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Research article

Substrate concentration: A more serious consideration than the amount of 5-hydroxymethylfurfural in acid-catalyzed hydrolysis during bioethanol production from starch biomass

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ABSTRACT

5-hydroxymethylfurfural (5-HMF) yield during bioethanol production from starch was determined using spectrophotometry and chromatography. Increasing acid concentration and time favored 5-HMF production with HCl while yield decreased after 45-minute hydrolysis time for HNO_3 and H_2SO_4 hydrolyzed samples. Impacts of glucose (substrate) concentration and produced 5-HMF on bioethanol yield were studied with different sulphuric acid concentrations and different α-amylase and amyloglucosidase activities. A central composite rotational design was utilized to determine the conditions of hydrolysis for optimum glucose production. The results showed that maximum glucose yield occurred at 0.5 M acid concentration and 45-minute hydrolysis time, while maximum yield was achieved at 120 and 280 units of α-amylase and amyloglucosidase activities respectively. It was shown that 5-HMF did not exhibit much inhibition on ethanol yield at low acid concentrations but became pronounced at higher acid concentrations, while high glucose concentrations had a pronounced negative effect on ethanol yield and fermentation efficiency.

1. Introduction

Biofuels produced from wide varieties of biomass have been found to have great potential as suitable alternatives to petroleum fuels with their attendant ability to reduce the emission of greenhouse gases [1, 2]. Large-scale replacement of petroleum-based fuels with renewable ones will have positive impacts on climate change, energy security and vehicle efficiencies. Also, the addition of bioethanol in conventional fuel enhances the octane number and minimizes the use of toxic, octane-enhancing additives like methyl tertiary butyl ether [3]. Major industrial production of bioethanol is centered on the use of first-generation biofuels (sucrose or starch-based biomass) such as corn ethanol in the US, sugarcane ethanol in Brazil, corn and cassava in China, cassava in Thailand and Nigeria, wheat in Europe. Several challenges have been encountered in biofuel (bioethanol) production from different biofuel generations. These challenges are in the area of (a) food security (b)production cost and (c) conversion technologies etc. [4].

The use of first-generation biofuels is faced with economic factors that influence food security while second-generation biofuels are faced with low product yield due to the recalcitrant nature of the biomass, high energy consumption needed for pre-treatment and other inhibitory effects that make the overall process less viable [5, 6]. In the hydrolysis of biomass, several by-products with inhibitory action on the fermentation/ethanol yield are generated. The inhibitors include; furfural, 5-HMF, acetic acid (AA), formic acid (FA), levulinic acid (LA) and phenolics (phenol and its derivatives). The furans (5-HMF and furfural) affect fermentation by inhibiting the *in-vitro* activity of the enzymes in the primary carbon catabolism such as the aldehyde dehydrogenase, hexokinase, triphosphate dehydrogenase, and pyruvate dehydrogenase [7, 8].

Among these by-products, 5-HMF seems to be the most impactful. 5-HMF can be produced via acid-catalyzed dehydration of diverse carbohydrates such as glucose, fructose, sucrose and cellulose or as an intermediate product in the Maillard reaction, [9]. The furan aldehydes (furfural and HMF) have been found to inhibit the growth of yeast and decrease ethanol productivity and yield by reducing the activities of

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several yeast enzymes such as aldehyde dehydrogenase, alcohol dehydrogenase and pyruvate dehydrogenase [7, 10].

Different analytical methods have been developed to determine the quantity of furan compounds (HMF and furfural) in starch hydrolysate from acid hydrolysis and food samples. The UV-Visible spectrophotometric method was reported to be fast and cheap but scarcely sensitive and specific, whereas, the high performance liquid chromatographic (HPLC) method was slow and expensive but more accurate [11, 12].

The UV–VIS Spectrophotometry was considered a green methodology because it involves the use of inexpensive instrumentation, and does not use much toxic solvents as chromatographic methods do. 5-HMF and furfural have their characteristic absorbance band at a wavelength of 284 nm and 277 nm, respectively, and hence can be determined precisely by UV-Visible spectral method only if one of them is present in the medium without the interference of the other contaminants [13, 14]. Unfortunately, in acid hydrolysis of starch, both are present in the hydrolysate or sugar syrup and their accurate determination becomes a great challenge. These interferences can be avoided through the use of different techniques such as the double-wavelength technique or simultaneous measurement of the spectra by absorption treatment of charcoal before and after reduction with sodium borohydride, two-phase reaction system with HCl in the presence of DMSO, poly(1-vinyl-2-pyrrolidine) (PVP) and salting-out technique with different organic solvents [12, 14, 15, 16].

The inhibitive effects of substrates like glucose have not been extensively studied and compared with other known fermentation inhibitors such as 5-HMF. This current work attempts to study the effect of different acid types, acid concentrations and time on HMF production from starch using the UV-Visible spectrophotometric method. To verify the data obtained from the UV spectra, a further precision study was carried out using the HPLC method. Also, the inhibitive effects of substrate and 5-HMFconcentrations on the ethanol yield and percentage efficiency were investigated.

2. Material and methods

2.1. Reagents/sampling of raw material

Fresh tubers of cassava were harvested from Alakahia, in Obio-Akpor, Local Government Area of Rivers state, Nigeria. The tubers were peeled, washed, sliced into sizes, oven-dried at 60 °C to a constant weight and milled. 200g each of the dried and milled tubers were soaked in 500ml of 1% sodium metabisulfite at 28 °C. The mixtures were allowed to stand for 24 h before they were filtered through a muslin cloth in a large volume of water. The supernatant was carefully decanted to afford the starch which was air dried for 24 h and was further dried to constant weight at 60 °C in an oven. The starch was characterized as reported in Adewumi *et al.* [17]. All chemicals and reagents used were of analytical grade. A standard stock solution containing 100 mg/L 5-hydroxymethylfurfural was purchased from Sigma-Aldrich, Germany and used to prepare the working standard solutions (0.06–0.12 mg/L). Sulphuric (H_2SO_4), Hydrochloric (HCl) and Nitric (HNO_3) acids were purchased from BDH chemicals, England. Acetonitrile (HPLC grade) and ultrapure water were obtained from the biotechnology laboratory of Covenant University Ota, Ogun state.

2.2. Statistical analysis

Data collected from the assays were subjected to analysis of variance (Anova) using IBM SPSS statistical editor (windows version 23). The test of significance was determined by Duncan test at a significance level $P \leq 0.05$.

2.3. Statistical design

The experimental design was developed using Response surface methodology (Design ExpertR 11.0.0 Stat-ease, Inc. Minneapolis, USA). A two-factor and 3-level Central Composite Rotational Design (CCRD) consisting of a set of 12 and 9 experimental runs for acid and enzymatic hydrolysis respectively were designed for the study at three separate levels of low (−1) central point (0) and high (+1) as presented in Tables 1 and 2. The levels of the independent variables (factors) adopted using the CCRD design for optimization are also presented in Tables 1 and 2. The second-order model that was selected for predicting the optimal point is expressed in Eq. (1).

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

Where, Y is the predicted response (glucose yield). X_1 and X_2 are variables (factors).

β_o is the constant term. β_1, β_2 are coefficients of linear terms. β_{12} is coefficient of cross term.

β_{11} and β_{22} are coefficients of quadratic term and e is the error term.

2.4. Enzymatic hydrolysis of cassava starch

Enzymatic hydrolysis of the starch was carried out using the combined methods of Liu [18], and Mojovic *et al.* [4]. The reaction conditions were substrate concentration (10%W/V), α-amylase (60–180 enzyme activity unit/g starch) and amyloglucosidase (140–420 enzyme activity unit/g starch).

The hydrolysis process was carried out using a thermostated water bath with a shaker (Shz-88 Thermostatic Digital Shaking Water Bath). The starch was gelatinized in a water bath at 90 °C for 10 min at a substrate to liquid ratio of 1:10. The gelatinization temperature of the cassava starch is 80 °C. Thereafter, the gelatinized, mixture was liquefied with 2 ml of α-amylase at different activities of 60–180 unit/g starch at 75 °C for 60 min. On completion of the reaction, the medium temperature was lowered to 55 °C and pH adjusted to 5.0 using 1% HCl. Subsequently, 2 ml of different activities of amyloglucosidase (140–420 unit/g starch) was added to the solutions, and saccharification was further carried out for 3 h at 55 °C [19]. To deactivate the enzyme, the temperature of the medium was raised to 100 °C after which the solution was allowed to cool and the residue removed by filtration.

2.5. Acid hydrolysis of starch

This was carried out in an autoclave (Techmel and Techmel U.S.A. model TT-280A) in accordance with the method of Gupta *et al.* [1]. Starch slurry was made in a 1: 20 substrates to liquid ratio in a 250 ml flask and the mixture was transferred into the hydrolyzing vessel. The operating condition was: sulphuric acid concentration (0.25, 0.5, 0.75 moldm-3), Temperature: 121 °C and Time (15, 30, 45 and 60 min). At the end of the hydrolysis, the solution was neutralized, filtered using filter paper, and the resultant hydrolysate was analyzed for total reducing sugar (TRS) concentration using the DNSA method [20].

2.6. Fermentation of hydrolysate

Fermentation of the hydrolysate obtained from both acidic and enzymatic hydrolysis processes was carried out using 1 g/L *Saccharomyces cerevisiae.* The media used for fermentation consisted of yeast extract (2 g/L), peptone (3 g/L), glucose (2 g/L), starch hydrolysate and distilled water up to 1000 ml. The media was enhanced with $MgSO_4.7H_2O$ (1 g/L), KH_2PO_4 (2 g/L), $CaCl_2$ (0.1 g/L) and NH_4Cl (1 g/L) and subjected under anaerobic conditions of pH 5.0 at 30 °C for 72 h.

Table 1. CCRD variables and their coded levels for enzymatic hydrolysis of starch.

Variables	Units	Symbols	Coded Levels		
			−1	0	+1
α-Amylase	Enzyme units	X_1	60	120	180
Amyloglucosidase	Enzyme units	X_2	140	280	420

Table 2. CCRD variables and their coded levels for acid hydrolysis of starch.

Variables	Units	Symbols	Coded Levels		
			−1	0	+1
Acid concentration	$Moldm^{-3}$	X_1	0.25	0.5	0.75
Time	Minutes	X_2	15	30	60

Before use as an inoculum (2ml). The media was initially sterilized in an autoclave for 15 min and cooled before the addition of yeast. The media was then aerobically cultured in a 250 ml conical flask in a water bath with a shaker at 30 °C for 24 h before it was separated by centrifugation.

2.7. Determination of total reducing sugars (TRS)

The hydrolysate obtained from both hydrolysis methods were analyzed for total reducing sugar (TRS) concentration using 3, 5-dinitrosalicylic acid (DNSA) method. A standard curve was obtained by measuring the absorbance of known concentrations of glucose solutions at a wavelength of 540 nm using a UV-Visible spectrophotometer (Metash UV-5200 spectrophotometer, Shanghai Metash Instruments Co Ltd).

2.8. Determination of ethanol concentration using potassium dichromate method

The method of Sayyad *et al.* [21] was used to determine the concentration of ethanol with slight modification. Three standard ethanol concentrations in percentages 2.5%, 5.0%, and 10.0%V/V were prepared, 1ml each of the ethanol concentrations was transferred into a 100 ml conical flask containing 3 ml of 0.25 M $K_2Cr_2O_7$, 3 ml of 6 M H_2SO_4 and the solutions were allowed to stand for 10 min after thorough shaking at 150 rpm for color change. At the expiration of the 10 min, 39 ml of distilled water was added into the solution and the absorbance was obtained against a blank containing (1ml of water, 3 ml of 0.25 M $K_2Cr_2O_7$, 3 ml of 6 M H_2SO_4, and 39 ml of water) at a wavelength of 595 nm using UV-visible spectrophotometer. A plot of absorbance against standard ethanol concentration was thus obtained. This method was used to determine the unknown ethanol concentration from the fermentation broth by adding 1ml of the broth to the flask containing 3 ml of 0.25 M $K_2Cr_2O_7$, 3 ml of 6 M H_2SO_4.

The percentage (v/v), actual (g/L) and fermentation efficiency (%) of the ethanol produced from each hydrolysate were determined from the absorbance readings, ethanol density formula and the initial glucose concentration before fermentation respectively through the following sets of equation (Eq. 2–6).

$$1.0\ \text{g starch} + H_2O\ 1.1\ \text{g glucose}\ 0.56\ \text{g ethanol} \quad (2)$$

The amount of ethanol (gL^{-1}) obtained from the fermentation broth was evaluated from the following sets of equations (Eq. 3–6)

$$\text{Volume of ethanol (ml/100ml)} = \text{CE } (\%VV^{-1})/100 \times \text{AVB (ml)} \quad (3)$$

$$\text{Amount of ethanol (g/100ml)} = \text{Vol of Ethanol (ml)/ 1ml} \times \text{Density of Ethanol } (gml^{-1}) \quad (4)$$

$$\text{Amount of ethanol } (gL^{-1}) = \text{Mass of Ethanol (g)/ 100 ml} \times 1000\text{ml /1L} \quad (5)$$

Where CE = concentration of ethanol obtained from spectrophotometric absorbance readings.

AVB = Actual volume of the fermentation broth.

The fermentation efficiency (Eq. 6) is calculated from the theoretical ethanol yield obtained from the Gay Lussac equation (Eq. 2).

$$\text{EFF (\%)} = \text{Actual Ethanol yield } (gL^{-1}) \text{ / Theoretical Ethanol yield } (gL^{-1}) \times 100 \quad (6)$$

2.9. Analysis of 5-hydroxymethyl furfural (5-HMF)

Three standard solutions of 5-HMF; 0.06, 0.09 and 0.12 mg/L were prepared from a stock solution of 1.0 mg/L and their absorbance values were obtained from a UV-spectrophotometer at the wavelength of 284 nm. To determine the unknown concentration of each hydrolyzed sample, a standard calibration curve of absorbance against concentration of standard 5-HMF solutions was plotted. To determine the concentration of each sample, 3 ml of each hydrolyzed sample (after treatment with activated carbon) was analyzed for its 5-HMF concentration using UV-spectrophotometer and the absorbance was obtained at 284 nm. The concentration of each sample was obtained from the plot of absorbance against standard 5-HMF concentration [22].

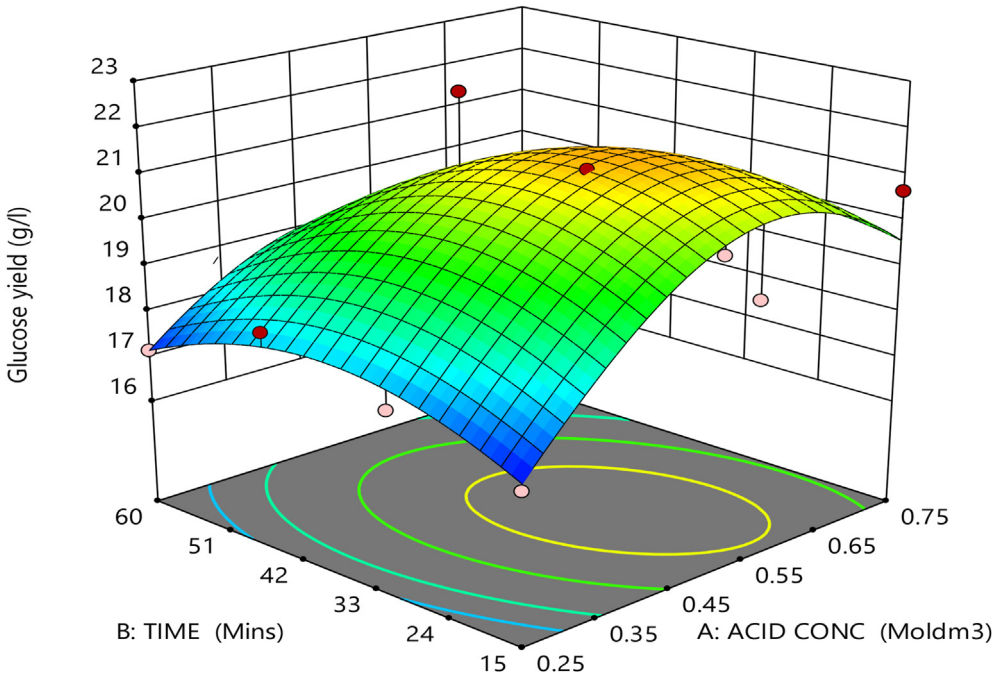

Figure 1. Response surface plots of Time and Acid concentration on the glucose yield (g/L) during acid hydrolysis of cassava starch.

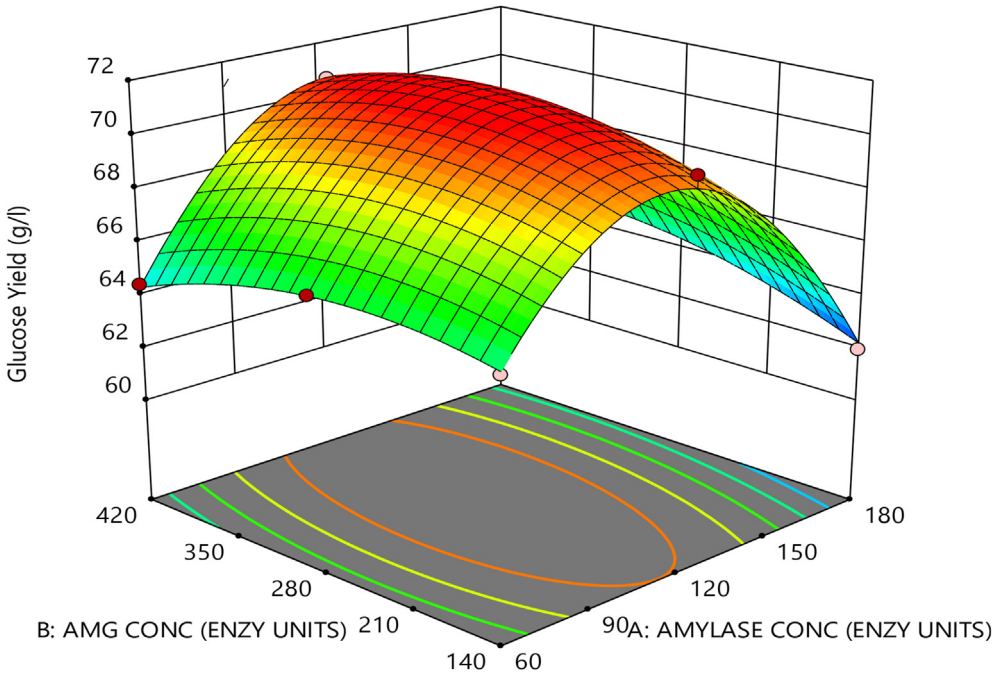

Figure 2. Response surface plots of α-amylase and amyloglucosidase activities on the glucose yield (g/L) during enzymatic hydrolysis of cassava starch.

2.10. Determination of 5-hydroxymethyl furfural (HMF) with high performance liquid chromatography (HPLC)

The method of Hu *et al.* [23] was used to obtain the HMF concentration in the starch (1g) samples hydrolyzed with different concentrations of sulphuric acid (0.25–0.75 M) at different times (15–60 min). HPLC (Agilent 1290 infinity LC System) equipped with photodiode array detector (DAD- G4212A), binary pump (G4220A), auto sampler (G4226A), Agilent column (BEH C18, 150 mm × 2.1 mm, 1.7μm, 80 °C) and a Chem-station software (Agilent Technologies) were used to analyze the samples and determine the concentration of HMF. The mobile phase was made up of water/acetonitrile (70/30%) mixture at a flow rate of 0.250 mlmin-1 at pressure of 1200 bar. Twenty microliter (20.00 μL) of both the standard HMF and the sample (hydrolysate) were injected into the column and analysis was run for 15 min and the spectra recorded at a wavelength of 284 nm. The HMF spectra of the samples were identified by comparing the spectra of HMF standard based on the retention time. A sample of the chromatogram obtained from the HPLC analysis of both the sample and the standard 5-HMF presented below as Figure 6(A and B) is in good correlation.

Table 3. Ethanol yields (g/L) of starch hydrolysate at different H_2SO_4 concentrations and time.

Acid concentration (M)	Time (Minutes)			
	15	**30**	**45**	**60**
0.25	5.55	5.47	6.65	4.91
0.5	5.65	5.70	6.28	5.81
0.75	6.17	6.32	6.46	6.29

Table 4. Results of Ethanol yield (g/L) of cassava starch hydrolysate at different α-amylase and amyloglucosidase activities (units).

α-Amylase (Units)	Amyloglucosidase (Units)		
	140	**280**	**420**
60	21.16 ± 1.53^{a}	23.73 ± 0.20^{b}	27.30 ± 0.23^{c}
120	19.83 ± 1.15^{a}	25.34 ± 0.17^{b}	28.70 ± 0.20^{c}
180	20.04 ± 0.17^{a}	22.97 ± 0.34^{b}	25.44 ± 0.36^{c}

3. Results and discussion

The result of glucose yield from the acid hydrolysis of cassava starch through the experimental design is presented in Table 2. The analysis showed that the most efficient condition in terms of response yield (glucose) occurred at 0.5 M acid concentration and 4-minute hydrolysis time (Figure 1). Under this condition, 22.46 g/L glucose yield was obtained corresponding to a hydrolysis efficiency of 44.96%.

The resulting responses (Figure 1) showed that both acid concentration and time have positive influence on glucose yield at lower conditions of hydrolysis. It was observed that there was a progressive increase in the glucose yield as acid concentration and time increases from 0.25-0.65 M and 15–51 min respectively. Further increase beyond 0.65 M and 0.51 min resulted in a decrease in glucose yield.

The acid concentration was observed to have a positive effect on glucose yield as it is found to improve on an increase in the hydrogen ion concentration which enhances its catalytic ability to break more

Table 5. Effect of acid types on HMF (mg/L) production at different acid concentrations and time.

PARAMETERS	Acid conc ($moldm^{-3}$)	Hydrolysis Time (mins)	Acid Types		
			H_2SO_4	HNO_3	HCl
	0.25	15	0.06	0.109	0.12
		30	0.071	0.116	0.124
		45	0.079	0.116	0.139
		60	0.072	0.113	0.15
	0.50	15	0.081	0.114	0.081
		30	0.084	0.127	0.084
		45	0.111	0.136	0.111
		60	0.107	0.133	0.107
	0.75	15	0.097	0.116	0.143
		30	0.101	0.131	0.21
		45	0.126	0.144	0.218
		60	0.113	0.137	0.255

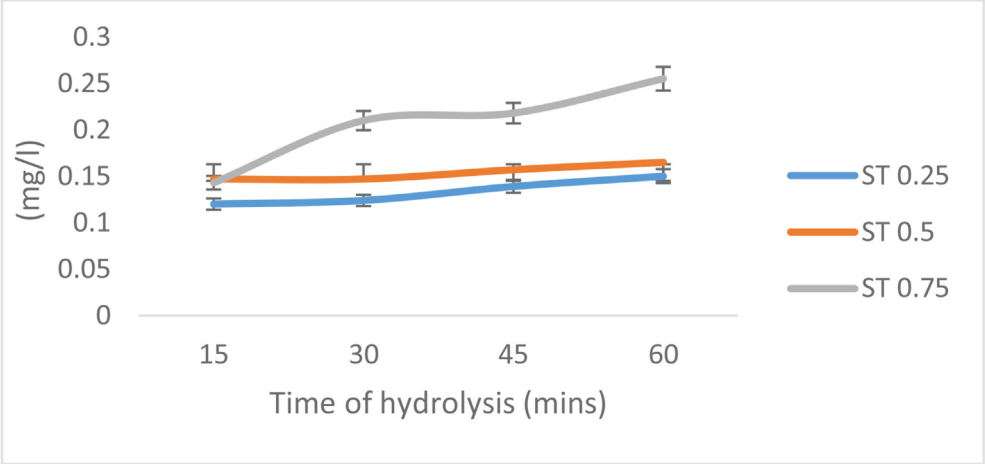

Figure 3. Effects of acid concentration and time on HMF production from HCl hydrolyzed starch.

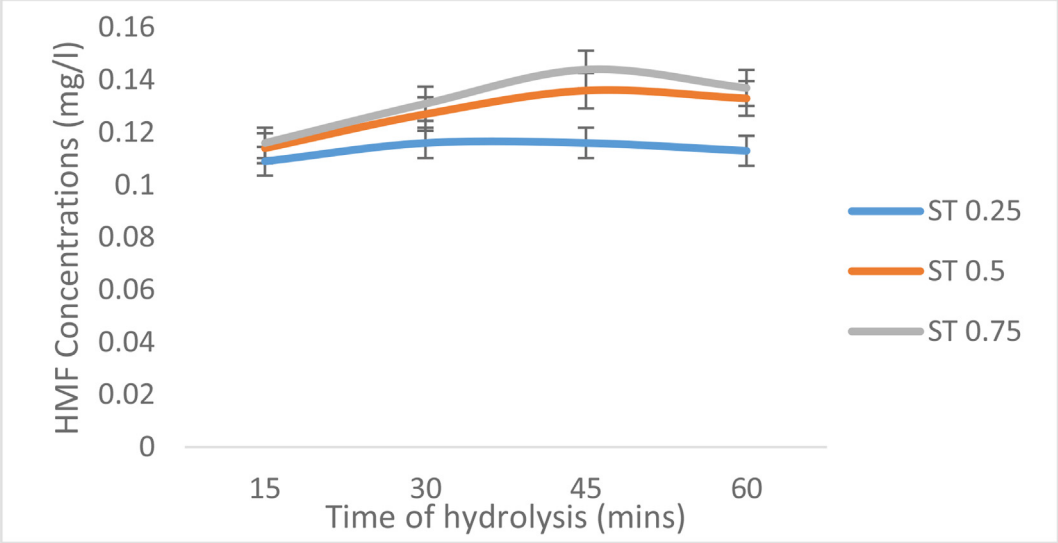

Figure 4. Effects of acid concentration and time on HMF production from HNO_3 hydrolyzed starch.

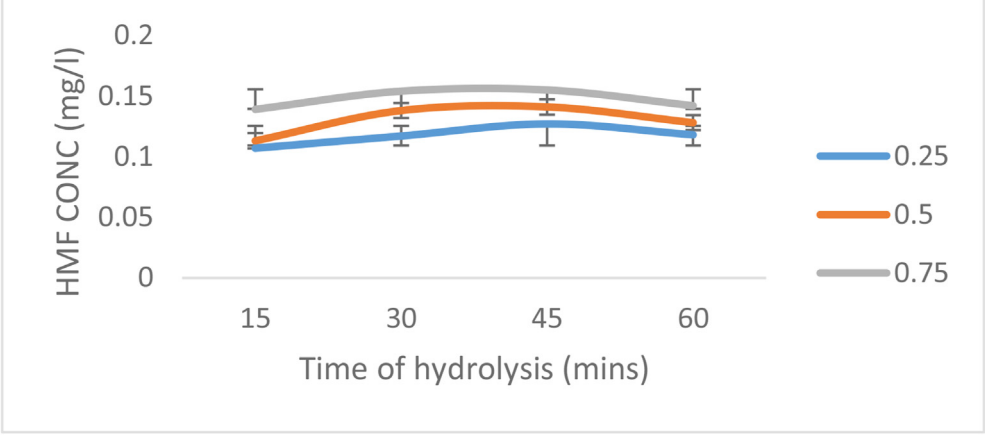

Figure 5. Effects of acid concentration and time on HMF production from H_2SO_4 hydrolyzed starch.

glycosidic bonds within the biomass structure thereby causing an increase in glucose yield [23]. The reduction in glucose yield as observed at higher acid concentrations and time might be a result of the degradation of the glucose to inhibitory by-products such as 5-HMF etc.

The effect of the interaction between α-amylase and amyloglucosidase on the glucose yield during enzymatic hydrolysis of the cassava is presented in the response yield (Figure 2). As observed, the glucose yield increased as α-amylase and amyloglucosidase activities increased from

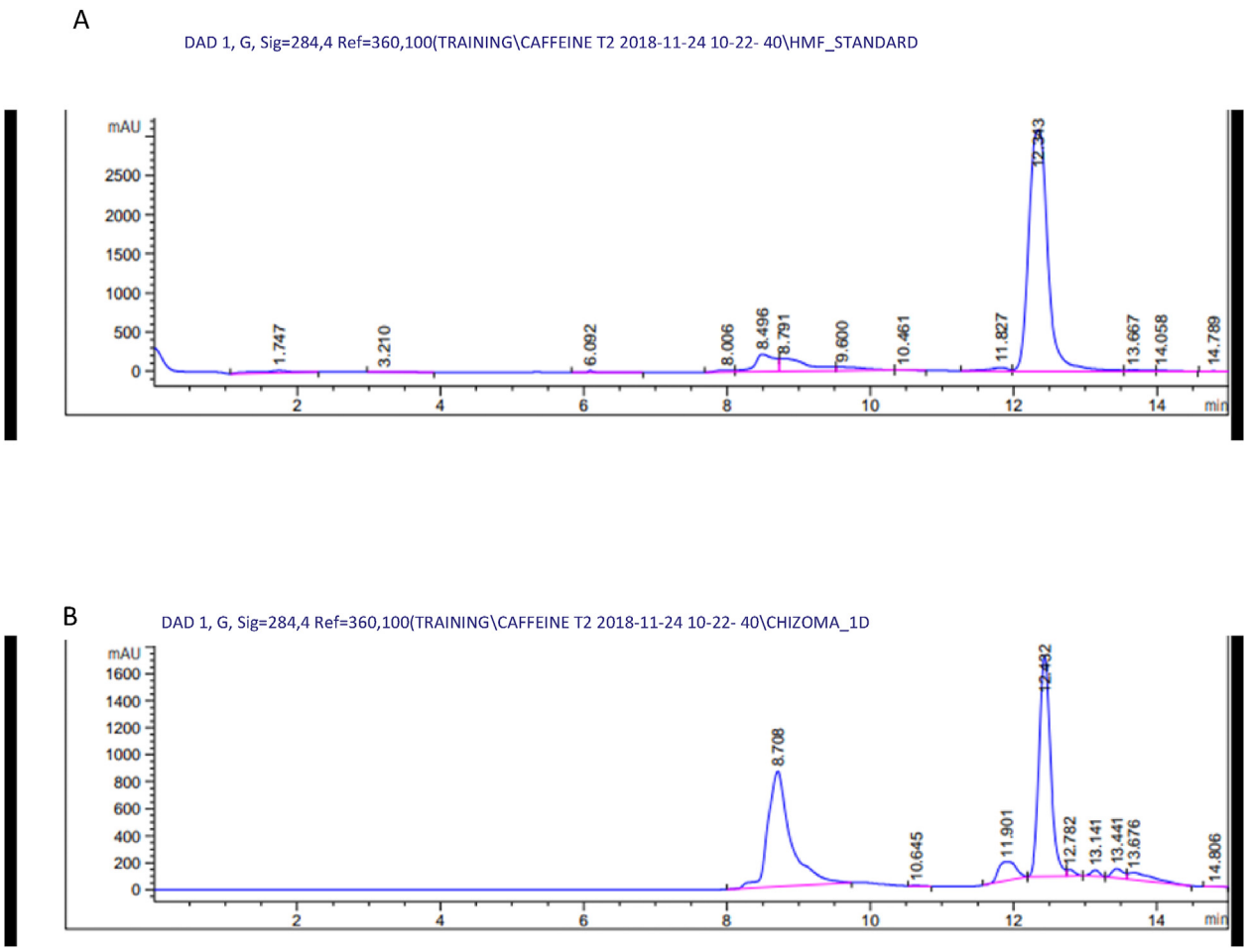

Figure 6. HPLC Chromatogram of (A) Standard 5-HMF, (B) 5-HMF in acid hydrolyzed sample.

60-120 and 140–420 enzyme units respectively. In all conditions of hydrolysis, it was observed that the glucose yield exceeded 70 g/L when α-amylase concentration was 120 units, and yield decreased as activity was further increased to 180 units. The decrease may be due to enzyme stress at high concentrations [24]. However, amyloglucosidase was found to have a positive influence on glucose yield as yield increased linearly with concentration.

The analysis of variance from the 12 and 9 experimental runs of acid and enzymatic hydrolysis respectively, showed that the model is good and presents a strong correlation between the variables evaluated. The analysis of variance of the result of acid hydrolyzed samples shows that there was no significant difference with a P-value of 0.136 and a coefficient of determination (R^2) value of 0.686. However, the trend differed in the opposite direction with enzymatic hydrolysis in which the analysis showed a significant difference with a P-value of 0.002 and an R^2 value of 0.993. Thus there is a stronger correlation between α-amylase and amyloglucosidase than acid concentration and time on glucose yield.

3.1. Fermentation of starch hydrolysate from acid and enzymatic hydrolysis

From the result of the acid hydrolysis, it was observed that among the different acid concentrations, glucose concentration at 45-minute hydrolysis time offers the most efficient fermentation condition in terms of ethanol yield. Under this condition, a maximum ethanol yield of 6.65 g/L with a fermentation efficiency of 70.82% was obtained from an initial glucose concentration of 18.38 g/L (Table 3). At 60-minute hydrolysis time, it was evident that ethanol yield decreased in all acid concentrations. On the other hand, a maximum ethanol yield of 28.70 g/L with a fermentation efficiency of 81.28% was achieved with an initial glucose concentration of 70.61 g/L under enzymatic hydrolysis at 120 and 420 α-amylase and amyloglucosidase activities respectively (Table 4). On the whole, the ethanol yield obtained from this study is in tandem with that from other studies like: 34.45 gl-1 (74.98%) from de-oiled Pongamia pinnata seed [25], sugarcane substrate [26], 6.9%ww-1 (62.4%) of 20% ww-1 corn meal [4]. Yi-Huang *et al.* [27], evaluated the effect of substrate inhibition on bioethanol yield and observed that substrate inhibition is

Table 6. Result of HPLC analysis showing effect of time and acid concentration on HMF production from H_2SO_4 hydrolyzed starch sample.

Code	Acid conc ($moldm^{-3}$)	Hydrolysis Time (mins)	Retention Time (mins)	Area (mAU*s)	Recovery (%)
1	0.25	15	12.332	16073	35.98
2		30	12.333	71167	69.42
3		45	12.316	63649	75.30
4		60	12.324	71731	81.51
5	0.50	15	12.327	63189	78.81
6		30	12.334	56950	81.53
7		45	12.326	87511	82.25
8		60	12.351	91655	80.64
9	0.75	15	12.335	74664	86.57
10		30	12.338	83990	80.06
11		45	12.343	90435	79.65
12		60	12.322	55327	47.65

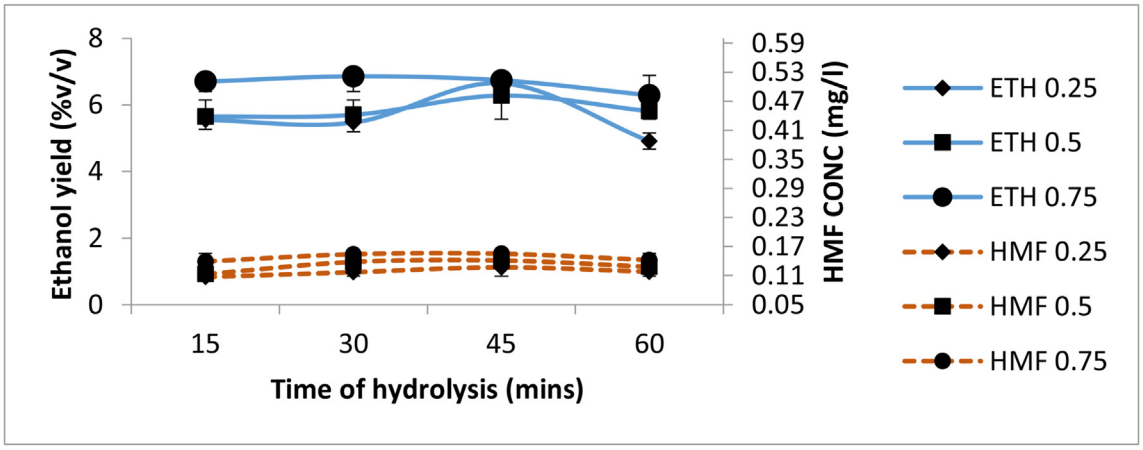

Figure 7. Effect of 5-HMF on Ethanol yield during acid hydrolysis.

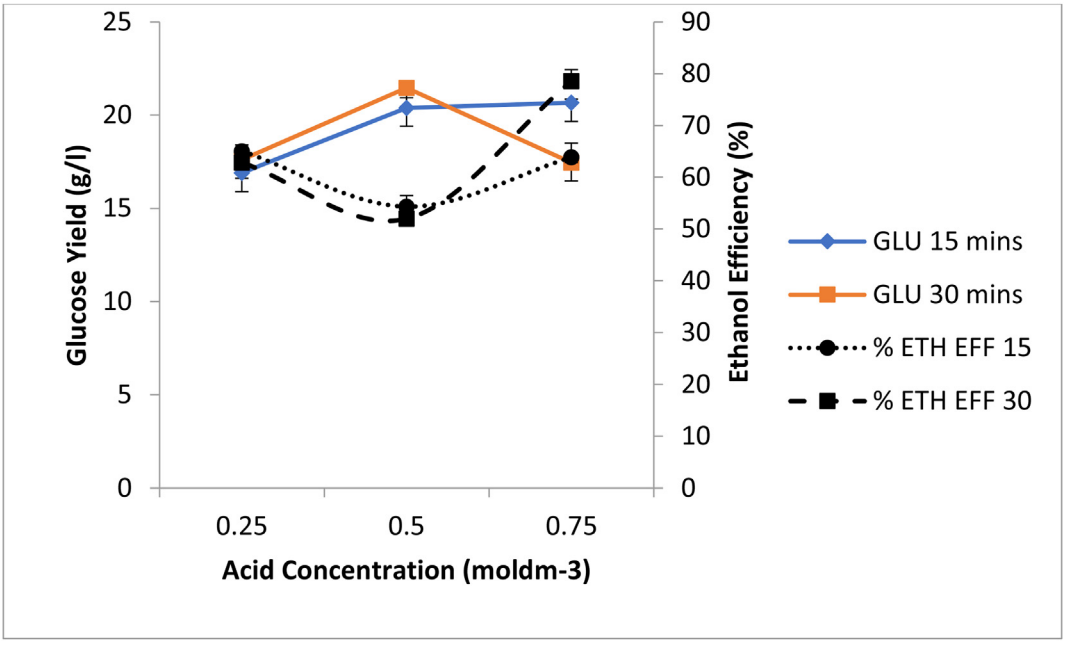

Figure 8. Effects of substrate (glucose) concentration on Ethanol yield of acid hydrolyzed samples at 15 and 30 min hydrolysis time.

evident at initial glucose concentrations above 200 g/L with a maximum ethanol yield of 8.3 g/L for 180 g/l glucose concentration obtained. Erkan *et al.* [28], reported that the highest ethanol yield of 32.07 g/L (43.867%) was obtained when HMF in the fermentation medium is 6 g/L while the lowest ethanol yield of 3.993 g/L (32.14%) was obtained when HMF is 10 g/L.

The low ethanol yield from H_2SO_4 hydrolyzed samples could be attributed to the presence of inhibitors such as 5-HMF and high salt concentration (excess sodium sulphate in the medium) which has been reported to cause osmotic and ion toxicity stress to yeast [29]. Also, from the present study, the cost of enzymes and energy (due to longer hydrolysis time) necessary for the conversion of feedstock (cassava) to bioethanol was found to be ten (10) times higher than that encountered in the acidic process and this agrees with the report of Prasoulas et al. [30].

3.2. Effect of acid type on 5-hydroxymethylfurfural (HMF) production

Table 5 illustrates the effect of acid types (H_2SO_4, HCl, HNO_3) at different concentrations (0.25–0.75 M) and time (15–60 min) on HMF production from acid hydrolysis of the starch samples. The activities of the different acid types were investigated to determine the trend of glucose degradation to HMF at different concentrations and times at 120 °C. The study showed that at 120 °C the catalytic actions of the acid towards HMF formation are in the following decreasing trend HCl > HNO_3 > H_2SO_4. The highest HMF yield of 0.255 mg/L was achieved at 0.75 M and 60 min of hydrolysis time for the HCl hydrolyzed sample. Conversely, the highest HMF yield of 0.144 and 0.126 mg/L were achieved at 45-minute hydrolysis time for HNO_3 and H_2SO_4 hydrolyzed samples respectively, and yield decreased as time increased to 60 min. The basic strength of the dissociated anions within the solution was attributed to the selectivity of HMF and its yield [31]. The trend in the degradation activity of the acids obtained in this study is in excellent agreement with other related work like Roman-leshkov *et al.* [16].

3.3. Effects of acid concentration and time on HMF production

Time and acid concentration are some of the reaction parameters that affect the yield of not only sugars but also sugar degradation products [32]. It was observed from this work (Figures 3, 4, and 5) that an increase in concentration (from 0.25-0.75 M) and time (from 15-60 min) favored HMF formation and yield for starch samples hydrolyzed with HCl.

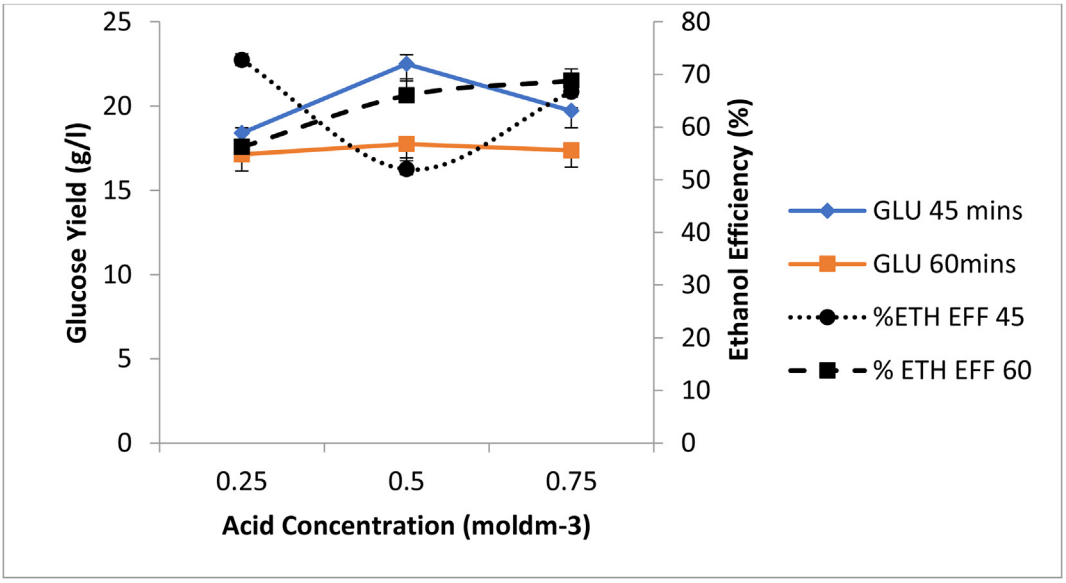

Figure 9. Effects of substrate (glucose) concentration on Ethanol yield of acid hydrolyzed samples at 45 and 60 min hydrolysis time.

However, for samples hydrolyzed with H_2SO_4 and HNO_3, HMF formation increased as time increased from 15 – 45 min but yield decreased as time was further increased to 60 min. The decrease in HMF yield at a longer time may be attributed to the breakdown of HMF to levulinic acid (LA) and formic acid (FA) [9].

In order to validate the effect of time and acid concentration on HMF production, the starch was hydrolyzed with different concentrations of H_2SO_4 at different time intervals and the analysis was this time performed with high performance liquid chromatography (HPLC). The result obtained (Table 6) is in excellent agreement with those obtained from spectrophotometric analysis for samples hydrolyzed with sulphuric acid (Figure 5). At 0.25 M acid concentration, HMF yield increased as time increased from 15-60 min. However, for 0.5 M concentration, HMF yield decreased after 45 min of hydrolysis while the reverse was the case for 0.75 M concentration in which yield decreased as time increased. The highest HMF production of 86.57% was achieved at 0.75 M acid concentration at 15 min of hydrolysis time. The observed reduction in HMF yields especially at 0.75 M concentration over time may be attributed to HMF degradation to FA and LA. Therefore, in designing any industrial production process (either for biofuel or polymer production), the product choice (glucose, HMF or ethanol) will determine the type of acid or concentration and time that would be utilized to obtain optimum yield.

Figure 6A and B below depicts the chromatograms from the HPLC analysis of the standard 5-HMF and the 5-HMF in the acid hydrolyzed sample. The retention times of the signals in the two chromatograms are in perfect correlation confirming the integrity of the results obtained.

3.4. Effect of 5-HMF and substrate (glucose) concentrations on ethanol yield

The effect of 5-HMF concentration on ethanol yield is shown in Figure 7, while Figures 8, 9, and 10 show the effect of substrates (glucose) concentration on ethanol yield.

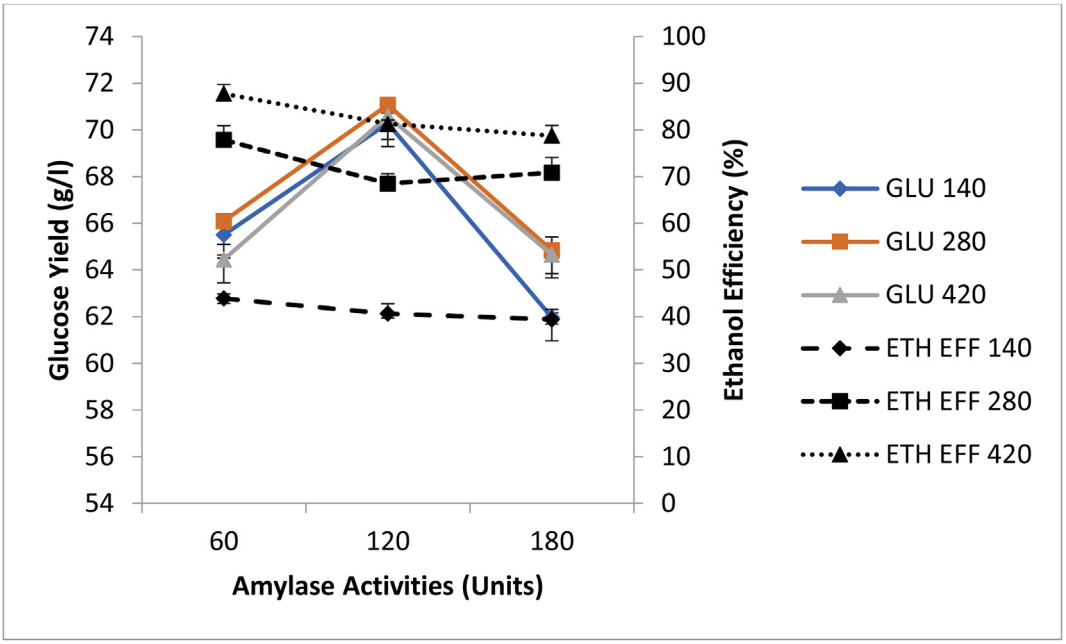

Figure 10. Effects of substrate (glucose) concentration on Ethanol yield from sample hydrolyzed with different α-amylase and amyloglucosidase activities.

The results obtained which compared very well with other works that determined ethanol with dichromate oxidation method [33, 34, 35] show that ethanol yield increased simultaneously as 5-HMF concentration increased from 15-45 min and decreased as time increased to 60 min in almost all samples hydrolyzed with 0.25 M and 0.5 M acid concentrations, whereas, the reverse trend was the case for samples hydrolyzed with 0.75 M concentration. In almost all conditions for samples hydrolyzed with 0.25 and 0.5 M acid concentrations, the maximum ethanol yield was achieved at 45-minute hydrolysis time while with 0.75 M, the maximum ethanol yield was achieved at 15 min. From the experimental data, it was observed that 5-HMF concentration had a slight inhibitory effect on ethanol yield because both 5-HMF and ethanol increased and decreased almost simultaneously with time, especially at 0.25 and 0.5 M concentrations. The reduction in ethanol yield observed at 60 min of hydrolysis time may be due to the collective effects of HMF, carboxylic acids (AA, FA, LA) and phenolic (colored compounds) present in the medium. In order to reduce the inhibitive nature of 5-HMF in large-scale (real application) bioethanol production, hydrolysis time should not be more than 45 min.

Apart from hydrolysis by-products [36], the rate of fermentation enzyme growth can be inhibited by the product (ethanol) or substrate (glucose) during the fermentation process. In the presence of these inhibitors, fermentative enzymes especially yeast experience a reduction in cellular stability, microbial contamination due to low cell density, low tolerance to substrate concentration and ethanol yield [37]. It was found in the course of this study that initial glucose (substrate) concentration has a pronounced effect on the fermentation process (Figures 8, 9, and 10). As observed, the maximum ethanol yield (g/L) and fermentation efficiency (%EFF) were achieved at lower glucose concentrations. Thus increase in glucose concentration was found to have a negative effect on the ethanol yield (g/L) and fermentation efficiency (%) in almost all conditions of fermentation for both acid and enzyme hydrolyzed samples.

4. Conclusion

The data obtained from this study showed that glucose (substrate) concentration has a pronounced effect on the ethanol yield (g/L) and fermentation efficiency (%) during bioethanol production from starch in almost all conditions of fermentation for both acid and enzyme-hydrolyzed samples. This is a serious indication that substrate concentration is a very important and sometimes a greater inhibitor of the fermentation process than by-products like 5-HMF. Acid (HCl, HNO_3, and H_2SO_4), as well as enzymatic (amylase and Amyloglucosidase) hydrolysis of cassava starch, can be carried out at different acid concentrations, time and enzyme activities. The results obtained showed that optimum hydrolysis and eventual ethanol yield occurred at 0.5M HCl acid concentration, 45 min hydrolysis time, and 120 and 280 units of α-amylase and amyloglucosidase activities respectively. H_2SO_4 hydrolyzed samples produced the lowest ethanol yield in comparison to HCl and HNO_3 acid. Therefore, to enhance the efficiency of glucose conversion in the fermentation process for first-generation bioethanol production, enzymes or yeast strains that can convert high substrate concentrations to ethanol should be developed and utilized.

Declarations

Author contribution statement

Chizoma Nwakego Adewumi: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Effiong Idongesit Ekpo: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ozioma Achugasim: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Regina Enyidia Ogali; Onyewuchi Akaranta: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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