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Optimisation of enzymatic fermented glucose production of wild cocoyam starch using response surface methodology

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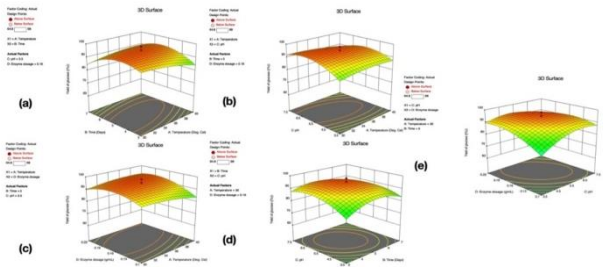
Graphical abstract



Abstract

The enzymatic fermentation of starch from non-edible sources using *Aspergillus niger* could help supplement the increasing glucose demand within the Nigeria food supply chain. Also, response surface methodology could help optimise the glucose production of starch hydrolysed from cocoyam tubers. In this work, we evaluated and optimized the enzymatic-fermented glucose production of wild cocoyam starch using response surface methodology. Wild cocoyam starch was hydrolyzed using *A. niger* isolated from the soil. The optimization process involved temperature, time, pH, and enzyme dosage, alongside the kinetics and thermodynamics of enzymatic hydrolysis. Optimum conditions for glucose yield of 95 % by enzyme hydrolysis included: temperature = 35 °C; time = 5 days; pH = 5.5; and enzyme dosage =0.16 g/mL, with wild cocoyam showing promising as substrate. The F-value of quadratic model terms appeared statistically significant (F= 65.42, p<0.0001). Fourier Transform Infra-Red Spectrometer (FTIR) and Gas Chromatography-Mass Spectrophotometer (GC-MS) analyses confirmed characteristic bands of glucose as industrially promising with 60% purity. Further, the enzymatic hydrolysis adhered to Michaelis-Menten kinetic model with maximum reaction rate of 82.6 ml/day at 35 °C.

Keywords: Amylase enzyme, *Aspergillus niger*, Michaelis-Menten kinetic model, response surface methodology, wild cocoyam

1. Introduction

Globally, several tropical crops serve as a source of starch production, which include cassava, sweet potato, yams, cocoyam, and rice (Okafor and Okeke, 2018). Starch is primary raw material, mainly utilized in the brewery, sugar industries for glucose and maltose production (Yamamoto *et al.*, 2016; Ali *et al.*, 2020). Located in the endosperm of grains, roots, and tubers of some crops (Zainab *et al.*, 2011; Mathew *et al.*, 2015), cocoyam starch has to be at acceptable quality for pharmaceutical purposes, where hydrolysis of starchy waste from cocoyam processing to sugar is equally possible (Okafor and Okeke, 2018). Starch combines two polymers of glucose: amylose and amylopectin. Amylose comprises linear (1 → 4) α - D glucan, which possess a degree of polymerization (D.P.), which refers to the number of glucose molecules of ~ 400, with few branched residues linked with (1 → 6) bondings. Amylopectin comprises branched D-glucan with predominantly α - D (1 → 4) linkages and ~ 4% of the α - D (1 → 6) type, having amylose-like chains of D.P. 12–50 linked in diverse possible ways. Additionally, starch could be converted into different sweeteners by an acidic or enzymatic process (Okafor and Okeke, 2018).

The industrial conversion of polysaccharide to monosaccharide and disaccharide involve processes like saccharification, gelatinization, and liquefaction (Saggi *et al.,* 2016) employing microbial enzymes (Zainab *et al.*, 2011). Prior to saccharification, the starch mix with water, and gelatinized, prior to exposure to the attack of

saccharifying agents like dilute acids/enzymes from malt or microorganisms (Okafor and Okeke, 2018). Several enzymes essential in starch hydrolysis include: α-amylase (enzymes able to hydrolyse α-1, 4 bonds and by-pass α-1, 6 bonding), Beta amylase (enzymes able to hydrolyse the α-1, 4 bonding, but unable to by-pass the α-1, 6 bonds), amyloglucosidase or glucoamylase (enzymes able to hydrolyze α-1, 4 and α-1:6 bonds), pullulanase (de-branching enzyme able to bring about hydrolysis of α-D-(1, 6) linkages in amylopectin), iso-amylase (de-branching enzyme where the three glucose units within the group are attached to the rest of the molecules through the α-D-(1, 6) bonding for it to function), and glucosidases (enzymes that prefer to attack the α-1, 4 linkages) (Okafor and Okeke, 2018). Recent years, microbial synthesized enzymes for the hydrolysis of starch are among areas of interest to researchers. For instance, amylases have been useful in the hydrolysis of starch to simple sugar. Besides the increased attention of amylase enzyme could involve technological and economic benefits, there is evidence regarding amylase and its capacities, particularly in yeast, fungi, bacteria, and moulds (Sethi *et al*., 2016, Mathew *et al*., 2016).

Aspergillus niger is among amylolytic enzyme sources, primarily aerobic, prevails over a wide range of hydrogen ion concentrations, cultivated and maintained easily in the laboratory given their capacity to utilise various simple to complex food sources (Mathew *et al*., 2016). Besides its availability to industrial production of many substances, the fermentation process of *A. niger* in food has been considered "generally recognized as safe" (G.R.A.S.) (Cairns, Nai, and Meyer, 2018). The fungal amylase enzymes are widely produced by solid-state fermentation (S.S.F.) in developing countries because of their simple operations, affordability, and high enzyme yield (Sethi *et al*., 2016, Mathew *et al*., 2016). In the last two decades, S.S.F. has involved developing bioprocesses, for instance, bio-detoxification of agro-industrial residues, bio-transformation of crops /crop-residues for nutritional enrichment, and production of value-added products (Pandey *et al*., 2000). The application of fungal amylase enzymes from *A. niger*to produce simple sugar from agricultural waste can be an economic prospect to a country like Nigeria. Glucose is among simple sugars, widely used as a primary sweetener in the confectionery industry (Zainab *et al*., 2011), food manufacturing (Khoshnoud, 2018), and pharmaceutical companies (Ansari *et al.,* 2015). The extraction of glucose from different starchy sources has been reported, like in maize (Bušić*et al*., 2018), banana (Itelima, 2013; Bello-Perez *et al*., 2002), cassava (Ansari *et al.*, 2015), and sweet potato (Mathew *et al*., 2016).

Wild cocoyam is a tuberous, heart-shaped leaf varying in length from 15 cm to 60 cm and red and white blotches. It has 88.42 % dry matter containing about 85.14 % metabolisable energy (carbohydrate) (Oladije *et al*., 2011). Optimising the utilisation of natural starch cocoyam is needed in food bioprocessing, as it serves as raw materials and as additives like gelling, stabilising, and thickening agents (Lopulalan *et al*., 2020). Meanwhile, to achieve process improvement and optimisation of operational factors associated with agro-food products, statistical approaches such as response surface methodology (R.S.M.) continues to hold a compelling track record of relevance to the food industry. By combining statistical and mathematical methods, Boudechiche *et al*. (2017) showed that R.S.M. would evaluate the relative significance of various process parameters in the presence of complex interaction. The R.S.M. provides a platform that identifies optimum conditions within the multi-variable system(s), to reduce the number of experiments, save cost as well as time (Shojaei, and Shojaei, 2018; Shojaei *et al*., 2020). Further, R.S.M. helps establish the changing effects and trends of different factors over each other. Such designed changes in the process input variables and resultant variations could be detected in output responses (Shojaei, and Shojaei, 2018; Shojaei *et al*., 2020). However, there is paucity of relevant information about glucose production specifically from wild cocoyam tubers in Nigeria. The enzymatic fermentation of starch from non-edible sources using *A. niger* could be a way to support the increasing glucose demand within the food supply chain in Nigeria, which by population, remains the largest country in Africa. On the other hand, R.S.M. can help to actualise the optimisation of glucose production, which is applicable to the hydrolysis of starch obtained from cocoyam tubers. The specific objective of this current work was to evaluate and optimise the enzymatic-fermentated glucose production of wild cocoyam starch using response surface methodology. Specifically, the wild cocoyam starch was hydrolyzed with *A. niger* isolated from the soil. Essentially, the optimisation process on temperature, ime, pH, and enzyme dosage, alongside kinetics and thermodynamics of enzymatic hydrolysis were performed.

2. Materials and methods

2.1. Procurement and Processing of wild cocoyam samples

The wild cocoyam was obtained from the open market situated at Oboloafor, Nsukka, of Enugu State, Nigeria (6.90236° N, 7.36869° E), subsequently authenticated by the Department of Plant Science and Biotechnology University of Nigeria Nsukka, Enugu State, Nigeria. The freshly harvested wild cocoyam tubers were sorted, washed with distilled water, sun-dried for four days, peeled and pulverized into different smaller sizes, and milled to flour using a manual grinder to obtain a stock sample. The milled sample was sieved through a fine-mesh sieve size of 200 μm. The wild cocoyam samples were kept in a dry, cool place of ambient temperature 28±2°C until required. Essentially, all the chemicals and reagents employed in this study were of analytical grade standard.

2.2. Determination of carbohydrate content

The carbohydrate content of ground cocoyam was determined using the Official Methods of Analysis, O.M.A. (2019).

2.3. Synthesis of microbial enzyme

2.3.1. Isolation of an Amylolytic Aspergillus niger

Isolation of amylolytic fungal strain for hydrolysis of starch was determined by Mathew *et al*. (2016). The amylolytic fungal strain was isolated from the soil using a medium and autoclaved at 121 °C for 15 min and 15 psi. The suspension of the soil sample (2 g) in 20 mL of treated distilled water was centrifuged at 3,000 g with a portable centrifuge (Hettich E.B.A. 8S, Tuttlingen, Germany). Then 0.1 mL of the supernatant was pour plated, and incubated at room temperature 28±2°C for 48 hours. The medium was observed to be transparent around the microbial colonies inferring the production of amylase. The territory with colourless zones was determined by subtracting the radiuses (mm) of a settlement from the clear zone. The more colourless colony zones of *A. niger* were then selected and streaked on the malt extract agar to purify them. They were subsequently identified microscopically based on the type of cell and features of colonial morphology with the procedure reported in Mathew *et al*. (2016).

2.3.2. Crude amylase synthesis

The amylolytic*A. niger* isolated and selected was cultured on a wild cocoyam base medium using the procedure stated in Mathew *et al*. (2016). The medium is comprising 12 g of wild cocoyam placed in a 250 ml Erlenmeyer flask. Aqueous solution of mineral salts of $MgSO_4.7H_2O$, 0.1%, KH_2PO_4, 0.1 %; $CaCl_2$, 0.1 %; $FeSO_4$, 0.05 % and $(NH_4)_2 SO_4$, 0.1 % was used to moistened it to 54% moisture content. The medium was autoclaved at 121°C for 15 min and 15 psi. The isolated *A. niger* (2 x 10^7 spores / 1000 mL) was inoculated on the sterilized media plates and incubated at room temperature for four days.

2.3.3. Enzyme extraction and purification

The buffer of citrate phosphate pH of 7.4 was added to the moisturized cocoyam mould generated from incubation in the ratio 10:1 (v/w) in a 500 ml Erlenmeyer flask at 30 °C for 60 min, filtered, and the supernatant became the source of crude enzyme. The enzyme was purified by adding ammonium sulfate to the filtrate (250 ml) to reach 80% saturation. After centrifugation at 9,000 × *g* for 15 min, the residue was dissolved in 50 ml of 20 mM potassium phosphate buffer (pH 7.4) and dialyzed thoroughly against this buffer overnight.

2.3.4. Enzyme assay

The amylase activity in a reaction mixture (containing 2 ml of the extracted enzyme, 2 ml of 2 % (w/v) starch solution of cocoyam, and 0.2 ml of buffered solution (pH 7.4) of citrate) was stabilized using the assay method described by Mathew *et al*., (2016). The reacting mixture was incubated at 60 °C for 60 min. The tube containing the reacting mixture was immersed in boiling water at 100 °C for 2 min to stop the reaction. The DNS methods described in Mathew *et al*. (2016) to estimate the reducing sugar evolved. The amylase activity (U) was expressed as the quantity of enzyme that catalyzed the production of one micromole of glucose per minute under the specified conditions of the assay.

2.3.5. Determination of amylase enzyme molecular weight

The molecular weight characterization of the amylase enzyme was carried out using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) according to techniques employed by Sadhu *et al*. (2013) and Imran *et al*. (2018) with slight modification. 12 % separating gel was used to determine the molecular weight of the amylase enzyme.

2.4. enzymatic hydrolysis of Colocasia *spp (wild cocoyam) tuber flour with amylolytic* A. niger

The hydrolysis of starch from the wild cocoyam by enzyme was performed in a 300 cm^3 conical flask containing 60 cm^3 of 5 % inocula of amylolytic *A. niger* with 60 ml dosage of the wild cocoyam (enzyme dosage of 0.16mg/ml) and incubated on a stirrer with the speed of 350 rpm at various temperatures for different time interval and different pH. The yield of the soluble sugar was measured with a refractometer (Model RF M960) after filtration, while the product of reducing sugars was determined using the DNS method (Kongkiattikajorn, 2012).

2.5. Optimization of enzymatic hydrolysis

The Central Composite Rotatable Design (C.C.R.D.) was selected as most fitting for the optimization experiment because Sun *et al*. (2014) understood that its polynomial surface works well for this kind of process. Sun *et al*. (2014) showed that it was possible to evaluate the influence of the variables on the response functions and establish the optimal conditions. Therefore, for this work, optimizing the parameters for enzymatic hydrolysis of wild cocoyam starch to produce glucose was performed. The initial steps involved screening the parameters to ensure their appropriateness. Subsequently, the runs of the experimental design were completed. In total, there were 30 runs for the four-factor experimental design, which were needed to determine the response function, and evaluate the (design) performance through the analysis of experimental data. In this, the optimisation process will require coefficient evaluation, predicting as well as verifying the response of the adjusted model. The operational factors (coded and actual values of the independent variables) used to establish the optimization of the enzymatic hydrolysis are shown in Table 1. The experimental design matrix for enzyme hydrolysis of wild cocoyam starch is shown in Table 2. The empirical Equation is represented as shown in Equation (1):

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \quad (1)$$

The selection of the levels for each factor was based on the experiments performed to study the effects of process variables on the enzymatic hydrolysis of wild cocoyam starch to produce glucose. Therefore, by using the developed model, it would be possible to have the predicted responses compared with the observed values. Where x represents the independent variables (temperature, time, pH, and enzyme dosage) and y stands for the yield of glucose.

Table 1. Operational factors used to establish the optimisation of the enzymatic hydrolysis

Factor	Units	Low level	High level	-α	+α	0 level
Temperature (A)	°C	30(-1)	40(+1)	25(-2)	45(+2)	35
Time, (B)	Days	3(-1)	7(+1)	1(-2)	9(+2)	5
pH (C)		3.5(-1)	7.5(+1)	1.5(-2)	9.5(+2)	5.5
Enzyme dosage (D)	(g/ml)	0.1(-1)	0.22(+1)	0.04(-2)	0.28(+2)	0.16

Table 2. Experimental design matrix for enzyme hydrolysis of wild cocoyam starch

Run order	Temperature (°C) A		Time (Days) B		pH (C)		Enzyme dosage (g/ml) D		Yield of glucose (%) Y
	Coded	Real	Coded	Real	Coded	Real	coded	Real	
1	-1	30	-1	3	-1	3.5	-1	0.1	71
2	+1	40	-1	3	-1	3.5	-1	0.1	64.8
3	-1	30	+1	7	-1	3.5	-1	0.1	69.6
4	+1	40	+1	7	-1	3.5	-1	0.1	73
5	-1	30	-1	3	+1	7.5	-1	0.1	94
6	+1	40	-1	3	+1	7.5	-1	0.1	75
7	-1	30	+1	7	+1	7.5	-1	0.1	76.8
8	+1	40	+1	7	+1	7.5	-1	0.1	76
9	-1	30	-1	3	-1	3.5	+1	0.22	80
10	+1	40	-1	3	-1	3.5	+1	0.22	80.5
11	-1	30	+1	7	-1	3.5	+1	0.22	78
12	+1	40	+1	7	-1	3.5	+1	0.22	88
13	-1	30	-1	3	+1	7.5	+1	0.22	82
14	+1	40	-1	3	+1	7.5	+1	0.22	77
15	-1	30	+1	7	+1	7.5	+1	0.22	73.4
16	+1	40	+1	7	+1	7.5	+1	0.22	79
17	-2	25	0	5	0	5.5	0	0.16	89
18	+2	45	0	5	0	5.5	0	0.16	88.5
19	0	35	-2	1	0	5.5	0	0.16	70
20	0	35	+2	9	0	5.5	0	0.16	70
21	0	35	0	5	-2	1.5	0	0.16	70
22	0	35	0	5	+2	9.5	0	0.16	75
23	0	35	0	5	0	5.5	-2	0.04	70.3
24	0	35	0	5	0	5.5	+2	0.28	82
25	0	35	0	5	0	5.5	0	0.16	94
26	0	35	0	5	0	5.5	0	0.16	94
27	0	35	0	5	0	5.5	0	0.16	95
28	0	35	0	5	0	5.5	0	0.16	94
29	0	35	0	5	0	5.5	0	0.16	98
30	0	35	0	5	0	5.5	0	0.16	95

2.6. Characterization of Glucose

2.6.1. Fourier Transform Infra-Red Spectrometer (FTIR) Analysis of Extracted Glucose

The extracted glucose sample from wild cocoyam was analysed using FTIR. The purpose was to use FTIR to determine the functional group present in the simple sugar. The infrared spectrum was recorded in the mid-infrared region (500-5000 cm^{-1}) in an empty chamber of Shimadzu FTIR-8400S spectrophotometer using KBr discs as matrices. A spectral resolution of $2cm^{-1}$ was used, and the spectral was accumulated over the scans.

2.6.2. Gas Chromatography-Mass Spectrophotometer (GC-MS) Analysis of Extracted Glucose

The percentage purity of the glucose samples was determined with the help of GC-MS. The glucose analysis was carried out with GC-MS (Thermo Finnigan Trace GC/Trace DSQ/A1300 E.I. Quadropole) equipped with an SGE-BPX5 MS fused silica capillary column (film thickness 0.25 µm).

2.7. Michaelis-Menten kinetic model for Enzymatic Hydrolysis Process

The Michaelis-Menten kinetic model has been used for the kinetic aspect of this current work - Equation (2) according to Schnell (2014).

$$-r_s = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

Where r_s = the rate of the enzymatic reaction; Vmax = the maximum rate of the reaction for a given amount of the total enzyme concentration; Km = the Michaelis-Menten constant; [S] = the substrate concentration, the linear form of the model is shown in Equation (3).

$$-\frac{1}{r_s} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\frac{1}{[S]} \quad (3)$$

The graph of $-\frac{1}{r_s}$ versus $\frac{1}{[S]}$ generates a straight line, which would have an intercept and slope that can be used to determine Vmax and Km, respectively.

The rate of the reaction was calculated from the substrate concentration using Equation (4) since the substrate concentration is low (K_m<< S), and the rate of the enzymatic reaction is assumed to be first order.

$$r_s = -\frac{d[S]}{dt} = k[S] \quad (4)$$

where [S] = the limiting reactant or substrate concentration [S]. The differential change $\frac{d[S]}{dt}$ can be determined using a numerical method (Schnell, 2014). This method is applied specifically when the data points of the independent variable are spaced equally, such as t_1-t_o = t_2-t_1= Δt. The three point-differential formulas were used in this work. The three-point differential formulae are presented in Equations (5) - (7):

Initial point:

$$\left(\frac{d[S]}{dt}\right)_{t_0} = \frac{-3[S]_0 + [S]_1 - [S]_2}{2\Delta t} \quad (5)$$

Interior point:

$$\left(\frac{d[S]}{dt}\right)_{t_1} = \frac{[S]_2 - [S]_0}{2\Delta t} \quad (6)$$

Last point:

$$\left(\frac{d[S]}{dt}\right)_{t_0} = \frac{[S]_3 + 4[S]_4 - 3[S]_5}{2\Delta t} \quad (7)$$

The rate of change of the amount of reacting species in terms of concentration $\frac{d[S]}{dt}$ with time was calculated using Equations (5) - (7). The A.O.A.C. (2005) method (Mc Cleary *et al*., 2002) was used to analyse the concentration of wild cocoyam starch. The approach involves the measurement of the mass of wild cocoyam starch.

Following the work of Jin *et al.* (2015), the changes in enthalpy of activation (ΔH), entropies of activation (ΔS), and Gibbs free energy (ΔG) of the enzymatic fermentation can be calculated using the following equations:

$$ln\frac{k}{T} = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (8)$$

$$\Delta G = \Delta H - T\Delta S \quad (9)$$

where k = Kmax = rate of constant, R is the universal gas constant (8.314 J/mol/K), while T is the Kelvin temperature (K). Also, Ea is the activation energy (J/mol), ΔG is the Gibbs free energy of activation (J/mol), ΔH is the enthalpy of activation (J/mol), and ΔS is the entropy of activation (J/mol */K).

2.8. Statistical Analysis

The enzymatic hydrolysis process data for glucose production were given to analysis of variance (ANOVA), and differences were considered statistically significant at $p<0.05$. The proposed model was evaluated using the Design-Expert® Software Version 12 (Stat-Ease Inc., Minneapolis, USA). The coefficients, validation of glucose production, kinetics, and thermodynamics of parameters have been reported accordingly.

3. Results

3.1. Characterization of the substrate and isolate

The wild ground cocoyam was characterized to establish the carbohydrate content. The wild ground cocoyam contains about 84% carbohydrate (data not shown). The *A. niger* streaked on the malt extract agar were subsequently identified. Microscopically, the layer of *A. niger* colonies appeared dense dark-brown with conidial blackheads, having a covering of white yellow like base, and seemed quite huge but roughly spherical, and yet, dark brown, somewhat flask-shaped projection supported on the partitioned metulae. The conidiophores appeared smooth-walled, quite translucent, or somewhat amor-phous towards the cell membrane. The quantity of amylase produced in the enzyme isolation by the process of solid-state fermentation is shown in Table 3. Six (6) strains of *A. niger* that generated numerous colourless zones on the medium of RBB-agar were randomly isola-ted. Among them, *A. niger* AMA05 was discovered as the best amylase that produced strain with a cleared zone of 9.0 mm (Table 3). The *A. niger* AMA05 obtained the lar-gest enzyme activity (1000 U/ml). Hence, it was selected for subsequent use in the starch hydrolysis to simple sugar.

Table 3. Amylase producing ability of A. niger strains

Total no. of isolates	SDS-PAGE pattern	Isolate code	Clear zones (mm)	Amylase activity (U/ml)
6	1	AMA05	9.0	1000
	2	AMA10	7.1	856
	3	AMA15	6.2	750
		AMA20	4.5	642
		AMA25	3.4	440
		AMA30	2.0	330

Key: AMA refers to the amylase isolate type used in this study.

Table 4: Coefficients in terms of coded factors

Factor	Coefficient Estimate	Df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	95.00	1	0.7033	93.50	96.50	
A-Temperature	-0.5208	1	0.3517	-1.27	0.2287	1.0000
B-Time	-0.4375	1	0.3517	-1.19	0.3121	1.0000
C-pH	1.60	1	0.3517	0.8463	2.35	1.0000
D-Enzyme dosage	2.55	1	0.3517	1.80	3.30	1.0000
AB	2.99	1	0.4307	2.08	3.91	1.0000
AC	-1.68	1	0.4307	-2.60	-0.7632	1.0000
AD	2.11	1	0.4307	1.19	3.02	1.0000
BC	-2.19	1	0.4307	-3.11	-1.28	1.0000
BD	0.5188	1	0.4307	-0.3993	1.44	1.0000
CD	-3.66	1	0.4307	-4.57	-2.74	1.0000
A^2	-1.47	1	0.3290	-2.18	-0.7728	1.05
B^2	-6.16	1	0.3290	-6.86	-5.46	1.05
C^2	-5.54	1	0.3290	-6.24	-4.84	1.05
D^2	-4.62	1	0.3290	-5.33	-3.92	1.05

Table 5. Analysis of variance (ANOVA) for enzymatic hydrolysis of wild cocoyam starch to produce glucose

Source	Sum of Squares	Df	Mean Square	F-value	p-value
Model	2718.59	14	194.18	65.42	< 0.0001
A-Temperature	6.51	1	6.51	2.19	0.0159
B-Time	4.59	1	4.59	1.55	0.0232
C-pH	61.12	1	61.12	20.59	0.0004
D-Enzyme dosage	155.55	1	155.55	52.41	< 0.0001
AB	143.40	1	143.40	48.31	< 0.0001
AC	45.23	1	45.23	15.24	0.0014
AD	70.98	1	70.98	23.91	0.0002
BC	77.00	1	77.00	25.94	0.0001
BD	4.31	1	4.31	1.45	0.2471
CD	213.89	1	213.89	72.06	< 0.0001
A^2	59.59	1	59.59	20.08	0.0004
B^2	1041.29	1	1041.29	350.83	< 0.0001
C^2	840.75	1	840.75	283.27	< 0.0001
D^2	586.45	1	586.45	197.59	< 0.0001
Residual	44.52	15	2.97		
Lack of Fit	32.52	10	3.25	1.36	0.3880
Pure Error	12.00	5	2.40		
Cor Total	2763.11	29			

3.2. Molecular weight of the amylase enzyme

The SDS PAGE of the synthesized enzymes from *A. niger* was shown in Figure 1. The molecular weight of amylase was ~ 75 KDa. The Lane M showing the molecular weight marker as well as the Lane P showing the purified amylase can be seen.

3.3. Statistical evaluation of glucose production

The coefficients of the generated empirical relationship between results of glucose produced (Y) and the four variables in coded values give optimum glucose yield as presented in Table 4. The responses changed with the unit value of the factor when other factors were constant. The general average response of the overall runs represented the intercept of the orthogonal design. The settings of the factors were used to adjust the coefficients. For an orthogonal element, variance inflation factors and V.I.F.s were equal to 1, while multi-collinearity had V.I.F.s more than 1. The ANOVA for enzymatic hydrolysis of wild cocoyam starch is shown in Table 5. A coefficient represented the effect of a factor with a single element. On the other hand, the coefficients with more than one factor represented the interaction between these factors. The F-value of the quadratic model terms is statistically significant (F= 65.42, p<0.0001). Further, all the linear terms A, B, C, and D and interaction term A*B, A*C, A*D, B*C, and C*D with all the quadratic terms A^2B^2,C^2 and D^2 equally statistically significant (p<0.05). After removing the insignificant terms except for the terms that supported the hierarchy, the final model was generated and presented in (Equation. 10).

$$\mathbf{Y = 95 - 0.521A - 0.438B + 1.60C + 2.55D + 2.99AB - 1.68AC + 2.11AD - 2.19BC - 3.66CD - 1.47}A^2 \mathbf{- 6.16}B^2 \mathbf{- 5.54}C^2 \mathbf{- 4.62}D^2 \quad (3)$$

The high regression coefficient (R^2 = 0.9839), and the adjusted R^2 (0.9688) were in agreement with the predicted R^2 (0.9260) value.

3.4. Multi-response and validation of glucose optimization

The multi-response surface and contour plots of tested variables towards glucose optimisation are shown in Figure 2. The interaction effect of time and temperature on glucose yield is shown in Figure 2a. Glucose yield increases with both time and temperature, as enzyme denatures (with higher temperature). The interaction effects of temperature and pH on glucose yield are shown in Figure 2b. The glucose yield increased with pH and temperature. The interaction effects of enzyme dosage and temperature on glucose yield are shown in Figure 2c. Glucose yield increased with enzyme dosage at temperature below 35°C. However, at a temperature above 35 °C, the glucose yield declines, probably because of enzyme denaturation. The interaction effects of pH and reaction time on glucose yield are presented in Figure 2d. Glucose yield increases with pH and reaction time. The interaction effect of pH and enzyme dosage on the yield of glucose is depicted in Figure 2e. Glucose yield increased with pH and enzyme dosage. The validation of the optimal values for glucose production is shown in Table 6. The experimental (optimal) yield of glucose production was 95 % at optimized temperature conditions of 35 °C, time of 5 days, pH of 5.5, enzyme dosage of 0.16 mg/mL, and the desirability of 0.9999 as against the 94 % glucose yield that would eventually become predicted by the model.

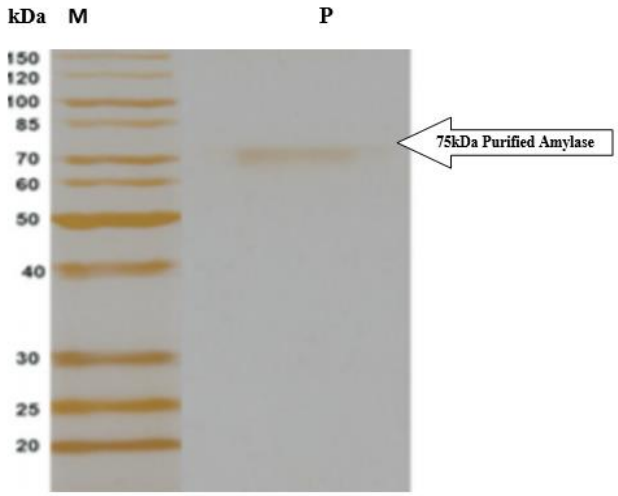


Figure 1: SDS PAGE (12%) for amylase enzyme synthesized from Aspergillus niger. Lane M: molecular weight marker; Lane P: Purified amylase.

3.5. FTIR and GC-MS analysis of glucose production

The FTIR of glucose produced via enzymatic hydrolysis is shown in Figure 3. Between the regions, 550/600 to 1500/1800 cm^{-1}, some peaks within the C=O and C-C groups could be seen. Additionally, between areas 2800/2900 and 3200 cm^{-1}, some peaks within the C-H bond of alkyl groups and O.H. groups involved in hydrogen bonds were seen. The GC-MS of the glucose produced from wild cocoyam is shown in Figure 4. The percentage purity of glucose produced was determined as 60 % for glucose and 20 % for fructose obtained at a retention time of 12 and 15 min, respectively. The remaining 20 % was most likely to be disaccharide (sucrose) and polysaccharide (unconverted starch).

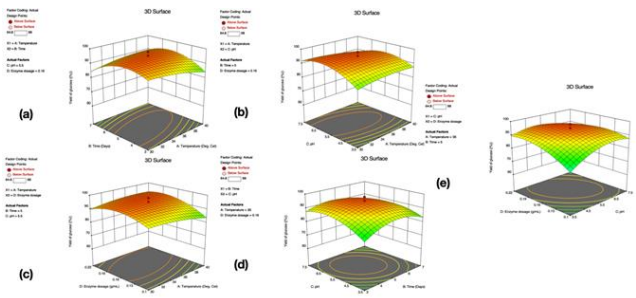


Figure 2: The surface and contour plots showing multi-responses of tested variables towards glucose optimisation. The surface and contour plots of: (a) time (days) and temperature (°C); (b) pH and temperature (°C); (c) enzyme dosage (g/mL) and temperature (°C); (d) pH and time (days); and (e) enzyme dosage (mg/mL) and pH.

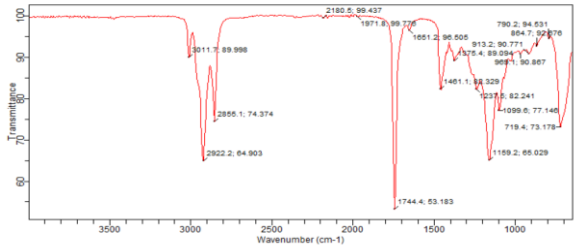


Figure 3: FTIR of the glucose produced from wild cocoyam via enzymatic hydrolysis.

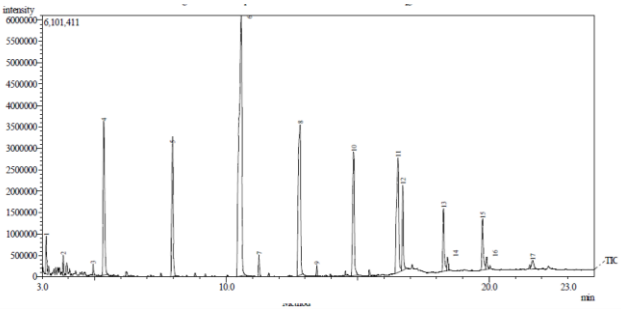


Figure 4: GCMS of the glucose produced from wild cocoyam via enzymatic hydrolysis.

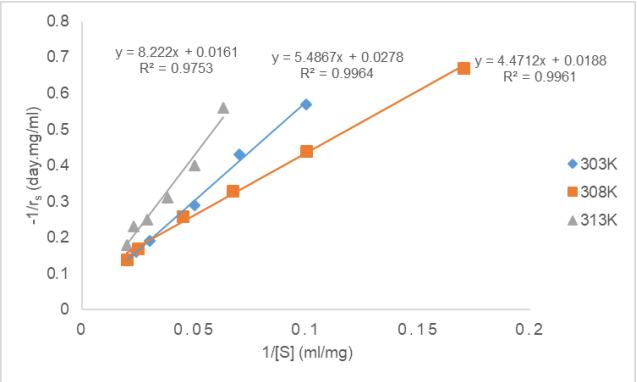


Figure 5: Kinetic plot showing fitting curves involving enzymatic hydrolysis at different temperatures.

3.6. Kinetics and thermodynamics of enzymatic hydrolysis

The kinetics and thermodynamics parameters for glucose production are shown in Table 7. The kinetic plot showing fitting curves involving enzymatic hydrolysis at different temperatures is shown in Figure 5. The experimental data have been fitted into the Michaelis-Menten Equation. The R^2 values of 0.9964, 0.9961, and 0.9753, which appeared close to unity, confirm that the data fitted well with the Michaelis-Menten Equation. From Table 7, the ΔG obtained increases with temperature, and Michaelis-Menten constant (Km), correspondingly.

4. Discussion

It is not a surprise that the wild ground cocoyam contained about 84% carbohydrate given starch comprises the main component (Lopulalan *et al*., 2020). Despite their nutritional role, carbohydrates remains the structural material that occupy a space among the members of cell membranes, and shown to participate in cellular recognition processes (Ruiz-Matute *et al*., 2011). After being streaked on the malt extract agar, the cell type and its features were of dense dark-brown with dense dark-brown with conidial blackheads, supported on the partitioned metulae, having a covering of white or yellow base with conidiophores that appeared smooth-walled, quite translucent, or somewhat amorphous towards the cell membrane. These features were all consistent of colonial morphology of *A. niger* (Mathew *et al*., 2016). It is important to reiterate that the *A. niger* AMA05 was considered the better amylase at this current study. This is because it had the largest enzyme activity (1000 U/ml), and therefore, was selected for subsequent use in the starch hydrolysis to simple sugar (Refer to Table 3).

The molecular weight of amylase (~ 75 KDa) (Refer to Figure 1) was not too far from those obtained by other workers that synthesized enzymes from *A. niger*. For instance, Sadhu *et al*. (2013) reported the molecular weight of 97 kDa for cellulase synthesized from *Bacillus* species, and obtained its refined band enzyme at 16 % gel formation. Iqbal *et al*. (2011) reported the molecular weight of 58 KDa for cellulase enzyme produced through *Trichoderma viride* using a 12 % separating gel. Spier *et al*. (2011) reported 108 kDa phytase from *A. niger FS3*, while Greiner *et al*. (2009) reported 85 kDa phytase from *A. niger 11T53A9*. More so, Imran *et al*. (2018) reported 71 kDa cellulose produced from *A. niger I.M.M.I.S*.

Table 6. Validation of the optimal values for glucose production

S/N	Responses	Temperature (oC)	Time	pH	Enzyme dosage (mg/mL)	Experimental Yield (%)	Predicted Yield (%)	% Error	Desirability
1	Yield of glucose	35	5days	5.5	0.16	95	94	0.071	0.9999

Table 7. Kinetics and thermodynamics parameters for glucose production

T(K)	K_m	V_{max} (mL/day)	ΔG (kJ/mol)
303	197.4	36.0	-1.36
308	237.9	53.2	-2.63
313	510.7	62.1	-3.90
Thermodynamics Parameters			
ΔH (kJ/mol)	75.6		
ΔS (kJ/mol.K)	0.254		

The statistical analysis of the responses from the design matrix was performed to optimize the process variables (temperature, time, pH, and enzyme dosage) (Refer to Tables 4 and 5). Predicting the yield of the glucose produced would be dependent on the significance of the interactions between the process parameters. From Table 4, the correlation of factors would go high with the V.I.F. Given this, the V.I.F.s that appear less than 10, would be considered as tolerable. Therefore, the V.I.F.s are less than 10 signals that the model would most likely be adequate to predict the glucose production. From Table 5, when the *p*-values get lower than 5% (i.e., α = 0.05: error or 95%: confidence), it was indicative of a significant model (Tripathi *et al*., 2009; Boudechiche *et al*., 2017). The statistically significant quadratic model terms corroborate the linear terms A, B, C, and D and interaction term A*B, A*C, A*D, B*C, and C*D with all the quadratic terms A^2B^2,C^2 and D^2 (p<0.05) (*Eqn 10*). The coefficient of variance (CV) was deduced as 2.13 %, which was less than 10 %, which suggests the model result could be reproduced.

The response surface, when combined with C.C.R.D., would optimize the factors as well as display the (response surface) orientations using three-dimensional (3D) response surface plots. This combination improved the understanding of the main factors and how the interactions provided a simple examination of the effects of independent variables on glucose production (Sivarajasekar and Baskar 2014; Boudechiche *et al*., 2017). The multi-response surface and contour plots of tested variables towards glucose optimisation (Refer to Figure 2) provided useful insights. For instance, the fact that glucose yield increases with pH and reaction time could probably be due to the adequate time provided for the hydrolysis of wild cocoyam starch. Possibly, a higher pH and reaction time might decrease the glucose yield, given by the quadratic terms (Refer to *Eq.* 10), where two factors showed a negative significant effect. Another instance is

where the glucose yield increased with pH and enzyme dosage. Probably, the enzyme present sufficed in converting the starch to glucose. The validation of the optimal values for glucose production (Refer to Table 6) was strengthened, given the error of the optimal results of the hydrolysis process, which was less than 2 % (0.0071). This demonstrated that the model was adequate in predicting the responses. It was important to reiterate that the yield of glucose produced has been optimized. Given that the optimized temperature conditions (of 35 °C, time of 5 days, pH of 5.5, enzyme dosage of 0.16 mg/mL), and the desirability of 0.9999 as against the 94 % glucose yield that would eventually become predicted by the model, the temperature, time, pH, and enzyme dosage would likely be constrained within the following ranges (30 – 40 ℃), (3 – 7 days), (3.5 – 7.5), and (0.1 - 0.22 mg/ml), respectively.

FTIR spectroscopy is among analytical tools used to qualitatively complement microscopic investigations (Ibrahim *et al*., 2011), which would help determine the specific changes in agro-food product compositions. In this current study, the qualitative characterization of glucose production was performed by FTIR spectroscopy. Through infrared light that excites the chemical bonds, the FTIR spectroscopy can identify organic materials. The different chemical bonds at this excited state can absorb the light energy at frequencies unique to the various bonds. The wavenumber of peak reflects the type of bond that is present (Refer to Figure 3). These characteristic bands appeared consistent with glucose (Ibrahim *et al*., 2006), consistent with those produced via enzymatic hydrolysis of cocoyam starch of this study. Moreover, the use of GC-MS continues to be among excellent facilities to analyze simple or complex mixtures. Given that carbohydrates occur as simple or complex mixtures as the current study showed percentage purity of glucose produced was determined as 60 % for glucose and 20 % for fructose (Refer to Figure 4), the use of chromatographic techniques becomes necessary for their determination (Ruiz-Matute *et al*., 2011).

By comparing Figure 5 and Table 7, the Michaelis-Menten constant increases with temperature. The R^2 values of 0.9964, 0.9961, and 0.9753, which appeared close to unity, confirm that the data fitted well with the Michaelis-Menten Equation. From Table 7, the ΔGobtained increases with temperature, and Michaelis-Menten constant (Km), correspondingly. However, the increases in ΔG with temperature, would not correspond to those of Vm. From Table 7, the maximum rate of the reaction appeared higher at a temperature of 35 °C. This probably might be the best reaction to take place at this specific temperature (of 35 °C) at this current study. Further, the thermodynamic parameters equally point to an enzymatic hydrolysis reaction, which took place during the glucose production at this current study, was likely endothermic, and spontaneous.

5. Conclusion

To a great degree, this current study provided useful evidence that wild cocoyam could be a suitable substrate in glucose production. Essentially, the microbial amylase enzyme (*A. niger*) was able to hydrolyze the starch. The wild ground cocoyam contains about 84% carbohydrate, with amylase molecular weight of ~ 75 KDa. Specifically, the optimum conditions by enzyme hydrolysis (temperature, 35 °C; time, 5 days, pH 5.5, and enzyme dosage, 0.16 g/ml) achieved a glucose yield of 95 %. Both FTIR and GC-MS analyses revealed characteristic bands of glucose with 60% percentage purity, which demonstrated some potential for industrial usage. Distinctive bands of glucose depicted functional groups of hydroxyl, aldehyde, ketone, and carbon-hydrogen bonds. The experimental data have been fitted into Michaelis-Menten kinetic model, showing outcome presented the enzymatic hydrolysis as conformable and hence, considered feasible. The direction of future studies should be to explore the optimisation of enzymatic fermented glucose production obtainable from starch of other unexplored tropical crops.

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Conflict of interest

The authors declare that there are no competing interests associated with this work.

Author contributions

N.N.U., C.N.U., and C.F.O. conceived and designed research. N.N.U., C.N.U., C.F.O., and O.M.M.N. conducted experiments, and contributed new reagents or analytical tools. N.N.U., C.N.U., C.F.O., and O.M.M.N. analysed data and prepared the initial draft of manuscript. W.A.R., C.U.O., S.M.H., and C.O.R.O. crosschecked/validated the analysed data, and revised the manuscript. All authors read and approved the manuscript.

Availability of data

The data that support the findings of this study are available from the corresponding authors, upon reasonable request.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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