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Research Article

Optimization and characterization of bioethanol production from *Icacina trichanta Oliv* and *Anchomanes difformis Blume* as non-food starch feedstocks

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Abstract. The growing competition between food and fuel remains the major drawback of first-generation bioethanol production. Despite their high conversion efficiencies and role as cleaner substitutes for fossil fuels, their cost implications in the production process have led to the search for utilization alternative non-food crops. This study investigates the use of two non-food starch crops, Anchomanes difformis Blume (ADB) and Icacina trichantha Oliv. (ITO), as sustainable alternatives to cassava (CAS) for bioethanol production. Enzymatic hydrolysis conditions were optimized using Response Surface Methodology (RSM), while the bioethanol was characterized by Fourier Transform Infrared Spectrophotometry (FTIR) and High-Performance Liquid Chromatography (HPLC). Results showed that optimum sugar yields were achieved at 180 Unit/g α-amylase and 480 Unit/g amyloglucosidase, where ITO, ADB, and CAS produced 74.29 g/L, 80.81 g/L, and 70.61 g/L reducing sugars, respectively. Correspondingly, ethanol yields were highest in ADB (34.08 g/L with 81.82% efficiency), followed by ITO (31.66 g/L with 84.04% efficiency) and CAS (30.38 g/L with 88.58%). Substrate inhibition was observed, indicating an inverse relationship between glucose concentration and ethanol conversion efficiency. The Michaelis–Menten model was employed to study the kinetics of the entire production process which demonstrated that ADB is the strongest performer overall (producing more ethanol per unit time). The study highlights that ADB and ITO, both non-edible and widely available in Nigeria, demonstrate superior sugar and ethanol yields compared to cassava, positioning them as promising candidates for sustainable, low-cost, and non-food feedstocks in bioethanol production. Their use can enhance energy security, reduce pressure on food crops, and contribute to climate change mitigation by providing renewable alternatives to fossil fuels.

Keywords: Bioethanol; Cassava; Enzymatic Hydrolysis; Non-food Starch crops; Optimization

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1. Introduction

Bioethanol production can only be sustainably maintained as a cleaner substitute for fossil fuels if non-food crops with better conversion efficiencies are used (Adewumi *et al.*, 2022; Efeovbokhan *et al.*, 2019). Although first-generation bioethanol has offered sustainability, sources such as corn, sugarcane, cassava, potatoes, and wheat are hampered by the conflict between food and fuel (Megala *et al.*, 2020; Bekele-Bayu *et al.*, 2022). In the meantime, issues including low yield, higher inhibition due to the recalcitrant nature of feedstocks, and high production costs plague second-generation (lignocellulose) and third-generation (algae) bioethanol production (Wang & Tester., 2023; Hou *et al.*, 2024). Because lignocellulosic biomass is not derived from food, it prevents conflicts between food and fuel while producing bioethanol (Anggoro and Oktavia, 2021). However, the intricacy of its structure, the high expenses of detoxification and pretreatment, and the comparatively poor yield of its products make it economically unviable (Mamudu, A. O., & Olukanmi, T. 2019; Jelani *et al.*, 2023; Adewumi *et al.*, 2024). Algae are examples of third-generation bioethanol feedstocks that do not affect food security. However, their wider use and development are constrained by the challenges of algae collection and the high expenses of conversion processes (Hou *et al.*, 2024; Tambat *et al.*, 2023).

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Due to these concerns, numerous governments have launched programs and provided incentives to promote and increase the production of biofuels, with an emphasis on utilizing crops that do not jeopardize the nation's food security (Perumal *et al*., 2024; Gupta *et a*l., 2024). Corn in the U.S.A., barley, and wheat in Europe, sugarcane and bagasse in Brazil, and barley and cassava in China and Taiwan have all been used in the bioethanol sector in recent years (Devi *et al*., 2021; Adewumi *et al*., 2025). However, Nigeria's bioethanol sector is still in its early stages, and most bioethanol is still imported. The government of Nigeria has recognized sugarcane, corn, and cassava as possible feedstocks for bioethanol; however, because these crops are also essential food sources, their availability and sustainability pose problems. The situation is made more difficult by the high price of these feedstocks on the market (Abila, 2014; Adewumi *et al*., 2018). The marginal cost and return analysis for cassava production per hectare is ₦77,500, according to Itam *et al.* (2018), who attributed high production costs to input costs and farmers' ignorance about improved cassava varieties, whether they are adopters or not.

Apart from Nigeria, cassava has been a key feedstock for bioethanol production in other regions of the world. Pervez *et al.* (2014) found that after hydrolyzing and fermenting 20.0 g/L of cassava starch, the ethanol conversion efficiency obtained was 84.0%. Similarly, Wangpor *et al.* (2017) achieved a fermentation percentage of 85.4% and a maximum bioethanol output of 43.5 g/L when cassava starch was hydrolyzed via enzymatic hydrolysis and ex-situ nanofiltration processes. Furthermore, Pradyawong *et al.* (2018) examined the ethanol yield from cassava starch compared to several corn starch types. The findings showed that cassava produced ethanol with a 2.8% greater yield than corn varieties. In order to reduce or completely eradicate the effects of cassava or other food crops on food security, the search for substitute feedstocks that can increase bioethanol production in Nigeria without endangering food security is required (Mamudu., & Olukanmi, 2019).

Icacina trichantha oliv (ITO) is a perennial shrub from the *Icacinacea* family that grows up to 2-3m above the ground. The plant is native to West Africa and is reported to have high resistance to drought. ITO plant produces a sizeable non-edible tuber weighing up to 3kg in southern Nigeria. ITO is known by different names in Nigeria; it is named gbe-gbe, which means "throw away" in Yoruba, ofo-ala, or Eriagbo, meaning "eat and vomit" by the Igbos. Studies have reported high carbohydrate content, ranging from 71.13 to 91.93% (Ayeni, A. O., 2013; Ogunwa et al., 2016; Adewumi et al., 2019).

Anchomanes difformis (Blume) Engl (ADB) is a wild herbaceous plant in the Araceae family known for its prickly stem and large divided leaves with a spathe that sprouts from a horizontal tuber. The plant grows about 2 meters high, with its tuber measuring up to 80cm long and 20cm wide. Afolayan *et al.* (2012) reported that the starch has an off-white coloration with a carbohydrate content of 88.9%. The tuber is non-edible, especially in southern Nigeria, where it grows as a wild plant due to irritations of the mouth, tongue, and throat when consumed. The Yorubas called the plant abrisoko (Adewumi *et al*., 2019).

In addition, the utilization of inexpensive, ecologically friendly, and renewable energy sources such as ITO and ADB would be vital on a global scale, especially in the transportation industry where there is rising energy demand for cleaner fuels to mitigate the effects posed by the production, consumption, and depletion of non-renewable fossil fuels—which have sparked serious worries about climate change (Sokan-Adeaga *et al*., 2024; Perumal *et al*., 2024). This study therefore applies response surface methodology to optimize enzymatic hydrolysis of *Anchomanes difformis* and *Icacina trichantha*, two indigenous non-food starch tubers that have received little to no attention in bioethanol research. By benchmarking them against cassava under identical conditions, this work provided the first comparative insight into their potential reducing sugar and ethanol yields. The findings highlight their ability to serve as alternative bioethanol feedstocks that do not compete with food security, offering both scientific and socio-economic novelty.

2. Material and method

2.1 Materials

The CAS tuber (TMS 326) and tubers of ITO and ADB, as shown in Figure 1, were gathered from a farm in the University of Port Harcourt, Nigeria. The tubers were peeled, washed, cut into pieces, and milled for starch extraction. All reagents used were of analytical grade. The enzymes were acquired from Sigma-Aldrich Germany: dry active yeast (*saccharomyces cerevisiae*), amyloglucosidase (10115-1G-F; with amyloglucosidase activity of 70U/mg), and α-amylase (10065-10G; with alpha-amylase activity of 30 U/mg). Analytical-grade sulfuric acid (95–97%), potassium dichromate (99.5%), and other reagents were purchased from BDH Chemicals in England.

2.2 Starch Extraction and Characterization

ITO, ADB, and CAS starch extractions were carried out following the methods described by Adewumi *et al.* (2019). The Association of Official Analytical Chemists (A.O.A.C., 1990) standard was used to calculate the starches' proximate content. Adewumi *et al.* (2019) employed a calorimetric iodine affinity technique to assess the starches' amylose concentration.

2.3 Experimental design

The experimental design was carried out by adopting the method described by Adewumi *et al.* (2022).

2.3.1. Enzymatic Hydrolysis procedure

The conditions for the hydrolysis of the starch samples were: α-amylase (60-180 units/g starch), amyloglucosidase (140-420 units/g starch), and substrate concentration (15%W/V). A thermostatic shaking water bath (Shz-88) was utilized for the

Fig 1. (a) ADB tuber (b) CAS tuber (c) ITO tuber

hydrolysis. At a solid-to-liquid ratio of 1: 10, the starches were first gelatinized for 10 minutes at 90°C. After that, the mixture was liquefied for one hour at 75°C using 2 ml of various α-amylase activity (60–180 units/g starch). After the procedure, 1% HCl was used to lower the pH to 5.0 and the medium temperature to 55°C. In addition, 2 ml of various amyloglucosidase activity (140–420 units/g starch) was added, and saccharification was continued for an additional three hours at 55°C. The enzyme was deactivated by raising the medium temperature to 100 °C. At this point, the solution was allowed to cool, and filtration removed the residue.

2.3.2. Fermentation of the hydrolysate

In a medium consisting of yeast extract (2 g/L), glucose (2 g/L), peptone (3 g/L), and distilled water (1000 mL), the hydrolysate was fermented with Saccharomyces cerevisiae (1 g/L). $MgSO_4.7H_2O$ (1 g/L), NH_4Cl (1 g/L), KH_2PO_4 (2 g/L), and $CaCl_2$ (0.1 g/L) were added to the media as supplements. The broth was then exposed to anaerobic conditions for 72 hours at 30 °C and a pH of 5.0.

2.3.3. Purification of the produced bioethanol

The quantity of ethanol in the fermentation broth was distilled by simple distillation. A two-step distillation method was used in order to achieve an ethanol concentration up to the azeotropic point (94.5%) from less than or equal to 12% (≤ 12%) in the fermentation broth.

The final purification (dehydration) above the azeotropic point was achieved with the use of a Type 3Å (Armstrong) molecular sieve (Sigma Aldrich, Germany) using a liquid phase dehydration method (thermal swing adsorption method) at a temperature of 79°C. The water molecules were allowed to enter the sieve's cavity (because its diameter is less than 3Å), while ethanol with a diameter above 3Å is blocked and thus distilled off

2.4. Statistical Design for Acid and Enzymatic Hydrolysis

Utilizing Response Surface Methodology and Design Expert software (version 11.0), the ideal conditions for enzymatic hydrolysis techniques were identified. The dependent variable, glucose production, was assessed regarding the effects of amylase and amyl glucosidase activity on enzymatic hydrolysis. Three coded levels (-1, 0, +1) of the Central Composite Rotational Design (CCRD), which included 13 experimental runs for the hydrolysis, were used to analyze the glucose yield. These conditions are shown in Table 1. Equation 1 represents the second-order model used to forecast the optimum position.

$$Y = \beta_0 + \sum \beta_1 X_1 + \sum \beta_2 X_2 + \sum \beta_{12} X_1 X_2 + \sum \beta_{11} X_1^2 + \sum \beta_{22} X_2^2 + e^{2} \quad (1)$$

Where Y = glucose yield (predicted response), X_1 and X_2 = variable factors (enzyme concentrations), β_o is a constant term. β_1 and β_2 are the coefficients of the linear terms, β_{12} is the coefficient of the cross term, β11, and β_{22} are the coefficients of the quadratic term and e is the error term.

2.5 Analytical methods

2.5.1. Quantification of total reducing sugars (TRS)

Adewumi *et al.* (2022) used the 3,5-dinitrosalicylic acid (DNS) technique to assess the TRS of the hydrolyzed samples. The hydrolyzed samples' sugar concentration against that of the standard was analyzed at 540 nm using a Metash UV-5200 spectrophotometer (Shanghai Metash Instruments Co Ltd).

2.5.2. Potassium dichromate method for Ethanol content analysis

To estimate the ethanol content in the broth, the combined chromic approach of Sayyad *et al.* (2015) and Adewumi et al. (2022) was used. A Metesh UV-5200 spectrophotometer at a wavelength of 600 nm was used to evaluate 1 ml of 10% ethanol (standard concentration) using potassium dichromate (0.2 M) in sulfuric acid (4 M). After color development, the absorbance was measured. The ethanol concentration of the samples' fermentation broth was ascertained using a similar process. The yield was computed using the following sets of equations (Eqs. 2–4), and the absorbance measurements were used to determine the actual (g/L), percentage (V/V) and fermentation efficiency (%) of the ethanol (EtOH) obtained.

$$\text{Starch (1g)} + H_20 \rightarrow \text{Glucose (1.1g)} \rightarrow \text{ethanol (0.56g)} \quad (2)$$

$$\text{Vol of EtOH} \left(\frac{\text{ml}}{100\text{ml}}\right) = \frac{\text{EC } (\%\text{VV}^{-1})}{100 \times \text{ AVF (ml)}} \quad (3)$$

Where EC is ethanol concentration obtained from the spectroscopic absorbance data, while AVF is the actual volume from the fermentation broth.

$$\text{Amount of EtOH} \left(\frac{\text{g}}{100\text{ml}}\right) = \frac{\text{Mass of EtOH (g)}}{100\text{ml} \times 10 \ (mlL^{-1})} \quad (4)$$

Theoretical ethanol yield from [Eq 4] was used to calculate the fermentation efficiency (EFF)

$$\text{FER Eff } (\%) = \frac{\text{Actual EtOH yield } \left(\text{gL}^{-1}\right)}{\text{Theoretical EtOH } \left(gL^{-1}\right) \times 100} \quad (5)$$

Where actual yield is the amount of ethanol obtained from the fermentation process in the current study, theoretical yield is the maximum amount of ethanol that could be obtained based on the initial glucose concentration.

2.5.3. Component analysis of the fermentation broth

The compositional content of the broth was estimated with an Agilent LC System HPLC equipped with an Aminex HPX-87H (Bio-Rad) ion-exclusion column and a refractive index detector (model 4212). The mobile phase consisted of 0.005 M sulphuric acid, delivered at a flow rate of 0.8 Ml/min.

Table 1
Coded levels of variable for CCRD for enzymatic hydrolysis

Variables	**Units**	**Symbols**	**Coded Levels**		
			-α	0	+α
α-Amylase	Enzyme units	X_1	60	120	180
Amyloglucosidase	Enzyme units	X_2	140	280	420

2.5.4. Fourier Transform Infrared Spectrophotometric (FTIR) Analysis

The FT-IR analysis was conducted using Nicolet iS10 FT-IR Spectrophotometer. The spectral frequency was within the range of 4000 and 350 cm^{-1} with a spectral resolution of 4 cm^{-1}. Win-IR Pro software was employed for IR spectra analysis, with a peak sensitivity adjusted to 2 cm^{-1}.

2.6. Kinetic Analysis of the Process

The Michaelis–Menten kinetics mathematical model a reliable model for a simple enzyme-catalyzed reaction was adopted for the kinetic analysis of the process. The equation can be expressed as follows

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (6)$$

In Eq. 6 the relationship between the initial rate of product formation and substrate concentrations can be expressed. The Michaelis constant (K_m) and maximal velocity (V_{max}) are constants that must be determined. The kinetic parameters were firstly determined with Lineweaver–Burk plot (a double reciprocal plot) which is simplified to linear form from the Michaelis–Menten equation. The Lineweaver–Burk plot is expressed as:

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (7)$$

where Vo represents rate of product formation, Vmax as the maximum product formation rate, Km as the Michaelis–Menten constant and [S] as the substrate concentration. From the Lineweaver–Burk equation, the maximum product formation rate and Michaelis-Menten constant were obtained from the intercepts and slopes of the respective graphs of $1/V_0$ against 1/[S] for amylase and amyloglucosidase (AMG) in ITO, ADB and CAS substrates.

Fermentation (ethanol production phase). In this process, logistic growth/modified Gompertz model for bioethanol production was adopted

$$P(t) = P_{max} . \exp(-exp\,[\frac{R\,max}{P\,max}\ (\lambda + t) + 1]) \quad (8)$$

Where P (t) is ethanol concentration at time t, Pmax Maximum ethanol produced (g/L), R_{max} Maximum ethanol production rate (g/L.h), λ Lag phase duration (h), Theoretical ethanol produced (P_{th}) = 0.51 G_{max}

Therefore,

$$P(t) = Pth\,(1 - e^{Kptf}) \quad (9)$$

Where,

$$Kp = -\frac{1}{tf}\,In\,(1 - \frac{Pobs}{Pth}) \quad (10)$$

Ethanol efficiency = P_{obs}/P_{th} , K_p = Fermentation rate, Pobs = Actual ethanol produced, t_f = Fermentation duration (72 hours)

3. Results and Discussions

3.1. Compositional Analysis of ITO, ADB and CAS Starches

Table 2 provides a summary of the starches' component analysis and physicochemical properties. The amylose-amylopectin ratio is a key factor determining the percentage of starch converted to simple sugars. Their physicochemical characteristics and applications are determined by the structural differences between amylose, an amorphous linear polymer with 20–30% starch, and amylopectin, a highly branched semi-crystalline polymer with 70–80% starch (Varghese *et al.*, 2022; Dike *et al.*, 2024). Amylose is well-known for its excellent resistance to digestion, while amylopectin's high glycemic index causes it to break down quickly (Ayeni, A. O. *et al.*, 2020). According to Varghese *et al.* (2022), starches with a greater amylose concentration (>30%) have a reduced conversion efficiency, which is explained by their strong enzyme resistance.

According to the current study's results, ADB (85.27%) and CAS (85.55%) have higher carbohydrate content when compared to that of ITO (80.89%). Although ITO yield is marginally lower than those of ADB and CAS, it however compares favourably with those reported in literature where ADB starch contains 73.09-88.87% while ITO starch and flour contain 88.91% and 71.13%, respectively (Ogunwa *et al.*, 2016; Adewumi *et al.*, 2019). Consequently, the amylose content of the starches differed, with CAS containing the highest and ITO containing the least. CAS is anticipated to produce more resistant starch than the ITO. Indeed, high amylose content was found to negatively impact the sugar yield of CAS, where the lowest yield is produced when compared to ITO with the lowest carbohydrate and amylose content. However, ADB, with higher carbohydrates and moderate amylose content, produced the highest sugar yield in the enzymatic process. The significance of the physiochemical properties of the starches in bioethanol production is summarized in Table 2. These findings indicate that a combination of lower amylose content, higher swelling capacity, and favourable carbohydrate content were key factors driving the higher conversion efficiency of ADB and ITO relative to cassava.

Table 2
Proximate composition and Physicochemical properties of the starches

Parameters (%)	ADB	ITO	Cassava	Significance
Moisture	10.10±0.10	7.40±0.08	8.58±0.33	Affects storage stability
Ash	0.30±0.19	1.80±0.12	2.17±0.38	High ash can reduce fermentability
Protein	2.36±0.25	4.38±0.18	1.01±0.09	Promotes enzymes metabolism
Fat	1.51±0.14	2.80±0.14	1.34±0.13	Lipids may form complexes, hindering enzyme access
Carbohydrate	85.27±0.08	80.89±0.38	85.55±0.12	Directly linked to ethanol yield
Fibre	0.56±0.15	0.64±0.24	1.30±0.24	High fibre may lower starch extractability
Amylose	15.10±0.11	11.28±0.63	20.43±0.56	Higher amylose lowers sugar yield
Water Absorption Capacity	83.3±0.2	71.0±0.16	78.5±0.22	Enhances hydrolysis
Swelling Power (90 °C)	12.1±0.09	14.5±0.06	13.8±0.14	Increases access to enzymes
Crystallinity	A-Type	A-Type	A-Type	Open structure and susceptible to enzymatic hydrolysis
Gelatinization Temperature (°C)	73	78	68	Higher temp requires more energy

3.2. Reducing Sugar (Glucose) Production and Optimization.

Response Surface Methodology (RSM) was employed to optimize enzymatic hydrolysis conditions using α-amylase and amyloglucosidase (AMG) concentrations for glucose production from three feedstocks: CAS, ITO, and ADB. The Central Composite Rotatable Design (CCRD) with 13 runs (Tables 6) enabled the generation of second-order polynomial models, with model fit and significance assessed using analysis of variance (ANOVA: Table 3-5). Table 1 displays the experiment design of the production process. The fitness of the quadratic model for the sugar yield of all the feedstocks is presented through ANOVA analysis. In contrast, the conditions of optimum sugar yield are presented in a 3D response surface plot.

The analysis of variance (ANOVA) for the enzymatic hydrolysis of CAS, ITO, and ADB, respectively revealed highly significant p-value of (< 0.0001) for all the starches. The analysis results proved the appropriateness and fitness of the quadratic model in predicting the sugar yield indicating that the selected variables adequately describe the system behaviour. These results indicate a low likelihood of noise affecting the outcome. The enzymes amylase (A) and amyloglucosidase (B) significantly increase the amount of sugar in both starches.

Table 3
ANOVA for Quadratic model of Enzymatic hydrolysis of CAS

Source	Sum of Squares	Df	Mean Square	F-value	p-value		
Model	303.24	5	60.65	74.61	< 0.0001	significant	
A-AMYLASE CONC	2.03	1	2.03	2.50	0.1580		
B-AMG CONC	147.11	1	147.11	180.99	< 0.0001		
AB	13.07	1	13.07	16.08	0.0051		
A^2	3.34	1	3.34	4.11	0.0823		
B^2	103.09	1	103.09	16.83	< 0.0161		
Residual	5.69	7	0.8128				
Lack of Fit	5.57	3	1.86	3.80	0.6008	not significant	
Pure Error	0.1165	4	0.0291				
Cor Total	308.93	12					
Fit Statistics	Std. dev	Mean	C.V(%)	R^2	Adjusted R^2	Predicted R^2	Adeq. Precision
	1.2016	67.10	1.34	0.9816	0.9684	0.8578	23.7548

Table 4
ANOVA for Quadratic model of Enzymatic hydrolysis of ITO

Source	Sum of Squares	Df	Mean Square	F-value	p-value		
Model	326.79	5	65.36	242.21	< 0.0001	significant	
A-AMYLASE CONC	62.53	1	62.53	231.74	< 0.0001		
B-AMG CONC	112.67	1	112.67	417.53	< 0.0001		
AB	0.0000	1	0.0000	0.0000	1.0000		
A^2	12.14	1	12.14	44.98	0.0003		
B^2	92.00	1	92.00	340.93	< 0.0001		
Residual	1.89	7	0.2698				
Lack of Fit	0.4772	3	0.1591	0.4507	0.7307	not significant	
Pure Error	1.41	4	0.3529				
Cor Total	328.68	12					
Fit Statistics	Std. dev	Mean	C.V(%)	R^2	Adjusted R^2	Predicted R^2	Adeq. Precision
	0.5195	69.57	0.7467	0.9943	0.9901	0.9797	46.5286

Table 5
ANOVA for Quadratic model of Enzymatic hydrolysis of ADB

Source	Sum of Squares	Df	Mean Square	F-value	p-value		
Model	485.07	5	97.01	1211.97	< 0.0001	significant	
A-AMYLASE CONC	117.66	1	117.66	1469.89	< 0.0001		
B-AMG CONC	213.13	1	213.13	2662.55	< 0.0001		
AB	14.82	1	14.82	185.17	1.0000		
A^2	26.60	1	26.60	332.28	0.0003		
B^2	61.75	1	61.75	771.38	< 0.0001		
Residual	0.5603	7	0.0800				
Lack of Fit	0.0926	3	0.0309	0.2640	0.8487	not significant	
Pure Error	0.4677	4	0.1169				
Cor Total	485.63	12					
Fit Statistics	Std. dev	Mean	C.V(%)	R^2	Adjusted R^2	Predicted R^2	Adeq. Precision
	0.2829	72.73	0.3890	0.9988	0.9980	0.9971	108.09

Table 6
Design of experiment for Cassava, ITO and ADB

		Factor 1	Factor 2	Response 1 (Glucose Yield) g/L		
Std	Run	A:AMYLASE CONC ENZY UNIT	B:AMG CONC ENZY UNIT	CASSAVA	ITO	ADB
8	1	120	480	70.61	71.54	77.54
10	2	120	310	70.07	73.77	76.71
3	3	60	480	68.45	66.47	68.17
11	4	120	310	70.35	72.89	76.22
7	5	120	140	59.65	63.54	65.48
2	6	180	140	60.78	64.08	65.11
6	7	180	310	70.18	74.29	77.63
1	8	60	140	55.46	57.47	60.17
4	9	180	480	66.54	73.08	80.81
12	10	120	310	70.17	72.76	76.09
13	11	120	310	69.99	72.54	76.11
9	12	120	310	69.91	73.81	76.8
5	13	60	310	70.1	68.14	68.64

Nevertheless, it was shown that amyloglucosidase had a more significant effect on sugar yield than amylase. Amyloglucosidase concentration ($p < 0.0001$) and its quadratic term ($p = 0.0161$ and 0.0001) had strong effects, suggesting AMG played a dominant role.

This indicates that each enzyme independently and synergistically (via quadratic terms) contributed significantly to glucose yield. The sugar production was significantly impacted by the two-factor (two-way) interaction AB, A^2, and B^2, with p-values below the threshold p-value (< 0.05). The model effectively captures the experimental values that established a connection between the variables under investigation, as evidenced by the high correlation (R^2) with adjusted R^2 values of 0.9816 (0.9684), 0.9943 (0.9901) and 0.9988 (0.9971) for CAS, ITO and ADB, respectively in addition to very low standard deviation (SD) of near unity of 1.20, 0.51 and 0.28 and CV% of 1.34, 0.75 and 0.39 for CAS, ITO and ADB with all signifying a strong agreement between the model results and experimental data as observed in the predicted versus actual plots (Figure 2).

In addition, Adequate precision values of CAS (23.75), ITO (46.53) and ADB (108.09) were greater than the minimum desirable value of 4 for all models, indicating good signal-to-noise ratios. The coefficient of variation (C.V.%) was lowest for ADB (0.3890%), followed by ITO (0.7467%) and CAS (1.34%), which further supports the precision of ADB's model. This study demonstrates the successful use of RSM to optimize enzymatic

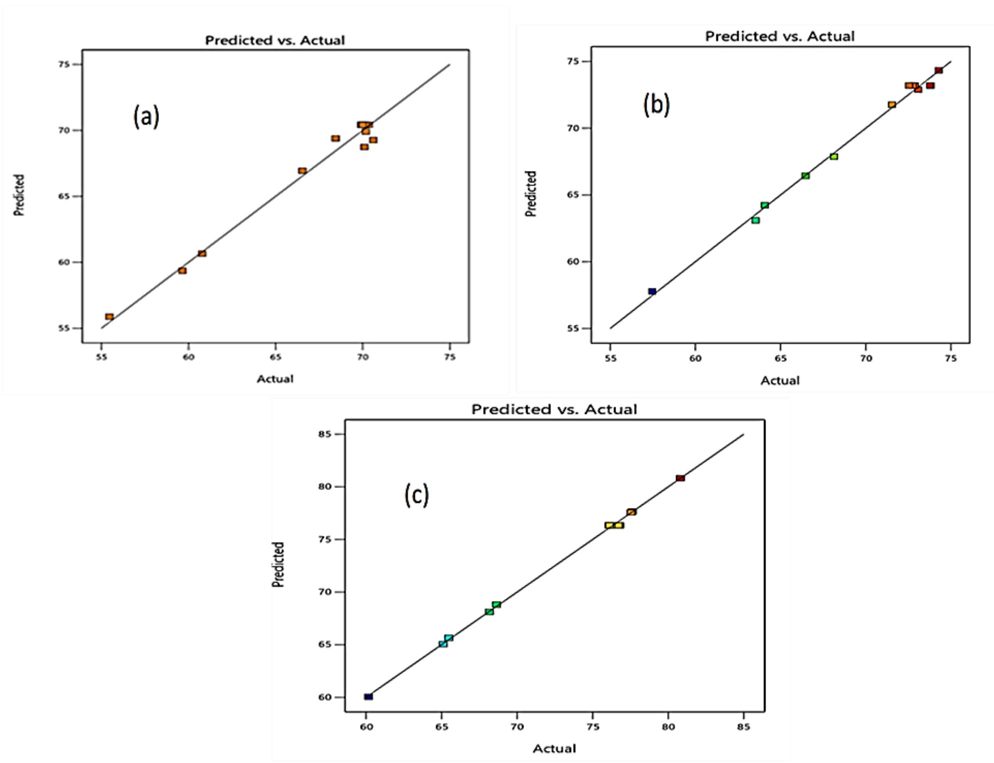


Fig 2. Predicted Vs actual yield of glucose for (a) CAS, (b) ITO, and (c) ADB after enzymatic hydrolysis

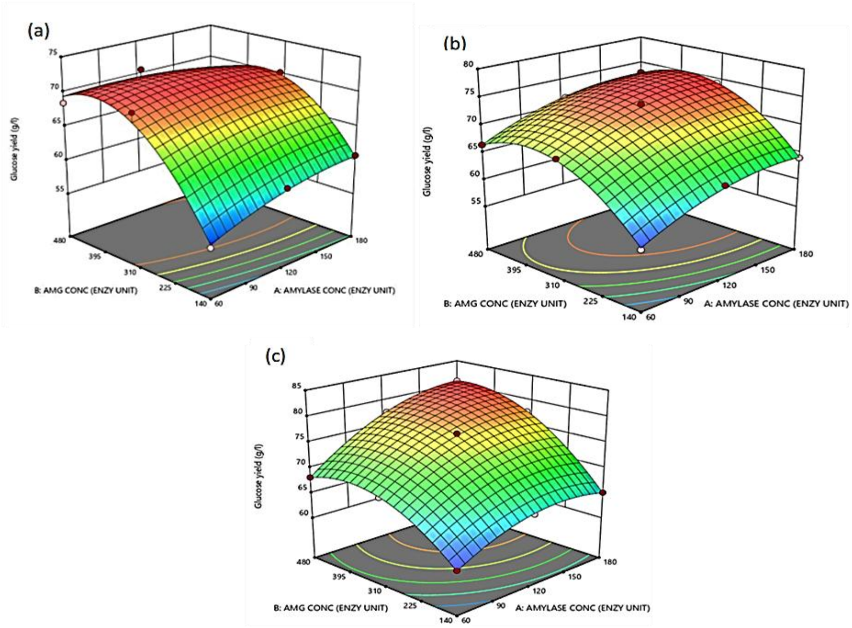


Fig 3. 3D Response surface plots on the effects of α-amylase and amyloglucosidase concentrations on the Glucose yield (g/l) of (a) CAS, (b) ITO, and (c) ADB starch samples after enzymatic hydrolysis.

hydrolysis parameters for glucose production from three biomass substrates with response and predictive model accuracy which is crucial for maximizing glucose yield and ensuring cost-effective bioconversion, offering significant implications for industrial-scale bioethanol production.

The model's ability to predict the response within the tested region with a 96.8%, 99.4%, and 99.9% confidence level for CAS, ITO, and ADB is demonstrated by the 3D response plot result (Figure 3). The 3D results showed that the variables (amylose and amyloglucosidase) had a favourable effect on the glucose yield of the samples. The optimum glucose production was obtained under all analysis conditions at 180 amylase activity and 480 amyloglucosidase activity for ITO and ADB,

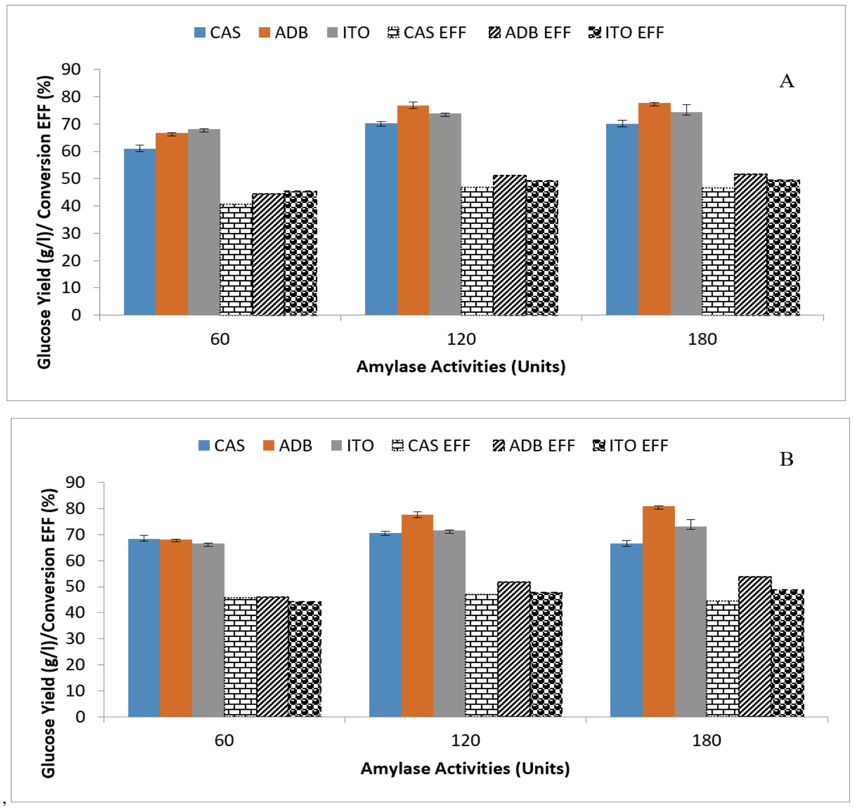


Fig 4. Glucose production (g/L) and conversion efficiency (EFF %) for ADB, ITO, and CAS starches at varying α-amylase activities, with a fixed amyloglucosidase activity at (A) 310 units/g starch and (B) 480 units/g starch.

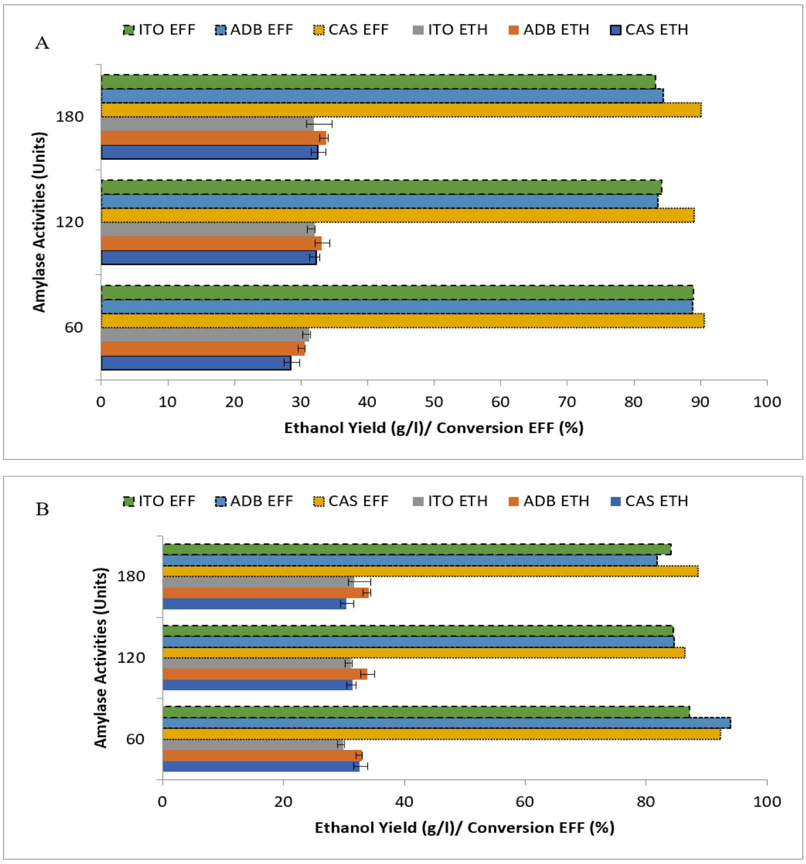


Fig 5. Ethanol yield (g/L) and conversion efficiency (EFF %) for ADB, ITO, and CAS starches at varying α-amylase activities, with a fixed amyloglucosidase activity of (A) 310 units/g starch and (B) 480 units/g starch.

with a yield of 74.29 g/L and 80.81 g/L, respectively. In contrast, CAS produced 70.61 g/L at an optimum condition of 120 amylase and 310 amyloglucosidase units.

The optimal conditions identified in this study are consistent with that of Ruiz *et al.* (2011), who examined the effects of glucoamylase (81.5 U/g of starch) and alpha-amylase (130.5 U/g of starch) enzyme doses on the hydrolysis of 100 g of cassava starch. According to the report, the best-reducing sugar yield was attained at 46°C, pH 4.5, and an enzymatic mixture dose of 16.4 U/g of starch. Similarly, when alpha-amylase and glucoamylase hydrolysed cassava pulp is used at pH 4.5, 60°C, and 24-hhydrolysis period, Virunanon *et al.* (2013) observed a total reducing sugar production of 99.6 mg/g of cassava pulp, beginning with an initial starch content of 50 g/100 mL.

3.3. Reducing sugar (glucose) yield of the samples

The glucose yield of CAS, ITO, and ADB from the hydrolysis process at different enzyme activities are displayed in Figures 4 (A and B). The analysis was performed at two fixed amyloglucosidase levels to compare feedstock performance under identical enzymatic conditions. In all the conditions analysed, amylose composition negatively impacted the glucose yield. CAS, with the highest carbohydrate and amylose content of 85.55 % and 20.43%, produced the lowest glucose yield and conversion efficiency when compared to ITO, which contained the least carbohydrate and amylose content of 80.89% and 11.28%, respectively. It has been found that α-amylase enzymes cannot hydrolyse native starches with a greater amylose concentration (Tester & Karkalas, 2006; Li *et al.*, 2004).

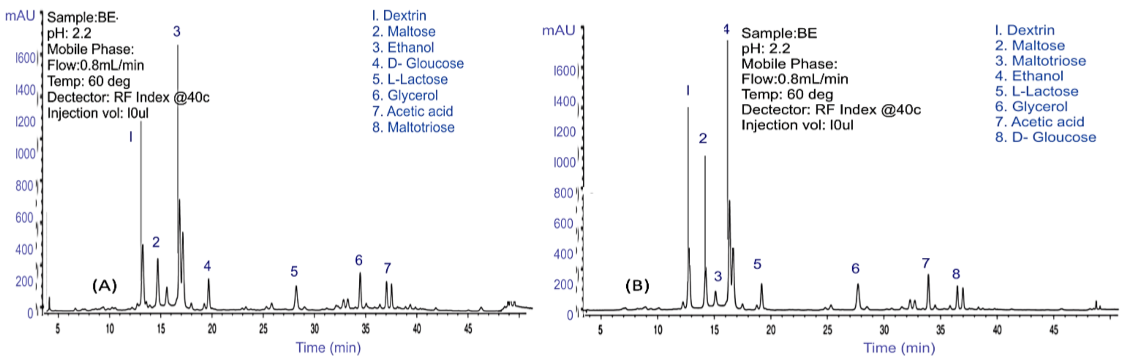


Fig 6. Component analysis of the fermentation broth of the samples (A) ITO, (B) ADB using HPLC.

Varghese *et al.* (2022) validated the finding and reported that starches with a greater amylose concentration are more resistant to enzyme degradation, reducing conversion efficiency.

This discovery is consistent with the lower conversion of CAS starch to glucose. The maximum glucose yield (conversion efficiency) of 70.1 g/L (46.73%), 77.63 g/L (51.75%), and 74.29 g/L (49.53%) for CAS, ADB, and ITO were achieved at 180 amylase activity. A similar trend was observed at 480 amyloglucosidase activity, where ADB with 80.81 g/L (53.87%) produced the highest yield and conversion, and ITO produced 73.08 g/L (48.72%) at 180 amylase activity. In comparison, CAS produced the least 70.61 g/L (47.07) at 120 amylase activity. The lower conversion rate (below 60%) could be justified since hydrolysis was carried out for 4 hours. The findings in this current study align with the report of Wangpor et al. (2017), who achieved a sugar yield of 75.0 g/L by hydrolysing 20% w/v cassava starch with a combination of 0.3 mg amylase and 0.5 mg amyloglucosidase per gram dry starch for a total of 150 mins. However, the limitation obtained in the conversion efficiency of the starches could be overcome by extending the hydrolysis time beyond three hours or further optimizing the enzyme cocktail which could further enhance sugar and ethanol yields, making ADB and ITO even more superior to cassava.

3.4. Ethanol yield of the samples

Consequently, the ethanol yield of ADB, ITO, and CAS were compared after the fermentation process with *saccharomyces cerevisiae* for 72 hours. Under all conditions (Figures 5 A and B), ADB produced the highest ethanol yield of 34.08 g/L with a conversion efficiency of 81.82%, while ITO produced 31.66 g/L (with 84.04% conversion) and CAS produced 30.38 g/L (with 88.58% conversion) at 180 is amylase and 480 amyloglucosidase

Substrate inhibition was observed to play a key role in the ethanol conversion efficiency; samples with higher glucose yields were observed to produce the least ethanol yield. For instance, at 180 amylases and 480 amyloglucosidase units, an ethanol yield of 34.08 g/L was achieved with an efficiency of 81.82% from ADB that produced an optimum glucose yield of 80.81 g/L. In contrast, with the lowest glucose yield of 66.54 g/L, CAS achieved an ethanol yield of 30.38 g/L, resulting in 88.58 % efficiency. A similar trend was observed at 310 amyloglucosidase units (Figure 6). Zhang *et al.* (2015) studied the effects of substrate (glucose) inhibition on yeast cells. They reported that a higher amount of substrate inhibited cell growth and ethanol yield above the threshold of 160 g/L glucose. In addition, Adewumi *et al.* (2022) found that substrate concentration has more inhibitory effect than 5-hydroxymethyl furfural during acid hydrolysis of cassava starch. The result obtained (Figure 8) revealed the presence of dextrin and maltose in the fermentation broth which may imply that S. cerevisiae may experience inhibitory effects on efficiency even at low glucose levels

3.5. Component analysis of the fermentation broth with HPLC

Eight (8) compounds were identified in the fermentation broth of both ITO and ADB samples (Figure 6), among which dextrin, maltose, and ethanol were the major components. Ethanol (peak 3 and 4 for ITO and ADB respectively) at a retention time of 16.8, was found to be the most abundant compound, with 32.7% and 38.5% abundance for ITO and ADB respectively. At the same time, dextrin and maltose are the second and third most abundant compounds in both samples. However, the presence of glucose, maltotriose, L-lactose, glycerol, and acetic acid in lower concentrations portrays that the process undergoes enzymatic hydrolysis. A higher amount of dextrin and maltose indicates an incomplete hydrolysis process because four hours of hydrolysis time was used. This implies that more time would be required to hydrolyse the starches effectively. The results obtained in this analysis agree with the conversion efficiency of glucose (less than 55%) obtained in the current study. The lower glucose concentration of 1.2% at a retention time of 37.5 showed that almost all glucose formed is converted into ethanol, which also agrees with higher ethanol conversion of above 80% obtained in the current study. Therefore, future process optimization in respect to time should be carried out to effectively convert the starches and fully harness the potential of ITO and ADB for optimum ethanol yield.

3.6. Characterization of bioethanol produced by FTIR-Spectrophotometry.

Figure 7 illustrates the absorption spectrum of the produced ethanol after 60 numbers of scanning using an FTIR

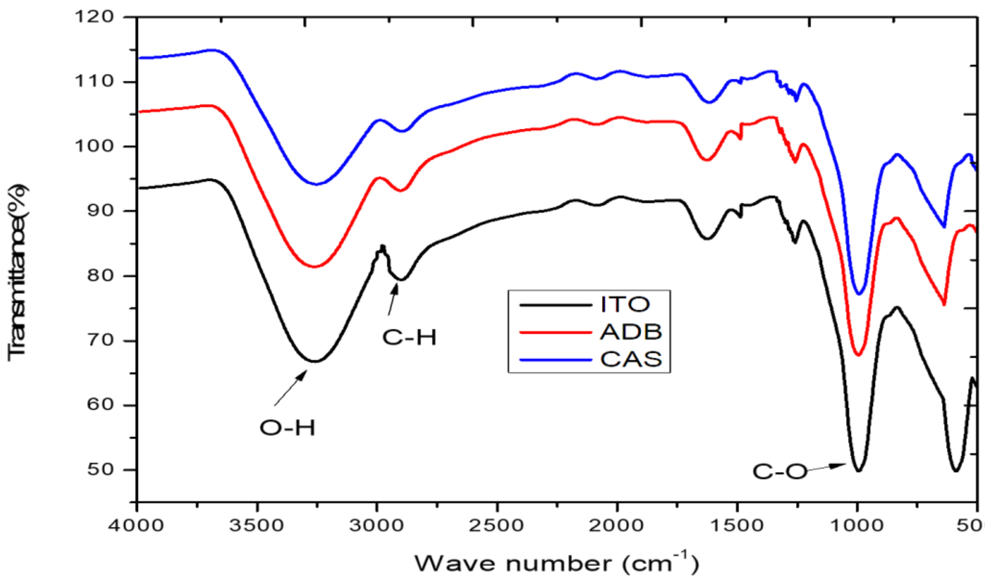


Fig 7. FTIR spectrum of the produced bioethanol.

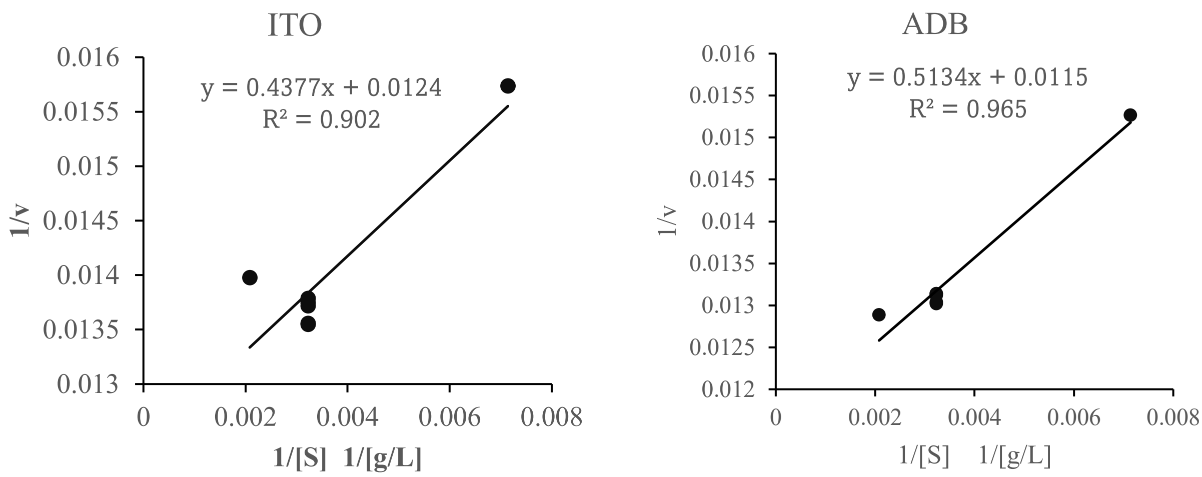
spectrophotometer. Two major absorption peaks were displayed in the spectrum for the produced ethanol, one at range 3500-3200 cm^{-1} characteristics of an O-H stretching vibration, another at 1100-995cm^{-1} characteristic of stretching vibration of the carbon-oxygen (C-O) end of an organic compound. The 500-750 cm^{-1} peak is characteristic of a compound's carbon-hydrogen (C-H) bending vibrations. Alcohols show a characteristic absorption band (medium band) between 3400 to 3600 cm^{-1}. The existence of C-O stretching vibration confirms the presence of an alcohol. Phenols and carboxylic acids are compounds that also show the band within this range, but the band sizes differ. Carboxylic acids display very broad bands, while phenols show sharp bands around 3600 cm^{-1} with a confirmation of double but equal peak (showing the isomeric structure) between 700 - 900 cm^{-1}. The strong presence of the C-O band and a weak or uncharacteristic C=O vibration around 1637 cm^{-1} of the carbonyl group validated that the compound produced is not a carboxylic group. Also, the absence of the broad O-H band and aryl C-H deformation band strongly confirmed that the O-H band observed belongs to the alcohols. This confirmed that the compound produced is ethanol. The results obtained are similar to the work of Ningthoujam *et al.* (2023) and Bekele-Bayu *et al.* (2022).

3.7. Kinetics of the ethanol production process

The kinetic study was carried out to understand the enzyme-substrate interactions, enzyme efficiency and the effects of substrate concentration on reaction rates. This is modelled using Michaelis–Menten kinetics in starch hydrolysis and fermentation studies for the three feedstocks at optimum sugar and ethanol yield. Figures 8 represents the individual plots of 1/V0 versus 1/[S] with which the Michaelis constant (Km) and maximal velocity (Vmax) are obtained from the slopes and intercepts of each feedstock.

Analysis of the Lineweaver–Burk plots reveal that both AMG and Amylase (Table 7) conform closely to Michaelis–Menten kinetics, as evidenced by strong linear regressions (R^2 = 0.902 for ITO, 0.965 for ADB and 0.9466 for CAS at different AMG and constant amylase concentrations). In the ITO sample, amylase showed stronger substrate affinity (Km = 9.015) than AMG (Km = 35.298), while both enzymes had similar Vmax values. In the ADB sample, AMG demonstrated much higher affinity (Km = 1.000) and a slightly higher Vmax than amylase, making it the more efficient enzyme. In the CAS sample, amylase results were unreliable due to very low R^2 (0.0167) and reduced Vmax, whereas AMG showed a better fit (R^2 = 0.9466) but weaker affinity (Km = 45.936). Overall, amylase was more efficient in ITO, AMG performed best in ADB, and AMG was the more reliable catalyst in CAS conditions. Detailed kinetic analysis for sugar production is available as a supplementary file.

The impacts of the kinetic studies of the entire bioethanol process are summarized in Tables 8, showing that ADB is the strongest performer for throughout (more ethanol per unit time), while ITO balances good hydrolysis kinetics with high fermentation efficiency. Cassava trails in throughput but



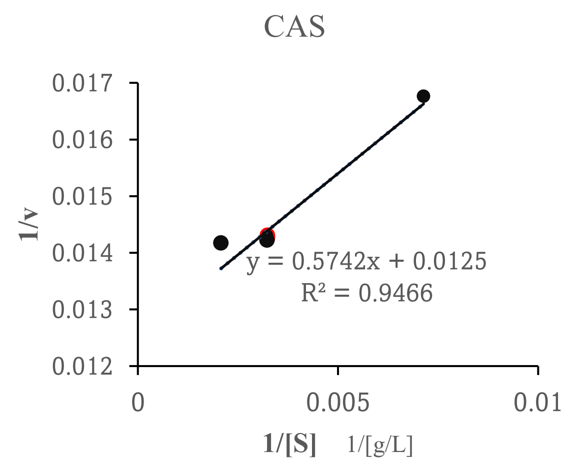


Fig 8. Lineweaver–Burk plots for ITO, ADB and CAS substrates at different AMG concentrations

Table 7
Summarized kinetic studies on glucose production from the feedstocks

Sample	Substrate	R^2 value	K_m value	V_{max}
ITO	Amylase	0.941	9.015	78.740
	AMG	0.902	35.298	80.645
ADB	Amylase	0.967	13.864	84.745
	AMG	0.965	1	86.957
CAS	Amylase	0.0167	0.0769	69.930
	AMG	0.9466	45.936	80.000

Table 8
Summarized kinetic studies on bioethanol production from the feedstocks

Parameter	ADB	ITO	Cassava	Implication
Gmax (g/L)	80.81	74.29	70.61	ADB produced the highest glucose yield at highest conversion rate
Hydrolysis conversion, Xg	0.485	0.446	0.424	
Hydrolysis rate, Kh (h^{-1})	0.166	0.148	0.138	ADB hydrolyzed fastest due to moderate amylose & high carbohydrate content
Fermentation lag phase, λ (h)	4.2	3.8	5.1	ITO adapted fastest to yeast fermentation
Pth = 0.51Gmax (g/L)	41.21	37.89	36.01	ADB > ITO > CAS
Pobs (g/L)	34.08	31.66	30.38	Matches experimental results
Ethanol efficiency, (η)	0.827	0.836	0.844	Cassava's hydrolysate reaches the highest efficiency
Fermentation rate, kp (h^{-1})	0.02436	0.02508	0.02577	CAS yielded the slightly highest apparent rate
Avg. productivity, $\bar{r}$ (g/L.h)	0.473	0.440	0.422	ADB delivers the highest average ethanol productivity

**(Values are computed from the equations 6-10 using data obtained from the experimental work*

maintains strong conversion efficiency relative to its theoretical maximum.

4. Conclusion

Efficient, accessible, renewable, and environmentally friendly feedstocks, including technologies that maximize conversion while reducing inhibition, are essential to the growth of the bioethanol sector. The use of non-food crops, *Anchomanes difformis Blume (*ADB) and *Icacina trichantha Oliv* (ITO), as resource-efficient and low-cost raw materials for bioethanol production was optimized in this study using CCRD design of Response surface methodology, with cassava (CAS) starch as a reference feedstock. Response surface optimization predicted the model's significance, with R^2 values of 0.9988, 0.9943, and 0.9816 for ADB, ITO, and CAS, respectively, at an optimum enzymatic hydrolysis of 180 amylase and 480 amyloglucosidase activities per gram of starch. Under these conditions, ADB, ITO, and CAS produced 80.81 g/L, 74.29 g/L, and 70.61 g/L, reducing sugar yields, respectively. Similarly, ADB produced 34.08 g/L yield of ethanol with a conversion rate of 81.82%. ITO and CAS produced 31.66 g/L (84.04%) and 30.38 g/L (88.58%), respectively, demonstrating that ADB produced the highest reducing sugar and ethanol yield while CAS produced the least. Substrate inhibition inversely impacted the conversion efficiencies among all the samples analysed, where samples with higher sugar concentrations produced lower ethanol yields. In addition, high amylose content was found to negatively impact the sugar yield of CAS, where the lowest sugar yield is produced when compared to ITO with the lowest carbohydrate and amylose content. The HPLC analysis revealed eight compounds in the fermentation broth, among which dextrin, maltose, and ethanol were the major components, with percentage abundance of 26.2%, 17.3%, and 38.5%, respectively. The presence of dextrin and maltose signify incomplete hydrolysis; hence, future process optimization in respect to time should be carried out to effectively convert the starches and fully harness the potential of ITO and ADB for optimum ethanol yield. The high glucose and ethanol yield obtained from the non-food crops ADB and ITO compared to cassava from both the RSM Model and the kinetic studies confirmed that they are potential feedstocks and efficient substitutes for food crops in bioethanol production. In addition, the low-cost reduction potential of the non-food feedstocks would enhance large-scale bioethanol production while helping to alleviate the effects of climate change due to global warming.

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Authorship contribution statement

Adewumi Chizoma Nwakego: Conceptualized, Methodology, Investigation, Data curation, writing original draft; Vincent E. Efeovbokhan: Designed the experiments; Formal Analysis; Adeleke A. Adekunle, Rasheed, A. Hauwa, and Olabode Oluwasanmi: Validation and Editing. Dike, Humphrey N., and Olaniyan D. Deborah: Provided software and Data Visualization; Achugasim, O: Contributed reagents, materials, and supervision.

Declarations

The authors declare no financial or personal relationships with other organizations; hence, there is no conflict of interest. We also declare that no grant was received from any funding source.

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