



Response Surface Optimization of Bioethanol Production from Roselle (*Hibiscus Sabdariffa* L.) using *Saccharomyces Cerevisiae*

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DOI: <https://doi.org/10.68050/JAMS.2026.629>

Received: 06 September 2026; Accepted: 12 September 2026; Published: 01 October 2026

ABSTRACT

Bioethanol production from renewable biomass has gained increasing attention due to the growing demand for alternative forms of energy and environmental issues from fossil fuels. This study investigated the production and optimization of bioethanol from zobo (*Hibiscus sabdariffa* L.) extract using *Saccharomyces cerevisiae* isolated from palm wine. The roselle extract was characterized for physicochemical properties, fermentable sugars, organic acids, and mineral composition. Fermentation was conducted using one-factor-at-a-time (OFAT) screening followed by response surface methodology (RSM) based on central composite design (CCD) to evaluate the effects of fermentation time, initial pH, substrate concentration, and temperature on ethanol production. The extract contained glucose (22.90 mg/g), fructose (16.50 mg/g), and sucrose (11.70 mg/g), with malic acid (45.65 mg/g) as the predominant organic acid. The extract had a pH of 3.56, moisture content of 10.80 g/100 g, and ash content of 9.70 g/100 g, with calcium (0.55 mg/g), potassium (0.46 mg/g), and iron (0.22 mg/g) as major mineral components. Preliminary screening identified 60 h, pH 6, substrate concentration of 60 g/L, and temperature of 40 °C as favorable conditions. RSM produced a statistically significant quadratic model ($F = 17.81$, $p = 0.0010$) with $R^2 = 0.9765$, adjusted $R^2 = 0.9217$, and adequate precision of 13.6882. The lack of fit was non-significant ($p = 0.0924$), with a coefficient of variation of 5.16%. Fermentation time, initial pH, and substrate concentration had significant linear effects on ethanol production, while temperature was not significant as a linear term. The pH $\times$ temperature interaction and the quadratic effects of fermentation time and pH were also significant. The model predicted optimum conditions of 60 h, pH 6, substrate concentration of 80 g/L, and temperature of 40 °C, yielding an experimental ethanol concentration of 5.10 g/L compared with predicted value of 5.31 g/L, corresponding to a deviation of 3.95%. Diagnostic plots indicated that the model assumptions were reasonably satisfied; however, the negative predicted R^2 (-1.1933) indicated limited predictive reliability for new observations. The findings demonstrate the potential of roselle extract as a substrate for bioethanol production and the applicability of RSM for optimizing the fermentation process.

Keywords: Bioethanol; *Hibiscus sabdariffa*; roselle; *Saccharomyces cerevisiae* ; fermentation; response surface methodology; optimization; renewable biomass.

INTRODUCTION

The rising demand for fossil fuels, combined with concerns regarding greenhouse-gas emissions and energy security, has driven interest in renewable energy sources like bioethanol (Apuyor et al., 2026; Dadi et al., 2025; Kant et al., 2025;). Bioethanol is a renewable liquid fuel, which can be biologically produced by microbial fermentation of carbohydrate containing feedstock (Tse et al., 2021; Rozina et al., 2022). This process can be either extraction of carbohydrates from the feedstocks or hydrolysis of carbohydrates, and then follows fermentation with *Saccharomyces cerevisiae* (Bušić et al., 2018; Juneja & Kumar, 2024) to convert sugars

into ethanol. Therefore, using agricultural biomass for ethanol production can offer an alternative way of valorization of the resource to value added fuel products (Mwanyesya et al., 2025). The *Saccharomyces cerevisiae* has been well studied and is one of the most commonly used microorganisms for industrial ethanol fermentation due to its fermentative capabilities and proven use in bioethanol production (Parapouli et al., 2020). It is affected by fermentation conditions, such as temperature, pH, sugar concentration, fermentation duration, nutrient conditions, osmotic pressure, and ethanol concentration (Chen et al., 2025; Thuy et al., 2024). Temperature, ethanol tolerance and efficient sugar usage were still underscored as desirable attributes of yeast strain for industrial ethanol production as reported in recent reviews (Fentahun, 2026; Tsegaye et al., 2024). The influence of the fermentation variables is frequently inter-dependent. For instance, higher concentrations of substrates might lead to greater substrate amounts that are available to yeast, but very high substrate concentrations can cause osmotic stress and influence yeast performance (Douradinho et al., 2023; Verhagen et al., 2025). Likewise, yeast growth and enzyme activity, membrane properties and ethanol tolerance are affected by temperature (Sahana et al., 2024). Experimental research in recent years has shown that the best fermentation conditions vary depending on a yeast strain and a substrate. For instance, ethanol production by *S. cerevisiae* isolates was optimized by Altınışık et al., (2024) and they reported that optimum conditions for the isolates studied were approximately 30–35 °C and pH 4–5.5. However, one factor at a time experimentation is suitable for screening, and it is inefficient to use when multiple factors are tested simultaneously because it does not adequately describe interactions between factors (Ramin & Mir Taher, 2026). Response surface methodology offers a statistical approach for the assessment of linear, quadratic and interaction effects, as well as the generation of mathematical models associating process variables with a measured response (Apuyor et al., 2026; Mondal et al., 2021). In recent research of bioethanol, RSM has still been used. Thatiyamanee et al. (2024) optimized sugar concentration, urea concentration and yeast concentration using sweet sorghum and validated the predicted optimum in the experiment by using Box–Behnken design. Likewise, in 2021, a study of the production of bioethanol from sugar-beet molasses was developed by Beigbeder et al. (2021), which used *S. cerevisiae* as the yeast and the concentration of the substrate, pH, temperature, and fermentation time as independent variables and obtained a quadratic model. Roselle (*Hibiscus sabdariffa* L.) is rich in sugars, organic acids, minerals and other phytochemicals, with malic acid and minerals like calcium and iron being relatively high while the amount of glucose was low (Edo et al., 2023; Idowu-Adebayo et al., 2021). The above characteristics make roselle a good plant material for fermentation studies, depending on the availability and accessibility of the carbohydrates that can be fermented in the plant (Ibrahim et al., 2026). Previously, it has been shown that roselle and other plant-based substrates can be used to produce alcoholic beverages and bioethanol using *Saccharomyces cerevisiae*. In a passion fruit-roselle beverage, Zhang et al. (2025) optimized the fermentation process by immobilizing *S. cerevisiae*, obtaining an alcohol content of 10.0% (v/v). In the same way, Nwogwugwu et al. (2019) optimized bioethanol production from calabash (*Crescentia cujete*) by *S. cerevisiae* and RSM. In their study they considered the process variables substrate concentration, pH, temperature and fermentation time and obtained an optimized concentration of bioethanol of 6.19% (v/v). The results show that statistical experimental design can be used to assess and improve the fermentation conditions affecting the production of ethanol. While previous studies focused on ethanol production from roselle and other plant-based substrates, it is still necessary to systematically study the effects and interactions of key fermentation variables on ethanol production using roselle (*Hibiscus sabdariffa* L.) under well-controlled experimental conditions. Hence, the current work explore the production and optimization of bioethanol from roselle by *S. cerevisiae*. Specifically, the study characterized the roselle extract, studied the effect of fermentation time, pH, substrate concentration and temperature on ethanol production, formulated a response surface model, identified the significant process variables and interactions, and determined the optimum process conditions for maximizing ethanol production..

MATERIALS AND METHODS

Materials

The materials used in this study included roselle calyces (*Hibiscus sabdariffa* L.) purchased from Abraka Main Market, Delta State, Nigeria, and *Saccharomyces cerevisiae* isolated from palm wine obtained from local palm wine tapper in Abraka. The roselle plant material was authenticated by a botanist at Delta State

University, Abraka. A voucher specimen of *Hibiscus sabdariffa* L. was prepared and deposited in the university herbarium under voucher number DELSUH-235. Other materials used included distilled water, malt extract agar (MEA), peptone, sodium chloride, fermentable sugars, hydrochloric acid, sodium hydroxide, sodium metabisulphite, analytical-grade reagents, and laboratory glassware. The instruments and equipment employed in the study included a pH meter, analytical balance, drying oven, muffle furnace, atomic absorption spectrophotometer, and thermostatic water bath.

Preparation of Roselle (Zobo) Juice

The method for the preparation of roselle (zobo) juice was adapted from Opara and Nwahia (2012), with some modifications. The mature dried roselle calyces were sorted to remove foreign materials and damaged portions and were then washed briefly with distilled water. Approximately 250 g of the cleaned roselle calyces was extracted with 1000 mL of boiling distilled water at a solid-to-liquid ratio of 1:4 (w/v) for 20 min. The resulting extract was allowed to cool to room temperature and was subsequently filtered to remove the calyx residues. The filtrate obtained was used as the fermentation medium for ethanol production.

Isolation and Propagation of *Saccharomyces cerevisiae*

The method used by Olurunfemi et al. (2005) was followed for yeast isolation. In brief, Approximately 1 g of palm wine was diluted with 10 mL of sterile distilled water and mixed well. Acidified Malt Extract Agar (MEA) was acidified to around pH 4.0 and 1 mL of the diluted sample was inoculated and incubated on the medium at 37 ± 1 °C for 24 h. Isolates resembling yeast were picked and repeatedly subcultured on acidified MEA to ensure the apparent purification of the yeast cultures. The presumptive identification of the yeast isolate was further assessed using sugar fermentation tests with maltose, sucrose, and glucose. The composition of the test medium was 1.0% peptone, 0.1% NaCl, and 1.0% of the respective fermentable sugar. The isolate that showed the expected positive fermentation reactions with the three sugars (M+S+G) was selected as the presumptive *Saccharomyces cerevisiae* isolate. The confirmed (M+S+G) yeast was then cultured on acidified MEA slant and kept at 37 °C as a stock culture. The acidified MEA slants were used as stock culture for the confirmed isolate. Four MEA slants of the stock culture were used for propagation. The cultures were transferred into 200 mL of standard must in an Erlenmeyer flask and incubated on an orbital shaker for 24 h. An additional 200 mL of standard must was added at 48 and 72 h. The viability of the propagated yeast culture was $98 \pm 2\%$, while the initial viable cell concentration ranged from 5×10^6 to 10×10^6 cells / mL. The standardized yeast inoculum was added at 10% (v/v) to each fermentation vessel, corresponding to an initial cell density of approximately 1×10^7 cells/mL. The vessels were sealed to minimize the escape of CO₂ during fermentation.

Sterilization and Aseptic Handling

All glassware, culture media, and materials that came into contact with the yeast culture were sterilized by autoclaving at 121 °C and 15 psi for 15 min. Following sterilization, the materials were allowed to cool under aseptic conditions before use. The roselle extract was pasteurized at 80 °C for 15 min and subsequently cooled to room temperature before inoculation with the propagated *Saccharomyces cerevisiae* culture. All inoculation and fermentation procedures were performed aseptically to minimize the risk of microbial contamination.

Physicochemical Characterization of Roselle Extract

The roselle extract was characterized for pH, moisture, ash, sugar composition, organic acids and mineral composition using the procedures described below.

Determination of pH

Approximately 10 g of roselle material was soaked in 200 mL of distilled water and the pH of the resulting extract was measured using a calibrated digital pH meter (Jenway Model 3020).

Determination of Mineral Elements

Mineral elements were determined using atomic absorption spectrometry. The instrument reported in the original laboratory record was an AAS instrument (GBC 932). Metal concentration was calculated from the instrument reading after applying the appropriate dilution factor:

$$\text{Metal content (mg/L)} = \text{dilution factor} \times (\text{AAS}) \text{ reading} \quad (1)$$

Where conversion to mg/g of sample was required, the appropriate sample mass and final digest volume were incorporated into the calculation.

Determination of Moisture Content

Approximately 2 g of roselle sample was weighed into a previously dried and weighed porcelain crucible. The sample was dried in an oven at 105 °C for 3 h and subsequently transferred to a desiccator for cooling before weighing.

Moisture content was calculated as:

$$\text{Moisture content (g/100g)} = \frac{\text{difference in weight}}{\text{sample weight}} \quad (2)$$

Assessment of Ash Content

Approximately 2 g of roselle sample was weighed into a previously dried and weighed porcelain crucible. The sample was incinerated in a muffle furnace at 550 °C until a constant mass of light-grey ash was obtained.

Ash content was calculated as:

$$\text{Ash content (g/100g)} = \frac{\text{Mass of ash}}{\text{Mass of sample}} \quad (3)$$

Qualitative Test for Carbohydrates

A qualitative carbohydrate test was performed using Molisch's reagent. Approximately 5 mL of roselle extract was transferred into a test tube and two drops of Molisch's reagent were added. Concentrated sulfuric acid was carefully introduced down the side of the tube to form a separate lower layer. The formation of a violet ring at the interface was interpreted as a positive test for carbohydrates.

Determination of Organic Acids

Organic acid composition was determined using the colorimetric method adopted from Hemalatha and Anbuselvi (2013), as used in the original study.

Fermentation Study

The fermentation experiments were conducted in two stages: preliminary one-factor-at-a-time screening and response-surface optimization.

Preliminary Screening Experiments

The preliminary Screening experiments were performed to assess effects of fermentation time, pH, substrate concentration and temperature on ethanol production. The variables were varied individually while the remaining experimental conditions were maintained constant. Fermentation vessels were washed and sterilized using a 3% sodium metabisulphite solution for 5 min. approximately 1000 cm³ of roselle juice was conditioned by heating at 90 °C and subsequently adjusted to the required pH using 0.1 M HCl or 0.1 M NaOH. For the fermentation-time experiment, seven vials containing substrate samples were investigated at, 20, 40, 60, 80,

100, 120 and 140 min. The samples were fermented in the scaled polymeric vial with connected tubes for the estimation of gas production. Approximately 1 g of yeast was added to each fermentation vessel. The vessels were sealed to minimize the escape of CO₂ during fermentation. The CO₂ produced was collected and quantified by titration using 0.1 M NaOH with phenolphthalein as the indicator.

Effect of Temperature on Fermentation

The effect of temperature was investigated by varying temperature while maintaining substrate concentration, pH and fermentation time constant. The experimental temperature range was 30 –70 °C using a thermostatically controlled water bath.

Effect of Substrate Concentration

The effect of substrate concentration on ethanol production was evaluated by varying the substrate concentration within the preliminary experimental range of 20–100 g/L, while maintaining temperature, pH, and fermentation time at constant levels.

Effect of Initial pH

The effect of initial pH on ethanol production was evaluated by varying the pH while maintaining the substrate concentration, temperature, and fermentation time at constant levels. The initial pH was adjusted using 0.1 M hydrochloric acid (HCl) or 0.1 M sodium hydroxide (NaOH). The pH meter was calibrated using an appropriate standard buffer before measurements were taken. The broad screening range was useful for identifying the response profile, although subsequent RSM optimization was conducted within the narrower range of pH 4–8.

Effect of Fermentation Time

The effect of fermentation time was investigated by varying the fermentation duration while keeping the other process variables constant.

2.4.6 Determination of Rate of Ethanol Fermentation

The rate of fermentation was measured as the volume of CO₂ produced at 20mins time interval. The evolved CO₂ was quantified by titration with 0.1 M NaOH using phenolphthalein as the indicator. The measured CO₂ evolution was used as the response variable for evaluating the effects of the fermentation conditions on ethanol product

RESPONSE SURFACE METHODOLOGY

Experimental Design

RSM is a statistical modelling approach that is basically empirical to assess the relationship between the controllable experimental factors and the responses received from a process. CCD was used in this study because it is suitable for developing and fitting a second-order (quadratic) model. The design has sufficient number of experimental runs to test the model for statistical significance and adequacy, even considering the lack of fit of the model (Li et al., 2002). To find the optimum conditions of the process variables that affect ethanol fermentation, RSM was used. The factors taken into account were temperature (X₁), pH (X₂), Concentration of substrate (X₃), and Fermentation time (X₄) as shown in the Table 1. The experimental data were correlated with a second order polynomial model having linear, quadratic and interaction terms of the independent variables (Equation (4)).

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ij} x_i^2 + \sum \sum_j \beta_{ij} x_i x_j + \epsilon \quad (4)$$

In the equation, **Y** represents the predicted response, **i** and **j** denote the linear and quadratic terms, respectively,

β represents the regression coefficients, and k indicates the total number of independent variables investigated in the experiment.

RSM was used to investigate the combined effects of incubation time, initial pH, substrate concentration and temperature on the measured fermentation response.

The four independent variables were:

X_1 = incubation time (h);

X_2 = initial pH;

X_3 = substrate concentration (g/L); and

X_4 = temperature (°C).

The experimental ranges were:

Table 1: Range of alpha values for variables in the experimental design

Variables	Coded levels		
	-1	0	1
Temperature	Low	Medium	High
Ph	Low	Medium	High
Substrate concentration	Low	Medium	High
Time	Low	Medium	High

RESULTS AND DISCUSSION

Physicochemical Properties, Sugars, Organic Acids and Minerals of Roselle Extract

The physicochemical properties, sugar profile, organic acid composition, and mineral content of the roselle extract are presented in Table 2.

Table 2: Physicochemical properties, sugars, organic acids and mineral composition of roselle extract

Composition	Parameters	Dark red roselle variety
Ash content (g/100g)		9.7
Sugar (mg/g)	Sucrose	11.7
	Glucose	22.9
	Fructose	16.5
Organic acid (mg/g)	Tartaric	0.11
	Citric	0.45
	Oxalic	0.06
	Succinic	0.8
	Malic	45.65
pH		3.56
Moisture content (g/100g)		10.8
	Na	0.33
	Mg	0.21
	K	0.46

Minerals (mg/g)	Ca	0.55
	Fe	0.22
	Mn	0.001
	Cu	0.03

The roselle extract contained measurable amounts of sucrose, glucose, and fructose, demonstrating the presence of fermentable sugars that can potentially support yeast fermentation. Glucose was present at 22.90 mg/g, followed by fructose at 16.50 mg/g and sucrose at 11.70 mg/g. Malic acid was the predominant organic acid detected, with a concentration of 45.65 mg/g, followed by succinic acid at 0.80 mg/g and citric acid at 0.45 mg/g. The dominance of malic acid is consistent with previous characterization of roselle, in which malic acid was reported as an important organic acid constituent (Izquierdo-Vega et al., 2020; Li et al., 2024). The extract had a pH of 3.56, indicating an acidic medium. Such acidity may influence microbial growth and fermentation performance. The optimum pH for ethanol production is strain- and substrate-dependent; recent studies using *S. cerevisiae* have frequently reported optimum conditions around pH 4–5.5, although higher values may occur depending on the fermentation system (Tadesse et al., 2024; Sewsynker-Sukai & Gueguim Kana, 2022). The mineral composition showed calcium as the most abundant mineral among those measured (0.55 mg/g), followed by potassium (0.46 mg/g), sodium (0.33 mg/g), iron (0.22 mg/g), magnesium (0.21 mg/g), copper (0.03 mg/g), and manganese (0.001 mg/g). Minerals can contribute to microbial metabolism because several mineral ions function as enzyme cofactors (Jung et al., 2013). Every enzyme requires cofactors to function properly. These results support the use of *H. sabdariffa* as a nutrition source for the human body (Jung et al., 2013). The pH of the water extract was found to be 3.56. This indicates that the extract has a reasonable taste for drinking. The moisture content was found to be 10.80 g/100g and the ash content was found to be 9.7 g/100g.

Effect of Parameters Variation on Ethanol Production from Zobo Leaves (*H. sabdariffa*)

The data on the effect of time, temperature, pH and subtract concentrate on the production rate of ethanol from zobo leaves (*H. sabdariffa*) are presented in the figures 1- figure 4 below;

Effect of Fermentation Time on Ethanol Production Rate

The result for the effect of time in ethanol production rate is show in the figure 4.1 below;

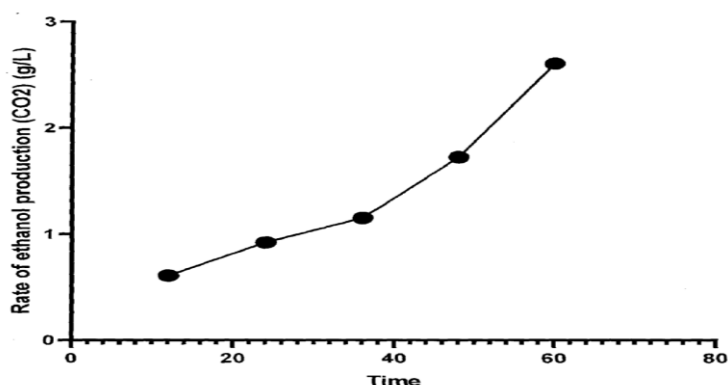


Figure 1: Effect of time on ethanol production rate

Figure 1 shows the effect of time on the rate of ethanol production from *H. sabdariffa* extract. From the figure, it was observed that at the 12-hour fermentation, ethanol production was detected from the extract with a yield of 0.61 g/L, and this amount increased with increase in incubation time up to 60 h with a maximum yield of 2.60 g/L. The production of ethanol from *H. sabdariffa* extract during the fermentation period could be attributed to the breakdown of leaf polysaccharide to glucose and the eventual conversion of the glucose into ethanol by microorganisms in the fermentation broth (Jawad et al., 2013). Therefore, the increase in the rate of

ethanol production observed at an incubation time of 60 h could be associated with the conversion of available fermentable sugars into ethanol by *S. cerevisiae*.

Effect of pH on the rate of ethanol production

The result for the pH on the ethanol production rate is shown in the figure 2 below;

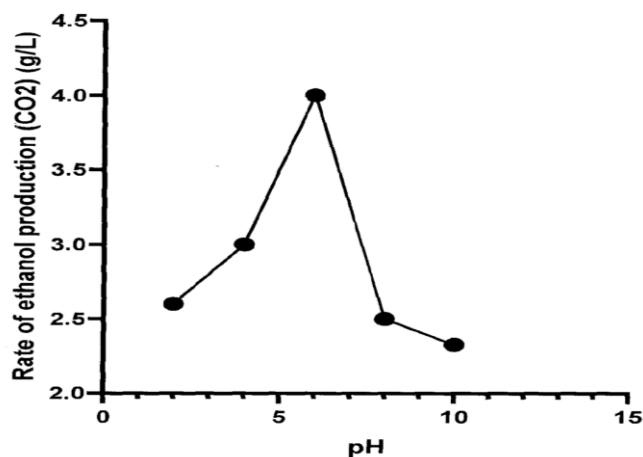


Figure 2: result of the effect of pH on ethanol production rate

The effect of pH on the rate of ethanol production from *H. sabdariffa* extract can be seen in Figure 2 above. From the figure, ethanol production increased as the initial pH increased from the lower levels investigated and reached a maximum reported response of 4.00 g/L at pH 6. Thereafter, the response decreased, with the lowest reported value of 2.33 g/L occurring at pH 10. This finding is consistent with the optimal pH range reported for *Saccharomyces cerevisiae*. 4.0–6.0 (Egharevba et al., 2014). Outside the optimum pH range, the enzyme cells are less tolerant to the pH environment and expectedly less active and efficient in substrate conversion (Asiagwu, 2017). The observed optimum around pH 6 is higher than the approximately pH 4–5.5 range reported for several *S. cerevisiae* ethanol systems. This difference may reflect the particular yeast strain, substrate composition, and experimental conditions used in the present study. Recent RSM studies have similarly demonstrated that optimum pH is dependent on the particular yeast and fermentation substrate (Tadesse et al., 2024; Phong et al., 2022).

Effect of Temperature on Ethanol Production Rate

The result for the impact of temperature on ethanol production rate is shown in the Figure 3 below:

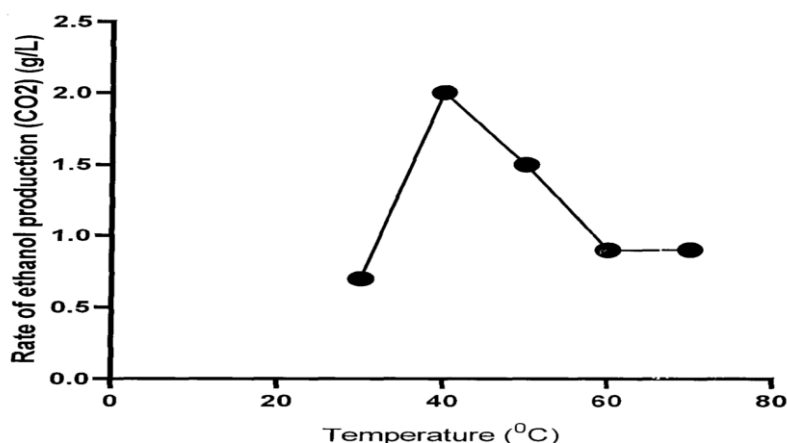


Figure 4.3: Result of the impact of temperature on ethanol production rate

The effect of temperature on the rate of ethanol production is shown in Figure 4.3 as the rate of CO₂ evolution is displayed as a function of the temperature (°C). The result indicated that the rate of CO₂ production increased as the temperature increased and at 40 °C, the highest rate of CO₂ production was obtained, after which the rate decreased. This implies that the conditions of the experiment were optimum at 40 °C. The observed trend indicates that *Saccharomyces cerevisiae* have metabolic activity over a relatively wide temperature range, but fermentation activity is optimal within a narrower range of temperature. This is in agreement with the activity of yeast fermentation as seen in Rinki et al., 2024 which relies on temperature. The amount of CO₂ evolved was first increased then decreased, suggesting that temperature played a significant role in the amount of fermentation. The CO₂ production rate during the initial stage of fermentation could be related to an increase in the metabolic activity and use of the substrate, while the rate of CO₂ production in the later stages of fermentation could be related to a decrease in yeast metabolic activity due to the temperature rising above optimum. The first rise in fermentation rate with temperature is explained by the increase in the molecular kinetic energy that increases the enzymatic reactions and the rate of conversion of the substrate. The temperatures above the optimum however, will have a negative effect on yeast cells and fermentation enzymes as it would increase thermal stress and disrupt cellular and enzymatic function (Asiagwu, 2017). As a result, the CO₂ production above 40 °C will decrease, suggesting that too high temperatures could negatively affect the metabolism of *S. cerevisiae* and the effectiveness of ethanol fermentation.

Impact of Substrate Concentration on the Rate of Ethanol Production

The data for the impact of substrate concentration on the rate of ethanol production is shown in the Figure 4 below:

Rate of ethanol production (CO₂) (g/L) 50 100 Substrate conc. 150

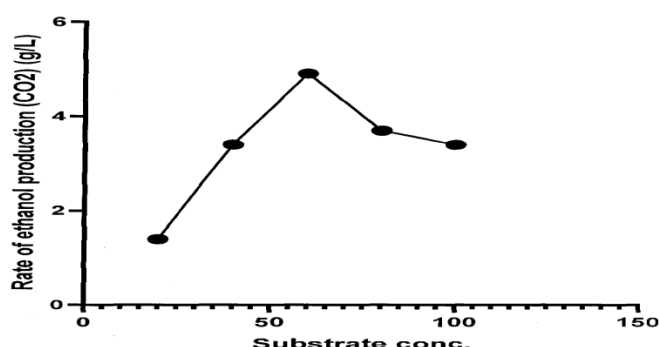


Figure 4: Impact of substrate concentration on the rate ethanol production

The results of the substrate concentration effect on the rate of ethanol production from *H. sabdariffa* extract are shown in Figure 4. The results showed that the higher the concentration of substrate the higher the rate of ethanol production and also the CO₂ evolution increased with increase in the concentration of substrate (Asiagwu, 2017). However, when the concentration of substrate was increased beyond 60 g/L the rate of fermentation did not increase significantly. The first boost of ethanol is due to more fermentable sugars in the medium, which can be used to generate more ethanol and stimulate yeast growth and metabolism. The fermentation system looks like it has reached a concentration where it would not be possible to increase the reaction rate by adding more substrate. May be related to the times when the enzyme and/or cell's metabolic capacity is saturated, meaning more substrate would not be metabolized at a proportionally greater rate. Hence, the most optimum concentration for substrate was 60 g/L for the conditions that were studied.

Optimization of experimental results for ethanol production from Zobo (Roselle) (*H. Sabdariffa*) Extracts RSM

The results obtained from the screening experimental phase were further analyzed for optimization and a model was generated using Design Expert 13 software adopting RSM with CCD. The variables investigated are shown below with their coding in Table 3 below:

X1- Incubation Time (Hrs)

X2- pH

X3- Substrate concentration (g/L)

X4- Temperature(°C)

Table 3: Variables coding

Variables	Coded and actual values		
	-1	0	1
X ₁ - Incubation Time [Hrs]	48	60	72
X ₂ - pH	4	6	8
X ₃ - Substrate conc. [g/L]	40	60	80
X ₄ - Temperature [°C]	30	40	50

The values from the table above were selected from the optimum range in the OFAT phase and were used to generate the matrix for the Response Surface Optimization experiment.

Table 4: Optimization matrix of ethanol production from zobo leaves via fermentation

Std	Run	Factor 1 A:X1- Incubation Time [Hrs]	Factor 2 B:X2- pH	Factor 3 C:X3- Substrate conc. [g/L]	Factor 4 D:X4- Temperature [°C]	Response 1 Rate of ethanol prod. From zobo leaves g/L
10	1	60	8	60	40	2.7
1	2	60	6	60	30	4.51
3	3	60	6	60	40	4.97
16	4	72	4	80	50	2.97
2	5	60	6	80	40	5.1
20	6	60	6	60	40	4.9
11	7	60	6	60	40	4.94
6	8	60	4	60	40	4.28
12	9	72	4	40	50	2.1
8	10	48	8	40	50	1.72
4	11	72	6	60	40	1.49
14	12	48	8	80	50	2.1
5	13	60	6	60	40	4.9
15	14	72	8	40	30	1.2
18	15	48	6	60	40	4.17
7	16	60	6	60	40	4.39
13	17	60	6	40	40	4.37
21	18	60	6	60	50	4.2
17	19	48	4	80	30	3.21
9	20	72	8	80	30	2.9
19	21	48	4	40	30	3



The responses from the matrix in Table 4 above showed that the optimum rate of ethanol production was observed at standard random order of 2 and standard run order of 5. This gives the response in terms of rate of ethanol production of 5.10 g/L, and it occurred at optimum values of incubation time of 60 h, initial pH of 6, substrate concentration of 80 g/L, and temperature of 40 °C. However, the substrate concentration and the response observed are quite different from that of the previous OFAT experiment. This may be due to human error in the manual experimental run and the effects of variable interactions (Aquinas et al., 2024)

Results from Table 4 were subjected to various mathematical models so as to determine the best one that fits the experimental result and this is shown in Table 5 below.

Table 5: Model Summary Statistics

Source	Std. Dev.	R ²	Adjusted R ²	Predicted R ²	PRESS	
Linear	1.31	0.1676	-0.0405	-0.7344	56.97	
2FI	1.55	0.2687	-0.4626	-29.404	998.66	
Quadratic	0.3586	0.9765	0.9217	-1.1933	52.04	Suggested
Cubic	0.2422	0.9929	0.9643		*	Aliased

From Table 5, it was observed that out of the four models given, quadratic has the best fit of R². This shows that it has the best description of the experimental results. Cubic also has a high R² but it was aliased, therefore not suitable. This was also confirmed by the predicted residual sum of squares (PRESS) value of 52.04 being the lowest.

Table6: Fit Statistics

Std. Dev.	0.3586			R ²	0.9765
Mean	3.53			Adjusted R ²	0.9217
C.V. %	10.16			Predicted R ²	-1.1933
				Adeq Precision	13.6882

A negative **Predicted R²** in Table 6 implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher order model may also predict better. **Adeq Precision** measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 13.688 indicates an adequate signal. This model can be used to navigate the design space. Sequel to this fit summary analysis, the quadratic model was chosen for this work as shown in the equation 5;

$$Y \text{ (Rate of ethanol production)} = \beta_0 + \sum_{i=1}^n \beta_i X_i + \sum_{i=1}^n \beta_{ii} X_i^2 + \sum_{i < j}^n \beta_{ij} X_i X_j + \varepsilon \quad (5)$$

This research model is in line with that of Karuppaiya et al. (2010), where ethanol was produced from cashew apple juice via fermentation. The model that best describes the fermentation process was the quadratic model.

Table 7: Sequential Model Sum of Squares

Response 1: Rate of ethanol prod. From zobo leaves

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	261.61	1	261.61			
Linear vs Mean	5.51	4	1.38	0.8056	0.5394	
2FI vs Linear	3.32	6	0.5533	0.2304	0.9571	
Quadratic vs 2FI	23.25	4	5.81	45.19	0.0001	Suggested
Cubic vs Quadratic	0.5371	2	0.2686	4.58	0.0924	Aliased
Residual	0.2346	4	0.0587			

Total	294.45	21	14.02			
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In Table 7, Quadratic vs 2FI model has the highest sum of squares (SS), mean square, and Fischer value of 23.25, 5.81, and 45.19 respectively resulting in a p-value of <0.0001 being the lowest. The suggested model therefore should be a second-order polynomial with interaction effects.

Table 8: Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Linear	27.11	12	2.26	38.51	0.0015	
2FI	23.79	6	3.96	67.59	0.0006	
Quadratic	0.5371	2	0.2686	4.58	0.0924	Suggested
Cubic	0	0				Aliased
Pure Error	0.2346	4	0.0587			

The model with the lowest F-value and highest p-value forms the most desirable model. From Table 8 above, the quadratic model satisfied these conditions hence it can fit any data outside this experimental work. A similar view has been made by Shankar et al. (2015). The selected model has an insignificant lack-of-fit as shown in the analysis of variance table below.

Table 9: Analysis of Variance (ANOVA) for Quadratic Model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	32.07	14	2.29	17.81	0.001	significant
A-X1- Incubation Time [Hrs]	3.59	1	3.59	27.92	0.0019	
B-X2- pH	1.25	1	1.25	9.7	0.0207	
C-X3- Substrate conc. [g/L]	1.51	1	1.51	11.77	0.014	
D-X4- Temperature [°C]	0.048	1	0.048	0.3736	0.5635	
AB	0.0008	1	0.0008	0.0063	0.9393	
AC	0.4901	1	0.4901	3.81	0.0988	
AD	0.219	1	0.219	1.7	0.2397	
BC	0.125	1	0.125	0.9719	0.3623	
BD	2.43	1	2.43	18.9	0.0048	
CD	0.0545	1	0.0545	0.4233	0.5394	
A ²	6.4	1	6.4	49.78	0.0004	
B ²	2.18	1	2.18	16.93	0.0063	
C ²	0.2637	1	0.2637	2.05	0.2022	
D ²	0.0088	1	0.0088	0.0682	0.8027	
Residual	0.7717	6	0.1286			
Lack of Fit	0.5371	2	0.2686	4.58	0.0924	not significant
Pure Error	0.2346	4	0.0587			
Cor Total	32.85	20				

The ANOVA showed that the overall quadratic model was statistically significant ($F = 17.81$, $p = 0.0010$). Incubation time, initial pH, and substrate concentration were significant linear terms at $p < 0.05$. Temperature was not significant as an isolated linear term ($p = 0.5635$).

Among the interaction terms, only the pH $\times$ temperature interaction (BD) was statistically significant at the 5% level ($p = 0.0048$). The incubation-time $\times$ substrate-concentration interaction (AC) had $p = 0.0988$ and

therefore should not be described as statistically significant at $\alpha = 0.05$. It could be described as marginal at the 10% level if the study explicitly adopted $\alpha = 0.10$. The quadratic effects of incubation time (A^2) and pH (B^2) were significant, whereas substrate concentration (C^2) and temperature (D^2) were not.

Thus, the statistically significant terms at $\alpha = 0.05$ were: A, B, C, BD, A^2 , and B^2 .

Regression Coefficients

Table 10: Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	4.6	1	0.118	4.31	4.89	
A-X1- Incubation Time [Hrs]	-1.34	1	0.2536	-1.96	-0.7195	5
B-X2- pH	-0.79	1	0.2536	-1.41	-0.1695	5
C-X3- Substrate conc. [g/L]	0.389	1	0.1134	0.1115	0.6665	1
D-X4- Temperature [°C]	-0.155	1	0.2536	-0.7755	0.4655	5
AB	0.0225	1	0.2835	-0.6713	0.7163	5
AC	0.2475	1	0.1268	-0.0628	0.5578	1
AD	-0.37	1	0.2835	-1.06	0.3238	5
BC	0.125	1	0.1268	-0.1853	0.4353	1
BD	-1.23	1	0.2835	-1.93	-0.5387	5
CD	-0.0825	1	0.1268	-0.3928	0.2278	1
A^2	-1.58	1	0.2245	-2.13	-1.03	2.05
B^2	-0.9236	1	0.2245	-1.47	-0.3744	2.05
C^2	0.3214	1	0.2245	-0.2279	0.8706	2.05
D^2	-0.0586	1	0.2245	-0.6079	0.4906	2.05

The coefficient estimates show the expected change in the response if any other factors were held constant and the factor being estimated is changed by one. An orthogonal experimental design would have a different intercept that would be the average response for all runs of the experiment and the individual levels of the factors would be the deviations from this average. VIF = 1 for orthogonal factors, implying that there is no multicollinearity. A VIF > 1 indicates a higher degree of correlation among the explanatory variables, with the higher the VIF the more severe the multicollinearity. In general a VIF value of less than 10 is regarded as acceptable.

The regression coefficients indicate the direction and magnitude of the effects within the coded experimental region. The negative coefficients for incubation time, pH, and temperature indicate that increasing these variables from their coded centre toward their high levels was associated with decreasing response after accounting for the other terms in the model. The positive coefficient for substrate concentration indicates a positive linear contribution within the coded model, although its quadratic effect was not statistically significant.

The large negative coefficient for the BD interaction indicates that the combined effect of pH and temperature was lower than would be expected from their separate linear effects alone. Importantly, the statistical

significance of an interaction should be interpreted together with the response-surface plot rather than from the coefficient magnitude alone.

Final Quadratic Equation in Coded Variables

The corrected regression equation in terms of coded factors is:

$$Y \text{ (Rate of ethanol production)} = 4.60 - 1.34A - 0.790B + 0.389C - 0.155D + 0.0225AB + 0.2475AC - 0.370AD + 0.125BC - 1.23BD - 0.0825CD - 1.58A^2 - 0.9236B^2 + 0.3214C^2 - 0.0586D^2$$

where: A = coded incubation time;

B = coded pH;

C = coded substrate concentration; and

D = coded temperature.

The model in the equation above fitted the data adequately since the value of coefficient of determination (R^2) is high. The value for R^2 for ethanol production is 0.9765. The matching between the predicted and observed values indicated that the regression model fitted well with the observed data for ethanol production. The model also revealed that A (incubation time) mostly affected the rate of ethanol production, followed by C (substrate concentration), then B (initial medium pH), and finally D (process temperature). This was reflected in their coefficient magnitude. The negative sign in all the major terms indicates that increase in their level above the optimum level leads to decrease in the rate of ethanol production. It was also observed that interaction term AD possesses the highest magnitude (0.3700). This implies that the rate of ethanol production is negatively affected by the combination of factor level changes in incubation time and process temperature. A curvature is indicated among the quadratic terms, hence the negativity of terms A^2 (incubation time²), B^2 (pH²), and D^2 (process temperature²) depicts a concave quadratic curve while the positivity of term C^2 (substrate concentration²) reflects a convex quadratic curve. This implies that increase in incubation time, initial medium pH, and temperature causes an increase in the rate of ethanol production up to the optimum point after which the rate decreased. However, there was an outright decrease in rate of ethanol production as substrate concentration increased. The steepness of the curvature is a function of the quadratic term coefficients' magnitude, therefore A^2 , B^2 , and D^2 have the steepest curvature than C^2 .

Equation in Actual Variables

Using the experimental factor units, the regression equation supplied by the data is approximately:

$$Y = -50.8329 + 1.26386A + 4.59714B - 0.141086C + 0.610904D + 0.000938AB + 0.001031AC - 0.003083AD + 0.003125BC - 0.061625BD - 0.000413CD - 0.010997A^2 - 0.230908B^2 + 0.000803C^2 - 0.000586D^2$$

where A, B, C and D represent incubation time, pH, substrate concentration and temperature, respectively, in their original units.

The regression model represents the effect of the fermentation time (A), the initial pH (B), substrate concentration (C) and the fermentation temperature (D) on the response variable - ethanol production. Linearity, interaction, and quadratic terms are included in the model to represent the effect of each of the fermentation variables as well as the interaction and quadratic effect on ethanol production. The positive linear parameter for fermentation time, pH and temperature suggest that there are positive linear contributions to the predicted response in the fitted model; the negative linear parameter for substrate concentration suggests that the other parameters in the fitted model are held constant and a negative linear contribution would be made to the predicted response. The interaction terms are used to describe the interaction effect between pairs of



process variables, and the quadratic terms are used to account for the curvature in the response and to help the model determine the optimum process variables.

Table 11: Fit Statistics

Std. Dev.	0.3586	R²	0.9765
Mean	3.53	Adjusted R²	0.9217
C.V.%	5.16	Predicted R²	-1.1933
		Adeq Precision	13.6882

NB: Adeq Precision = adequate precision, C.V. % = coefficient of variation, Std. Dev. = standard deviation, R² = coefficient of determination, Adj R² = adjusted coefficient of determination, Pred R² = predicted coefficient of determination.

The goodness of the fit of the model was checked by the determination of Coefficient (R²) as shown in Table 11. The R² value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R² value is always between 0 and 1. The closer the R² value to 1, the stronger the model is and the better it predicts the response (Chen & Qi, 2023). In this case, the value of the determination coefficient (R² = 0.9765) indicates that 97.65% of the variability in the response could be explained by the model. However, the predicted R² of -1.1933 requires careful consideration. Unlike the R² and adjusted R² values, a negative predicted R² indicates poor predictive performance of the fitted model for observations not used in model fitting. Thus, the high R² value should not be interpreted as evidence that the model has strong predictive capability outside the experimental data used to construct it. The difference between the high R² (0.9756), adjusted R² (0.9186), and negative predicted R² (-1.1933) suggests that, although the quadratic model describes the experimental data well, its ability to predict independent or new observations within the design space is limited.

This discrepancy may be associated with the relatively small experimental design, experimental variability, the number of model terms relative to the available observations, or the distribution of the experimental runs within the design space. Therefore, the model is best interpreted as a useful empirical representation of the observed experimental response and for examining the effects and interactions of the investigated variables within the experimental region, rather than as a highly reliable predictive model for untested conditions. In addition, the value of the adjusted determination coefficient (Adj R² = 0.9217) is also very high to advocate for a high significance of the model. Also, a higher value of the Adequate Precision (Adeq Precision = 13.6882) justifies an excellent correlation between the independent variables. The large value of Adequate Precision of 13.6882, which is far more than 4, implies that the measurement of signal to noise is favorable. The predicted optimum levels of incubation time, pH, substrate concentration, and temperature were obtained by applying the regression analysis to Equation 4.2 above. The C.V. value of 5.16% for the quadratic model is low, which means the model has high precision and reliability since the value is less than 10%; it also stands that only 5.16% variation is unexplainable or due to error.

Table 12: comparison of actual and model prediction of bioethanol production

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	2.7	2.88	-0.1847	0.936	-2.041	-3.369	4.082 ⁽¹⁾	-12.915 ⁽¹⁾	10
2	4.51	4.69	-0.1847	0.936	-2.041	-3.369	4.082 ⁽¹⁾	-12.915 ⁽¹⁾	1
3	4.97	4.6	0.3717	0.108	1.097	1.121	0.01	0.39	3
4	2.97	2.92	0.0522	0.971	0.855	0.832	1.632 ⁽¹⁾	4.819 ⁽¹⁾	16
5	5.1	5.31	-0.2087	0.536	-0.855	-0.832	0.056	-0.895	2
6	4.9	4.6	0.3017	0.108	0.891	0.873	0.006	0.304	20

7	4.94	4.6	0.3417	0.108	1.009	1.011	0.008	0.352	11
8	4.28	4.46	-0.1847	0.936	-2.041	-3.369	4.082 ⁽¹⁾	-12.915 ⁽¹⁾	6
9	2.1	2.06	0.0402	0.971	0.658	0.624	0.967	3.610 ⁽¹⁾	12
10	1.72	1.68	0.0402	0.971	0.658	0.624	0.967	3.610 ⁽¹⁾	8
11	1.49	1.67	-0.1847	0.936	-2.041	-3.369	4.082 ⁽¹⁾	-12.915 ⁽¹⁾	4
12	2.1	2.05	0.0522	0.971	0.855	0.832	1.632 ⁽¹⁾	4.819 ⁽¹⁾	14
13	4.9	4.6	0.3017	0.108	0.891	0.873	0.006	0.304	5
14	1.2	1.16	0.0402	0.971	0.658	0.624	0.967	3.610 ⁽¹⁾	15
15	4.17	4.35	-0.1847	0.936	-2.041	-3.369	4.082 ⁽¹⁾	-12.915 ⁽¹⁾	18
16	4.39	4.6	-0.2083	0.108	-0.615	-0.58	0.003	-0.202	7
17	4.37	4.53	-0.1607	0.536	-0.658	-0.624	0.033	-0.671	13
18	4.2	4.38	-0.1847	0.936	-2.041	-3.369	4.082 ⁽¹⁾	-12.915 ⁽¹⁾	21
19	3.21	3.16	0.0522	0.971	0.855	0.832	1.632 ⁽¹⁾	4.819 ⁽¹⁾	17
20	2.9	2.85	0.0522	0.971	0.855	0.832	1.632 ⁽¹⁾	4.819 ⁽¹⁾	9
21	3	2.96	0.0402	0.971	0.658	0.624	0.967	3.610 ⁽¹⁾	19

Model Validation Plots for Ethanol Production from Zobo Leaves

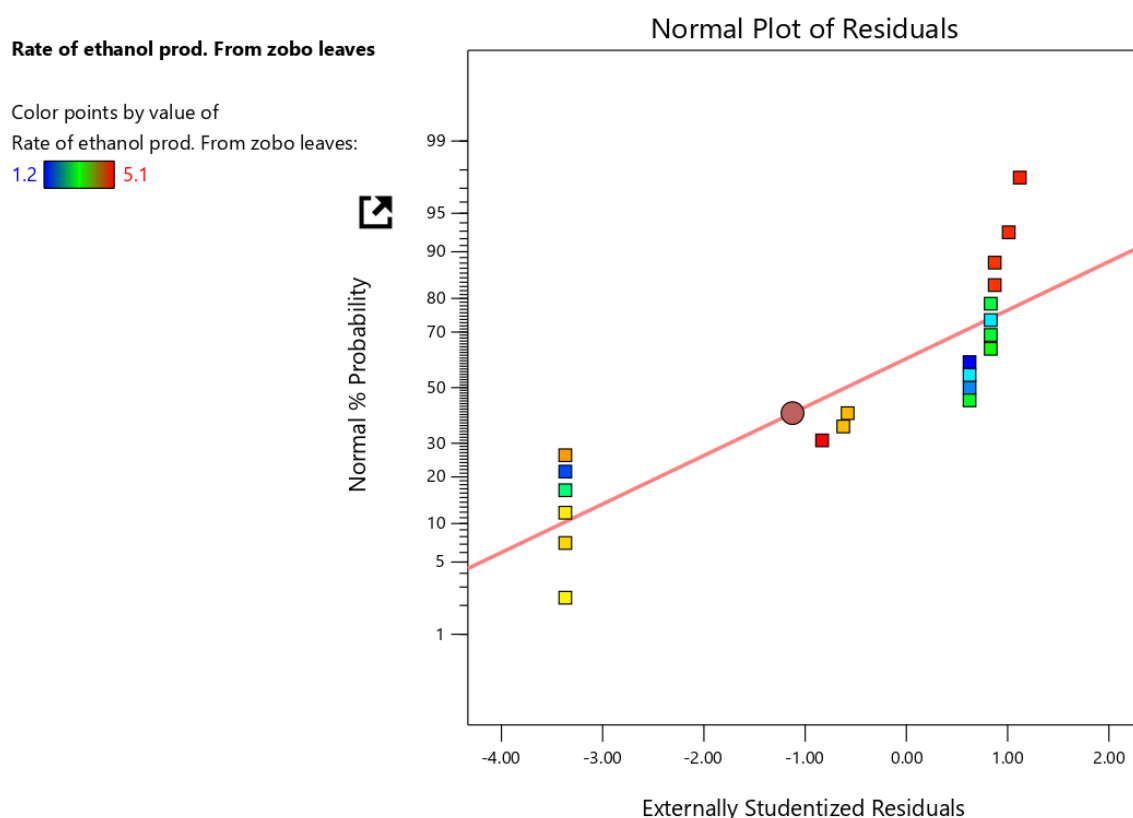


Figure 5: Normal plot of residuals for rate of ethanol production

From the normal plot of residuals graph, it is obvious that the normal % probability and externally studentized residuals data plots were normally distributed. They were evenly scattered and didn't assume any shape. This means that the model is satisfactory because all the data are within the predicted value. All experimental values are close to the predicted values by the model.

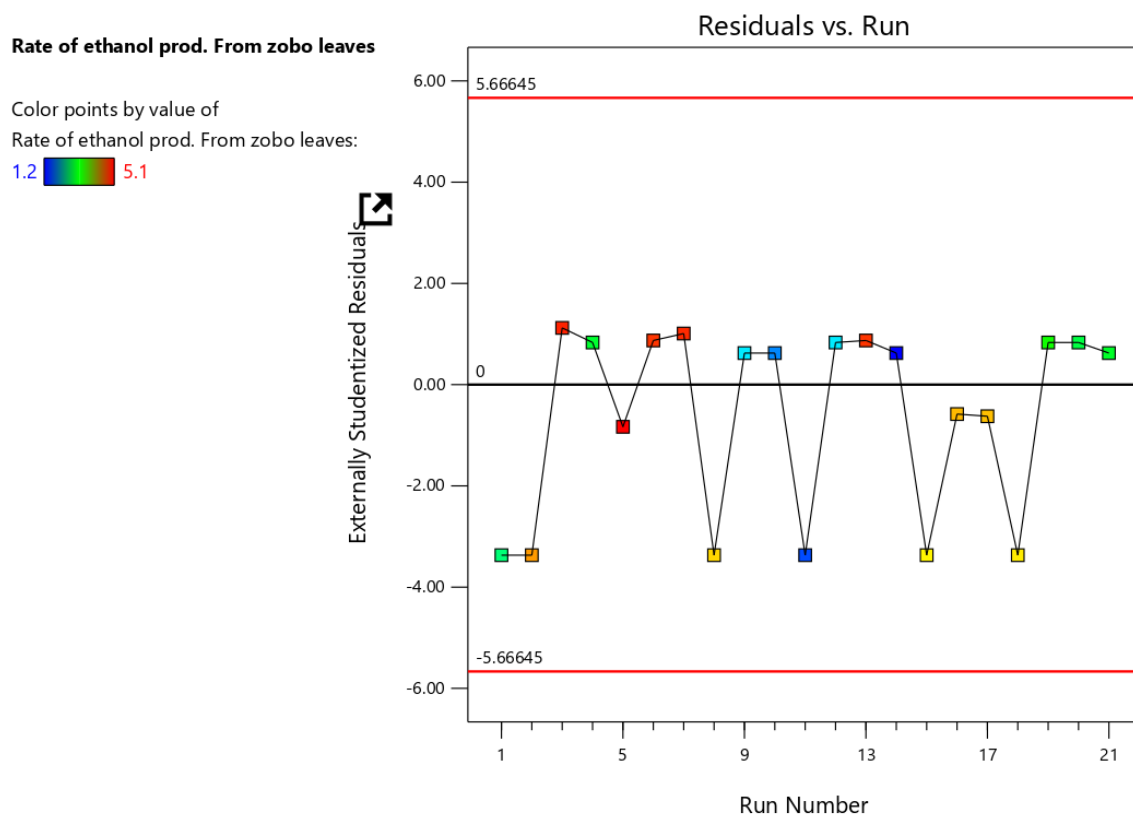


Figure 6: Residual plot against run number

The skewness observed in figure 6 above do not follow any pattern hence the model is a good one.

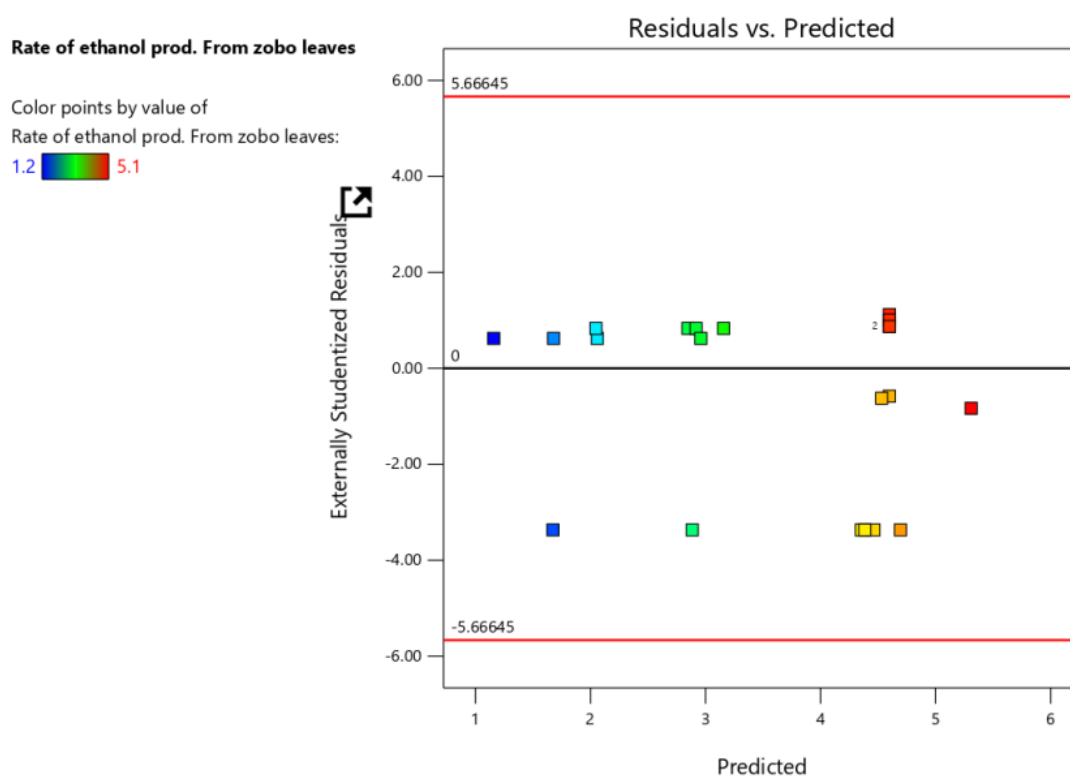


Figure 7: Residual plot against predicted values

Figure 7 plot shows no definite shape or pattern and the data are randomly scattered, therefore the model satisfied the residual plot criteria.

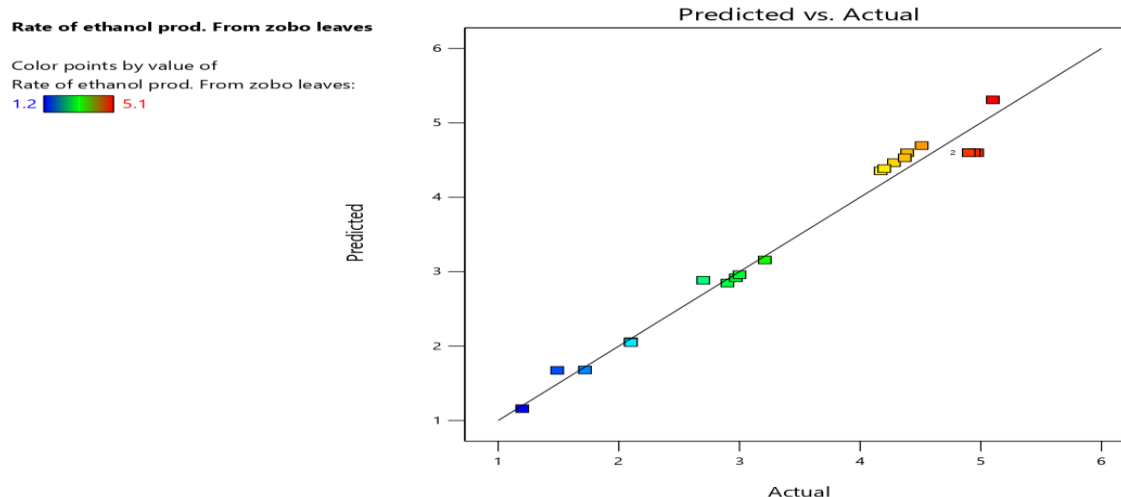


Figure 8: A plot of predicted values against observed values

From Figure 8, it was observed that the experimental values are in close proximity to the model-predicted values; hence the diagnostic flow is satisfactory. Based on the above diagnostic plots, the model is therefore a good fit for the experimental data.

One limitation of the study was the relatively poor predictive performance of the quadratic RSM model. Although the model showed a high coefficient of determination ($R^2 = 0.9765$) and adjusted (R^2) (0.9217), the predicted (R^2) was negative (-1.1933)). This indicates that, despite the model adequately explaining a large proportion of the variation observed within the experimental data, its ability to predict responses for new observations outside the experimental runs was limited. This limitation could be addressed in future studies by increasing the number of experimental runs, conducting independent validation experiments, and evaluating the model with additional observations outside the original experimental runs

Contour and 3D Surface Plots of Ethanol Production from Zobo Leaves

Three-dimensional (3D) response surface and contour plots are used to show the optimization of zobo extract fermentation conditions for ethanol production as shown in figures 9-20. The plots were created by fixing two process variables at the optimum values and changing the other two at the same time within the experimental ranges of the variables.

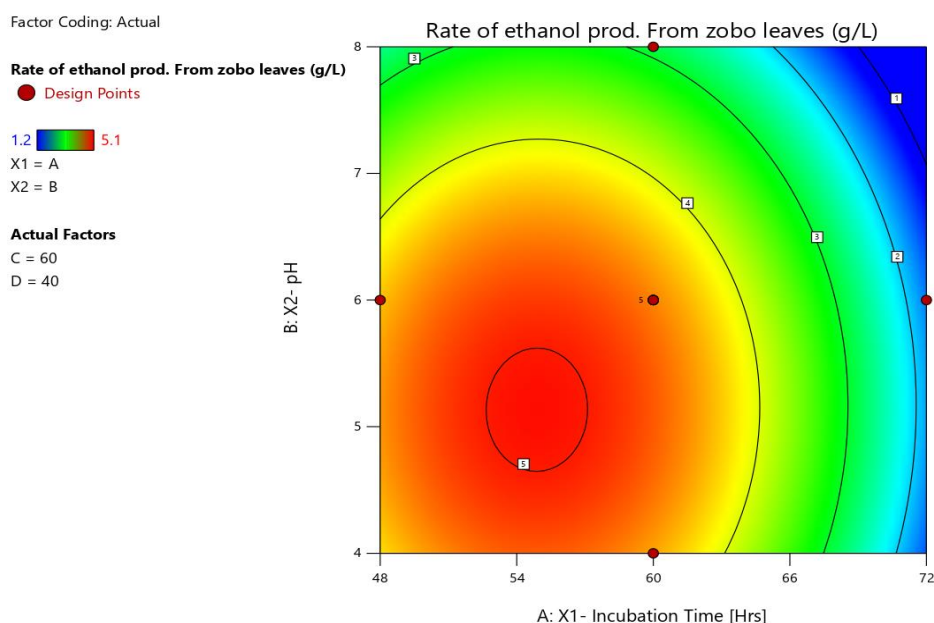


Figure 9: Contour plot of incubation time and pH interaction

The interactive effect of incubation time and pH on ethanol production is presented in Figure 9. The response surface plot indicates that the highest ethanol production was obtained at a pH of 6 and an incubation time of 60 h. The region corresponding to approximately 48–63 h of incubation and pH 4–7 suggests favourable conditions for ethanol production by *S. cerevisiae*. However, the optimum fermentation period may vary depending on the yeast strain, fermentation conditions, substrate characteristics, and other process parameters. Under the conditions investigated in the present study, the optimum combination was an incubation time of 60 h and pH 6.

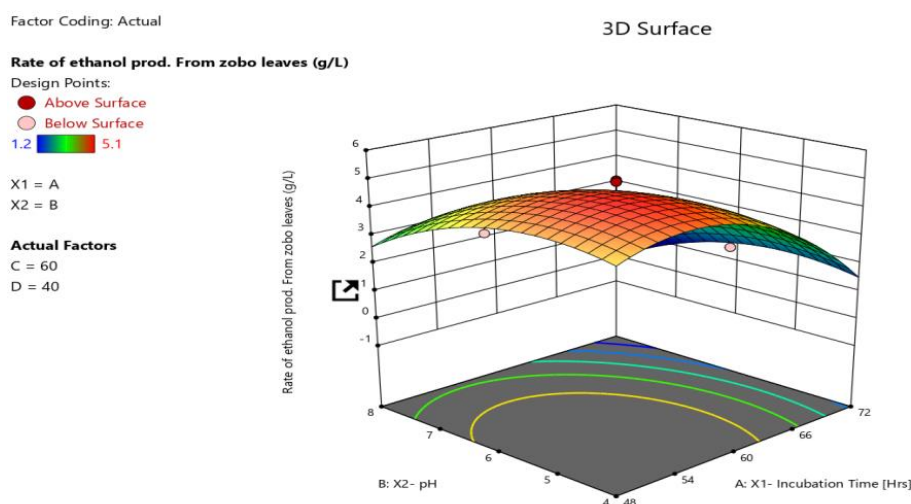


Figure 10: 3D plot for incubation time and pH interaction

The 3D plot in Figure 10, represents the interactive effect of incubation time and pH in a different view (i.e., in a 3-dimensional view) but has the same explanation as the contour plot for pH and temperature interactive effect stated above.

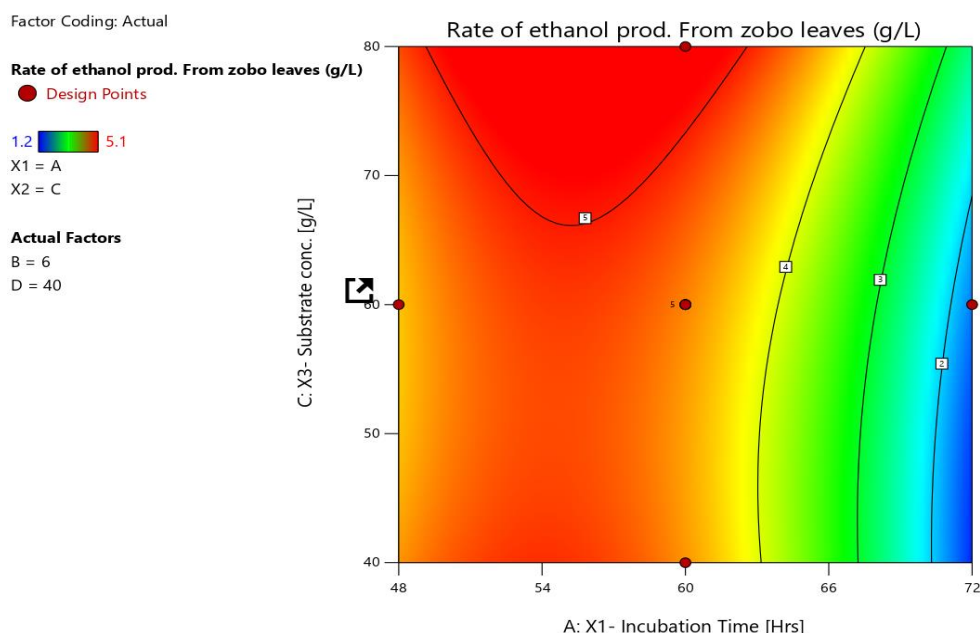


Figure 11: Contour plot of incubation time and substrate concentration interaction

The interaction between the incubation time and substrate concentration on ethanol production from zobo leaves and their optimum level can be determined from Figure 11. It was found that with increase in incubation time to its optimum point and substrate concentration to its highest range, ethanol production is increased. But beyond the optimum incubation time of 60 h, ethanol production starts decreasing. The midpoint of the curve

showed that maximum production of ethanol was obtained at an incubation time of 60 h and substrate concentration of 60 g/L.

Factor Coding: Actual

3D Surface

Rate of ethanol prod. From zobo leaves (g/L)

Design Points:

● Above Surface

○ Below Surface

1.2 5.1

X1 = A

X2 = C

Actual Factors

B = 6

D = 40

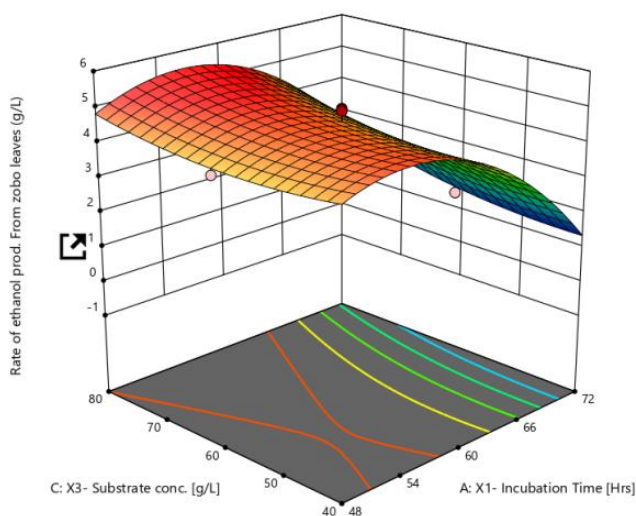


Figure 12: 3D plot for incubation time and substrate concentration interaction

The 3D plot in Figure 12 represents the interactive effect of incubation time and substrate concentration in a different view (i.e., in a 3-dimensional view) but has the same explanation as the contour plot for pH and temperature interactive effect stated above.

Factor Coding: Actual

Rate of ethanol prod. From zobo leaves (g/L)

● Design Points

1.2 5.1

X1 = A

X2 = D

Actual Factors

B = 6

C = 60

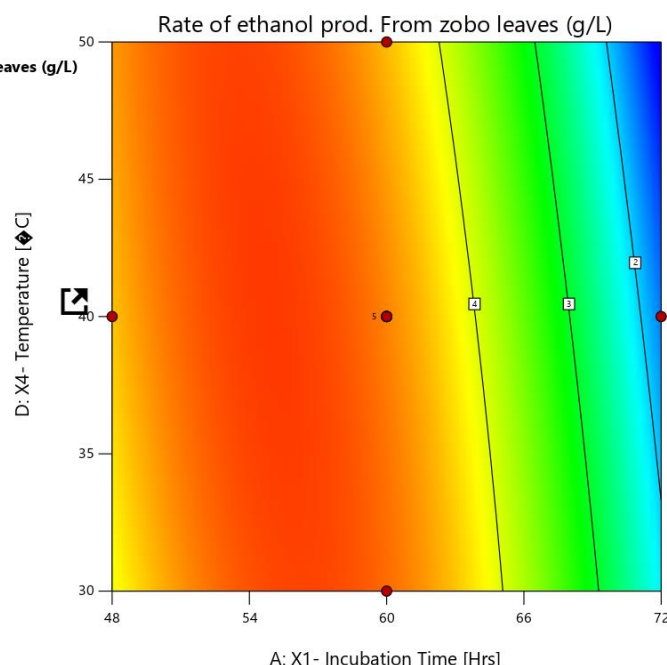


Figure 13: Contour plot showing the interactive effect of incubation time and temperature on ethanol production

Figure 13 shows the interaction between incubation time, temperature, and their effect on ethanol production. From the graph, incubation time of 60 h and temperature of 40 °C are the optimum conditions for highest ethanol yield. Longer incubation time from 64–72 h regardless of the temperature range, ethanol yield starts decreasing. This result is in line with the findings of Ghosh et al. (2012).

Factor Coding: Actual

3D Surface

Rate of ethanol prod. From zobo leaves (g/L)

Design Points:

● Above Surface

○ Below Surface

1.2 5.1

X1 = A

X2 = D

Actual Factors

B = 6

C = 60

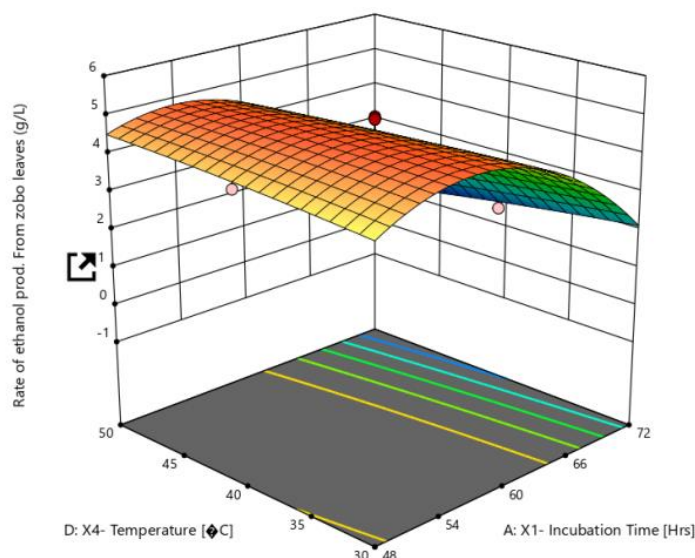


Figure 14: 3D plot for incubation time and temperature interaction

Also, the 3D plot in Figure 14 represents interactive effect of incubation time and temperature in a different view (i.e., in a 3-dimensional view) but has the same explanation as the contour plot for pH and temperature interactive effect stated above.

Factor Coding: Actual

Rate of ethanol prod. From zobo leaves (g/L)

Rate of ethanol prod. From zobo leaves (g/L)

● Design Points

1.2 5.1

X1 = B

X2 = C

Actual Factors

A = 60

D = 40

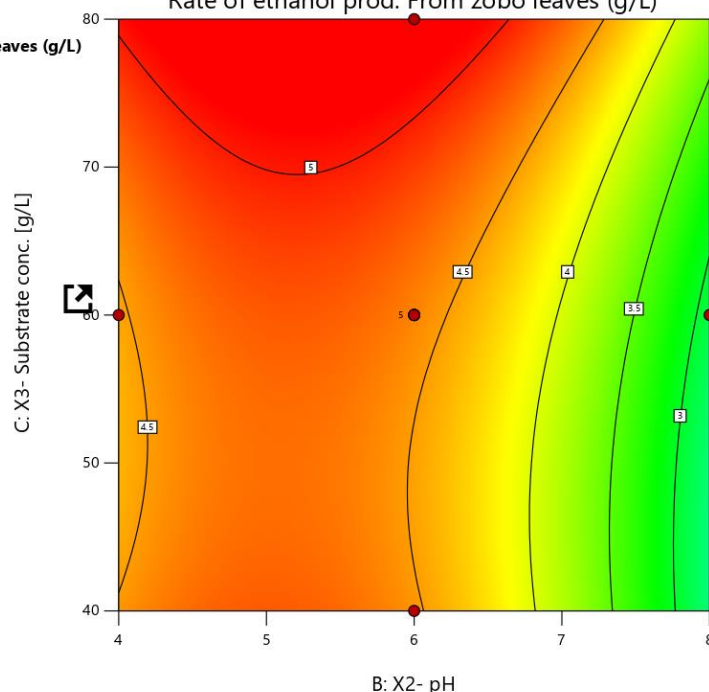


Figure 15: Contour plot of pH and substrate concentration interaction

In Figure 15, at pH 4-6 of fermentation, ethanol production was increased regardless of substrate concentration. A slight decrease in ethanol production was observed pH above 6. When initial medium pH was increased from 7 to 8, ethanol production dropped for all initial substrate concentration levels. A maximum ethanol yield was achieved with values of initial medium pH of 6 and 60 g/L substrate concentration.

Factor Coding: Actual

3D Surface

Rate of ethanol prod. From zobo leaves (g/L)

Design Points:

● Above Surface

○ Below Surface

1.2 5.1

X1 = B

X2 = C

Actual Factors

A = 60

D = 40

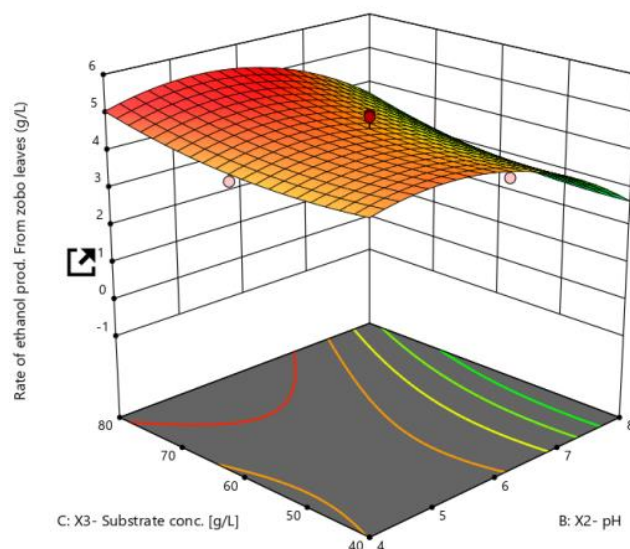


Figure 16: 3D plot for pH and substrate concentration interaction

The 3D plot in Figure 16 represents the interactive effect of pH and substrate concentration in a different view (i.e., in a 3-dimensional view) but has the same explanation as the contour plot for pH and temperature interactive effect stated above.

Factor Coding: Actual

Rate of ethanol prod. From zobo leaves (g/L)

● Design Points

1.2 5.1

X1 = B

X2 = D

Actual Factors

A = 60

C = 60

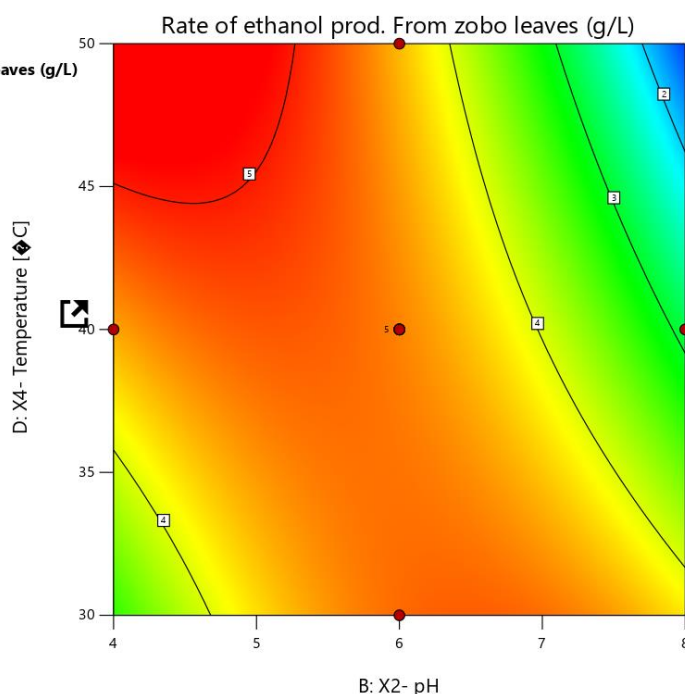


Figure 17: Contour plot of pH and temperature interaction

Figure 17 shows that the highest ethanol production was obtained at an optimum pH of 6 and a temperature of 40 °C. At lower pH and temperature levels, ethanol production was relatively low; however, the production rate increased progressively as the pH and temperature approached the optimum levels. This indicates that the interaction between pH and temperature significantly influenced ethanol production under the experimental conditions.

Factor Coding: Actual

3D Surface

Rate of ethanol prod. From zobo leaves (g/L)

Design Points:

● Above Surface

○ Below Surface

1.2 5.1

X1 = B

X2 = D

Actual Factors

A = 60

C = 60

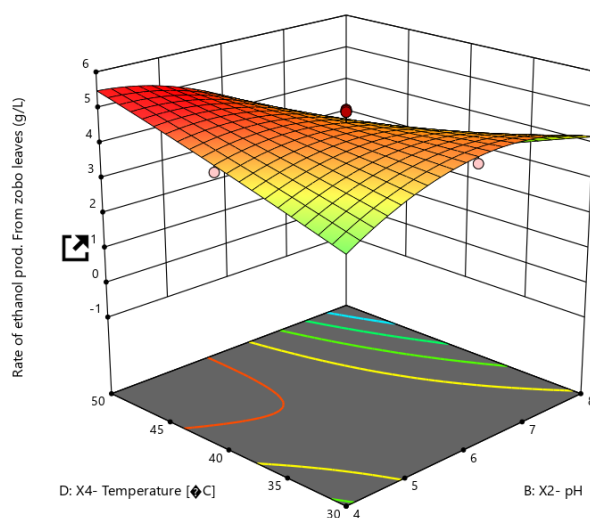


Figure 18: 3D plot for pH and temperature interaction

The 3D plot in Fig. 18 represents the interactive effect of pH and temperature in a different view (i.e in a 3 dimensional view) but has the same explanation as the contour plot for pH and temperature interactive effect stated above.

Factor Coding: Actual

Rate of ethanol prod. From zobo leaves (g/L)

Rate of ethanol prod. From zobo leaves (g/L)

● Design Points

1.2 5.1

X1 = C

X2 = D

Actual Factors

A = 60

B = 6

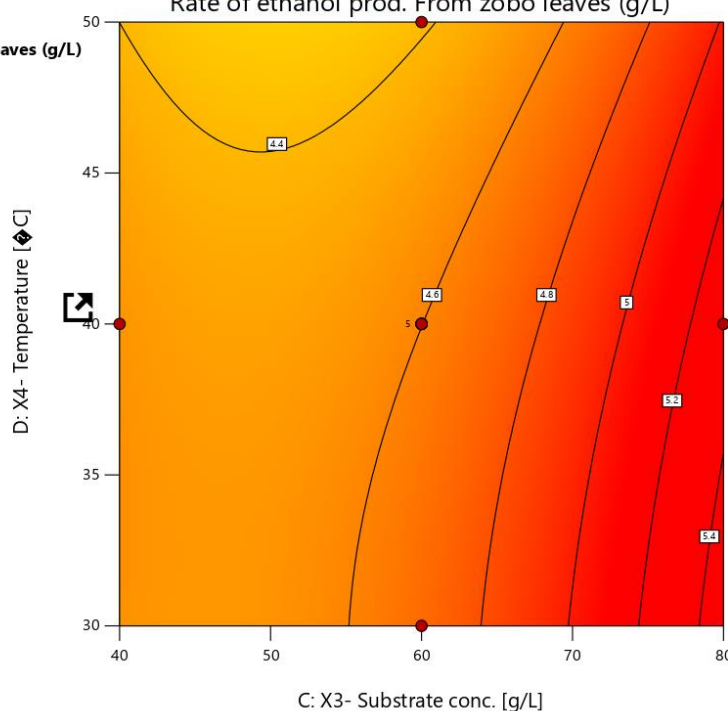


Figure 19: Contour plot of substrate concentration and temperature interaction

Figure 19 illustrates the interactive effect of substrate concentration and temperature on ethanol production. The results indicate that ethanol production increased with increasing substrate concentration up to the optimum level of 60 g/L, particularly at the optimum temperature of 40 °C. Beyond 60 g/L, however, further increases in substrate concentration resulted in a decline in ethanol production across the investigated

temperature range. This suggests that substrate concentrations above the optimum level did not enhance fermentation performance under the experimental conditions.

Factor Coding: Actual

3D Surface

Rate of ethanol prod. From zobo leaves (g/L)

Design Points:

● Above Surface

○ Below Surface

1.2 5.1

X1 = C

X2 = D

Actual Factors

A = 60

B = 6

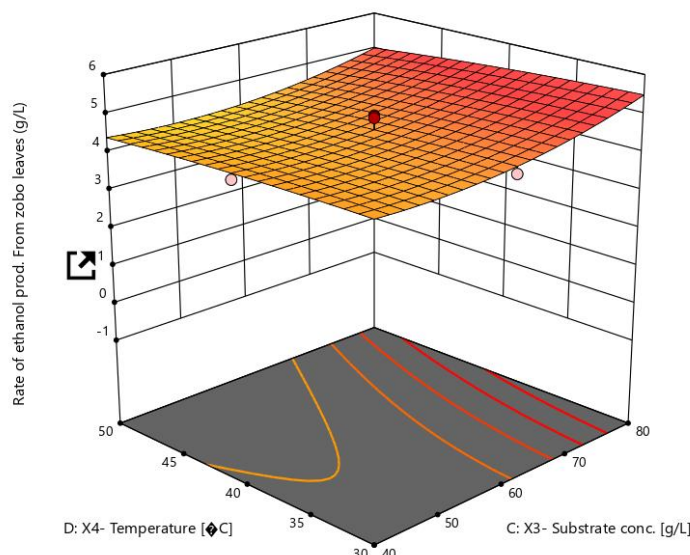


Figure 20: 3D plot for substrate concentration and temperature interaction

The 3D plot in Fig. 20 represents the interactive effect of pH and temperature in a different view (i.e in a 3 dimensional view) but has the same explanation as the contour plot for pH and temperature interactive effect stated above.

Table 13: Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:X1- Incubation Time [Hrs]	Within range	48	72	1	1	3
B:X2- pH	Within range	4	8	1	1	3
C:X3- Substrate conc. [g/L]	Within range	40	80	1	1	3
D:X4- Temperature [°C]	Within range	30	50	1	1	3
Rate of ethanol prod. From zobo leaves	Maximize range	1.2	5.1	1	1	3

Table 14: 10 out of 100 Solutions found

Number	X1- Incubation Time [Hrs]	X2- pH	X3- Substrate conc. [g/L]	X4- Temperature [°C]	Rate of ethanol prod. From zobo leaves	Desirability	
1	60.583	4.567	74.145	47.909	5.480	1.000	Selected
2	53.150	4.392	46.797	46.782	5.659	1.000	
3	55.835	6.442	75.742	32.001	5.364	1.000	
4	60.043	4.269	42.276	47.658	5.307	1.000	
5	53.700	5.794	69.188	42.094	5.143	1.000	
6	64.330	4.167	79.752	49.740	5.206	1.000	
7	52.865	4.964	41.964	47.938	5.685	1.000	



8	54.045	4.169	64.933	43.139	5.241	1.000	
9	59.379	4.096	79.055	39.877	5.104	1.000	
10	57.321	6.022	70.330	31.825	5.134	1.000	

Experimental Replicates and Data Analysis

All fermentation experiments were conducted in triplicate using independent fermentation vessels under identical experimental conditions. Independent experimental replicates were used to account for experimental variability and to improve the reliability of the measured ethanol production. The results obtained from replicate experiments were expressed as mean $\pm$ standard deviation (SD). For the experimental validation, three independent replicates were performed, and the resulting ethanol concentrations were used to calculate the mean and standard deviation.

Experimental Validation of the Selected Fermentation Condition

The selected experimental condition obtained from the response surface analysis was experimentally validated to compare the observed ethanol production with the model-predicted response. Three independent fermentation experiments were conducted under the selected process conditions, and ethanol concentration was determined using the same analytical procedure applied to the experimental runs. The three validation experiments produced ethanol concentrations of **4.95, 5.10, and 5.25 g/L**, respectively, giving a mean ethanol concentration of **5.10 $\pm$ 0.15 g/L (n = 3)**. The model predicted an ethanol concentration of **5.31 g/L** under the selected experimental condition. The difference between the observed mean and predicted response was therefore **0.21 g/L**, corresponding to a percentage deviation of **3.95%**.

The relatively small difference between the experimental and predicted responses indicates reasonable agreement between the selected experimental condition and the model estimate within the investigated design space

Table 15. Experimental validation of the selected fermentation condition

Validation parameter	Value
Predicted ethanol concentration (g/L)	5.31
Replicate 1 (g/L)	4.95
Replicate 2 (g/L)	5.10
Replicate 3 (g/L)	5.25
Observed ethanol concentration (mean $\pm$ SD, g/L)	5.10 $\pm$ 0.15
Number of replicates (n)	3
Absolute difference (g/L)	0.21
Percentage deviation (%)	3.95

CONCLUSION

The findings of this study demonstrate that roselle (*Hibiscus sabdariffa* L.) has considerable potential as a substrate for bioethanol production using *Saccharomyces cerevisiae*. The extract contained fermentable sugars (glucose, fructose, and sucrose), organic acids (predominantly malic acid), and essential minerals (calcium, potassium, and iron) that support yeast fermentation. Response surface methodology (RSM) with central composite design (CCD) was successfully applied to optimize the fermentation process. The quadratic model was statistically significant ($F = 17.81$, $p = 0.0010$) and provided a good fit to the experimental data ($R^2 = 0.9765$; adjusted $R^2 = 0.9217$; C.V. = 5.16%), with a non-significant lack of fit ($p = 0.0924$). Incubation time, initial pH, and substrate concentration had significant linear effects on ethanol production, while temperature was not significant as a single linear variable. The pH $\times$ temperature interaction and the quadratic effects of incubation time and pH were also significant. The optimum fermentation conditions were: incubation time of 60 h, initial pH of 6, substrate concentration of 80 g/L, and temperature of 40 °C, yielding an ethanol concentration of 5.10 g/L, which closely agreed with the model-predicted value of 5.31 g/L (3.95% deviation).



Diagnostic plots confirmed the model satisfied the assumptions of normality, independence, and constant variance. One limitation was the negative predicted R^2 (-1.1933), indicating limited predictive reliability for new observations outside the experimental runs. Future studies should increase experimental runs and conduct independent validation experiments.

Acknowledgements

The authors acknowledge the Department of Chemistry, Delta State University, Abraka, for analytical support.

Competing interests

The authors declare no competing interests.

Funding

The authors declare that this research was conducted without any external financial funding

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