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The Effect of Time, pH, and Starter Concentration on Bioethanol Content in the Tobacco Stem Fermentation Process

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Abstract. The depletion of fossil fuels is occurring in various parts of the world while fuel energy needs continue to increase. This condition encourages the search for alternative fuels with high availability of raw materials. Bioethanol, an environmentally friendly renewable energy from biomass, can be a solution to replace fuel oil. Tobacco stems, with high cellulose and hemicellulose content, can be used as raw materials for bioethanol. This study aims to optimize bioethanol production from tobacco stems through the influence of fermentation time, pH, and starter concentration. The research method involves base pretreatment, hydrolysis, fermentation, and distillation, with the results analyzed using the Response Surface Methodology (RSM) approach and the Central Composite Design (CCD) model. The independent variables used during fermentation include fermentation time (72 – 168 hours), pH (4 – 5), and starter concentration (0.1% – 0.3% w/v). Based on the Analysis of Variance (ANOVA), the variables that significantly affect ethanol content are fermentation time and starter concentration. The results of the CCD analysis showed optimum conditions at a fermentation time of 120 hours, pH 4.5, and starter concentration of 0.2% (w/v), producing a bioethanol content of 23.007% (v/v). This study shows the potential of tobacco stems as a sustainable source of bioethanol.

Keywords: *bioethanol, fermentation, hydrolysis, tobacco stem, renewable energy*

1. Introduction

The need for fossil fuels in industry, transportation, and households increases yearly [1]. Fossil-based fuels are non-renewable fuels that can run out if used continuously [2]. Meanwhile, the demand for energy in various countries is growing. Even the Environmental Impact Assessment (EIA) estimates global energy demand will increase by 25% by 2050 [3]. Dependence on and depletion of fossil-based fuels has prompted researchers to look for

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alternative fuels with abundant availability [4]. Bioethanol is a renewable fuel that is adequate as a substitute for fossil fuels[5].

Bioethanol, with the chemical formula C_2H_5O, is a single-chain alcohol with an octane number of 108, does not evaporate quickly, has a low calorific value, and is flammable [6]. Bioethanol has advantages such as being biodegradable, non-toxic, has a higher oxygen content and octane number compared to other fuels, and is environmentally friendly because it can produce lower CO_2 gas [7]. Bioethanol has several categories that are differentiated based on the raw materials used for production, some of which are first-generation and second-generation [8]. First-generation bioethanol is a conventional biofuel made from raw materials from food crops such as barley, wheat, corn, and sugar cane [9]. First-generation bioethanol is unsuitable for commercial and large-scale production because raw materials are essential as Indonesia's main food commodity [10]. This is supported by the Republic of Indonesia Law No. 18 of 2012, which states that using food commodities as raw materials for energy sources is not permitted if it threatens food security [11]. Second-generation bioethanol comes from non-edible lignocellulosic biomass [12]. Second-generation bioethanol is appropriate for large-scale and commercial development because it comes from highly available biomass and does not compete with food needs [13].

One of the biomass that can potentially be used as raw material for bioethanol production is tobacco (*Nicotiana tabacum* L.) [14]. Tobacco biomass is abundantly available in Indonesia [15]. This is supported by data stating that in 2022, 96.6% of tobacco was produced through smallholder plantations in Indonesia, with a harvest of 225.7 thousand tons [16]. High tobacco production, not accompanied by optimal processing, can cause environmental pollution [17]. Lack of knowledge, innovation, and technology results in farmers not processing tobacco stalks but burning them directly or burying them in the environment [18]. Meanwhile, tobacco stems have a high cellulose and hemicellulose content, namely 56.10% and 22.44% so that they can be processed into products such as bioethanol [19].

Bioethanol is produced in three basic stages: pretreatment, hydrolysis, and fermentation [20]. Pretreatment is the initial step in breaking down lignocellulose to make it more accessible to polymers in biomass and reduce cellulose crystallinity, porosity, and surface area of the material [21]. Hydrolysis, as the second stage, aims to minimize cellulose and convert it into sugar to ferment it [22]. The primary sugars biomass hydrolysis produces are glucose and xylose [23]. The next stage, fermentation, is carried out to convert sugar from raw materials

into ethanol [24]. Fermentation is the third step in the second-generation ethanol production process [25]. In fermentation, sugar, glucose, and xylose are metabolized by microorganisms in the previous stage, resulting in metabolism, namely ethanol [26]. The fermentation stage of bioethanol production generally uses the help of yeast [27].

Yeast or khamir is a fungus that consists of one cell, does not form hyphae, and is included in the Ascomycotina fungus group [28]. Bread yeast can be used in fermentation because it is stable, not dangerous or toxic, and easy to obtain and maintain [29]. One of the bread yeast species, namely *Saccharomyces cerevisiae*, is the right choice for bioethanol production fermentation because it can produce large amounts of alcohol and has a tolerance for high alcohol levels (12% - 18% abv) [30]. The characteristics of *Saccharomyces cerevisiae* are that it is facultatively anaerobic, grows well at a temperature of 30 ^{o}C and pH 4.0-4.6, is resistant to high temperatures, and can tolerate a wide range of pH, so the process is less susceptible to infection [31]. In addition, this microbe has a very economical price and is easy to find compared to other types of yeast [32].

Several studies have been conducted on bioethanol from tobacco raw materials. Yuan et al. [33] produced tobacco-based biobutanol using the base and acid pretreatment method, enzymatic hydrolysis, fermentation with *Saccharomyces cerevisiae* to obtain an alkaline yield of 2.75 kg/10 kg of tobacco stem (72.7%), acid-catalyzed yield = 2.69 kg of ethanol/10 kg of tobacco stem (70.6%). Sophanodorn et al. [34] produced a tobacco-based bioethanol yield of 12.47 g/L using pretreatment and enzymatic hydrolysis, yeast culture, and fermentation using *Saccharomyces cerevisiae*. Saccharomyces cerevisiae's concentration affects the fermentation time length [35]. The longer the fermentation time, the higher the bioethanol content produced [36]. However, the bioethanol levels decrease when they reach the optimal point because the productivity of *Saccharomyces cerevisiae* decreases, and the nutrients start to run out [37]. The addition of yeast can affect the ethanol content. Guo et al. [38] conducted bioethanol production from tobacco with operating conditions of 5 grams of starter, 100 mL of 2% glucose solution, pH 4.8 sodium acetate buffer solution, obtained simultaneous saccharification and ethanol fermentation results of 106.6 mg/g ethanol, which increased by 138.0% compared to tobacco stems that were not given additional.

Based on this background, an innovation in making bioethanol with optimal levels and a cheap process is needed. Although research on making bioethanol has been conducted, there has never been a discussion on the effect of time, pH, and Starter concentration on bioethanol

levels simultaneously. These three variables need to be discussed regarding optimal conditions so that the bioethanol levels obtained are promising [39]. Therefore, this study will discuss "The Effect of Time, pH, and Starter Concentration on Bioethanol Levels in the Tobacco Stem Fermentation Process," with a focus on the fermentation process to determine the effect of time, pH, and starter concentration on tobacco stem fermentation. By knowing the operating conditions of these three variables, this research method is expected to be more efficient and economical, and the bioethanol levels obtained are more optimal.

2. Research Methods

2.1 Materials

The materials used in this research included alkaline hydrolysate, sulfuric acid (H_2SO_4, analytical grade, obtained from Merck), baker's yeast (*Saccharomyces cerevisiae*, food grade, from Sigma-Aldrich), urea ($(NH_2)_2CO$, analytical grade, from Merck), distilled water, and filter paper (Whatman Grade 1, from Sigma-Aldrich). Sample preparation began with tobacco stems collected from PTPN 10, Ajung, Jember Regency, which were subsequently processed for use in bioethanol production.

2.2 Pretreatment

The sample is dried tobacco stems cut into small pieces (3-4 cm) and dried in the sun for 3 days, then oven-dried at a temperature of 130 – 190°C for 20 minutes with an interval of 10 minutes [40]. The dried stems are ground using a blender until they become a fine powder, then sieved using a 120 mesh sieve to reduce the size and increase the surface area.

2.3 Hydrolysis

The sample used in the fermentation process was a base hydrolysate produced through an initial pretreatment. This pretreatment involved treating the sample with 6% NaOH at a temperature of 140°C and an agitation speed of 150 rpm for 1 hour. Following this, the base hydrolysis process was conducted under optimal conditions: using 2% NaOH solution, microwave power of 350 watts, and a treatment time of 15 minutes. This process yielded a base hydrolysate with a 7.40 mg/mL reducing sugar concentration.

In the fermentation stage, 20 bottles of base hydrolysate samples were prepared according to variable data generated by the Design Expert 11 Software and the Response Surface Methodology (RSM) Central Composite Design (CCD) model. The pH of the hydrolysate was adjusted to the range of 4-5 using diluted H2SO4. Then, baker's yeast was

added as the starter at concentrations of 0.1%–0.3% (w/v) along with urea ($(NH_2)_2CO$) at 2% of the hydrolysate volume to support fermentation.

2.4 Fermentation

Fermentation was carried out for 72–168 hours. The fermented hydrolysate sample is shown in Figure 2.1. After fermentation, the sample was distilled at 78°C to separate bioethanol. The results of the distillation process in the form of bioethanol were stored in vials for each experiment, as shown in Figure 2.2. The distillation results obtained were then analyzed for bioethanol content using an alcohol meter. After that, the bioethanol content was analyzed using an alcohol meter. In addition, to further characterize the obtained bioethanol, infrared spectroscopy was used to analyze its chemical composition.

Figure 1. Fermentation Hydrolysate Sample

Figure 2. Bioethanol From Tobacco Stems

2.5 Results Analysis

Analysis of the results was carried out with the stages of sample preparation, where pH was set according to the treatment, bread yeast was added, and mixing was performed. The fermentation process was carried out for 72 - 168 hours with starter concentrations varying from (0.1% to 0.3% w/v). Afterward, the distillation process was conducted to produce the distillate

product, and the bioethanol content was analyzed. The results of this study were analyzed using the Response Surface Methodology (RSM) approach with the Central Composite Design (CCD) model. The Derringer method was applied to optimize the experimental results, determining the optimal conditions for fermentation time, pH, and starter concentration to maximize bioethanol content while reducing costs and experimental time.

2.6 Data Analysis

This study was conducted in 20 runs with variations in data obtained from the Design Expert 11 Software, adjusted to the Response Surface Methodology (RSM) approach of the Central Composite Design (CCD) model, as seen in Table 1.

Table 1. Fermentation Data Variation

Standard Deviation	Run	Factor 1	Factor 2	Factor 3
		pH	Time (hour)	Concentration (%)
5	1	4.00	72.00	0.30
17	2	4.50	120.00	0.20
7	3	4.00	168.00	0.30
12	4	4.50	200.72	0.20
9	5	3.60	120.00	0.20
15	6	4.50	120.00	0.20
4	7	5.00	168.00	0.10
13	8	4.50	120.00	0.03
11	9	4.50	39.27	0.20
16	10	4.50	120.00	0.20
3	11	4.00	168.00	0.10
19	12	4.50	120.00	0.20
2	13	5.00	72.00	0.10
8	14	5.00	168.00	0.30
14	15	4.50	120.00	0.36
6	16	5.00	72.00	0.30
10	17	5.35	120	0.20
18	18	4.50	120	0.20
1	19	4.00	72	0.10
20	20	4.50	120	0.20

Actual Data Analysis of ethanol content from sample fermentation variation data will be calculated for accuracy using Mean Absolute Percentage Error (MAPE), which measures the prediction error percentage. The formula used can be seen in Equation 1:

$$MAPE = \sum_{t=1}^{n} \left| \frac{y - \hat{y}}{y} \right| \times 100\% \quad \text{.....(1)}$$

Each formula element has the description n as the amount of data, y as the actual result value, and ŷ as the predicted result value. MAE measures the average prediction error on the same scale as the observed variable. The lower the MAE value, the better the model's prediction performance [41].

3. Result and Discussion

3.1 Analysis of Variance (ANOVA) Bioethanol Content

Ethanol content test data were processed using ANOVA with Software Design Expert 11 software, following the Response Surface Methodology (RSM) approach of the Central Composite Design (CCD) model. This analysis aims to determine the effect of independent variables (pH, time, and concentration) on the dependent variable (ethanol content). Testing was carried out randomly on 3 independent and 1 dependent variable with 20 runs. The results of the ANOVA analysis are shown in Table 2.

Table 2. ANOVA Results of Ethanol Content

Source	*Sum of Squares*	*df*	*Mean Square*	*F-value*	*P-value*	
Model	25.60	9	2.84	5.32	0.0076	*significant*
A-pH	0.0019	1	0.0019	0.0035	0.9542	
B-Time	13.08	1	13.08	24.48	0.0006	
C-Concentration	3.59	1	3.59	6.72	0.0269	
AB	0.1250	1	0.1250	0.2340	0.6390	
AC	0.0000	1	0.0000	0.0000	1.0000	
BC	3.13	1	3.13	5.85	0.0361	
A^2	1.37	1	1.37	2.56	0.1404	
B^2	4.74	1	4.74	8.87	0.0138	
C^2	0.0268	1	0.0268	0.0502	0.8272	
Residual	5.34	10	0.5342			
Lack of Fit	4.34	5	0.8684	4.34	0.0665	*not significant*
Pure Error	1.0000	5	0.2000			
Cor Total	30.94	19				

ANOVA data in Table 2 shows a Lack of Fit value of 0.0665. This value is more significant than α, and hypothesis H1 is accepted, indicating the suitability of the selected model. If the P-value in Lack of Fit is minor than α, the data is significant and suggests the model is unsuitable. Conversely, if the P-value is more significant than α, the data is insignificant, indicating the model is suitable [42]. After the results of the ANOVA test of ethanol content were obtained, the optimum response variable value was calculated using the Derringer method. This method determines the optimal conditions for the optimum value of ethanol content from the variables of time, pH, and starter concentration. The Response Surface Methodology (RSM) optimization in Table 3 shows the optimum value.

Table 3. Derringer's Desirability

pH	Time	Starter Concentration	Etanol Content	Desirability
4.5	120	0.2	23	1

Table 3 shows the Desirability (D) value reaching 1 at optimum conditions with pH 4,5, 120 hours, and a starter concentration of 0.2%, producing ethanol content of 23%. Next, the Cook's Distance Plot is carried out to determine the influence of data points in the least squares regression, check validation, and show the optimal experimental design space. The Cook's Distance vs Response Plot plot is shown in Figure 1 [43], and the results show that most values are between 0 and 1.

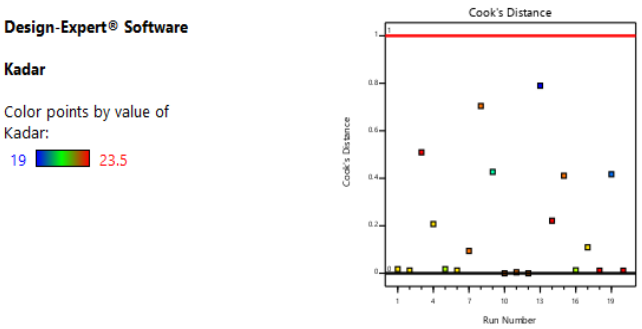

Figure 3. Cook's Distance Plot

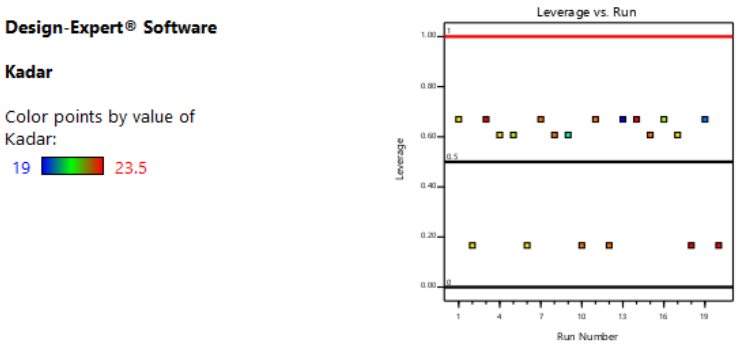

Figure 4. Leverage vs. Run Plot

The Leverage Test measures the influence of each point on model fit, with a leverage of 1.00 indicating a complete fit [43]. The average leverage is the number of terms in the model divided by the number of trials, shown in Figure 2. ANOVA analysis also presents the mathematical equations between the independent and dependent variables. Two equations from Software Design Expert 11, namely the coded and the actual equations, are used to predict the dependent variable [44]. The coded equation (equation 2) shows the relative impact of factors, while the actual equation (equation 3) is used to predict the response at a specific factor level.

Equation 2 (Coded):

$$Y = 23{,}01 - 0{,}0117A + 0{,}9785B + 0{,}5126C + 0{,}1250AB + 0{,}0000AC - 0{,}6250BC - 0{,}3083A^2 - 0{,}5735B^2 - 0{,}0432C^2 \quad (2)$$

Equation 3 (Actual):

$$Y = -9{,}40335 + 10{,}45124A + 0{,}082728B + 22{,}47685C + 0{,}005208AB - 6{,}55977E\text{-}14AC - 0{,}130208BC - 1{,}23328A^2 - 0{,}000249B^2 - 4{,}31554C^2 \quad (3)$$

3.2 Effect of Variables on Ethanol Content

Three independent variables were used in fermentation: 72-168 hours, pH 4-5, and starter concentration 0.1%-0.3%. The actual data of ethanol content from running 1-20 are shown in Table 4.

Table 4. Actual Data on Ethanol Content

Run	Time (hour)	pH	Starter Concentration (%)	Etanol Content (%)
1	39.3	4.5	0.2	20.5
2	72	4	0.3	22.5
3	72	5	0.1	19.0
4	72	5	0.3	22.0
5	72	4	0.1	19.5
6	120	4.5	0.2	22.5
7	120	3.7	0.2	22.0
8	120	4.5	0.2	22.5
9	120	4.5	0.03	23.0
10	120	4.5	0.2	23.0
11	120	4.5	0.2	23.0
12	120	4.5	0.4	23.0
13	120	5.3	0.2	22.5
14	120	4.5	0.2	23.5
15	120	4.5	0.2	23.5
16	168	5	0.1	23.0
17	168	4	0.1	23.0
18	168	5	0.3	23.5
19	168	4	0.3	23.5
20	200.8	4.5	0.2	22.5

Table 4 shows that increasing fermentation time and starter concentration increases ethanol content. Ethanol content (%v/v) can be known after the distillation results are analyzed for bioethanol content using an alcohol meter. Then, a content test will be carried out by running 20 times. Then, the bioethanol content will be obtained, as shown in Table 4. At a fermentation time of 200,726 hours, ethanol content decreased because nutrient concentrations decreased and microbial growth slowed [45]. The optimal activity of *Saccharomyces cerevisiae* was achieved at the right fermentation time, but fermentation that was too long depleted nutrients

and reduced the ethanol produced [46]. In ethanol fermentation, by-products such as lactic acid, acetic acid, and glycerol were produced, which could affect the final ethanol yield. The actual ethanol content data from Table 4 will be calculated for accuracy using the Mean Absolute Percentage Error (MAPE) using the formula in Equation 1. The results are obtained from the difference between the actual and predicted data divided by the actual data and expressed in absolute value so that the MAPE is always positive. The MAPE results that match the actual and predicted values from the RSM CCD method approach are shown in Table 5.

Table 5. Mean Absolute Percentage Error (MAPE) Results

Run	Actual Value	Prediction Value	Error Value |(y - ŷ)/y|
1	20.5	19.74	0.037073171
2	22.5	22.38	0.005333333
3	19	19.83	0.043684211
4	22	22.1	0.004545455
5	19.5	20.1	0.030769231
6	22.5	23.01	0.022666667
7	22	22.15	0.006818182
8	22.5	23.01	0.022666667
9	23	22.02	0.042608696
10	23	23.01	0.000434783
11	23	23.01	0.000434783
12	23	23.75	0.032608696
13	22.5	22.11	0.017333333
14	23.5	23.01	0.020851064
15	23.5	23.01	0.020851064
16	23	23.29	0.012608696
17	23	23.06	0.002608696
18	23.5	23.06	0.018723404
19	23.5	22.83	0.028510638
20	22.5	23.03	0.023555556
		MAPE Value	**0.06058458**
		MAPE Value (%)	**6%**

Table 5 shows that the MAPE value in this study is 6%. The calculation of MAPE involves subtracting the actual and predicted values, the absolute value of the difference, then dividing it by the actual value for each run and summing the results. The lower the MAPE value, the better the ability of the prediction model, with MAPE having a range of values to measure the accuracy of the prediction model [47].

3.3 3D Modeling Visualization

3.3.1 Effect of Time and pH on Bioethanol Content

Figure 3 shows the three-dimensional Response Surface curve for time and pH factors on bioethanol content, with five colored regions indicating bioethanol content. The deeper red color indicates higher bioethanol content. The contour plot's circular line shows the bioethanol

content's quadratic response value, indicating optimal conditions. Although pH has an interaction with time, the interaction is not significant on bioethanol content (P = 0.6390 > α = 0.05), as seen in Table 1, where the effect of time on bioethanol content is significant (P = 0.0006). Still, the effect of pH is not (P = 0.9542).

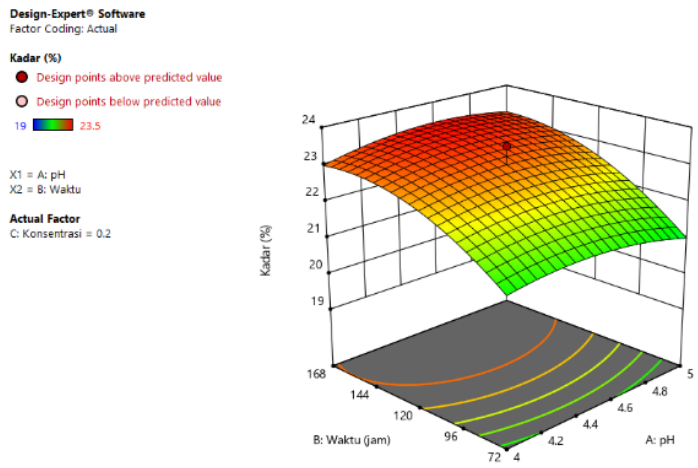

Figure 3. Effect Curve of Time and pH on Bioethanol Content

Supportive environmental conditions, including pH, influence high bioethanol levels. *Saccharomyces cerevisiae* grows optimally at pH 4-6, where pH below 3 reduces the speed of alcohol fermentation [48]. At a pH of 4.0-4.5, fermentation and growth of baker's yeast are optimal, while pH below 4.0 produces low ethanol levels. In addition, the length of fermentation time also affects the bioethanol content. Longer fermentation times increase ethanol levels, but after a specific time, nutrients are depleted, and the growth of microorganisms reaches the stationary phase, reducing ethanol production [49]. For example, at a fermentation time of 120 hours with a pH of 4.5, the ethanol content reaches 23.5%. Still, at a fermentation time of 200.726 hours, the ethanol content drops to 22.5% due to lack of nutrients and cessation of microorganism growth.

3.3.2 The Effect of Starter Concentration and pH on Bioethanol Content

Figure 4 shows the three-dimensional Response Surface curve for starter concentration and pH factors on bioethanol content, with five colored regions depicting bioethanol content. The deeper red color indicates higher bioethanol content. The contour plot's circular line shows the bioethanol content's quadratic response value, indicating optimal conditions. Although pH interacts with starter concentration, the interaction is insignificant on bioethanol content (P =

1.0000 > α = 0.05), as seen in Table 1. The effect of starter concentration on bioethanol content is significant (P = 0.0269), while pH's effect is insignificant (P = 0.9542).

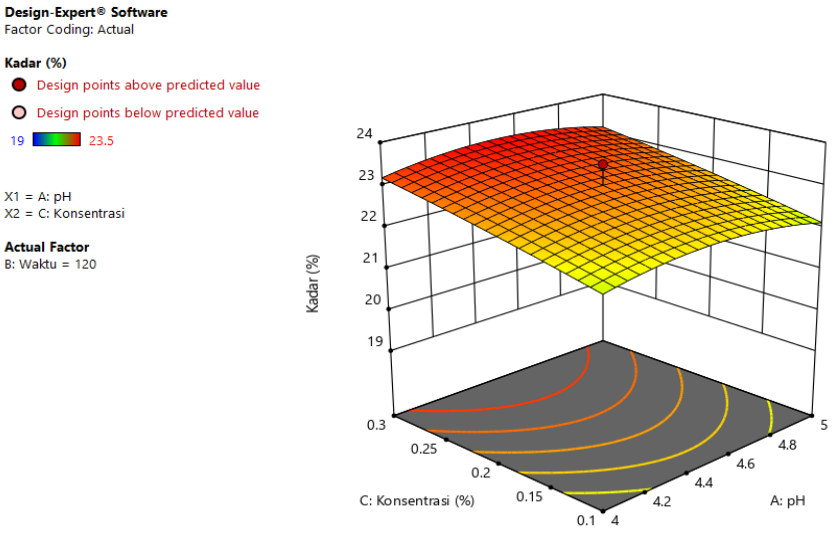

Figure 4. Effect Curve of Starter Concentration and pH on Bioethanol Content

Table 4 shows that the bioethanol content with pH 4 and a starter concentration of 0.1% at a fermentation time of 72 hours produces 19.5%, while pH 4.5 with a starter concentration of 0.368179% and a fermentation time of 120 hours produces 23%. Increasing the starter concentration accelerates fermentation due to the higher substrate, but pH 5.3409 with a starter concentration of 0.2% only produces 22.5% bioethanol because *Saccharomyces cerevisiae* is not optimal at that pH. At pH 5, glucose tends to be low, resulting in yeast being unable to decompose into ethanol and producing by-products such as acetic acid [45].

3.3. The Effect of Starter Concentration and Time on Bioethanol Content

Figure 5 shows the three-dimensional Response Surface curve for starter concentration and fermentation time factors on bioethanol content, with five colored regions depicting bioethanol content. The deeper red color indicates higher bioethanol content. The contour plot's circular line shows the bioethanol content's quadratic response value, indicating optimal conditions. The effect of fermentation time on bioethanol content was significant (P = 0.0006) and starter concentration (P = 0.0269), as seen in Table 1. There was a significant interaction between fermentation time and starter concentration on bioethanol content (P = 0.0361 <α = 0.05), which was observed in Figure 5.

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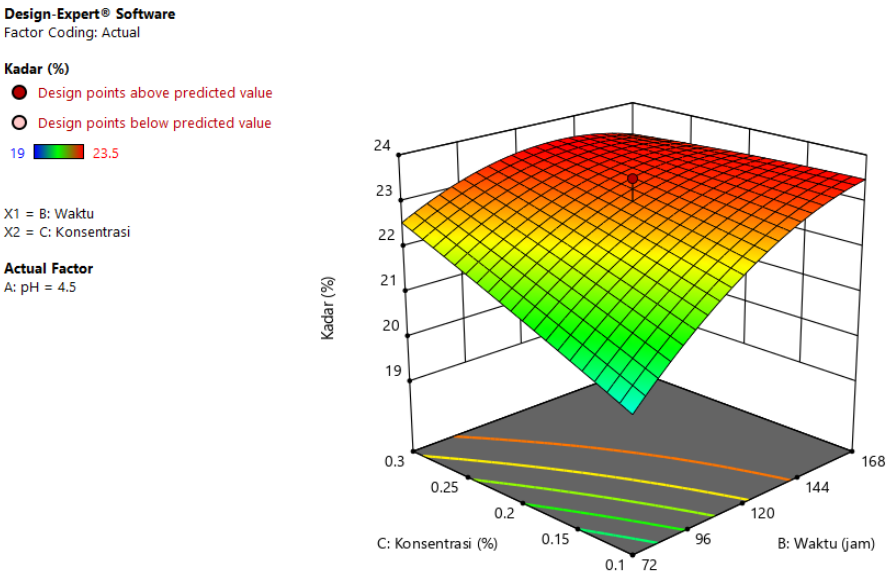

Figure 5. Effect Curve of Starter Concentration and Time on Bioethanol Content

The data in Table 4 shows that the longer the fermentation time and the higher the starter concentration, the higher the ethanol content. Increasing the starter concentration accelerates fermentation and increases the straightforward sugar content, allowing baker's yeast (*Saccharomyces cerevisiae*) to work optimally in producing ethanol [50]. However, the fermentation time has a maximum limit of 120 hours, after which the yeast activity decreases, especially at pH 5, where fermentation reaches the death phase. The decrease in ethanol levels is also caused by the depletion of the substrate and the reaction of changing to acetic acid. [49]. This study used a separate fermentation method than SSF because it used the results of alkaline pretreatment. Previous studies showed differences, where the highest ethanol content occurred in fermentation for 168 hours, but this study found the optimum at 120 hours. The results also showed that the optimal pH for *Saccharomyces cerevisiae* was 4.5, and the best starter concentration was 0.2%. Although there were differences with previous studies, this study recorded higher ethanol levels.

3.4 Parity Plot Analysis

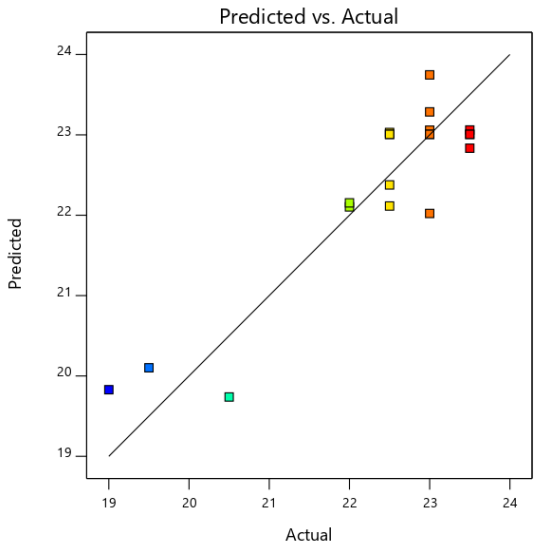

Figure 6. Predicted vs Actual Graph of Ethanol Levels

The straight lines on the graph are the predicted data, while the dots represent the actual data from each run. In Figure 6, the expected and actual ethanol content graphs show fairly good similarity with moderate scattering, indicating a significant approximation in the model analysis.

4. Conclusions

Based on this study, bioethanol content is mainly influenced by fermentation time and starter concentration, with optimal conditions at 120 hours of fermentation time, pH 4.5, and starter concentration of 0.2%. Fermentation time and starter concentration significantly affect ethanol yield, with an interactive effect between the two variables. pH also plays a role, although its impact is more minor. This study shows the potential of tobacco stems as a sustainable and renewable source of bioethanol, with a maximum ethanol content of 23.007% under optimized conditions. This information is essential for optimizing bioethanol production.

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