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Optimization of bioethanol production from papaya waste through fermentation using response surface methodology (RSM)

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Abstract. There is a growing pressure for the development of sustainable and environmental-friendly source of energy such as ethanol that could substitute the depleting fossil fuels. Papaya waste including papaya seed and papaya peel is one of the main fruit wastes in Southeast Asia which has great potential to be utilized as substrate for bioethanol production. In this study, papaya waste was fermented to produce bioethanol using *Saccharomyces Cerevisiae*. The effect of pH, temperature, and incubation time on bioethanol production was studied within the range of 3.0-6.0, 25-45°C and 24-96 h, respectively. These parameters were optimized using Response Surface Methodology (RSM) based Box-Behnken Design (BBD). It was found that a maximum ethanol concentration of 0.2224 g/ml was obtained from papaya waste at pH 4.5, 45°C and 24 hours. The significance of the parameters increased from incubation time, pH to temperature.

Introduction

Energy is a necessary component in life. According to the bp Statistical Review of World Energy 2020, oil (33.1%), gas (24.2%) and coal (27%) account for most of the global energy consumption, all of which are non-renewable energy sources [1]. The excessive consumption of fossil fuels has not only escalated the fuel price, but significantly elevated the environmental issues and public health concerns [2]. The issue has prompted the focus and development of biofuel technology as an alternative renewable energy, which has been developing rapidly since year 2000 [3]. Bioethanol which is mainly produced through fermentation of plant-based biomass is one of the environmentally friendly alternatives for the current energy issue.

To date, majority of bioethanol is still made from starch- or sugar-based crops which are known as first-generation biofuels such as potato and sugarcane due to high yield. However, increase in bioethanol production using first-generation biofuels has raised concerns about food security and fertile land. Thus, the exploration for second-generation biomass has mostly concentrated on cheap and abundant lignocellulosic municipal waste [4-6].

Papaya is one of the 15 most produced and marketed fruits in the world with a global production of 13.2 million metric tons in 2016 [7]. Papaya waste consisting of peels and seeds takes up approximately 20-25% of the fruit weight is one of the most abundant fruit wastes found in wet markets, main household solid municipal waste and processing industry waste [8-9]. These wastes are usually discarded in landfills or dumping site that result in land and water contamination and greenhouse gas emission [8]. Therefore, by utilizing papaya waste combining papaya peel and papaya seed to produce bioethanol through fermentation, several problems aforementioned could be overcome. However, it was found that there are limited studies on the capability of papaya waste, an abundant lignocellulosic solid food waste in bioethanol production through separated hydrolysis and fermentation method (SHF). Besides, papaya peel and papaya seed are believed to

be a promising source for bioethanol production based on its fibre composition, with high cellulose (24.6%, 25.4%) and hemicellulose content (20.4%, 3.4%) which can be converted into simple sugars for fermentation through hydrolysis, and relatively low lignin content (2.7%, 1.9%) [10,11] which acts as a barrier for the digestion of hemicellulose and cellulose into pentoses and hexoses [12]. Since the lignin content for both papaya peel and papaya seed are low, the pretreatment process can be carried out together with different pretreatment steps. Moreover, native microorganism existing on papaya waste during the decay process are proven to aid the bioethanol fermentation [9].

Bioethanol production from lignocellulosic waste is still not competitive to fossil fuels till date. Most of the researchers focus on the pre-treatment step to extract glucose in hydrolysis step for bioethanol production, but barely on fermentation process. A research gap for this topic was found to be the lack of study on the effects of fermentation parameters for lignocellulosic waste to maximize the yield of bioethanol. The variables in pH, temperature and incubation time highly affect the yield.

A research has been done to produce bioethanol using papaya peels through fermentation using the one-variable-at-a-time (OVAT) approach to optimize yield [13]. OVAT is often used in bioethanol production experiments. It was found that the results obtained using OVAT is not fully reliable as it does not take the interaction among the listed parameters into account [14]. Effective optimization of several independent fermentation parameters such as pH, temperature and incubation time using effective optimization tools are significant in maximizing the yield of ethanol.

In this study, papaya seed and papaya peel were used as the substrate to produce bioethanol through separated hydrolysis and fermentation method. The effect of fermentation parameters on the bioethanol yield was studied and Response Surface Methodology (RSM) was employed to optimize the bioethanol yield. The ethanol concentration product produced from the same amount of papaya reflects the yield of bioethanol.

Materials and Methods

Materials. Fresh papaya fruits were purchased from the local market in Ipoh, Perak, Malaysia. Only papaya waste comprising papaya seed and papaya peel was used in this study. The fermenting baker's yeast (*Saccharomyces cerevisiae*) used in the study was purchased from a food manufacturer in Malaysia and stored in a refrigerator until further use.

Design of Experiment. RSM based Box-Behnken Design (BBD) was utilized to optimize the fermentation pH, temperature, and incubation time for bioethanol production from papaya waste. A three-factor three-level BBD with 17 experimental runs were carried out. The design of experiment (DOE) was generated by Design Expert Software Version 8. The numerical factors were pH, temperature (^{o}C), and incubation time (h), while the response variable was bioethanol concentration (g/mL). The range of study for pH, temperature, and incubation time were 3.0-6.0, 25-45^{o}C and 24-96 h, respectively.

Experimental Methodology

The experimental methodology was adapted from M. R. A. Ghani and P. C. Oh [19].

Preparation of Substrate. 400 g of papaya peel and 400 g of papaya seed were collected and washed using water. Then, the waste was dried in oven at 70^{o}C for 24 h, and grinded into powder.

Acid Hydrolysis. 15 g of papaya waste was hydrolysed in 100 mL of 0.5 M sulphuric acid. The opening of the flask was covered with cotton plug and held at 121^{o}C for 15 mins in an autoclave (Hirayama) for sterilization. The hydrolysate was collected using vacuum filtration through coarse filter paper. The glucose concentration of hydrolysate was analysed using refractometer (Atago).

Propagation of Yeast Cell. 7 g of dried yeast powder was added into capped bottle containing 50 mL glucose yeast extract (GYE) and placed in an incubator water bath shaker (Julabo) at 30^{o}C

and 100 rpm. After 48 h, 10% of the sample was transferred into a 1 L capped bottle with 500 mL GYE to allow cell multiplication. Incubation was repeated at 35ºC and 100 rpm for 24 h. The cells were then transferred to sterilized 50 mL centrifuge tubes and centrifuged at 4°C and 10000 rpm for 10 mins using Centrifuge (Labogene). The supernatant (solution) was subsequently collected for fermentation.

Fermentation. The hydrolysate was neutralized using 1.0 M sodium hydroxide (NaOH), with 0.3% (w/v) yeast extract, and 0.2% (w/v) peptone. The flask containing the mixture was wrapped with foil and heated at 80ºC and 250 rpm for 30 mins in a water bath. The fermentation was performed at pH, temperature and incubation time based on the DOE obtained from Design Expert software. The pH of the mixture was maintained using 0.5 M H_2SO_4 and 1.0 M NaOH. After each fermentation cycle, the samples were analysed for ethanol concentration using refractometer.

Results and Discussion

Pretreatment. Six samples of glucose solutions with different concentrations (0, 25, 50, 75, 100 w/v%) were used as standard calibration to find the glucose content of the papaya waste hydrolysate. The calibration curve of glucose concentration vs refractive index can be represented by Eq. 1.

$$Y = 0.00008x + 1.3381 \quad (1)$$

The average refractive index for papaya waste hydrolysate is 1.3492 after 3 trial tuns, hence the average glucose concentration can be determined as 135.25 g/L by applying Eq. 1. Since glucose is simple sugar ready for fermentation, hence the higher the glucose content in the solution, the higher the ethanol amount that can be produced through fermentation, the higher the yield of bioethanol.

Ethanol Concentration. Five ethanol solutions with different ratio of 95% ethanol to distilled water were used as standard calibration to find the response ethanol concentration for each fermentation run. The calibration curve of ethanol concentration vs refractive index can be represented by Eq. 2.

$$Y = -(8 \times 10^{-11})\, x^3 + (5 \times 10^{-8})\, x^2 + (5 \times 10^{-5})\, x + 1.3329 \quad (2)$$

Model Fitting and Analysis of Variance (ANOVA). After completing 17 runs of experiment based on the DOE using BBD, the response (i.e. ethanol concentration) is tabulated in Table 1. The ANOVA with backward elimination regression was used to determine the significant model terms by reducing insignificant terms and is shown in Table 2. Based on Table 2, the Model *F*-value of 32.44 implies that the model is significant after reduction and has only 0.01% probability that the large *F*-value could occur due to noise. The low model *p*-value was a positive indication that shows the predictability of the model has over 95% confidence level. After backward elimination regression was done, only significant model terms with Prob>*F* less than 0.05 remained. Thus, linear factors A, B and C and quadratic factors A^2 and B^2 were maintained. The effect of pH and temperature were more significant than time on yield. Quadratic effect of time was not taken into considerations, while the quadratic effect of temperature was greater than that of pH. The Lack of Fit *F*-value of 4.23 implies that there is a 9.09% chance that a value this large could occur due to noise. Lack of Fit *p*-value of 0.0909 was not significant, which indicates that the model fits well and can be used to predict the response accurately.

Table 1. Experimental Response According to Experimental Design

Run	Factor 1 A: pH	Factor 2 B: Temperature [℃]	Factor 3 C: Time [h]	Response Ethanol Concentration [g/mL]
1	4.5	35	64	0.1954
2	6.0	25	64	0.1756
3	4.5	35	64	0.1999
4	3.0	35	96	0.1973
5	4.5	35	64	0.1974
6	4.5	45	96	0.2174
7	4.5	35	64	0.2016
8	4.5	25	24	0.1930
9	6.0	35	96	0.1754
10	4.5	45	24	0.2240
11	6.0	35	24	0.1920
12	3.0	25	64	0.1951
13	4.5	25	96	0.1976
14	3.0	45	64	0.2247
15	4.5	35	64	0.1971
16	3.0	35	24	0.2101
17	6.0	45	64	0.2118

Table 2. ANOVA for Variables After Backward Elimination Regression

Source	Sum of Squares	df	Mean Square	*F* Value	*p* value Prob > *F*
Model	2.92×10^{-3}	5	5.84×10^{-4}	32.44	< 0.0001[a]
A-pH	6.57×10^{-4}	1	6.57×10^{-4}	36.48	< 0.0001[a]
B-Temperature	1.70×10^{-3}	1	1.70×10^{-3}	94.44	< 0.0001[a]
C-Time	1.25×10^{-4}	1	1.25×10^{-4}	6.95	0.0231[a]
A^2	1.22×10^{-4}	1	1.22×10^{-4}	6.77	0.0246[a]
B^2	3.36×10^{-4}	1	3.36×10^{-4}	18.67	0.0012[a]
Residual	1.98×10^{-4}	11	1.80×10^{-5}		
Lack of Fit	1.74×10^{-4}	7	2.49×10^{-5}	4.23	0.0909[b]
Pure Error	2.36×10^{-5}	4	5.89×10^{-6}		
Cor Total	3.12×10^{-3}	16			

[a] significant
[b] not significant

The quadratic equations for ethanol yield (Y) in terms of coded factors and actual factors are given in Eq. 3 and Eq. 4, respectively.

$$Y = 0.200 - (9.059\times10^{-3})\,A + 0.015\,B - (3.943\times10^{-3})\,C - (5.373\times10^{-3})\,A^2 + (8.921\times10^{-3})\,B^2 \quad (3)$$

$$Y = 0.243 + 0.016\,\text{pH} - (4.787\times10^{-3})\,\text{Temperature} - (1.095\times10^{-4})\,\text{Time} - (2.388\times10^{-3})\,\text{pH}^2 + (8.921\times10^{-5})\,\text{Temperature}^2 \quad (4)$$

The model has a high R^2 value of 0.9365 which shows that the model is robust, and the experimental data can be well-fitted. Besides, the model has a low coefficient of variance (C.V. %) of 2.1179 (<10%) indicates that the model has high reproducibility.

Mutual Effects of Parameters. The correlation between each independent factor and their effects on the corresponding ethanol yield was plotted graphically and analyzed through Fig. 1, Fig. 2, and Fig. 3.

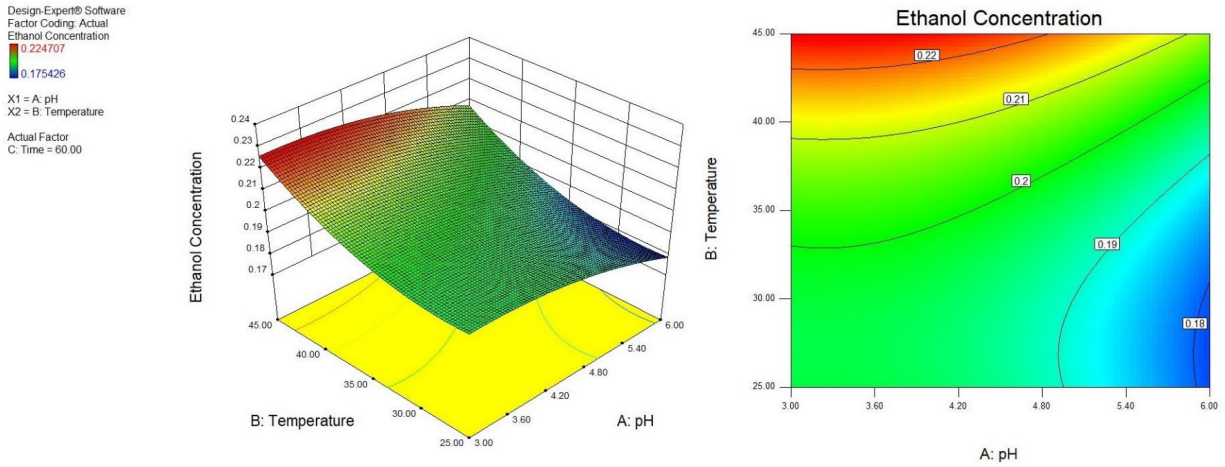

Fig. 1. (a) 3D Surface Plot (b) Contour Plot of Effect of pH & Temperature on Ethanol Yield

Fig. 1 illustrates the effect of fermentation pH and temperature on the ethanol concentration. These two parameters were varied in the range of pH 3.0 – 6.0, and 25 – 45°C, while the incubation time was kept constant at 60 h. It was observed that pH and temperature have great effect on the ethanol concentration. This was further proven by the ANOVA which shows both pH and temperature have *p*-values <0.0001.

The ethanol concentration decreased with increasing pH and decreasing temperature, which is in agreement with previous study by Abdulla et al. [13]. The maximum ethanol concentration of 0.2247 g/ml was achieved at 45°C with pH 3.0. The minimum ethanol concentration of 0.1754 g/ml was obtained at 25°C with 6.0. According to Abdulla et al. [13] , the yield of bioethanol will have an increasing trend until pH 5, which will then decrease beyond pH 5 [13, 14]. Hence, the response obtained partially agreed with the previous studies, where pH above 5 did not support fermentation process effectively. The analysis of the 3D surface plot and contour plot proved that the optimum fermentation temperature was at 45°C with mild acidic pH from 3.0 to 4.8 where the ethanol concentration was at its peak. Although pH generally has a greater effect than temperature on the production of bioethanol, in this range of study, temperature was observed to have a greater effect on the ethanol production from papaya waste since pH value within the range of 3.0 to 5.5 was suggested to be optimum for bioethanol production [14, 15] which is in line with what was observed from this work.

From the response obtained, it can be concluded that the higher the temperature, the higher the ethanol concentration produced through SHF using *S. cerevisiae*. This result was supported by G. Reed and H.J. Peppler [16] who suggested that the fermentation rate of yeast will grow with a factor of 1.5 to 2 for every 10°C increase in temperature, up until the temperature reaches 45°C [16]. Increase in fermentation temperature beyond 45°C will cause thermal deactivation of yeast and decrease the rate of ethanol production [14, 17]. Next, the increase in pH value decreased the production of bioethanol as fermentation process for biomass is favoured in slightly acidic condition. The optimum pH value to produce bioethanol from papaya waste through SHF was 3.0 to 4.8. The ethanol concentration was observed to be low at high pH value of 6.0 as this affect the shape of protein in enzymes by disturbing the bonds, which lowers the metabolic activity of the microorganisms [14]. In addition, high pH value also favoured the production of acid instead of ethanol during fermentation, hence lowered the ethanol production [17].

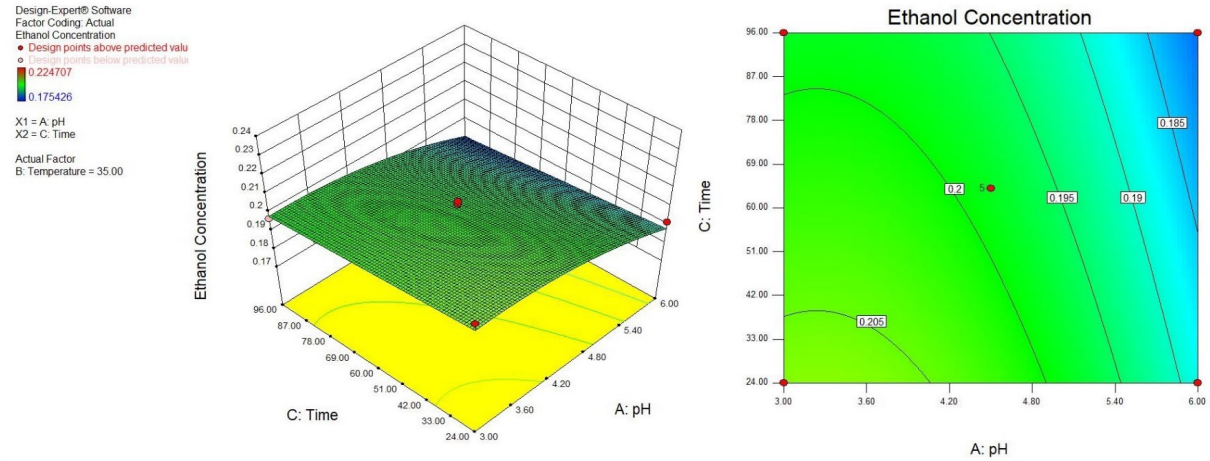

Fig. 2. (a) 3D Surface Plot (b) Contour Plot of Effect of pH & Time on Ethanol Yield

Fig. 2 shows the influence of fermentation pH and incubation time over the ethanol concentration. These two parameters were varied in the range of pH 3.0 – 6.0 and 24 – 96 h, at constant temperature of 35ºC. Based on Fig. 2, pH showed a greater effect on the ethanol concentration than temperature, which was proven by the low *p*-value of pH (<0.0001) compared to time (0.0231). The lower the *p*-value, the bigger the influence of the variable over the response of the model.

The ethanol concentration decreased with increasing pH which agreed with previous findings [15, 20]. On the other hand, the ethanol production decreased with increasing time beyond 24 h, which contradicted with previous studies, implying that optimum incubation time ranged from 36 to 72 h [6, 14, 15, 21]. At constant temperature of 35ºC, the maximum ethanol concentration of 0.2101 g/ml was achieved at 24 h with pH 3.0. The minimum ethanol concentration of 0.1754 g/ml was obtained at 96 h with pH 6.0. The analysis of Fig. 2 proves that the optimum fermentation time was at 24 h within pH range of 3.0 to 4.8, where the ethanol concentration was at its peak.

Ethanol concentration decreased with increasing pH as acidic environment favours the growth of yeast during fermentation. The ethanol production will increase with incubation time due to quick consumption of glucose by yeast cell if bioethanol is produced in bulk. Then, the production will remain constant at certain time, due to decrease in reducing sugar consumption by yeast cell [15]. However, prolonged fermentation may not be favoured as side products produced during the process may inhibit the yeast cell from further producing bioethanol at optimum rate [14]. In this study, the optimum incubation time was at 24 h instead of longer incubation time range from 36 to 72 h, which was suggested by other researchers [5, 14, 19, 22] . The contradiction may be due to the sample size of the lab-scale experiment was relatively small, where the available reducing sugar in the incubator was depleted and prolonged fermentation produced side products such as furfurals, via the degradation of the monosaccharides that further inhibited the ethanol fermentation process [19].

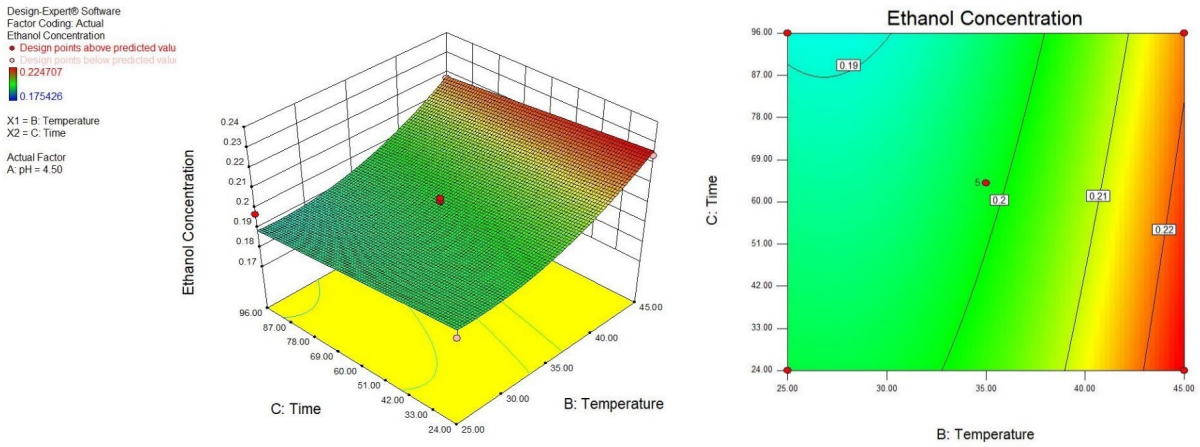

Fig. 3. (a) 3D Surface Plot (b) Contour Plot of Effect of Temperature & Time on Ethanol Yield

Fig. 3 illustrates the effect of fermentation temperature and incubation time over the ethanol concentration. These parameters were varied in the range of 25 – 45ºC and 24 – 96 h, while pH was kept constant at 4.50. Based on Fig. 3, temperature has a greater effect on the ethanol concentration than time which was in agreement with a several previous researches which have proven that temperature has a greater influence on ethanol yield [14, 20]. This was also validated by the *p*-value in ANOVA where temperature and time had values of <0.0001 and 0.0231, respectively. The ethanol concentration was observed to increase with increasing temperature and decreasing time. At constant pH of 4.5, the maximum ethanol concentration of 0.2240 g/ml was achieved at 24 h and 45ºC. The minimum ethanol concentration was obtained at 96 h and 25ºC. The analysis of Fig. 3 proved that the optimum fermentation time was at 24 h with temperature of 45ºC where the ethanol concentration was at its peak. The glucose available in the prepared solution was fully utilized by *S. Cerevisiae* at this condition.

Response Surface Optimization and Results Verification. Numerical optimization was used to determine the maximum ethanol concentration at the optimum condition of pH, temperature, and incubation time. The highest desirability shows the highest ethanol production at the optimum condition of all three varied parameters. Table 3 shows the most desirable operating condition suggested by RSM to produce bioethanol from papaya waste through fermentation.

Table 3. Numerical Optimization of Variables for Bioethanol Production

Run	pH	Temperature (°C)	Time (h)	Predicted Concentration (g/mL)	Desirability
1	4.50	45.00	24.00	0.2264	1[a]
2	3.00	45.00	64.00	0.2256	1
3	3.36	45.00	92.27	0.2226	0.987
4	5.92	45.00	24.00	0.2129	0.861

[a] selected

The higher the desirability, the higher the predicted ethanol concentration. Three confirmation experimental runs were carried out at pH 4.5, temperature of 45°C and incubation time of 24 h. The obtained responses for the confirmation runs are shown in Table 4.

Table 4. Verification of Response Obtained for Confirmation Experimental Run

Run	pH	Temperature (°C)	Time (h)	Predicted Concentration (g/mL)	Actual Concentration (g/mL)	Std Dev. (%)
1					0.2167	4.25
2	4.5	45.00	24.00	0.2263	0.2171	4.08
3					0.2224	1.75

Based on Table 4, the actual concentration of ethanol produced by the three confirmation runs was close with the predicted concentration with standard deviations below 5%. This result is satisfying and denotes that the numerical optimization is reliable to produce bioethanol with high concentration yield.

Conclusion

In this project, papaya waste including papaya seed and papaya peel was used as a substrate to produce bioethanol through fermentation process to enhance the development of green energy from lignocellulosic biomass. The fermentation conditions such as pH, temperature and incubation time were studied and optimized using RSM. This study was carried out within the fermentation condition of pH 3.0-6.0, temperature of 25-45°C and incubation time of 24-96 hours. According to ANOVA and model verification plots, the quadratic model was suitable to describe the interaction between variables with high reliability and reproducibility. The validation performed found that the predicted values fit the model. Based on the results obtained through the numerical optimization and verification run, the maximum ethanol concentration was produced at pH 4.5, 45°C and 24 h.

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