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Effect of Time, pH, and Yeast Concentration on Bioethanol Levels in the *Ulva* sp. Fermentation Process

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**Abstract.** Bioethanol is a form of renewable energy used to reduce dependence on fossil fuels, which have various adverse environmental impacts. *Ulva* sp. contains high amounts of carbohydrates, which can potentially be a raw material for bioethanol production. This study aims to determine the optimal conditions of the fermentation process, considering the variables of time, pH, and yeast concentration. This study used the hydrolysis results of *Ulva* sp. with optimum operating conditions of 0.1 N HCl concentration, 80 mesh particle size, and 450-watt microwave power. Measurement of bioethanol levels was performed using an alcohol meter. The results showed that the optimal conditions for fermentation were 7 days, pH 5.5, and a yeast concentration of 1.5%, resulting in a bioethanol content of 7.55%.

**Keywords:** bioethanol, fermentation, yeast, Ulva sp.

### 1. Introduction

Fuel is the energy that is needed by all countries in the world currently and is expected to increase over time. Meanwhile, oil and natural gas depend on fossil resources whose formation rate is inversely proportional to consumption. These fossil resources are included in non-renewable resources (non-renewable) if they are taken continuously; one day, their availability will run out [1]. Oil production over the last six months, up to June 2022, stands at 616.6 thousand barrels per day, according to the Special Task Force for Upstream Oil and Gas Business Activities (SKK Migas). The use of motorized vehicles continues to increase yearly, making energy scarce. According to the Indonesian Statistics Center (BPS), in 2021, the use of motorized vehicles in Indonesia is expected to reach around 270,688,529. Alternative fuels are

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needed to address these problems, specifically fuels that can be replenished for optimal use. Bioethanol is one of the potential fuels that can be used as an alternative to fossil fuels [2]. Indonesia is a tropical country with abundant biodiversity, diverse agricultural products, and plantation products, offering excellent opportunities for bioethanol development [3].

Ethanol has a high-octane number, high laminar speed, and high heat of vaporization, making it suitable for use as a transportation fuel [4]. Ethanol can be used as a substitute for transportation fuel, directly or mixed with gasoline. Gasoline mixed with ethanol up to 15% can be burned in a transportation combustion engine [5]. The carbon and hydrocarbon emissions levels in a mixture of gasoline and ethanol are lower than those of premium and Pertamax because ethanol contains 35% oxygen, which can increase combustion efficiency in vehicles [6]. Bioethanol, with the chemical formula C<sub>2</sub>H<sub>5</sub>O, is a single-chain alcohol with an octane number of 108, is difficult to evaporate, has a low-calorie value, and is flammable [7]. Bioethanol production for the first generation generally uses food as a raw material [8]. Sugar derived from carbohydrates (starch) is fermented with the help of microorganisms to produce bioethanol [9]. These microorganisms are Saccharomyces cerevisiae, Zymomonas mobilis, and Escherichia coli because they can convert simple sugars into ethanol [10]. Bioethanol is obtained by converting carbohydrates into glucose through various processes, including acid or enzymatic hydrolysis [11]. Bioethanol can be produced from multiple natural products; therefore, it can be referred to as bioethanol [12]. The residue biomass of aquatic plants can be used as a raw material for producing bioethanol, as is the case with the Algae *Ulva* sp.

Ulva sp. is a macroalga belonging to the class Chlorophyta. Ulva lactuca contains many chlorophyll cells, which give the algae its green color. Green algae generally store starch as a food reserve [13]. Ulva sp. is a type of macroalgae widespread in the sea and Indonesian fresh waters [14]. It generally inhabits rocks on dead coral fragments and varies in shape and size in response to changes in environmental factors [13]. The content contained in Ulva sp. per 100 grams of net weight, namely water 18.7%, protein 15.26%, fat 0.1-0.7%, carbohydrates 46-51%, fiber 2-5%, and ash content 16-23%, and contains vitamins B1, B2, B12, vitamin C, and vitamin E [15]. Ulva sp. They range up to 100 cm in length, are bright apple green in color, and have the shape of a folded sword with smooth but wavy edges. The middle of each strand is pale and dark, but it becomes more pronounced as it reaches the edges [16]. Ulva sp. can survive in the temperature range of 28-31 °C [15]. Ulva sp. is one of the species of the Ulvaceae tribe, systematically classified as Ulva sp., which can be seen in Table 1 as follows [17]

Table 1. Classification of Ulva lactuca

Kingdom	Plantae	
Division	Chlorophyta	
Class	Ulvophyceae	
Order	Ulvales	
Family	Ulvaceae	
genus	Ulva	
Species	Ulva lactuca	

The manufacture of bioethanol involves several stages, the first of which is hydrolysis, which converts polysaccharides into simple sugars and subsequently converts these sugars into ethanol [18]. Hydrolysis uses an enzymatic chemical method with an acid catalyst [19]. The hydrolysis process can use a catalyst in the form of an acid or an enzyme [20]. The acid used is strong, namely, hydrochloric acid (HCl). The effectiveness of the HCl catalyst type was higher in producing glucose at the same temperature, concentration, and time compared to H<sub>2</sub>SO<sub>4</sub>. This is because the nature of HCl is stronger with higher reactivity than H<sub>2</sub>SO<sub>4</sub> [21]. Hydrolysis of cellulose with acid using the microwave method can convert starch into simple sugars relatively quickly. Based on this, it can produce higher bioethanol yields with lower acid concentrations, making it environmentally friendly, cost-effective, and reducing production time [22]. Hydrolysis is a reaction process that utilizes water to break down a compound into its constituent parts [23]. The hydrolysis process can use a catalyst in the form of an acid or an enzyme [20]. One is hydrochloric acid (HCl), which can be used in starch hydrolysis because it is readily available. Still, hydrochloric acid must be handled properly because it is a corrosive liquid [24]. Although acidic, this compound contains chloride ions, which are non-toxic and non-reactive. Hydrochloric acid with an intermediate concentration of 30% to 34% is stable enough to be stored and maintained at this concentration [25]. Therefore, hydrolysis is essential in the manufacturing of bioethanol because it determines the amount of glucose obtained, which is then fermented into bioethanol. Termination of starch polymer chains to form dextrose or monosaccharide units, namely glucose, is the principle of starch hydrolysis [26]. The following reaction equation for the hydrolysis process is shown in Equation 1 [27].

$$(C_6H_{10}O_5)n_{(s)} + nH_2O \xrightarrow{HCl_{(l)}} nC_6H_{12}O_{6(l)}$$
 (1)

The second stage is fermentation, in which glucose is converted into ethanol and carbon dioxide (CO<sub>2</sub>). Fermentation is carried out by adding yeast to work at optimal temperatures [18]. Factors influencing the fermentation method are temperature, nutrition, pH, and fermentation time. The optimal temperature for the fermentation process ranges from 27 to 30

°C, and the optimal pH ranges from 4 to 7 [28]. Fermentation using glucose, starch, and fiber can produce liquid bioethanol [29]. Fermentation is one way to make alcohol by precipitating a carbohydrate-containing substance in an anaerobic state [30]. Fermentation is a process in which chemical changes occur in an organic substrate from the activity of enzymes produced by microorganisms [31]. Fermentation usually uses microorganisms such as yeast or mold, but can be done with bacteria and various organisms [32]. Saccharomyces cerevisiae is commonly used in bioethanol production because it can easily produce alcohol in large quantities and responds highly to alcohol content [33]. Saccharomyces cerevisiae yeast is used to increase the yield of the bioethanol production process because the process does not require sunlight. The alcohol produced from the fermentation process can contain up to 8-10% alcohol content [34]. In the fermentation process, yeast plays a role in converting glucose into ethanol and carbon dioxide gas [35]. The fermentation reaction for the formation of alcohol is shown in equation 2 [36]

$$C_6H_{12}O_{6(l)} + Saccharomyces cerevisiae \longrightarrow C_2H_5OH_{(l)} + 2CO_{2(g)}$$
 (2)

The next process is the distillation process, which is used to obtain purer ethanol with the aid of a distillation apparatus. Then conduct a test to determine ethanol levels [11]. Measurement of ethanol levels can be done using an alcohol meter.

Ulva sp. was hydrolyzed in this study using hydrochloric acid to form simpler molecules. Then, the ethanol fermentation process, carried out with yeast, involves hydrolysis. In several previous studies using various raw materials, it has been demonstrated that the fermentation time and Saccharomyces cerevisiae concentration both impact the bioethanol fermentation process. The longer the fermentation time, the higher the bioethanol content produced. However, bioethanol levels decreased when they reached the optimal point because the productivity of Saccharomyces cerevisiae decreased, and the available nutrients were depleted. High levels of bioethanol are also obtained from favorable environmental conditions, one of which is the influence of pH. This study aims to determine the optimal conditions for the fermentation process of Ulva sp. using the following variables: time, pH, and yeast concentration. References for this study were obtained from several journals, which are summarized in Table 2.

Raw Materials **Methods and Results** Ref Ulva reticulata Fermentation of Saccharomyces cerevisiae at pH 4.5 and [37] temperature 30 °C for 6 days. The results of the analysis of ethanol content have a purity of 5.02% Microalgae Saccharomyces cerevisiae fermentation at pH 4.5. The [27] Nannochloropsis sp. best fermentation time to produce ethanol is 72 hours, which is 8.9% Tetraselmis chuii Saccharomyces cerevisiae fermentation at 30 °C for 5 [38] Microalgae days. The resulting ethanol content of 1% Ferment at 27-30 °C. The highest ethanol content was in [39] Codium geppiorum fermentation with 20% yeast concentration for 7 days, Algae namely 3.03%. Saccharomyces cerevisiae fermentation at pH 4.5 and [40] Green Algae Spirogyra sp. temperature 30 °C. The highest yield of ethanol was 0.0613 in 5 days of fermentation with 1% fermipan Elephant Grass Saccharomyces cerevisiae fermentation at pH 4. The [36] highest bioethanol yield was 17.30% with 11% starter (Pennisetum purpureum content for 6 days Schumach) Microalgae Chlorella Fermentation Saccharomyces cerevisiae at pH 5 was [41] pyrenoidosa fermented for 3 days. The highest bioethanol concentration produced was 0.280% with 25% yeast concentration Sargassum Saccharomyces cerevisiae fermentation at 30 °C. The [1] crassifolium highest yield of bioethanol was obtained at pH 7 which was fermented for 72 hours, namely 67 ml [42] Powder Agar Fermentation of Saccharomyces cerevisiae at pH 7. Optimum ethanol content was achieved at 120 hours of Gracilaria verrucosa incubation with 0.1 M H<sub>2</sub>SO<sub>4</sub> concentration of 0.77% [43] Nira Aren Saccharomyces cerevisiae fermentation. The highest ethanol content was 45.70% in 6 days of fermentation using 7.5 ml of starter

Table 2. Research on Bioethanol Production

### 2. Research Methods

### 2.1 Materials

The materials used in this study included hydrolysis of *Ulva* sp., yeast (*Saccharomyces cerevisiae*), distilled water, HCl, NaOH, and urea.

### 2.2 Material preparation

Algae of the *Ulva* sp. species was washed thoroughly to remove sand and other impurities. Furthermore, the material is dried using an oven, after which it is mashed with a blender and sieved using an 80-mesh sieve to obtain *Ulva* sp. powder.

## 2.3 Hydrolysis Process

The hydrolysis process used 0.1N HCl, *Ulva* sp. powder, and 0.1N HCl was added. Furthermore, the hydrolysis process was carried out using a microwave with a power of 450

watts. After hydrolysis, the hydrolysate is cooled in the glass beaker until it reaches room temperature. The hydrolysate is then filtered using filter paper to separate the filtrate from the residue. The filtrate obtained was then put into a vial to test further, reducing sugar levels.

#### 2.4 Fermentation Process

The fermentation process uses the help of *Saccharomyces cerevisiae* with varying concentrations of 0.5%, 1%, and 1.5% of the total hydrolysate volume, [40] and added 2 M NaOH until the pH becomes 4, 5.5, and 7 for variations in pH. After that, 1% of the total hydrolysate volume was added as a nutrient for the culture into the fermentation vessel. Fermentation is carried out anaerobically. Tightly cover the fermentation container, then the container is perforated and given a hose that flows into a container filled with water and fermented with variations of 3, 5, and 7 days [39] at room temperature (30 °C).

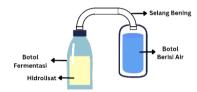


Figure 1. A series of fermentation equipment

### 2.5 Distillation Process

The fermentation results are then filtered to separate the filtrate from the residue. After that, the liquid is placed in the distillation flask for the distillation process and then heated to a temperature of 78 °C, corresponding to the boiling point of ethanol.

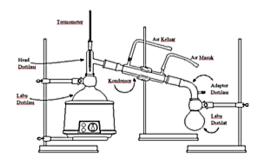


Figure 2. A series of distillation equipment [44]

## 2.6 Sample Analysis

This study investigated the levels of ethanol produced during the production of bioethanol. After the distillation process, cool the pure ethanol produced into a glass beaker and then test the levels by inserting an alcohol meter into the beaker. Then let stand for 5-10 minutes and see the scale read on the alcoholmeter.

## 2.7 Data Analysis

Data analysis used Design Expert software version 13, adapted to the Response Surface Methodology (RSM) approach and the 17 running Box Behnken Design (BBD) model according to Table 3.

No.	Fermentation time (days)	pН	Yeast Concentration (%)
1	7	4	1
2	3	5.5	1.5
3	3	4	1
4	5	7	1,5
5	7	7	1
6	5	5.5	1
7	5	5.5	1
8	3	5.5	0.5
9	7	5.5	1.5
10	5	5.5	1
11	5	4	0.5
12	3	7	1
13	5	5.5	1
14	5	5.5	1
15	5	4	1.5
16	5	7	0.5
17	7	5.5	0.5

**Table 3.** Variation of *Ulva* sp. fermentation data.

### 3. Results and Discussion

### 3.1 Analysis of Bioethanol Content Results

Based on the bioethanol content test measured using an alcoholmeter, the highest bioethanol content was 7.55% under the following operating conditions: 7 days of fermentation, pH 5.5, and yeast concentration of 1.5%. At the same time, the lowest bioethanol content was 1.50% under operating conditions with parameters of 3 days of fermentation time, pH 4, and 1% yeast concentration.

	5 5					
No.	Fermentation time (days)	pН	Yeast Concentration (%)	Bioethanol Content (%)		
1.	7	4	1	3.55		
2.	3	5.5	1.5	1.55		
3.	3	4	1	1.50		
4.	5	7	1.5	3.10		
5.	7	7	1	5.35		
6.	5	5.5	1	4.60		
7.	5	5.5	1	4.50		
8.	3	5.5	0.5	3.85		
9.	7	5.5	1.5	7.55		
10.	5	5.5	1	4.10		
11.	5	4	0.5	2.20		
12.	3	7	1	1.75		
13.	5	5.5	1	4.30		
14.	5	5.5	1	3.95		
15.	5	4	1.5	2.40		
16.	5	7	0.5	2.55		
17.	7	5.5	0.5	3.70		

Table 4. Results of Reducing Sugar Levels

## 3.2 Analysis of Variance (ANOVA)

Analysis of Variance (ANOVA) is a form of statistical hypothesis testing by drawing conclusions based on inferential statistical data or groups. Significant results can be seen from the p-value (probability value) <0.05. The results of the ANOVA can be seen in Table 4, and the results obtained are a p-value <0.05. This shows that the variables used in this study affect the levels of bioethanol. Lack of fit refers to a deviation from the model. The p-value for Lack of Fit was greater than 0.05 and showed insignificant results. In this study, the Lack of Fit value was 0.5177. This shows the suitability of the model. A significant relationship between the variables and the yield of bioethanol content is evident from the R² value. Table 5 shows that the R2 value is 0.9872, indicating a significant relationship between fermentation time, pH, and yeast concentration on bioethanol levels. In addition, there is a difference between the Predicted R² value and the Adjusted R² value <0.2, which indicates that the data is reasonable.

 Table 5. Results of Analysis of Variance (ANOVA)

Source	Sum of Squares	df	Mean square	F-value	p-value	
Model	37.87	9	4.21	60.40	< 0.0001	significant
A- Fermentation time	16.68	1	16.68	239.38	< 0.0001	
B-pH	1.16	1	1.16	16.69	0.0047	
C- Yeast Concentration	0.6612	1	0.6612	9.49	0.0178	

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Source	Sum of Squares	df	Mean square	F-value	p-value	
AB	0.6400	1	0.6400	9.19	0.0191	
AC	9.46	1	9.46	135.74	< 0.0001	
BC	0.0306	1	0.0306	0.4396	0.5285	
$A^2$	0.1181	1	0.1181	1.70	0.2340	
$\mathbf{B}^2$	8.64	1	8.64	124.03	< 0.0001	
$\mathbb{C}^2$	0.3664	1	0.3664	5.26	0.0555	
Residual	0.4876	7	0.0697			
Lack of Fit	0.1956	3	0.0652	0.8933	0.5177	not significant
Pure Error	0.2920	4	0.0730			
Cor Total	38.35	16				

The suitability between the experimental data and the model can be seen based on the parity plot graph in Figure 3. The straight lines on the graph are the predicted data, while the actual data from each run is shown as dots on the graph. The experimental data values cluster around the line, indicating a good match between the model and the experimental data, suggesting that the model is significant.

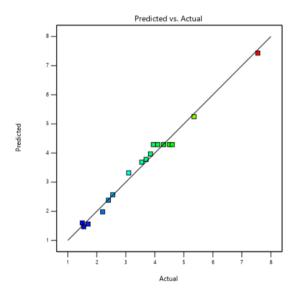


Figure 3. Graph of comparison of model data with experimental data on ANOVA

### 3.3 Effect of Parameters on Bioethanol Levels

## 3.3.1 Effect of Fermentation Time and pH on Bioethanol Levels

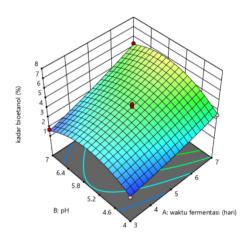


Figure 4. Effect of bioethanol content on fermentation time (days) and pH

Figure 4 is a graph illustrating the effect of fermentation time and pH on the production of bioethanol. The impact of fermentation time on significant bioethanol levels is shown in Table 5, where the p-value = <0.0001. The effect of pH on bioethanol levels is also substantial, as shown in Table 5, with a p-value = 0.0047. The interaction between fermentation time and pH for bioethanol is significant because p-value = 0.0191 < 0.005.

The graph in Figure 4 shows that the longer the fermentation time and the higher the pH, the higher the bioethanol content. High levels of bioethanol can be influenced by favorable environmental conditions, one of which is pH. However, the optimal pH condition is pH 5.5 because *Saccharomyces cerevisiae* has an optimal pH range for growth, specifically pH 4-5 [45]. Enzyme performance in yeast is affected by pH; if the pH is too acidic or alkaline, it disrupts enzyme activity. High pH conditions can cause the value of bioethanol levels to decrease because when the fermentation media conditions lead to a neutral pH, *Saccharomyces cerevisiae* enters a stationary phase or is no longer working and is experiencing growth again. During fermentation, the pH can decrease in the presence of organic acids produced by microorganisms [46]. This causes the pH four condition; yeast cannot work optimally, so the level of bioethanol produced is lower. The time of fermentation also affects the levels of bioethanol, as the yeast continues to reproduce over time in a fermenting solution medium capable of converting glucose into bioethanol [36]. As shown in Figure 4, a yeast concentration of 1.5% and a pH of 5.5 produced a bioethanol content of 1.55% over 3 days. Meanwhile, after

7 days of fermentation, with a yeast concentration of 1.5% and a pH of 5.5, a bioethanol content of 7.55% was produced.

### 3.3.2 Effect of Fermentation Time and Yeast Concentration on Bioethanol Levels

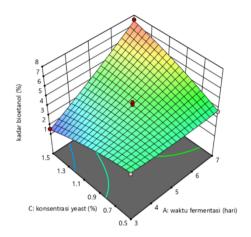


Figure 5. Effect of bioethanol content on fermentation time (days) and yeast concentration (%)

Figure 5 is a graph illustrating the effect of fermentation time and yeast concentration on the production of bioethanol. The impact of fermentation time on significant bioethanol levels is shown in Table 5, where the p-value = <0.0001. The effect of yeast concentration on bioethanol levels is also substantial, as shown in Table 5, with a p-value = 0.0178. The interaction between fermentation time and pH in relation to bioethanol is significant, as indicated by a p-value of <0.0001.

The bioethanol content with the operating conditions of 7 days fermentation time, pH 5.5, and 1.5% yeast concentration is 7.55%, whereas, in the operating conditions of 3 days fermentation time, pH 5.5, and 0.5% yeast concentration, it produces bioethanol 3.85%. Figure 5 shows that the bioethanol content increases with the length of time of fermentation and the high concentration of yeast. Yeast can develop and grow in fermented solution media to convert glucose into bioethanol. Thus, a high yeast concentration will increase the bioethanol level formed in the fermentation process. However, the fermentation time has a maximum limit of 7 days. Still, the fermentation time that exceeds the maximum number does not affect the increase in bioethanol levels because the yeast undergoes a death phase, resulting in a decrease in the yeast's activity in converting glucose to bioethanol [47].

# 3.3.3 Effect of pH and Yeast Concentration on Bioethanol Levels

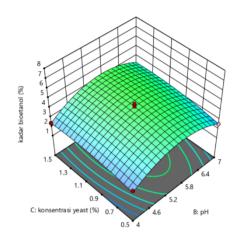


Figure 6. Effect of bioethanol content on pH and yeast concentration (%)

Figure 6 is a graph of the effect of the variable pH and yeast concentration on the bioethanol levels produced. The impact of pH on significant bioethanol levels is shown in Table 5, where the p-value = 0.0047. The effect of yeast concentration on bioethanol levels is also substantial, as shown in Table 5, with a p-value = 0.0178. However, the interaction between fermentation time and bioethanol pH was insignificant, as the p-value (0.5285) was greater than 0.05.

The bioethanol content at 5 days, pH 5.5, and 1% yeast concentration was 4.6%, while at 5 days, pH 4 and 0.5% were 2.2%. The high yeast concentration accelerates fermentation so that microorganisms can decompose glucose into ethanol maximally. This is because a higher yeast concentration increases the population of microorganisms that work in it, resulting in a greater level of bioethanol produced [41]. The pH variable in the fermentation process is crucial for yeast growth because yeast can only grow at certain pH conditions. Saccharomyces cerevisiae's optimal growth occurs in media with a pH of 4-5. However, the speed of the bioethanol fermentation process will decrease if the pH is below 3. Therefore, the level of bioethanol produced will be low if the pH is below 4 [45].

Table 6. Optimization of Maximum Bioethanol Content Expert Design

Fermentation time (days)	pН	Yeast Concentration (%)	Bioethanol Content (%)	Desirability
7	5,5	1,5	7,283	1,000

Table 6 shows the optimal results in response to bioethanol content of 7.283% when the operating conditions were 7 days of fermentation, pH 5.5, and yeast concentration of 1.5%, and the desirability value reached 1.000. The model's suitability for optimization is obtained when the desirability value is close to one.

No.	Raw material	Operating Conditions	Bioethanol Content Results	Ref
1.	Ulva reticulata	Fermentation time 6 days, pH 4.5	5.02%	[37]
2.	Mikroalga Chlorella pyrenoidosa	Fermentation time 3 days, pH 5	0.280%	[27]
3.	Alga Hijau <i>Spyrogyra</i> sp	Fermentation time 5 days, pH 4.5	0.281%	[40]
4.	Alga Merah Gracilaria verrucosa	Fermentation time 5 days, pH 7	0.77%	[39]
5.	<i>Ulva</i> sp.	Fermentation time 7 days, pH 5.5	7.55%	In this research

**Table 7.** Comparison of bioethanol levels with previous studies

Table 7 presents the results of earlier studies conducted under various operating conditions. From the table above, the lowest bioethanol content resulted from operating conditions, namely 3 days of fermentation with a pH of 0.280%. Meanwhile, the highest bioethanol content was achieved under operating conditions of 6 days of fermentation with a pH of 4.5, resulting in 5.02% bioethanol. However, judging from the operating conditions in Table 7, it is known that previous studies produced lower levels of bioethanol compared to this study. This can be due to differences in operating conditions used in the fermentation process.

#### 4. Conclusion

This research uses variables that include fermentation time (days), pH, and yeast concentration (%). This variable has a significant effect on the bioethanol content because it has a p-value <0.05. The results of the bioethanol content test are supported by the Analysis of Variance (ANOVA). The results show that the value of R2 is 0.9872. This study obtained the bioethanol content at 7.55% under operating conditions of 7 days of fermentation, pH of 5.5, and yeast concentration of 1.5%.

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