria die or only a few are alive. Besides that, it can also be because the glass rod used to spread the

bacterial diluent solution is still hot so that the bacteria all or part dies. The higher the dilution level, the fewer bacterial colonies that will form. This can be seen in the 10^{-6} dilution which has too many colonies and the 10^{-7} dilution has the least number of colonies in the dish. The number of colonies decreased as the dilution increased because the number of bacteria contained in each volume of inoculant transferred decreased because of the dilution being carried out. The appearance of a single colony that grows after the purification process on, can be seen in **Figure 2**.

Culturing and Harvesting of *Saccharomyces cerevisiae* and *Bacillus sp*

The biochemical activity was determined by fermenting various concentrations of ethanol. Positive test results in 1%, 3%, and 5% ethanol fermentation lasting 18 hours at 37°C (**Table 2**). The growing colonies were then inoculated into liquid media containing 1%, 3%, and 5% ethanol. The goal is to ensure that the bacteria can survive in a high concentration of ethanol solution. The incubation process was carried out at 37 °C for 18 hours. The length of incubation time used is different from the initial process because according to Trinh et al. (2015), 18 hours is a period of fast and active *S. cerevisiae* breeding or called the logarithmic phase. In this phase, the bacteria produce enzymes and reach their highest activity for the measurement process which is indicated by the presence of turbidity in the liquid media treated with 1%, 3%, and 5% ethanol. The effect of ethanol concentration was also observed on bacterial growth as well as its ability to produce acetic acid. Bacteria growing in liquid media added with 1% ethanol were relatively more numerous compared to 3% ethanol and 5% ethanol. Nonetheless, *S. cerevisiae* and *Bacillus sp* were relatively tolerant to 5% ethanol content. Ethanol with relatively higher levels gives a growth effect not so good. In this study, the ethanol fermentation process to acetic acid which lasted for 5 days at 30 ^{0}C could change the initial pH of the liquid media from 6 to 3. In ethanol fermentation, the decrease in pH was caused by the conversion of ethanol to acetic acid (Chakraborty et al., 2017).

The OD value shows how much bacteria grows. In the logarithmic phase, the more bacteria that grow, the more enzymes are produced, so that the enzyme activity increases, and the measurement results also increase. On solid media, the bacteria *Bacillus megatherium* and *Saccharomyces cerevisiae* were incubated for 24 hours at 37 °C until a single colony was formed to maintain their purity. The process of harvesting bacteria is carried out by growing isolates in liquid media to obtain bacteria that are still fresh and produce good measurements. After growing on solid media, the bacteria were inoculated into liquid media for 18 hours. The time of 18 hours was chosen based on references to 43 studies conducted by Trinh et al. (2015) which states that the logarithmic phase lasts 18 hours. The logarithmic phase is a period of rapid and observable culturing characteristics of highly active cells. In this phase, the bacteria produce primary metabolites such as carbohydrates and proteins. The composition of the cells and the concentration of the resulting metabolites are relatively constant. After 3-4 hours the initial stationary stage begins to occur. This phase is a state of balance between the rate of growth and the rate of death; so that a constant total number of living bacteria will be obtained. The suspension was washed with 50 mM phosphate buffer pH 7 three times using a vortex and centrifuged so that the bacteria were not mixed with other impurities which would affect the measurement results. The washed suspension was then dripped onto the surface of the SPCE and allowed to stand in a closed container for 3 days at room temperature. The bacterial suspension that had grown was measured for its optical density (OD) at a wavelength of 595 nm. The OD values obtained for *S. cerevisiae* averaged 0.258 at 3 hours of incubation and 1.278 at 24 hours of incubation. *Bacillus megatherium 23/6/22* has a growth value (OD) of 0.348 at an incubation time of 3 hours, *Bacillus megatherium 29/9/14* has an OD growth value of 0.32 after incubation for 24 hours, and *Bacillus 55* has an OD growth value of 0.384 after incubation for 24 hours. So, it can be concluded that the fastest growing OD value is *Bacillus megatherium 23/6/22*, which is only 3 hours. For *Bacillus 6* and *Bacillus 53* the OD growth value was very slow so no growth curve was observed (**Figure 3**).

Table 2. Biochemical test results on SCRF microbial cultures, *Bacillus megatherium 29/9/14*, *Bacillus megatherium 23/6/22*, *Bacillus 6*, *Bacillus 53*, and *Bacillus 55* with the addition of ethanol

Ethanol concentration	Result	Conclusion
Ethanol 1%	Bacteria grow, the pH of the medium drops, the media is cloudy	Positif
Ethanol 3%	Bacteria grow, the pH of the medium drops, the media is cloudy	Positif
Ethanol 5%	Bacteria grow, the pH of the medium drops, the media is cloudy	Positif

Condition: *Bacillus sp* bacteria were incubated in a test tube containing 10 mL of liquid heterotrophic media (24 hours, 30 °C), while the *S. cerevisiae* microbe was incubated in a test tube containing 10 mL of liquid YM medium (24 hours, 30 °C) using an incubator shaker with the addition of ethanol concentration variations

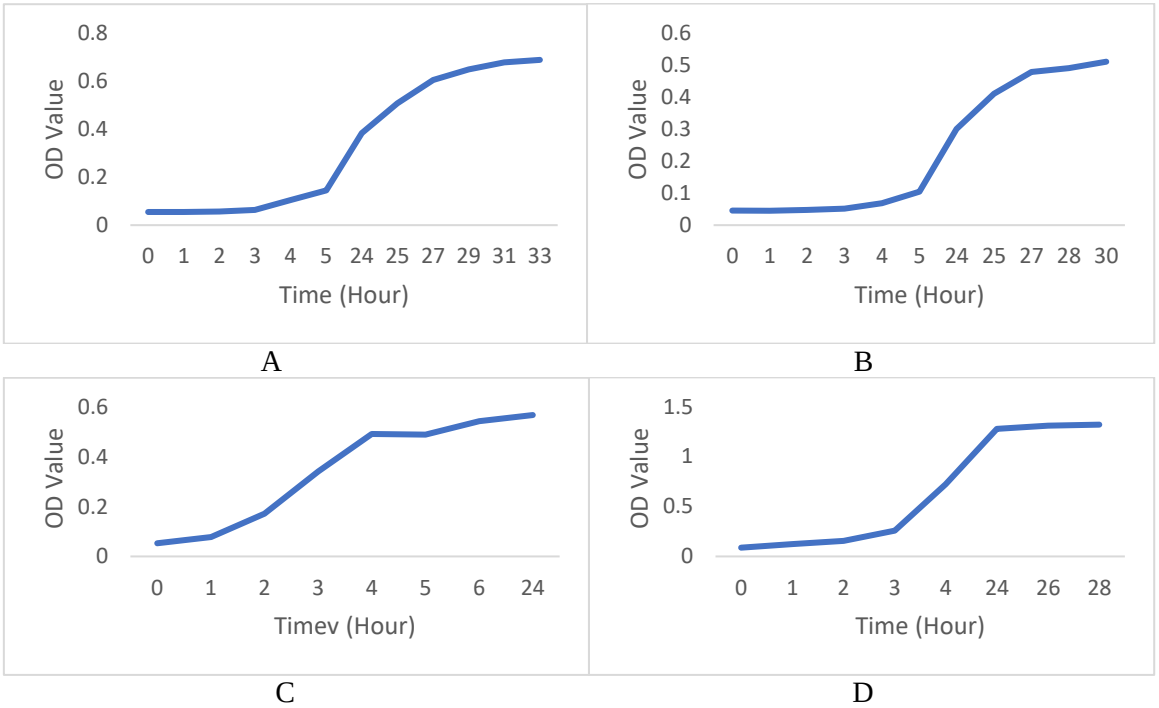


Figure 3. Growth curve of (A) *Bacillus 55*, (B) *Bacillus megatherium 29/9/14*, (C) *Bacillus megatherium 23/6/22*, (D) SCRF

Microbial Characteristics of *Saccharomyces cerevisiae* and *Bacillus sp*

Bacterial identification was carried out to ensure that the bacteria growing was *Saccharomyces cerevisiae* and *Bacillus megatherium* 23/6/22. Subsequent isolation is by scraping a single colony that grows in the first stage into another solid medium to obtain pure yeast colonies. The petri dish was then covered using plastic wrap to prevent contamination and the incubation process was carried out in the same way as the initial stage.

After obtaining a single colony, an identification process was carried out based on cellular morphology with a simple staining technique and observed using a microscope with a magnification of 1000 times. Yeast colonies that grow are yellowish white in color (**Figure 1F**) and are round tending to be oval (**Figure 4F**). This result was also confirmed based on research by Nurhakim et al. (2016) who stated that *Saccharomyces cerevisiae* is spherical with a large diameter between 5-10 µm and a small diameter between 1-3 µm to 1-7 µm, and its colonies are yellowish white.

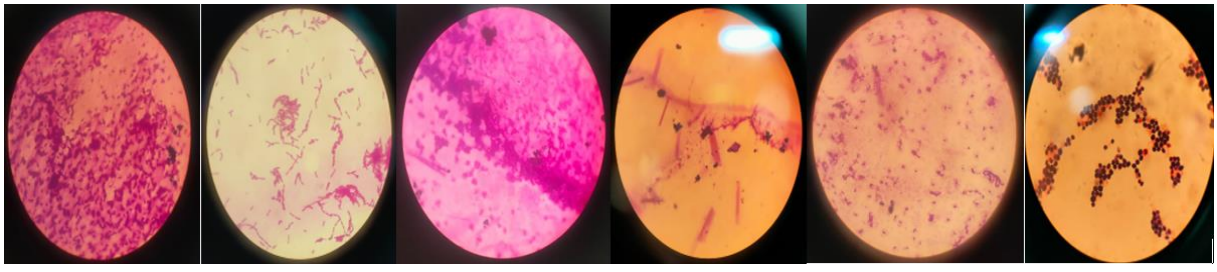
Morphological characteristics were carried out by means of gram staining. The function of gram staining is mainly to give color to parts of the cell structure which shows the chemical distribution of the parts of the cell so that it can distinguish it from other types of microbes. The results show that *S. cerevisiae* has an ellipsoidal shape, straight or slightly curved, single, and red in color as shown in **Figure 4F**. The *Saccharomyces* group is a group of gram-negative bacteria that will retain

the red color of safranin when gram staining is performed. The structure of the cell wall of gram-negative bacteria is dominated by lipids as the outer membrane, while the peptidoglycan layer lies between the inner and outer membranes. At the time of washing with ethanol, the outer layer of the cell wall, namely lipopolysaccharide, may dissolve with ethanol, so it cannot maintain the purple color of crystal violet. In addition, gram-negative bacteria cannot hold the dye after being stained with alcohol and will return to being colorless. After being given a contrasting color such as safranin, gram-negative bacteria will retain that color (Putri et al., 2018). The addition of the lead dye, safranin, makes the bacteria red.

Based on observations with a microscope, the *Bacillus sp* bacteria belongs to the Gram-positive, rod-shaped bacteria, which can grow under aerobic and anaerobic conditions. The spores are heat resistant (high temperature), and capable of degrading carbohydrates. Gram-positive bacteria are bacteria that retain methyl purple dye during the gram-staining process (**Figure 4A, B, C, D, E**). This type of bacteria will appear purple under a microscope. Gram-positive bacteria have a thick peptidoglycan cell wall. After the crystal violet staining process, the cell wall pores narrow which results in decolorization by alcohol, so that the wall retains the purple color. Possible positive bacterial cells will appear red if the decolorization time is too long. According to Ferid (2015), *Bacillus sp.* is known to have ADH enzymes in its metabolic system. The existence of this enzyme is beneficial if it is developed into an ethanol biosensor. ADH enzyme-based ethanol biosensors have better stability and specificity than biosensors using AOX. However, the response time is longer than the AOX enzyme-based ethanol biosensor (Kuswandi et al., 2014). Observations using a light microscope at 1000x magnification showed the morphology of the bacteria in the form of rods and purple in color when stained with crystal purple (**Figure 4**).

For five *Bacillus* isolates, it is necessary to determine one isolate that is selected as the best *Bacillus* bacterial isolate as a producer of the alcohol dehydrogenase enzyme. This can be seen from the peak of the highest oxidation current when measuring biofilm with a potentiostat. Based on the potentiostat measurements, current measurements were obtained for various types of *Bacillus sp* as listed in **Table 3**.

Based on the measurement results, Bacillus *megatherium* (BM) 23/6/22 has a higher current response compared to other *Bacillus* microbes, so it was determined that *Bacillus* microbes use *Bacillus megatherium 23/6/22*. In addition, from the results of the OD value growth curve measurements, which had the fastest growth time with an OD value of 0.348 was *Bacillus megatherium 23/6/22* with an incubation time of only 3 hours. The growth curve of *Bacillus megatherium 23/6/22* is like the growth curve of *Saccharomyces cerevisiae* which has an OD value of 0.254 with an incubation time of 3 hours.



A B C D E F

Figure 4. Staining of microbes growing on agar media: (A) *Bacillus megatherium 29/9/14*, (B) *Bacillus megatherium 23/6/22*, (C) *Bacillus 6*, (D) *Bacillus 53*, (E) *Bacillus 55*, (F) SCRF

Table 3. Data on ethanol oxidation current at SPCE modified with various *Bacillus sp* biofilm.

Ethanol	Oxidation current of ethanol measured at bacillus biofilm modified SPCE (µA)				
	BM 23/6/22	***BM 29/9/14***	***Bacillus 53***	***Bacillus 55***	***Bacillus 6***
0.1 %	100.8 ± 0.15	65.8 ± 1.94	76.0 ± 2.37	122.7 ± 2.39	65.7 ± 1.05
0.5 %	143.8 ± 0.84	104.7 ± 2.22	95.5 ± 1.41	81.3 ± 1.06	85.7 ± 1.08
1 %	133.1 ± 2.26	106.8 ± 2.80	112.8 ± 3.08	124.5 ± 1.05	92.2 ± 0.57
1.5 %	76.7 ± 1.86	66.2 ± 2.64	71.1 ± 0.91	83.1 ± 0.58	59.1 ± 1.39

Optimum Conditions for Consortium Biofilm of *Bacillus megatherium 23/6/22* and *Saccharomyces cerevisiae*

After the process of characterizing the bacteria, the process of selecting the optimum conditions for the formation of biofilms is carried out. Selection of the optimum conditions for the formation of biofilms is an initial step to look for some optimum conditions related to the formation and stability of biofilm activity of *Bacillus megatherium 23/6/22* and *Saccharomyces cerevisiae* to the closeness of the measurement results. The selection parameters to be tested are based on the pH of the buffer, the age of the biofilm, and the volume of the microbial suspension dripping. The SPCE that has been dripped with the bacterial suspension is stored for various days at room temperature and protected from sunlight to form a strong biofilm prior to measurement. Biofilms are formed in 5 stages, the first is reversible attachment or colonization. The second stage is the stage of biofilm growth. At this stage, the bacteria produce proteins to attach to surfaces. Furthermore, the bacteria produce a polymer matrix called EPS in the form of a hydrogel complex that attaches to the bacterial colony and forms it in 3 dimensions. The third stage is the maturation stage, the accumulated bacteria form several layers. The fourth stage is stage II maturation, at this stage, the microcolonies have changed to form microcolonies with a certain thickness. The fifth stage is the dispersion stage, at this stage, the biofilm that has been formed can experience cell release by erosion or sloughing. Erosion occurs periodically due to the shearing of flowing fluids. Sloughing is the release of many cells that occur randomly due to changes in the growth medium (Houdt & Michiels, 2005). On the 5th day of storage, the biofilm formed had not yet reached the maturation phase. The attachment and growth stages have occurred gradually, but the production of extracellular polymers which function to attach bacteria is not sufficient to form a biofilm layer that is permanently deposited on the SPCE surface. Biofilm formation requires a certain amount of time to reach the maturation stage. At the maturation stage, bacterial cells are naturally attached so that the activity of the enzymes in them is better protected from unfavorable external influences such as changes in temperature, pH, and the toxicity of the analyte matrix.

The morphology of the appearance of the SPCE blank without agarose, the SPCE blank with agarose, and the *Bacillus megatherium (BM) 23/6/22* and *Saccharomyces cerevisiae* SPCE biofilm consortium using a digital microscope can be seen in **Figure 5** which shows a clear difference that a consortium biofilm has formed in SPCE. when compared with the SPCE blank without agarose, and the SPCE blank with agarose. This was marked on the SPCE biofilm consortium where dots were visible showing the microbe *Bacillus megatherium (BM) 23/6/22* and the one shaped like an elongated rope was the microbe *Saccharomyces cerevisiae*.

The consortium technique used several techniques, namely by dripping each suspension of microbial *Saccharomyces cerevisiae* and *Bacillus megatherium 23/6/22* above SPCE and by mixing all microbial suspensions of *Saccharomyces cerevisiae* and *Bacillus megatherium 23/6/22* in one Eppendorf container with various variations as shown in Table 4. The potentiostat measurement results of the nine consortium techniques were carried out in various ethanol concentration series starting from 0.3%; 0.5%; 0.6 %; 0.7 %; 1.0 %; and 1.5% with data as in **Table 4**.

A B C

Figure 5. Morphology (A) SPCE blank without agarose, (B) SPCE blank with agarose and (C) SPCE biofilm consortium *Bacillus megatherium (BM) 23/6/22* and *Saccharomyces cerevisiae* using a digital microscope (400x magnification)

Table 4. Potentiostat oxidation current data from various consortium techniques

Ethanol concentration	Potentiostat oxidation current data from various consortium techniques (µA)								
	Mix 1:1	Mix 1:2	Mix 2:1	BM 23: SCRF 1:1	BM 23: SCRF 1:2	BM 23: SCRF 2:1	SCRF: BM 23 1:1	SCRF: BM 23 1:2	SCRF: BM 23 2:1
0.3%	188.2	280.9	20.9	19.92	64.36	209.48	117.12	79.36	171.82
0.5%	202.6	322.1	22.7	21.08	66.07	229.80	230.89	191.86	274.01
0.6%	206.6	329.2	30.4	21.39	83.07	240.43	274.12	227.44	251.10
0.7%	298.3	809.3	56.7	21.76	94.24	248.67	240.10	198.37	291.94
1%	346.3	896	70.5	22.12	131.28	256.94	110.35	142.88	349.03
1.5%	372.3	860	80.0	22.44	151.28	265.56	151.38	146.47	350

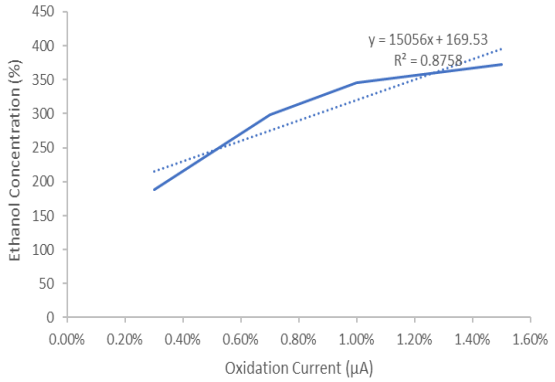


Figure 6. Measurement curve of oxidation current of ethanol biosensor on consortium techniques 1:1

For the dripping technique of each microbial suspension onto SPCE with the microbial *Bacillus megatherium 23/6/22* dripping technique, then continued by dripping the microbial *Saccharomyces cerevisiae* with various variations 1: 1, 1: 2; and 2: 1 shows small current and voltage values, namely in the range of 0.03-0.04 volts except for 2: 1 mixing. For the dripping technique, each microbial suspension onto SPCE is done using the *Saccharomyces cerevisiae* microbial dripping technique then followed by microbial dripping *Bacillus megatherium (BM) 23/6/22* with various variations 1: 1, 1: 2; and 2: 1 shows unstable current and voltage values, as well as ups and downs. Based on current data and curve stability as well as voltage, the best consortium technique is the technique by first mixing it in an Eppendorf container with a ratio of 1: 1. For the 1: 2 mixing technique it also provides a high current but at an ethanol concentration of 0.7% above the detected current exceeds 500 µA. According to Bilgy and Ayranci, (2016), the ADH peak was at 100−500 µA, while 600−800 µA indicated that was detected the alcohol oxidase enzyme. So, the 1: 2 mixing technique was not chosen for the consortium technique but the consortium technique by mixing it first in an Eppendorf container with a ratio of 1: 1.

After the consortium technique is established, the next step is optimizing the biofilm consortium *Bacillus megatherium (BM) 23/6/22* and *Saccharomyces cerevisiae* using the Response Surface Methode Box Behnken method with variations in pH buffer (6-9), biofilm age (5-15 days), and the droplet volume of the microbial suspension (50-100 µL). The optimal conditions for measuring the ethanol biosensor with the microbial consortium *Bacillus megatherium* and *Saccharomyces cerevisiae* are conditions of pH buffer 7.5, biofilm age of 10 days, and volume of microbe suspension dripping 75 µL. The volume of the microbial suspension of 50 µL, 75 µL, and 100 µL after the thickness measurement with a digital microscope was obtained, the average biofilm thickness was 0.042 nm, 1.977 nm, and 2.556 nm respectively. The results of the analysis of the effect of the variables on the current and the relationship between the two variables that affect the magnitude of the current can be seen in the contour (**Figure 7**). The color on the contour indicates the magnitude of the measuring oxidation current. The dark green contour indicates that the measured current is the maximum current.

The biofilm age optimization variable starts from 5 days to 10 days old and produces the optimum biofilm age, on the 10th day (**Figure 7B**). When the biofilm age value is high, the current increases because the high biofilm age causes the ADH enzyme to increase resulting in a catalytic reaction. The pH optimization variable starts from pH 6–9 which results in an optimum pH of 7.5. The effect of buffer pH (**Figure 7C**) shows the optimum condition of 7.5; because at that pH the enzymes will work well to provide a high current

response, this happens because enzyme activity is very dependent on pH. The resulting optimum pH is slightly alkaline because the positive surface of the electrode attracts anions to the surface to neutralize the charge so that negative charges tend to collect on the surface and change the optimum pH to be more alkaline. In addition, at a pH of 7.5, the microbial consortium can form biofilms well so that the availability of ADH also increases. This results in the catalytic reaction between the substrate and the enzyme going well so that the resulting current response is maximum. For the variable volume optimization, the microbe suspension dripping starts from a volume of 50 µL up to a volume of 100 µL and produces an optimum volume of 75 µL which is equivalent to a thickness of 1.977 nm (**Figure 7A**).

The appearance morphology of the SPCE biofilm consortium *Bacillus megatherium (BM) 23/6/22* and *Saccharomyces cerevisiae* with variations in the volume of the microbial suspension dripping, namely 50 µL, 75 µL, and 100 µL can be seen in **Figure 9**. **Figure 9** shows the more the volume of the microbial suspension dripping consortium, the more mixture of *Bacillus megatherium (BM) 23/6/22* and *Saccharomyces cerevisiae* microbial suspensions that form biofilms on SPCE.

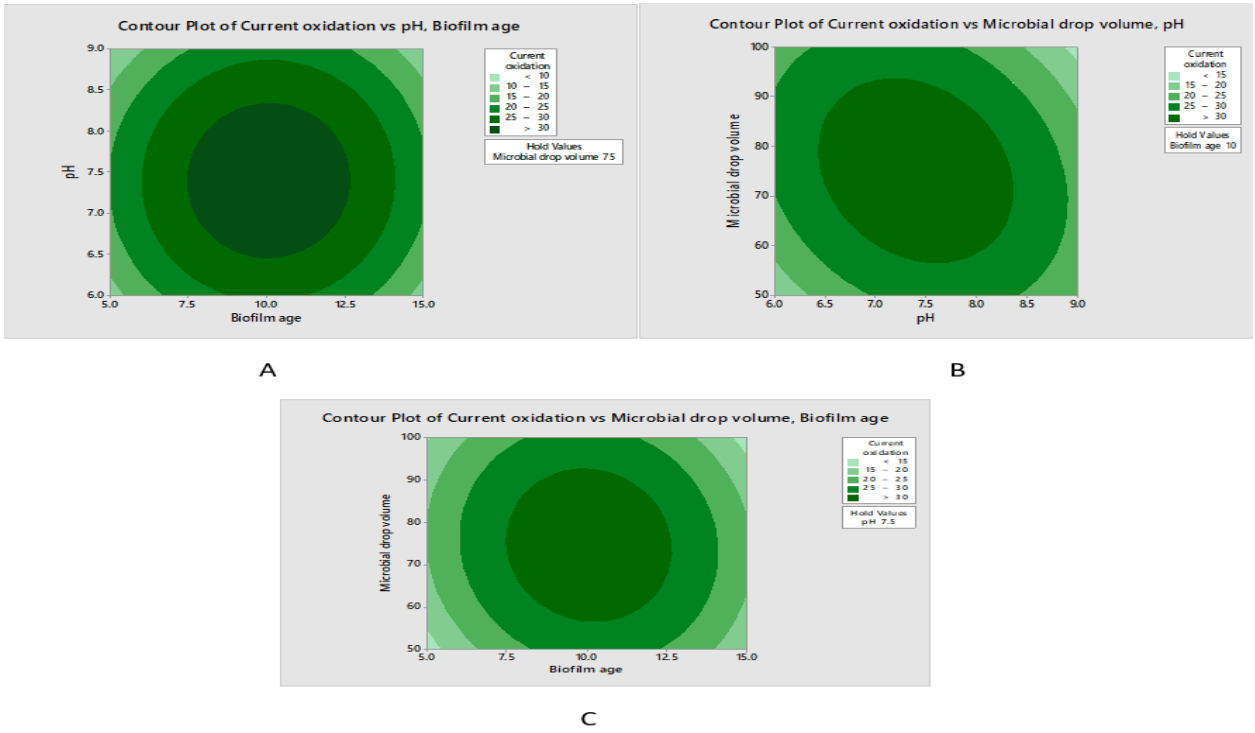


Figure 7. The contour of the relationship between the two variables on the current (A) effect of biofilm age and pH buffer on current (B) effect of pH buffer and microbial drop volume on current (C) effect of biofilm age and microbial drop volume on current

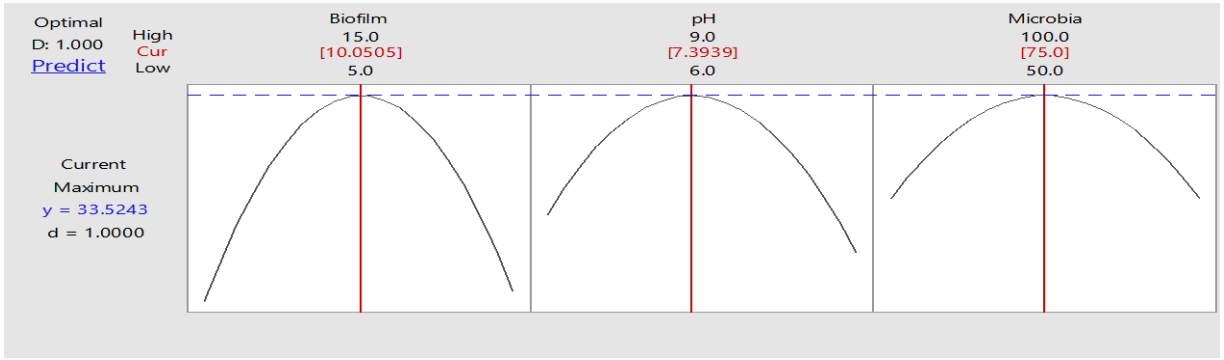


Figure 8. Optimized response of the microbial consortium biofilm

A B C

Figure 9. SPCE biofilm morphology of the *Bacillus megatherium (BM) 23/6/22* and *Saccharomyces cerevisiae* consortium with variations in the volume of microbial suspension dripping namely (A) 50 µL, (B) 75 µL, (C) 100 µL with a digital microscope (400x magnification)

Table 5. Several kinds of research for optimizing conditions for biofilm formation.

Bioreceptors/biofilm	The optimum time for biofilm formation (days)	pH Phosphate buffer solution	Microbial suspension dripping volume (µL)	Reference
Peroxidase coupled ferrocene activated alcohol oxidase	5	7.5	50	Somasekhar *et al* (2014)
AOX enzyme immobilized onto polyaniline film had carbon nanotubes (MWCNTs), gold nanoparticles (AuNPs), polyneutral red (PNR) film, and alcohol dehydrogenase immobilized on SPCE surface	49	7.0	100	Kuswandi *et al* (2014)
Bacillus sp biofilm immobilized on carbon paste electrode	7	7.75	150	Bilgi and Ayranci (2016)
A. aceti biofilm immobilized on SPCE surface	12	7.0	100	Iswantini *et al* (2017)
Acetobacter aceti biofilm immobilized on SPCE surface	14	6.8	100	Ninik *et al* (2019)
Acetobacter aceti biofilm immobilized on SPCE surface	14	6.8	100	Nurhidayat *et al* (2020)
Consortium microbe *S. cerevisiae* and *Bacillus megatherium* immobilized on SPCE surface	3	7.0	100	Iswantini *et al* (2020)
	10	7.5	75	This research

Several kinds of research for optimizing conditions for biofilm formation can be seen in **Table 5**. In **Table 5** the average volume of microbial suspension dripping is at a volume of 100 µL, whereas in this study the optimized volume was only 75 µL. This can save the volume of microbial suspension and the time needed to dry the biofilm. In this research, the optimum time required for biofilm formation was also relatively faster namely 10 days,

compared to other research that took more than 10 days, except for Balgi and Ayranci (2016) and Iswantini's et al. (2017) research which only took 7 and 3 days for biofilm formation.

4. CONCLUSIONS

In screening 14 microbial isolates of *S. cerevisiae* and 5 isolates of *Bacillus sp*, it can be concluded that 14 isolates of yeast *S. cerevisiae* that have the potential to produce ADH enzymes is one isolate with the SCRF code. For the 5 bacterial isolates of *Bacillus sp* that have the potential to produce ADH enzymes, all Bacillus isolates with the code *Bacillus megatherium 29/9/14, Bacillus megatherium 23/6/22, Bacillus 6, Bacillus 53, and Bacillus 55*. Based on the oxidation current data, *Bacillus megatherium 23 /6/22* produces the highest current compared to other *Bacillus* isolates. The consortium technique that provides the highest current is the method of mixing 1:1 (µL) microbial suspension in an Eppendorf container. Optimization of the consortium's biofilms using the Response Surface Methode produced biofilms aged 10 days, pH 7.5, and 75 µL of microbial suspension dripping volume.

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