Optimization of Bioethanol Production from Cassava Wastewater Using Response Surface Methodology.

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Abstract- ***Nigeria, a leading global producer of cassava, generates substantial amounts of processing waste, including wastewater. This readily available feedstock presents an opportunity to address the environmental challenges associated with improper waste disposal. Cassava wastewater, if not managed effectively, can contaminate water sources and contribute to soil degradation. This study investigated the optimization of bioethanol production from cassava wastewater using Response Surface Methodology (RSM). Four independent variables, namely temperature (25-45 °C), pH (6-8), incubation time (3-7 days), and wastewater concentration (25-100%), were evaluated using a Central Composite Design. A two-factor interaction (2FI) model was found to be statistically significant ($p < 0.0001$), with an adjusted R^2 of 0.9463 and a predicted R^2 of 0.8809, indicating a good fit. The model predicted maximum ethanol yield (17.34% v/v) at 45 °C, pH 8, 6 days' incubation, and 100% wastewater concentration. Validation experiments confirmed the model's accuracy, with an observed ethanol yield of 16.50% ± 0.87% (v/v), which was within the 95% prediction interval. Concurrent analysis revealed significant reductions in heavy metal concentrations (e.g., lead from 0.032 mg/L to 0.0021 mg/L) and total solids (from 119.65 mg/L to 3.71 mg/L) in the wastewater after fermentation. These findings demonstrate the feasibility of bioethanol production from cassava wastewater, offering a sustainable solution for waste management and a promising avenue for renewable energy generation.***

Indexed Terms- ***Bioethanol, cassava, waste water, palm wine, saccharomyces cerevisae, RSM, Optimisation, and renewable energy***

I. INTRODUCTION

Cassava (Manihot esculenta Crantz) is a vital staple food crop in many developing countries, particularly in sub-Saharan Africa, where it plays a crucial role in ensuring food security for millions (Ferraro et al., 2016). Nigeria, a leading global producer, has prioritized cassava cultivation, with the crop deeply integrated into the nation's agricultural and economic landscape (Otekunrin & Sawicka, 2019). However, the significant production volumes generate substantial amounts of waste, primarily in the form of peels and wastewater (Onyediako & Adiele, 2022; Ikuemonisan et al., 2020). These by-products, if not managed effectively, pose significant environmental challenges, including water pollution, soil degradation, and greenhouse gas emissions (Oghenejoboh, Ikhiamhe, & Oghenejoboh, 2021).

This study addresses these environmental concerns by investigating the potential of bioethanol production from cassava wastewater. Bioethanol, a renewable biofuel, offers a sustainable alternative to fossil fuels, aligning with global efforts to mitigate climate change and enhance energy security (Pothiraj et al., 2015). Furthermore, utilizing cassava wastewater for bioethanol production promotes a circular economy, minimizing waste disposal and creating value from a previously underutilized resource (Ekop, Ekanem, & Okon, 2019; Nizzy & Kannan, 2022).

This research employs Response Surface Methodology (RSM), a powerful statistical technique, to optimize bioethanol production. By systematically investigating the effects of key process parameters, RSM will identify optimal conditions that maximize ethanol yield while minimizing process variability. The findings of this study have significant implications for sustainable cassava processing, promoting environmental stewardship, enhancing economic viability, and contributing to a more sustainable energy future.

Methodology:

Collection of Cassava Wastes, Palm Wine and Enzymes

Cassava peels and wastewater were collected from cassava processing plants in Oyigbo. The peels were stored in sack bags, while the wastewater (45 L) was collected in 50 L-capacity Jerrycan. Both samples were immediately transported to the University of Port Harcourt Microbiology Laboratory to preserve their microbial integrity. The cassava wastewater was intended for bioethanol production, while the peels were designated for fish feed production.

Palm wine, produced from the Raffia palm, was purchased from a local tapper in Ahoada. It was collected in a clean five-litre plastic container and transported to the laboratory for the isolation of yeast strains. The focus was on isolating high-alcohol-tolerant yeast from the palm wine for potential fermentation processes.

The enzymes used in this study—Cereflo (β-gluconase + α-amylase), AMG (Amyloglucosidase), and Termamyl (Amylase)—were obtained from the Department of Microbiology and Brewing laboratory at Nnamdi Azikiwe University, Awka, Anambra State.

Isolation, Characterisation and Identification of Yeast

Isolation

Using palm wine as a reference sample for high alcohol-tolerant strains, yeast isolation was performed. The palm wine was fermented for 21 days to allow the proliferation of yeast. The fermented sample was centrifuged at low speed for five minutes, and the sediment was serially diluted. One millilitre of the diluent was streaked onto Glucose Yeast Agar (GYA) plates to isolate yeast colonies. The plates were incubated at 28°C for 24 hours, allowing for the growth of yeast colonies. Pure isolates were obtained through successive streaking on GYA plates to ensure the isolation of the fermentative yeast strains suitable for bioethanol production (Amoikon et al., 2019; Ejimofor et al., 2021; Frances et al., 2023).

Characterisation and Identification

The yeast isolates were characterised and identified based on their morphological traits, physiological characteristics, and fermentation patterns. The identification process followed the keys provided by Amoikon et al. (2019), Ejimofor et al. (2021) and Frances et al. (2023), focusing on distinguishing yeast species with strong fermentative abilities. Identification involved Gram staining and biochemical tests to ascertain yeast suitability for ethanol production.

Gram Staining

The Gram staining technique was applied to differentiate between Gram-positive and Gram-negative yeast cells, an essential step in identifying yeast that could withstand industrial fermentation conditions. A smear of yeast cells was heat-fixed on a microscope slide, followed by staining with crystal violet and Gram iodine. After decolourisation with alcohol and counter-staining with safranin, the cells were examined microscopically at ×100 magnification to confirm their Gram characteristics, which are linked to cell wall structure and potential fermentation efficiency (Amoikon et al., 2019; Ejimofor et al., 2021; Frances et al., 2023).

Sugar Fermentation Test

Yeast fermentation ability was evaluated using different sugars: glucose, lactose, sucrose, maltose, and mannitol. Each sugar was prepared in phenol red broth medium and inoculated with yeast isolates. The tubes were incubated at 30°C for 48 hours. A colour change from red to yellow indicated successful sugar fermentation, signalling the yeast's potential for bioethanol production from cassava wastewater. This test provided insights into the yeast’s ability to utilise diverse sugars present in cassava waste (Amoikon et al., 2019; Ejimofor et al., 2021; Frances et al., 2023).

Ethanol Tolerance Test

To determine the ethanol tolerance of the isolated yeast strains, they were inoculated into YPG broth containing varying concentrations of ethanol (5%, 10%, 15%, and 20%). The cultures were incubated at 30°C for two days, and yeast growth was assessed by plating serial dilutions on YPG agar. The resulting colonies were counted to evaluate the yeast's ability to survive and thrive in high ethanol concentrations, which is critical for efficient bioethanol production ().
Sedimentation Rate Determination

The sedimentation rate of the yeast isolates was measured to assess their flocculation ability, an important trait for yeast reuse in fermentation. Cultures grown on malt yeast extract glucose peptone medium were centrifuged, and a standard cell suspension was prepared. The decrease in optical density over two hours at 650 nm was monitored using a colourimeter. The sedimentation rate was calculated to evaluate the yeast's settling properties during fermentation, which affects its usability in continuous processing systems (Amoikon et al., 2019; Ejimofor et al., 2021; Frances et al., 2023).

$$\text{Sedimentation rate} = \frac{Total\ drop\ in\ reading}{Colimeter\ reading\ at\ 0\ hour} \times 100\%$$

Inoculum Development
A loopful of the isolated yeast culture was inoculated into 100 ml of sterilised YPG broth and incubated at 35°C for 24 hours. This culture served as the inoculum for the fermentation of cassava wastewater. The vegetative cells obtained after incubation were critical for initiating large-scale fermentation experiments for bioethanol production (Amoikon et al., 2019; Ejimofor et al., 2021; Frances et al., 2023).

Determination of Heavy Metals
Heavy metals in cassava wastewater were determined using Atomic Absorption Spectroscopy (AAS) following the method outlined by Olaoye et al. (2020). To prepare the sample, 50 mL of cassava wastewater was digested by adding 5 mL of concentrated nitric acid (HNO_3) and heating the mixture at 95°C for 30 minutes to ensure complete digestion. After cooling, the sample was filtered through the Whatman No. 1 filter paper, and the filtrate was diluted to 50 mL with deionised water. The sample was analysed using an AAS (PerkinElmer AAnalyst 400), and the concentrations of lead, cadmium, and arsenic were determined by comparing the absorbance values against standard calibration curves. Mercury, which was found to be below detectable limits (BDL), was also measured with the AAS but reported as BDL due to its concentration falling below the sensitivity threshold of the instrument.

Determination of Cyanide
The cyanide concentration in the cassava wastewater was determined using the Alkaline Picrate Method as described by Ezeigbo et al. (2015). A 20 mL aliquot of cassava wastewater was mixed with 10 mL of alkaline picrate reagent and incubated in a water bath at 37°C for 1 hour. The reaction produced a reddish-brown colour, indicating the presence of cyanide. The absorbance of the coloured solution was measured at 510 nm using a UV-Vis spectrophotometer (Thermo Scientific Evolution 201), and the cyanide concentration was determined by comparing the absorbance to a standard curve generated using known concentrations of potassium cyanide (KCN).

Determination of Total Solid Content
The total solid content of the cassava wastewater was measured using the Gravimetric Method Gilmore & Luong, 2016). A 100 mL sample of cassava wastewater was poured into a pre-weighed porcelain dish and placed in an oven at 105°C for 24 hours to evaporate the water and leave behind only the solid content. After drying, the dish was cooled in a desiccator and weighed again. The total solid content was calculated by subtracting the weight of the empty dish from the weight of the dish containing the dried residue, providing the concentration of suspended and dissolved solids in the wastewater.

Determination of Ethanol Concentration
The ethanol concentration in the cassava wastewater was measured using the Distillation and Dichromate Titration Method (Srimuang & Polprasert, 2019). First, 100 mL of the cassava wastewater was distilled to collect the ethanol vapour and condensed into a receiving flask. The distillate was treated with potassium dichromate solution and concentrated sulphuric acid, and the mixture was heated to allow ethanol to reduce the dichromate to chromium (III). The amount of remaining dichromate was then titrated with ferrous ammonium sulphate. The ethanol

concentration was calculated and expressed as a percentage (% v/v) based on the titration results.

Determination of Starch Content

The starch content in the cassava wastewater was determined using the Enzymatic Method (Ezeigbo et al., 2015). A 10 mL wastewater sample was hydrolysed with 5 mL of α-amylase enzyme at 90°C for 30 minutes to convert the starch to glucose. The glucose concentration was then measured using the dinitrosalicylic acid (DNS) method. One millilitre of the hydrolysate was mixed with DNS reagent and boiled for 10 minutes, after which the absorbance was read at 540 nm using a UV-Vis spectrophotometer. The starch content was calculated by back-calculating from the glucose concentration, using the stoichiometric relationship between glucose and starch.

Digestion of Cassava Wastewater and Estimation of Reducing Sugar

The digestion of cassava wastewater and estimation of reducing sugar was adapted from Archibong et al. (2016) as a preliminary assessment of the combined activity of the three enzymes—Cereflo (β-gluconase + α-amylase), AMG (Amyloglucosidase), and Termamyl (Amylase)—to produce reducing sugar, which can be fermented by yeast to produce ethanol.

Digestion of Cassava Wastewater

Fifty millilitres (50 ml) of cassava wastewater were measured into six 60 ml-capacity conical flasks. Three flasks were heated to boiling for 10 minutes to break down the solids in the wastewater, while the other three served as controls without enzyme addition. After cooling, 3 mL of the combined enzyme solution—Cereflo (β-gluconase + α-amylase), AMG (Amyloglucosidase), and Termamyl (Amylase)—were added to each of the three experimental flasks. All flasks were incubated in a water bath at 50°C for two hours with intermittent stirring every 30 minutes to facilitate enzyme activity. Following incubation, the temperature was briefly increased for 5 minutes to deactivate the enzymes. The mixtures were filtered using Whatman No. 1 filter paper, and the liquid fraction was collected for further analysis.

Estimation of Reducing Sugar

The reducing sugar content in the filtrates was estimated using the dinitrosalicylic acid (DNS) method described by Archibong et al. (2016). In test tubes, one millilitre of DNS reagent was added to 1 mL of each filtrate, including those from the experimental and control groups. The mixtures were heated in boiling water for 10 minutes, and then rapidly cooled under tap water. The final volume of each sample was adjusted to 12 mL with distilled water. A blank solution, containing 1 mL of distilled water and 1 mL of DNS reagent, was prepared for calibration. The samples' optical density (OD) was measured at 540 nm using a spectrophotometer against the blank. The concentration of reducing sugars in the filtrates was calculated using a glucose standard curve, and the average was determined from the three concentrations for both the experimental samples and the controls, providing insight into the effectiveness of the enzyme treatment.

Preliminary Evaluation of Cultural Parameters on Ethanol Yield

To assess the yeast's fermentative potential in cassava wastewater, the effects of pH, temperature, wastewater (substrate) concentration and incubation time on ethanol production were tested to obtain critical values of the parameters for optimising ethanol production as shown in Table 3.1.

The effect of temperature on ethanol production was assessed by adjusting the temperature of the culture medium (between 25 and 65^{o}C) while keeping other parameters such as pH (at 8), incubation time (5 days) and substrate concentration (100%) constant.

Similarly, the effect of different pH on ethanol production was assessed by adjusting the pH of the culture medium (between 5 and 9) while keeping other parameters such as temperature (at 30^{o}C), incubation time (5 days) and substrate concentration (100%) constant.

Likewise, the effect of incubation time on ethanol production was determined by adjusting the incubation time of the culture medium (between 1 and 7 days) while keeping other parameters such as temperature (30^{o}C), pH (at 8) and substrate concentration (100%) constant.

Finally, the effect of the wastewater (substrate) concentration on ethanol production was studied by adjusting the substrate concentration in the culture medium (between 25 and 100%) using sterile distilled water while keeping other parameters such as temperature (30ºC), pH (at 8) and incubation time (5 days) constant.

For each experiment to determine the effects of pH, temperature, wastewater (substrate) concentration and incubation time on ethanol production, 500 ml of the cassava wastewater was measured into a 1000 ml-capacity Erlenmeyer flask and heated to 90°C for ten minutes. The temperature was then reduced to 50°C, after which 8 mL of the enzyme solution containing a combination of Cereflo (β-gluconase + α-amylase), AMG (Amyloglucosidase), and Termamyl (Amylase) was added. The solution was left to stand for one hour to allow enzymatic digestion and then filtered using Whatman No. 1 filter paper.

The filtrate was sterilised at 121°C (15 psi) for 15 minutes. After cooling, the filtrate was inoculated with 5% (v/v) of the prepared yeast inoculum (standardised to 0.5 McFarland, equivalent to approximately 1.5 x 10^8 cells).

The fermentation of cassava wastewater was conducted at 30°C and pH 8 for five days, and under the varying conditions above, with continuous agitation at 100 rpm. After fermentation, the cell-free broth was analysed for ethanol production, providing insights into the yeast's efficiency in converting cassava wastewater into bioethanol.

Optimisation of Ethanol Production and Statistical Analysis

The optimisation of ethanol production from cassava wastewater was performed using Response Surface Methodology (RSM) with a Central Composite Design (CCD) (Ugwuodo et al., 2021). Four independent variables—pH, temperature, incubation time, and cassava wastewater concentration—were examined across a defined range, as presented in Table 3.1. These variables were chosen based on preliminary findings in section 3.6.2, which identified their influence on ethanol production. The factor levels were set as follows: pH (6 to 8), temperature (25 to 45°C), incubation time (3 to 7 days), and wastewater concentration (25 to 100%). Agitation and enzyme volume were controlled at 100 rpm and 8 ml for all experimental runs.

Design Expert version 13 generated 30 experimental runs based on CCD, ensuring the inclusion of minimum and maximum factor levels. A Two-Factor Interaction (2FI) model was used to describe the relationship between the independent variables and ethanol yield, as this model was suggested based on statistical evaluation. The system's behaviour was modelled using the following equation:

$$Y = \beta_0 + \sum_{i=1}^{K} \beta_i x_i + \sum_{i=1}^{K-1} \sum_{j=i+1}^{K} \beta_{ij} x_i x_j + e$$

Where:
Y represents the response (ethanol Yield),
x_i and x_j are the independent variables,
β_0, β_i, and β_{ij} are the model's regression coefficients, and
e represents the model's error term.

The adequacy of the 2FI model was assessed using analysis of variance (ANOVA), which included evaluating key indicators such as the coefficient of determination (R^2), adjusted R^2, and predicted R^2. A non-significant lack of fit and a significant model F-value were used to confirm the validity of the model. Additionally, the comparison of actual and predicted ethanol yields was made to ensure the model's accuracy and predictive capability. The 2FI model was selected over the quadratic model due to its higher predictive performance and the non-significant lack of fit, as confirmed by the ANOVA results.

Table 1a: Experimental Design for Optimising Ethanol Production

Factor	Name	Units	Type	Sub-Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Temperature	ºC	Numeric	Continuous	25	45	-1 ↔ 25.00	+1 ↔ 45.00	35.00	10.00
B	pH		Numeric	Continuous	6	8	-1 ↔ 6.00	+1 ↔ 8.00	7.00	1.00
C	Time	Days	Numeric	Continuous	4	6	-1 ↔ 4.00	+1 ↔ 6.00	5.00	1.00
D	Wastewater Concentration	wt%	Numeric	Continuous	50	100	-1 ↔ 50.00	+1 ↔ 100.00	75.00	25.00

Table 1b: Actual Composition of the Experimental Design

Std	Run	Temperature (ºC)	pH	Incubation Time (Days)	Wastewater (wt%)	Concentration
11	1	25	8	4	100	
17	2	25	7	5	50	
27	3	35	7	5	75	
18	4	35	7	5	50	
28	5	35	7	5	50	
30	6	45	7	5	75	
2	7	45	6	4	75	
7	8	25	8	6	100	
9	9	25	6	4	100	
25	10	35	7	5	50	
29	11	45	7	5	75	
16	12	45	8	6	100	
21	13	35	7	4	50	
12	14	45	8	4	100	
20	15	35	8	5	50	
26	16	35	7	5	50	
24	17	45	7	5	100	
5	18	25	6	6	50	
8	19	45	8	6	75	
10	20	45	6	4	100	
4	21	45	8	4	50	
3	22	25	8	4	75	
14	23	45	6	6	100	
19	24	35	6	5	50	
13	25	25	6	6	100	
1	26	25	6	4	75	
23	27	45	7	5	50	

6	28	45	6	6	100
15	29	25	8	6	100
22	30	45	7	6	50

Validation of the Statistical Model

To validate the statistical model for optimising the conditions used to maximise ethanol production, the cassava wastewater was fermented in triplicates under the influence of the optimum conditions (for temperature, pH, incubation time and cassava wastewater concentration) predicted by the RSM – CCD to ensure reliability. During validation, key parameters such as total solids, cyanide content, heavy metal concentrations, starch content, ethanol concentration and distillate volume were estimated and recorded before and after the fermentation period. However, foam formation was evaluated at 12-hour intervals for the predicted optimum period (72 hours), as an indicator of yeast activity. These assessments provided a comprehensive evaluation of the changes in the medium and confirmed the effectiveness of the optimised conditions.

The ethanol fermentation was carried out in Erlenmeyer flasks under the predicted optimum conditions, with samples taken at designated intervals. The concentration of the ethanol produced was quantified and the results were compared with the concentration of ethanol predicted by the model under the optimum conditions. This comparison allowed for assessing the model's accuracy and robustness, confirming its validity for optimising ethanol production from cassava wastewater.

Ethanol Quantification

Ethanol Assay Using Potassium Dichromate and Sulphuric Acid: To quantify ethanol production, 1 mL of the cell-free culture obtained after centrifugation was diluted to 5 mL with distilled water. Then, 1 mL of potassium dichromate ($K_2Cr_2O_7$) solution and 4 mL of concentrated sulphuric acid (H_2SO_4) were added. The absorbance of the resulting colour change was measured at 660 nm using a VIS spectrophotometer (AXION 721). A blank sample was prepared by replacing the culture supernatant with distilled water. Ethanol concentration was determined by comparing the absorbance values with a standard ethanol calibration curve (Zhang et al., 2019).

Ethanol Distillation and Measurement of Distillate Volume

Following fermentation in the YPG medium under optimised conditions, ethanol was recovered from the fermented broth using fractional distillation. The distillation process was conducted by heating the fermented broth in a distillation apparatus until ethanol vaporised. The ethanol vapour was condensed into liquid form and collected as the distillate. The volume of the ethanol distillate was measured using a calibrated volumetric cylinder to ensure precision in quantifying the amount of ethanol produced (Sukasem et al., 2017).

Determination of pH, Temperature and Total Solids

The pH was measured using a calibrated pH meter. A 5 g sample of wastewater was mixed with 20 mL of distilled water, stirred for 10 minutes, and left to stand for 30 minutes before measurement. The pH electrode was rinsed with distilled water and immersed in the sample, and readings were recorded once stabilised.

Temperature was recorded using a glass thermometer with 0.1°C graduations or an electronic thermometer. The thermometer was immersed in the sample until a stable reading was achieved, and the result was recorded to the nearest 0.1°C.

Total solids were determined using a multipurpose meter (EC400, Taiwan) to measure the concentration of dissolved and suspended solids in the wastewater.

Foam Formation Volume

Foam formation was observed as a visual indicator of fermentation activity during ethanol production (Umo et al., 2013). To quantify foam formation, the height of the foam layer in the fermentation flask was measured at regular intervals using a hand-held digital calliper. The foam volume was calculated based on the cross-sectional area of the flask and the measured foam height using Equations 3.2b and 3.2c. This parameter was recorded to monitor the fermentation's progress, as increased foam formation typically

correlates with active yeast metabolism and ethanol production.

Cross-Sectional Area = π × (Flask Diameter / Diameter)^2

2

Foam Volume (cm^3) = Cross-Sectional Area × Foam Height

Results:

Table 2.0: Characteristics of Yeasts Isolated from the Wastewater

Properties of the Yeasts Isolated from the Wastewater		
Morphological Characteristics of the Yeast Isolates (YI)		
Morphological Parameter	YI_1	YI_2
Surface	Smooth	Smooth
Margin	Entire	Entire
Colony Color	Cream	Cream
Size (mm)	0.5	0.3
Shape	Spherical	Ellipsoidal
Vegetative Reproduction	Budding	Budding
Probable Organism	S. cerevisiae	S. cerevisiae
Carbohydrates Fermentation by the Yeast Isolates (YI)		
Carbohydrate	YI_1	YI_2
Glucose	+	+
Lactose	-	-
Sucrose	+	+
Maltose	-	-
Mannitol	+	+
Ethanol Tolerance and Sedimentation Rate of the Yeast Isolates (YI)		
Ethanol Concentration (%)	Sedimentation Rate of YI_1	Sedimentation Rate of YI_2
5	65.4	60.1
10	67.3	75.1
15	66.9	69.2
20	68.4	73.9

Table 3: Physicochemical Parameters of Cassava Wastewater Before Fermentation

Parameter	Amount
Lead (mg/l)	0.032
Cadmium (mg/l)	0.0015
Arsenic (mg/l)	0.007
Mercury (mg/l)	BDL
Cyanide (mg/l)	0.029
Total Solid (mg/l)	119.65
Ethanol Concentration (v/v%)	2.28
Starch Content (%)	46.84

Key: BDL = Below detectable limit

Table 4: Reducing Sugar Yield of Enzymatic Digestion of Cassava Wastewater

Sample Group	Replicates	Optical Density (OD) at 540 nm	Reducing Sugar Concentration (g/L)	Notes
Experimental	Sample 1	0.35	15.0	Enzyme solution added
	Sample 2	0.30	12.5	Enzyme solution added
	Sample 3	0.32	13.0	Enzyme solution added
Average		0.32	13.5	-
Control	Control 1	0.10	4.0	No enzyme solution added
	Control 2	0.08	3.5	No enzyme solution added
	Control 3	0.09	3.8	No enzyme solution added
Average		0.09	3.77	-

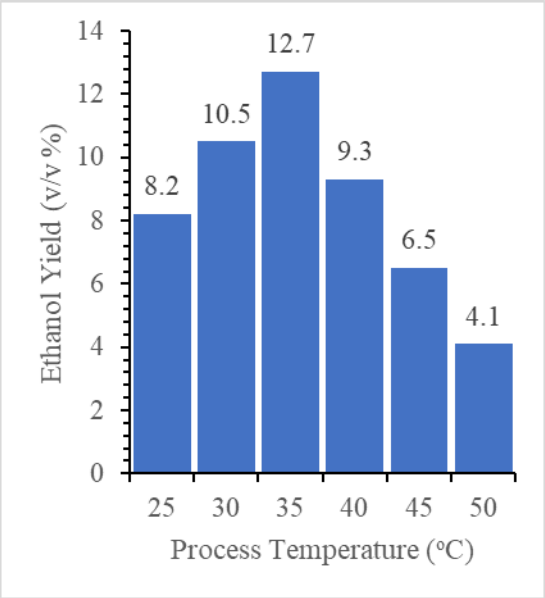


Figure 1: Effect of temperature on ethanol production

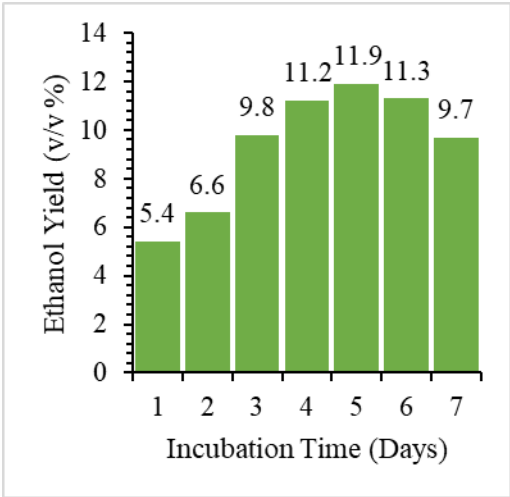


Figure 3: Effect of incubation time on ethanol production

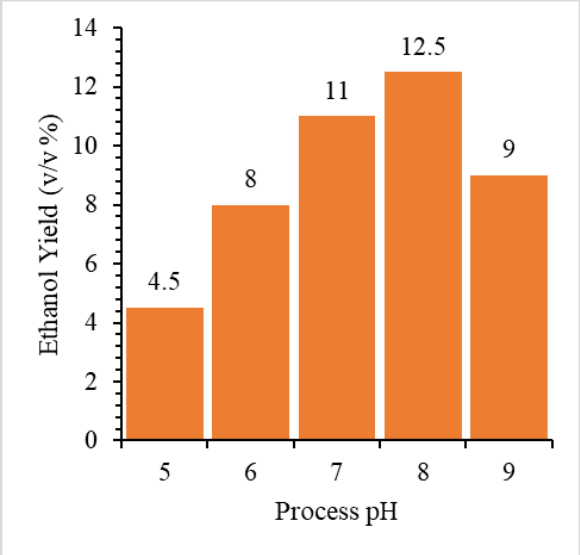


Figure 2: Effect of pH on ethanol production

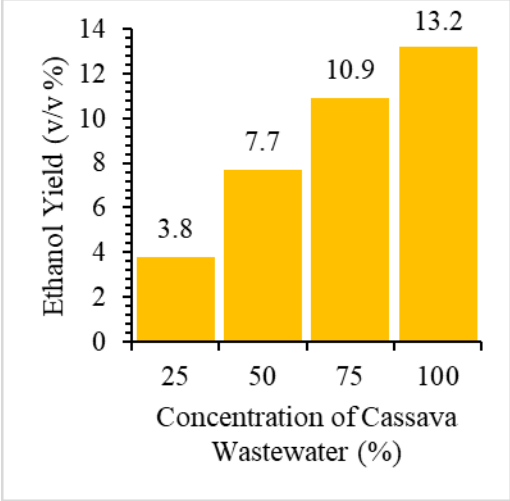


Figure 4: Effect of cassava wastewater concentration on ethanol production

Table 5 Composition of Experiments Optimising Ethanol Production

Run Order	Temperature (°C)	pH	Incubation Time (Days)	Wastewater Concentration (wt%)	Ethanol Yield (v/v%)	Predicted Value
1	25	8	4	100	5.5	6.23
2	25	7	5	50	8.2	8.32
3	35	7	5	75	11.9	11.25
4	35	7	5	50	9.6	10.19
5	35	7	5	50	9.8	10.19
6	45	7	5	75	15.2	14.58
7	45	6	4	75	13.7	14.07

8	25	8	6	100	8.8	7.36
9	25	6	4	100	7.9	7.32
10	35	7	5	50	10.4	10.19
11	45	7	5	75	13.2	14.58
12	45	8	6	100	16.4	17.34
13	35	7	4	50	11.4	10.99
14	45	8	4	100	17.8	16.91
15	35	8	5	50	11.7	11.09
16	35	7	5	50	10.9	10.20
17	45	7	5	100	18.3	17.12
18	25	6	6	50	6.9	7.32
19	45	8	6	75	14.3	14.76
20	45	6	4	100	15.6	16.55
21	45	8	4	50	13.9	14.47
22	25	8	4	75	7.5	7.95
23	45	6	6	100	17.3	17.66
24	35	6	5	50	9.4	9.28
25	25	6	6	100	8.6	9.13
26	25	6	4	75	7.9	7.78
27	45	7	5	50	12.6	12.05
28	45	6	6	100	18.5	17.66
29	25	8	6	100	6.6	7.36
30	45	7	6	50	11.2	11.07

Table 6: Fit Summary for the Response, Ethanol Yield

Source	Sequential p-value	Lack of Fit P-value	Adjusted R^2	Predicted R^2	
Linear	8.9093e-11	0.1460	0.8506	0.8077	
2FI	0.00015	0.7883	0.9463	0.8809	Suggested
Quadratic	0.7398	0.6996	0.9398	0.8638	
Cubic	0.6996		0.9265		Aliased

Table 7: Sequential Model Sum of Squares [Type I] for the Response, Ethanol Yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	4106.7	1	4106.7			
Linear vs Mean	354.4669	4	88.6167	42.2684	8.9093e-11	
2FI vs Linear	38.0875	6	6.3479	8.4192	0.00015	Suggested
Quadratic vs 2FI	1.6699	4	0.4175	0.4948	0.7398	
Cubic vs Quadratic	6.4681	9	0.7187	0.6969	0.6996	Aliased
Residual	6.1875	6	1.03125			
Total	4513.58	30	150.4526			

Select the highest-order polynomial where the additional terms are significant and the model is not aliased.

Table 8: Model Summary Statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	1.45	0.8712	0.8506	0.8077	78.25	
2FI	0.8683	0.9648	0.9463	0.8809	48.46	Suggested
Quadratic	0.9185	0.9689	0.9399	0.8638	55.42	
Cubic	1.02	0.9848	0.9265		*	Aliased

Table 9: Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Linear	46.23	19	2.43	2.36	0.1461	
2FI	8.14	13	0.6260	0.6070	0.7884	Suggested
Quadratic	6.47	9	0.7187	0.6969	0.6996	
Cubic	0.0000	0				Aliased
Pure Error	6.19	6	1.03			

Table 10: ANOVA of 2FI Model for the Response, Ethanol Yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	392.55	10	39.26	52.06	< 0.0001	significant
A-Temperature	63.82	1	63.82	84.64	< 0.0001	
B-pH	2.32	1	2.32	3.07	0.0958	
C-Incubation Time	1.62	1	1.62	2.14	0.1596	
D-Wastewater Concentration	23.72	1	23.72	31.46	< 0.0001	
AB	1.98	1	1.98	2.63	0.1213	
AC	0.4891	1	0.4891	0.6487	0.4305	
AD	26.36	1	26.36	34.96	< 0.0001	
BC	0.4324	1	0.4324	0.5735	0.4582	
BD	3.58	1	3.58	4.74	0.0422	
CD	4.74	1	4.74	6.28	0.0214	
Residual	14.33	19	0.7540			
Lack of Fit	8.14	13	0.6260	0.6070	0.7884	not significant
Pure Error	6.19	6	1.03			
Cor Total	406.88	29				

Factor coding is Coded.

Table 11: Fit Statistics

Fit Statistic	Value
Standard Deviation (Std. Dev.)	0.8683
Mean	11.70
Coefficient of Variation (C.V. %)	7.42
R^2	0.9648
Adjusted R^2	0.9463
Predicted R^2	0.8809
Adequate Precision (Adeq Precision)	21.7543

Table 12: Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	10.72	1	0.1851	10.33	11.11	
A-Temperature	2.60	1	0.2826	2.01	3.19	2.27
B-pH	0.5921	1	0.3379	-0.1151	1.30	2.72
C-Incubation Time	-0.9288	1	0.6345	-2.26	0.3991	2.40
D-Wastewater Concentration	1.60	1	0.2844	0.9999	2.19	1.10
AB	0.3639	1	0.2244	-0.1057	0.8335	1.07
AC	-0.3497	1	0.4341	-1.26	0.5590	1.06
AD	2.20	1	0.3728	1.42	2.98	2.24
BC	-0.3410	1	0.4504	-1.28	0.6016	1.08
BD	-0.9448	1	0.4337	-1.85	-0.0370	2.72
CD	2.05	1	0.8189	0.3385	3.77	2.41

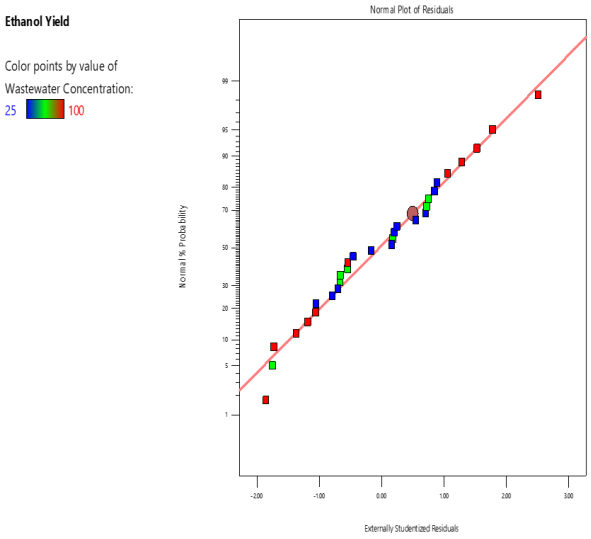


Figure 5: Normal probability plot

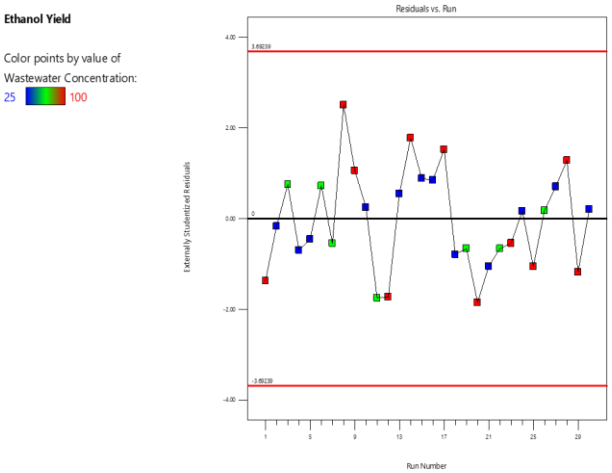
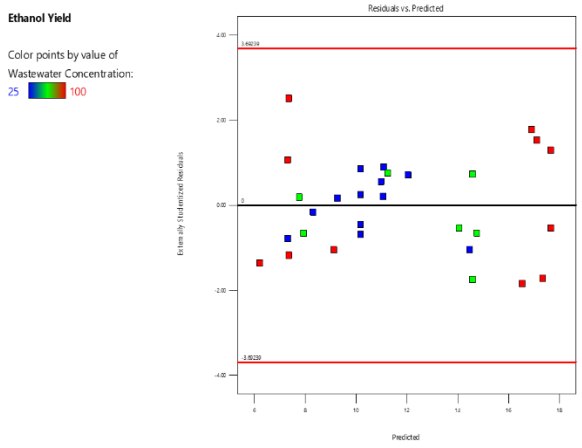


Figure 6.: A plot of residuals vs the experimental run numbers



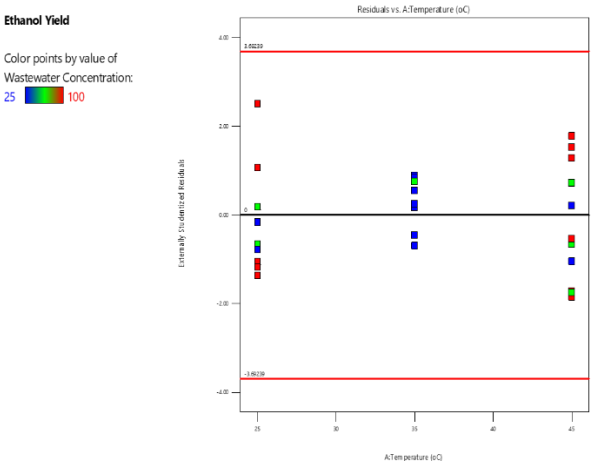


Figure 7: A plot of residuals vs the factors

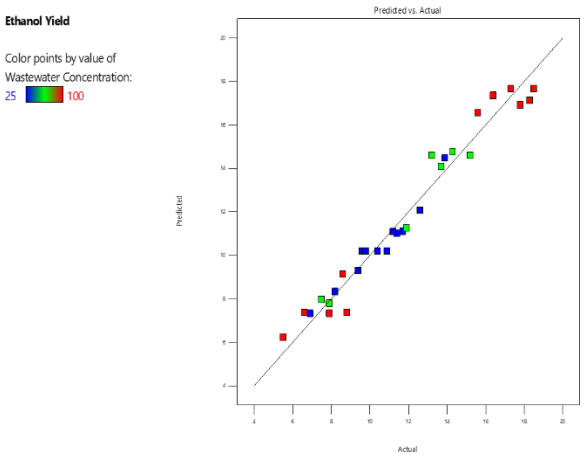


Figure 8: A plot of the predicted vs the actual ethanol yield

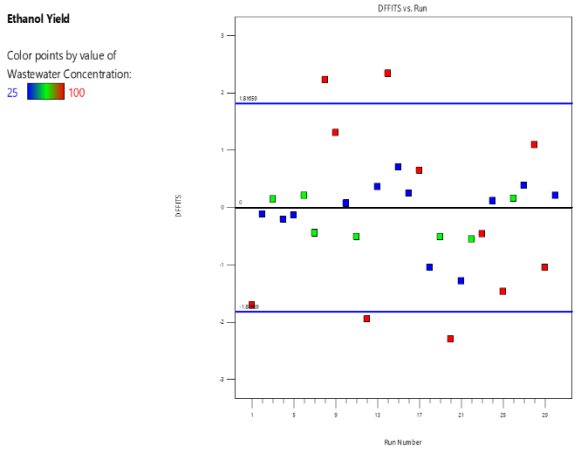


Figure 10: A plot of DFFITS (DFFITS vs Experimental runs)

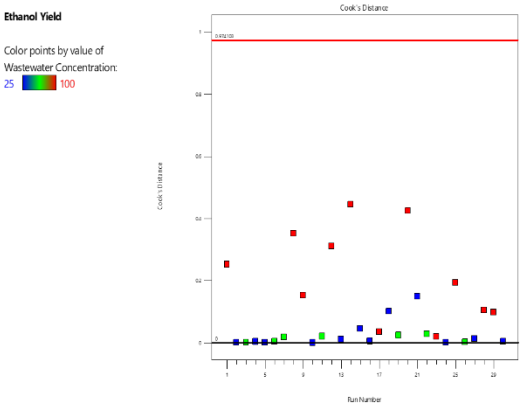


Figure 9: A plot of Cook's distance

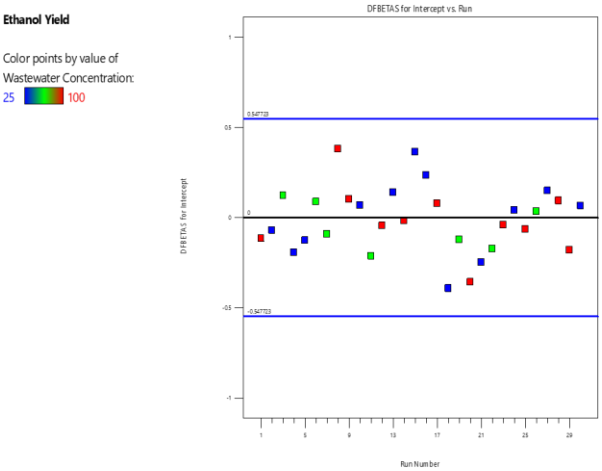


Figure 11: A plot of DFBETAS for intercept vs run

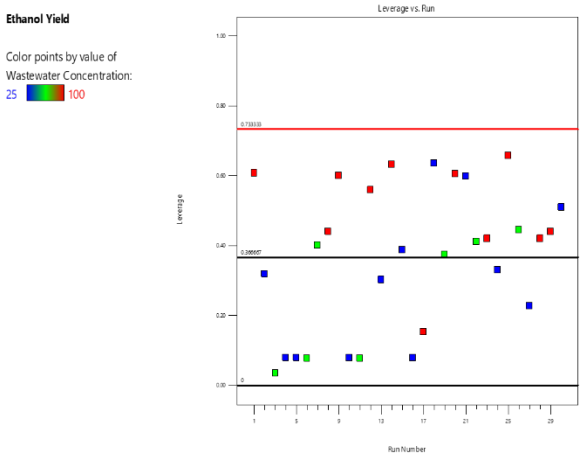


Table 13: Constraints Selected During Optimisation of Ethanol Production

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A: Temperature	is in range	25	45	1	1	3

B: pH	is in range	6	8	1	1	3
C: Incubation Time	minimise	3	7	1	1	3
D: Wastewater Concentration	is in range	25	100	1	1	3
Ethanol Yield	maximise	5.5	18.5	1	1	3

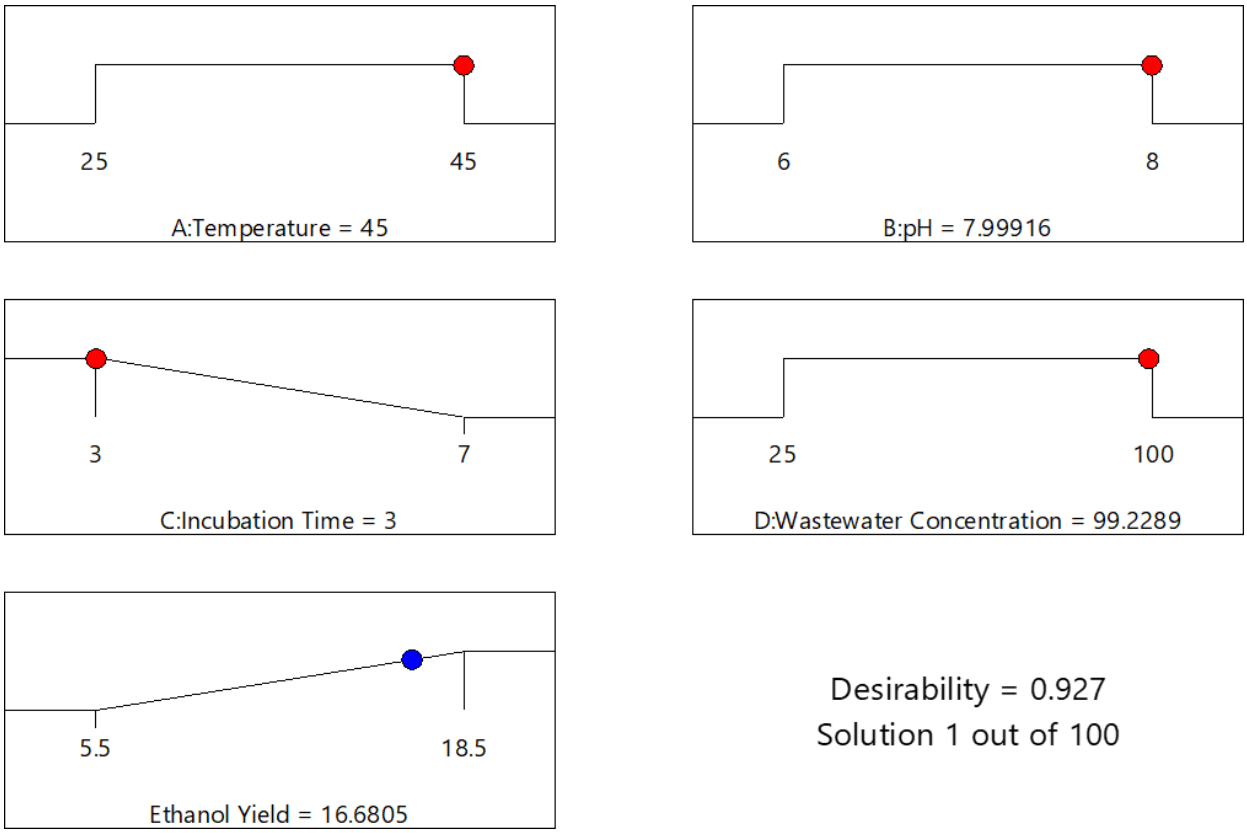


Figure 12: Predicted optimum conditions and the corresponding predicted ethanol yield

Table 14: Confirmation Tests Showing the Actual Observed Ethanol Yield Under the Predicted Optimum Conditions

Solution 1 of 100 Response	Predicted Mean	Predicted Median	Observed	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
Ethanol Yield (v/v%)	16.6952	16.6952	16.50	0.86832	3	1.12677	14.3369	16.5	19.0536

Two-sided Confidence = 95%

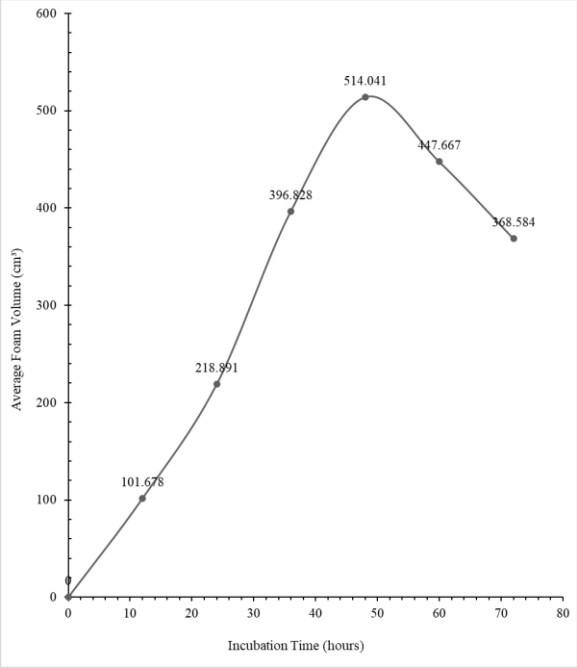


Table 13: Average Volume of Foam Formed During Fermentation Under the Predicted Optimum Conditions in the Confirmation Tests

Table 15: Changes in Physicochemical Parameters of Cassava Wastewater Before and After Fermentation Under the Predicted Optimum Conditions in the Confirmation Tests

Parameter	Before Fermentation	After 3 Days
Lead (mg/l)	0.032	0.0021
Cadmium (mg/l)	0.0015	0.0004
Arsenic (mg/l)	0.007	0.0013
Mercury (mg/l)	BDL	BDL
Cyanide (mg/l)	0.029	0.0015
Total solid (mg/l)	119.65	3.71
Ethanol concentration (v/v%)	2.28	16.50
Starch content (%)	46.84	9.63
Ethanol Distillate (ml)	11.40	82.50

Key: BDL = Below detectable limit

DISCUSSION

- Characteristics of Yeast Isolates and Cassava Wastewater

The yeast isolates from palm wine, identified as *Saccharomyces cerevisiae*, displayed ethanol tolerance and carbohydrate fermentation abilities, indicating their suitability for ethanol production. The physicochemical analysis of cassava wastewater revealed a high starch content and trace amounts of heavy metals, confirming its potential as a fermentation substrate.

The yeast isolates exhibited strong fermentative capabilities, with YI1 demonstrating a more stable ethanol tolerance across varying concentrations, making it a more promising candidate for industrial ethanol production. The ability of both isolates to ferment specific carbohydrates, including glucose and sucrose, highlights their metabolic suitability for converting the starch present in cassava wastewater into ethanol. YI2's fluctuating response to ethanol may indicate sensitivity at higher concentrations, suggesting the need for optimisation. The physicochemical composition of the cassava wastewater, particularly the high starch content, provided an ample substrate for fermentation. Still, the presence of trace heavy metals and cyanide underscores the importance of wastewater management and safety monitoring during production. The isolation of Saccharomyces cerevisiae from palm wine is consistent with previous studies, such as those by Antia et al. (2018) and Ejimofor et al. (2023), which have successfully identified *S. cerevisiae* from palm wine for fermentation purposes. The stable ethanol tolerance shown by YI1 aligns with research by Olaniyi et al. (2019), which demonstrated the high ethanol tolerance of *S. cerevisiae* strains isolated from palm wine. However, YI2's variable response mirrors findings from Ejimofor et al. (2023), where ethanol tolerance varied across different S. cerevisiae strains under specific conditions.

The physicochemical composition of cassava wastewater, especially the high starch content, is comparable to studies by Izah (2018) and Srimuang and Polprasert (2019), which found cassava wastewater to be a highly effective substrate for bioethanol production due to its carbohydrate richness. However, detecting heavy metals, such as lead and cadmium, aligns with environmental studies like those by Chidubem-Nwachinemere et al. (2023) and Antia et al. (2021), highlighting the need for stringent

wastewater management practices to avoid contamination during fermentation.

The successful isolation of fermentative yeast from palm wine, particularly the consistent ethanol tolerance exhibited by YI1, suggests that *S. cerevisiae* isolated from traditional sources can be effectively utilised for ethanol production from cassava wastewater. This demonstrates the potential of using locally sourced yeasts for bioethanol production, reducing reliance on commercial strains. The high starch content in cassava wastewater underscores its potential as a cost-effective substrate for ethanol production. However, the presence of heavy metals and cyanide raises concerns about environmental and safety risks, necessitating careful wastewater treatment and monitoring to ensure safe and sustainable ethanol production.

Optimisation and Validation of Ethanol Production

The study assessed various factors affecting ethanol production, including temperature, pH, incubation time, and wastewater concentration. The results revealed the effect of enzymatic digestion on reducing sugar production, the influence of fermentation parameters on ethanol concentration, and the outcomes of the optimisation experiments. Predictive models were also developed to describe the behaviour of ethanol yield under varying experimental conditions.

Furthermore, the diagnostics report assessed the model's fit and the influence of various factors on ethanol yield, confirming a strong fit between predicted and actual values. Optimum conditions for ethanol production were identified, with predicted and observed yields closely aligned. Physicochemical changes in cassava wastewater were observed during the confirmation fermentation tests, showing a reduction in heavy metals, total solids, and starch content, along with an increase in ethanol concentration and foam formation.

The enzymatic digestion of cassava wastewater significantly increased reducing sugar concentration in the experimental samples, providing a better substrate for fermentation. The results of the fermentation experiments highlighted temperature, pH, incubation time, and wastewater concentration as critical factors influencing ethanol yield. Ethanol concentration peaked at a temperature of 35°C, a pH of 8, an incubation time of 5 days, and a 100% wastewater concentration, demonstrating the importance of these parameters in optimising bioethanol production.

The Central Composite Design (CCD) provided insights into the interactions between these variables. The predicted ethanol yields closely matched the actual yields, confirming the accuracy of the mathematical model. The positive coefficient for temperature in the coded equation suggested a strong relationship between temperature and ethanol production, with the highest yields occurring at elevated temperatures. Similarly, the wastewater concentration had a positive impact, with higher concentrations leading to increased ethanol yield. However, in the actual factors equation, temperature had a slight negative effect on yield, highlighting the complexity of optimising multiple variables simultaneously.

The diagnostics report provided valuable insights into the model's performance. The normal probability plot and residuals vs. predicted ethanol yield plot confirmed that the model residuals followed a normal distribution, with no significant pattern in residuals, indicating a reliable fit between predicted and actual values. The predicted vs. actual ethanol yield plot further confirmed this, as the points closely followed the diagonal line, representing minimal deviation. The Cook's distance plot, leverage plot, and DFFITS analysis identified a few influential runs, but most experimental runs had minimal influence on the model. This suggests that the model was robust and not overly sensitive to any particular data points.

The predicted optimum conditions for ethanol production—45°C, pH 8, 3 days of incubation, and 99.792% wastewater concentration—resulted in a predicted ethanol yield of 16.693%. The observed yield of 16.50% closely matched this, confirming the model's accuracy. The reduction in heavy metals (lead, cadmium, arsenic), cyanide, total solids, and starch content during fermentation reflected the conversion of cassava wastewater components into ethanol. Foam formation during fermentation indicated vigorous microbial activity, peaking after 48 hours and

subsiding by 72 hours, correlating with the progression of fermentation.

The findings from this study are consistent with previous research on bioethanol production using Saccharomyces cerevisiae in some aspects but differ in others. For instance, studies by Ugwuodo et al. (2021) and de Gois Araújo Tavares et al. (2023) demonstrated similar results, with optimal ethanol production occurring within a temperature range of 30°C to 35°C but with slight differences in pH levels ranging from 5.0 - 5.5. In this study, increased ethanol yield was recorded at pH levels ranging from 7 to 8. This may be attributed to the differences in the strain of yeast involved as different strains may adapt and perform better at different pH levels than others. The increase in reducing sugar concentration following enzymatic digestion of cassava wastewater aligns with research by Chantawan et al. (2022), who reported that enzyme treatment enhances the fermentable sugar content in starchy substrates.

In contrast to Hawaz et al. (2022), where ethanol yields were more variable across different *Saccharomyces cerevisiae* strains, this study found consistent results using a single strain, further supporting the robustness of the yeast isolate used. The model developed in this study also mirrors findings from Niyomvong (2019), where a Central Composite Design was employed to optimise ethanol production, producing accurate predictions of ethanol yields based on temperature, pH, and substrate concentration.

Moreover, the performance of the ethanol yield model aligns with findings from other optimisation studies, such as those by de Gois Araújo Tavares et al. (2023) and Ugwuodo et al. (2021), who also demonstrated a strong correlation between predicted and actual ethanol yields using *Saccharomyces cerevisiae*. The robustness of the model, as shown by the normal distribution of residuals and the minimal impact of individual runs, supports similar findings from Niyomvong (2019), where ethanol production models exhibited high predictive accuracy.

The physicochemical changes observed during the confirmation fermentation tests are consistent with other studies. For instance, Bartošová and Blinová (2017) and Saggi and Dey (2019) also reported significant reductions in starch and heavy metals during the bioethanol production process, with increasing ethanol concentration over time. The decrease in heavy metal concentrations, such as lead and cadmium, and the reduction of cyanide reflect findings from studies by Izah et al. (2017), which emphasised the detoxification potential of fermentation processes. Finally, the foam formation observed during the process corresponds to microbial activity commonly reported in fermentation studies, indicating successful bioconversion (Kotoka et al., 2017; Zheng et al., 2019).

The optimisation of ethanol production from cassava wastewater demonstrates the potential for using agro-industrial waste as a valuable substrate for bioethanol production. The findings highlight the importance of controlling fermentation parameters to maximise yield, with temperature, pH, and wastewater concentration playing pivotal roles. The developed models provide a reliable tool for predicting ethanol yield under various conditions, which could be valuable in scaling up the fermentation process for industrial applications. Additionally, the successful conversion of cassava wastewater into bioethanol contributes to waste reduction and offers a sustainable approach to energy production.

The results of the diagnostics report and the confirmation of the model's accuracy suggest that the optimised conditions identified for ethanol production are reliable and can be applied in industrial processes. The consistency between predicted and observed yields indicates that the model can be used to guide ethanol production from cassava wastewater with high confidence. The reduction of heavy metals, cyanide, and starch content during fermentation implies that this process produces ethanol and reduces the environmental impact of cassava wastewater. This has significant implications for waste management and biofuel production, as it promotes a sustainable approach to converting agricultural waste into valuable energy resources.

CONCLUSION

The study successfully isolated fermentative yeast, identified as *Saccharomyces cerevisiae*, from palm wine and characterised cassava wastewater. The high

starch content and low levels of heavy metals confirmed the wastewater's suitability as a substrate for ethanol production, while the yeast's ability to ferment specific carbohydrates indicated its potential for effective bioethanol generation.

Through rigorous optimisation using Response Surface Methodology (RSM) and Central Composite Design (CCD), the optimal conditions for bioethanol production were determined. Key parameters such as temperature, pH, incubation time, and wastewater concentration were optimised, achieving an impressive ethanol yield of 16.50%.

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