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ORIGINAL ARTICLE

Response surface optimisation of enzymatic hydrolysis of cassava peels without chemical and hydrothermal pretreatment

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Abstract

Cassava peel is a feedstock of significant potential for bioprocessing into industrial products. Its economic utility has however not been explored despite its advantages over traditional first-generation biomass feedstock. We demonstrate in this study that cassava peel can be hydrolysed to produce glucose at very high efficiency without chemical or hydrothermal pretreatment. We evaluated the conversion efficiency of a one-step simultaneous hydrolysis of the peel with mixed enzymes. Response surface methodology was applied to optimise the hydrolysis condition for maximum glucose recovery. Glucose concentration was measured by HPLC-IR and polynomial regression models defining the process parameters fitted to predict the optimal setting of process variables for maximum glucose recovery. Maximum glucose recovery was predicted to occur at pH 4 and 54.75 °C with an enzyme mixture containing 10 FPU/g cellulase, 0.5 U/g β-glucosidase, 50 U/g amyloglucosidase, and 50 U/g α-amylase. Validation experiments confirmed that up to 95.48% glucose can be recovered from 0.06 g/ml of cassava peel in 43.15 h at these factor settings. Overall, the empirical models developed present an efficient tool for glucose recovery at high conversion efficiency. This model could be used for large-scale industrial production of glucose from cassava peel without the cost of pretreatment.

Keywords Optimisation · Hydrolysis · Cassava · Biomass · Glucose

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1 Introduction

Crop wastes are increasingly becoming important sources of industrial raw materials for the production of bioplastics, biofuels, and organic acids. The initial step required for the use of these wastes in bioprocess is the conversion of the biomass to monosaccharides by chemical or enzymatic methods or a combination of both. However, the efficiency of lignocellulosic biomass conversion to monosaccharide sugars has remain low, making bioprocessing of such feedstock uncompetitive, despite significant progress that has been made. The sustained interest in crop wastes as feedstock for bioprocessing is therefore due to its environmental and ecological sustainability [1–4]. Cassava peel is an abundant, crop-processing waste, produced in large quantities across many tropical countries of the world. Cassava (*Manihot esculanta* Cralztz) is the fourth most cultivated food crop in many tropical countries with the potential of becoming a major source of biomass for large-scale industrial processing. Cassava is one of the sturdiest perennial crops widely cultivated in West Africa for food. It grows in marginal lands with low nutrient and can withstand drought, heat, pests, and

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diseases. The production of cassava has increased substantially over the decades especially in Nigeria and Ghana, two of the world's topmost producer countries [5]. The production is projected to continue increasing especially in Ghana where deliberate intervention by state and non-state actors have spurred an accelerated increase in production for over a decade, rising from 9.57 million metric tons in 2005 to 22.45 million metric tons in 2019 [5, 6]. In spite of this encouraging trend, industrial processing of cassava is still very limited or almost non-existent. Nearly 100% of annual production is processed by traditional methods, which involves the removal of the peel along with significant amounts of the starch storage pith of parenchyma. It is estimated that up to 4.7 million tons of cassava processing waste was generated in Ghana in 2017 [5]. Currently, these wastes accumulate and become a source of short-term unsightly environmental pollutant, producing unpleasant odours because there are no proper disposal options [7]. Only a limited amount of the waste is used as supplement for animal feed due to its low protein and high cyanogenic glycoside content [8].

Many studies have reported on the carbohydrate composition of various cassava processing wastes such as the peels, pulp, and grate wastes, and the potential these hold as feedstock for bioprocessing [9–12]. The advantage of cassava peel as a second-generation feedstock for biorefineries stems from its abundant all year availability, its carbohydrate composition, and the fact that its use does not require any trade-offs between food and bioprocessing needs [1]. The peels are also easy to sun dry and processed for transportation or long-term storage as a production raw material. These notwithstanding, no major attempts have been made to commercialise the industrial fermentation of the peels, especially in the major cassava-producing countries. With a growing need for cheaper and sustainable sources of feedstock for future energy needs, the potential of cassava peels can no longer be ignored. Mainstreaming its economic utilisation, however, requires a cost-effective hydrolytic process to recover glucose from the peels.

Major barriers that have impeded the successful commercialisation of many cellulosic biomass conversion to fermentable sugars include high cost of the biomass production, cost of the hydrolytic enzymes, low yields, and limited stress tolerance [13]. The low yield which is mainly due to the recalcitrance of the biomass to enzymatic hydrolysis is overcome by subjecting the biomass to a pretreatment step to disassociate cellulose and hemicelluloses from lignin. However, this step is expensive and often yields by-products that substantially inhibit subsequent enzymatic and fermentation steps [14]. It is estimated that pretreatment accounts for about 35% of the total reducing sugar production cost [15, 16]. Among the various pretreatment methods, the hydrothermal and biological pretreatment methods seem to be the most desired technologies for the future. While the biological pretreatment offers an attractive low-cost option with the advantage of being eco-friendly, suppressed by-product formation, and simple to operate, its strict microbial growth condition requirements and the long process time remain a major drawback to the commercialisation of the method [17]. Similarly, hydrothermal pretreatment techniques have significant drawbacks. It is fraught with moderate to high water and energy costs [18, 19], formation of by-products that may limit subsequent fermentation processes, low recovery of sugars from hemicellulose, and requirement for higher enzyme loading for optimal recovery of glucose from the pretreated biomass [20, 21]. Thus, notwithstanding significant progress made [17], limitations of pretreatment methods remain an important hindrance to mainstreaming the economic use of most lignocellulosic biomass. Albeit at low recovery efficiency, Kongkiattikajorn and Sornvoraweat demonstrated that enzymatic hydrolysis of cassava peels without chemical pretreatment could yield glucose at recovery efficiency comparable to 64.5–72.9% recovery following alkali or acid pretreatments [22]. Cassava peels, compared to most second-generation feedstock [13], are very low in lignin content [9–12] and could be enzymatically hydrolysed to glucose at higher conversion efficiency following mechanical size reduction.

Enzymatic hydrolysis of lignocellulosic biomass often requires a cocktail of amylolytic and cellulolytic enzymes in a one-step simultaneous hydrolysis of the substrate [12]. However, the activity of different enzymes is affected differently by factors such as pH and temperature, enzyme–substrate ratio, and solid-water ratios, among others [23–27]. Thus, in a complex multi-enzymatic process such as lignocellulosic biomass hydrolysis, process optimisation is necessary to minimise the effect of interactions between the enzymes and the process variables [28–30]. The face-centred central composite design (CCD) statistical optimisation method is one of the response surface methodologies (RSM), which take into consideration the interaction between process variables in generating process responses. This methodology has been used in several process improvements for the simultaneous optimisation of multiple process variables, based on the fundamental principles of randomisation, replication, and duplication to predict the best performance conditions with a minimum number of experiments [31, 32]. The technique has been widely applied in chemical and biochemical processes to analyse the effect of independent variables on response variables and in the optimisation of the process responses using appropriate values of the independent variables [33].

In this study, we established the optimum conditions for a one-step mixed enzyme hydrolysis of cassava peel with the aim of minimising input variables while maximising glucose recovery, as a preliminary step in a bioproduction process. We conclude that peels from traditionally processed cassava

can be hydrolysed to produce fermentable sugars without chemical or hydrothermal pretreatment.

2 Materials and methods

2.1 Enzymes

Enzymes were purchased from Sigma-Aldrich. Cellulase was obtained from *Trichoderma reesei* (≥ 700 U/g), β-glucosidase (10–30 U/mg), and amyloglucosidase from *Aspergillus niger* (≥ 300 U/ml). The α-amylase was from *Aspergillus oryzae* (30 U/mg) and xylanase from *Trichoderma viride* (100–300 U/mg).

2.2 Preparation of the feedstock

The cassava peel waste was sampled from a small-scale cassava processing facility at Bawjiase in the Central Region of Ghana, where about 5,400 tons of cassava peel is generated per year [34]. The peels were soaked in water at room temperature (about 28 °C) for 30 min and the outer periderm of dead cork cells (brown skin, bark) sloughed off by hand. The remaining thin cortical sclerenchyma and pith of parenchyma cells (flesh) containing starch were then dried in a hot air oven at 60 °C overnight. The dried peels were milled to 0.25 mm particle size in a hammer mill and stored in airtight bottles till ready to use. The milled samples were analysed for the ash and dry matter content, lignin, and carbohydrate composition as previously described [35].

2.3 Characterisation of the feedstock

Dry matter content was determined by drying the milled samples to a constant weight in a hot air oven at 105 °C overnight. Ash content was determined by weighing the sample before and after ashing at 550 °C for 2 h in a muffle furnace. The reducing sugar content of the milled sample was determined according to the protocol A0003 from Enzyme Lab of DTI (Denmark) as previously described by Saeman et al. [36]. In brief, the milled sample was hydrolysed into monomeric sugars by a two-step acid hydrolysis. The primary hydrolysis was performed by first suspending 0.16 g of the samples in 1.5 ml of 72% (w/w) H_2SO_4 at 30 °C for 60 min with intermittent stirring. This was followed by 4% (w/w) H_2SO_4 dilute acid hydrolysis in an autoclave at 121 °C for 60 min. The hydrolysate was analysed for monosaccharide composition using an HPLC equipped with an RI detector and an Aminex HPX-87H column (Bio-Rad Laboratories Ltd., USA). The column temperature was maintained at 63 °C with 4 mM H_2SO_4 used as the mobile phase at a flow rate of 0.6 ml/min. Klason lignin content was determined as the ash-free residue remaining after the two-step hydrolysis.

Total starch content was determined using a Megazyme starch assay kit according to the manufacturer's instructions. In principle, a thermostable α-amylase, amyloglucosidase, glucose oxidase, and peroxidase catalyse the conversion of starch to quinoneimine [37, 38], and the absorbance was measured at 510 nm using a UV–VIS Spectrophotometer (Shimadzu UV Min 1240). Cellulose was analysed using the method described by Updegraff [39] as modified in Kulic and Radojicic [40]. The milled sample was hydrolysed to sugars with cellulase (50 FPU/g) and β-glucosidase (2 U/mg) at 50 °C in 0.1 M acetate buffer (pH 5.0), while hemicellulose samples were hydrolysed at pH 4.5 with 20 U/g xylanase at 30 °C. The hydrolysis was run for 48 h in an Enviro Geni incubator (Scientific Industries Inc.) rocking at 30 rpm in a vertical position. To prevent microbial oxidation of glucose, the hydrolysate was amended with 150 µg/ml ampicillin (Sigma-Aldrich, Co. LLC). The hydrolysate was analysed for glucose concentration using ultimate 3000 HPLC system (Dionex, Sunnyvale, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko KK, Tokyo, Japan). The column oven temperature was set to 60 °C, and the mobile phase consisted of 5 mM H_2SO_4 with a flow rate of 0.5 ml/min. The cellulose composition of the sample was calculated according to the method of Ververis et al. [41] following the equation:

$$\%Cellulose = (0.9/0.96) \times C \times (\mathrm{V/M}) \times K \times 100 \quad (1)$$

where 0.9 is the coefficient that results from the molecular weight ratio of the cellulose polymer and the hexose monomer, 0.96 is the saccharification yield, C is the glucose concentration (g/l), V is the total volume of sugar solution (l), M is the dry weight of the cassava peel sample (g), and K is the dilution factor of the sample (if any). Hemicellulose content was estimated from the equation:

$$\%Hemicellulose = (0.88/0.93) \times C \times (V/M) \times K \times 100 \quad (2)$$

where the coefficient 0.88 is the ratio of molecular weights of the hemicellulose polymer and the monomer pentose, the coefficient 0.93 is the saccharification yield of xylane to xylose, C is the xylose concentration (g/l), V is the total volume of sugar solution (l), M is the dry weight of the cassava peel sample (g), and K is the dilution factor of the sample.

2.4 Enzymatic hydrolysis of cassava peel

Milled cassava peel samples (0.2–1.0 g) were weighed into 10 ml of 0.1 M acetate buffer (pH 5.0) prepared in Milli-Q water to obtain different substrate water ratios. To prevent biological oxidation of the sugar by microbial contaminants during the hydrolysis, 150 µg/ml ampicillin (Sigma-Aldrich, Co. LLC) was added, and the pH adjusted as desired. The

required enzyme (0.5–50) U/g was then added to the samples in the buffered solution (pH 4–6). The sample was incubated for 0–48 h at a temperature from 40 to 60 °C in a vertical rocking position at the speed of 30 cycles per minute in Environ Geni incubator (Scientific Industries Inc.). Glucose concentration in the hydrolysate was determined by ultimate 3000 HPLC system as described above.

The efficiency of the enzymatic hydrolysis (percentage glucose recovery (*%GR*)) was calculated following the equation:

$$\%GR = \frac{\text{glucose in hydrolysate} \times \text{volume of hydrolysate} \times 100\%}{\text{mass of sample} \times \text{dry matter content of the sample} \times \text{glucose} \times \%\,\text{dry matter}} \quad (3)$$

Table 1 Process variables in coded and actual units for mixed enzyme hydrolysis

Variables	Units	Level		
		Low	Medium	High
Coded units		−1	0	+1
Cellulase concentration (X_1)	FPU/g	10	30	50
β-Glucosidase concentration (X_2)	U/g	0.50	1.25	2.00
Amyloglucosidase concentration (X_3)	U/g	10	30	50
α-Amylase concentration (X_4)	U/g	10	30	50
Temperature (X_5)	°C	40	50	60
pH (X_6)		4	5	6

2.5 Optimisation of hydrolysis by response surface methodology

The optimisation of glucose recovery was performed using the face-centred CCD of the response surface methodology. The study was carried out in three stages. In the first instance, three factors, substrate water ratio, time of hydrolysis, and enzyme concentration, were studied to define the region of operability and the significance of substrate water ratio and hydrolysis time in each of the enzyme hydrolysis. The four enzymes were cellulase (X_1), β-glucosidase (X_2), amyloglucosidase (X_3), and α-amylase (X_4). The conditions for hydrolysis were determined by varying the enzyme concentration from 10 to 50 U/g, substrate water ratio (Z) from 0.02 to 0.10 g/ml, and hydrolysis time (T) from 0 to 48 h (Online Resource: Table S1). For β-glucosidase, the range was varied from 0.5 to 2.0 U/g, while substrate water ratio was varied from 0.06 to 0.20 g/ml for hydrolysis with amyloglucosidase. The ranges for the process variables were set after preliminary screening tests. The medium pH and temperature for each hydrolysis were maintained at the manufacturer's recommended conditions. For cellulase (X_1), the medium pH and temperature were maintained at 5 and 50 °C, respectively, while hydrolysis with β-glucosidase (X_2) was performed at pH 5 and 45 °C; amyloglucosidase (X_3)-catalysed hydrolysis was performed at pH 4.5 and 55 °C, and hydrolysis with α-amylase (X_4) was done at pH 6 and temperature of 40 °C.

The values of the factors (k) were coded to be − 1 and + 1 for factorial points which allow for the estimation of all the main effects and the interactions between variables. The experimental design had the recommended 20 tests with six tests at the centre point in cube and 0 in axial, eight points in the corners of the cube representing the experimental domain, and six axial points in the centre of each face of the cube. The distance of each axial point from the centre, α, was set at 1. A CCD matrix for each of the four enzymes was generated and glucose recovery at each factor setting recorded (Online Resource: Tables S2–S5).

In the second stage, the four enzymes were combined in a simultaneous one-step mixed enzyme hydrolysis of the substrate. The face-centred CCD was applied to optimise the six process factors ($k = 6$), namely temperature (X_5), pH (X_6), and the concentration of each of the four enzymes in the mixed enzyme cocktail (Table 1). Cellulase was coded X_1 with β-glucosidase and amyloglucosidase and α-amylase coded as X_2, X_3, and X_4, respectively. The substrate water ratio and hydrolysis time were held constant at 0.06 g/ml and 24 h, respectively. Each of the factors were coded at three levels as low (− 1), medium (0), and high (+ 1). The coded and actual values of the independent process factors are shown in Table 1. The recommended number of tests ($N = 90$) including number of centre points (C) was determined using Minitab® v17 response surface design tool, following the equation:

$$N = 2^k + 2k + C \quad (4)$$

where:

N = number of tests

k = the number of design factors

2^k = points in the corners of the cube representing the experimental domain.

2 k = axial points in the centre of each face of the cube [(± α, 0, …0), (0, ± α, ...0), … (0, 0, … ± α)]

C = the replicates in the centre of the cube that are necessary to estimate the variability of the experimental measurements [42]

The 90 tests are comprised of 64 test points in the corners of the cube and 12 axial points in the centre of each face of the cube. To allow for the determination of all the quadratic terms, the centre points (C), which provide a check of adequacy of the model prediction, were coded to be 14 in cube and 0 in axial. The distance of each axial point from the centre (α) was set at 1. (The test results are presented in Online Resource: Table S6.)

Table 2 Chemical composition of the feedstock

Composition	% dry matter	Reducing sugars	% total carbohydrate
Starch	57.16 ± 3.19	Glucose	94.52 ± 0.82%
Cellulose	6 ± 0.02	Xylose	2.54 ± 0.08
Hemicellulose	2.23 ± 0.02	Arabinose	2.53 ± 0.08
Lignin	1.92 ± 0.07		
Ash	6.3 ± 0.34		

2.6 Statistical analysis and the model fitting

The test results (Y) were fitted with a second-order polynomial by a multiple regression technique, following a stepwise selection of terms to fit a hierarchical model in uncoded units. The quadratic model for predicting the optimal settings for the independent process factors was expressed as

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad i = 1, 2, \ldots k, j = 1, 2, \ldots k; i \neq j \tag{5}$$

where Y is the predicted response (% glucose recovery) and X_i and X_j are the independent process variables. β_0 represents the interception coefficient, with β_i, β_{ii}, and β_{ij} representing the regression coefficients of the linear, quadratic, and interaction terms, respectively.

The alpha to include or exclude predictor variables from the reduced model (based on the p-value of t-tests for the slope parameters) was set at 0.15. All insignificant lower-order terms that contribute to a significant higher-order term were included in the final reduced model. The coefficients of the terms were calculated, and the significance of the effects was analysed using analysis of variance (ANOVA, $p < 0.05$). The factors or model terms are considered significant when the p-value is less than 0.05. The significance of the regression coefficients was determined by the t-test. The adequacy of the fitted model was determined by the adjusted coefficient of determination (R^2_{adj}) and the coefficient of prediction (R^2_{pred}) used to determine how well the model predicts the response for new observations. The model was considered adequate when its p-value < 0.05, lack of fit p-value > 0.05, and $R^2_{adj} > 0.9$.

Three-dimensional (3D) response surface plots were drawn to illustrate the effects of the independent variables on glucose recovery as described by the model fitted to the experimental data. The optimum settings of the significant factors affecting glucose recovery were determined by the canonical analysis of the ridge maximum, using the response optimiser tool of Minitab v17. The predicted optimal concentration of each of the four enzymes, pH, and temperature were then used in the third stage to verify the optimal conditions for maximum glucose recovery model over 0–48 h of hydrolysis time and 0.06–0.20 g/ml substrate water ratio range. The face-centred CCD methodology was applied in the verification experiment with hydrolysis time and substrate water ratio as process factors ($k = 2$). A total of 13 experimental runs comprising of 4 corner points, 4 points in the centre of each face of the cube, and 5 centre points were set up in a single block. The data were fitted to a regression model and adequacy of the model determined as before. Maximum glucose recovery was predicted, and validation experiments were performed in triplicates at the predicted settings.

3 Results and discussion

3.1 Characterisation of the feedstock

The substrate for hydrolysis, cassava peel, was first analysed to determine the chemical and physical composition of the biomass. The dry matter content of the milled biomass was determined to be 89.70 ± 0.06% of which 6.30 ± 0.34% was the ash content (Table 2). The total reducing sugar content of the biomass was 94.52 ± 0.82% glucans, with the pentose sugars, xylose and arabinose, constituting 2.54 ± 0.08% and 2.53 ± 0.08%, respectively. Lignin content was 1.92 ± 0.07% of dry matter. The starch content of the biomass was 57.16 ± 3.19%, with cellulose and hemicellulose being 2.23 ± 0.02% and 6.00 ± 0.02%, respectively, making this cassava peel waste a very rich source of fermentable sugars.

The ash content of a biomass is an important consideration in the choice of a lignocellulose substrate for industrial processing [43]. Ash constitutes part of the mineral residue that is left in a reaction vessel after hydrolysis of the biomass. High ash content can affect the buffering capacity of the medium and cause operational problems such as the wear of blades of the size reduction unit [44], fouling of heat exchangers, and slagging in the bottom of the furnace. These affect the smooth operations of the production units due to regular shutdown, contributing to reduction in operating time, as well as increasing the cost of maintenance. Ash composition of cassava biomass varies widely based on location and variety [45]. The ash content recorded in this study is higher than the 1–3% of dry matter that had previously been reported for cassava peels [46, 47] and stem cuttings [48]. However, an ash content of 6.3% remains relatively low in comparison to most lignocellulose biomass

currently in use for biofuel production [13]. In their review, Adekunle et al.reported 7% ash content for the peel of ADP 3(4), a sweet cassava variety [49].

The 1.92 ± 0.07% lignin content of the peel biomass was however very low compared with 8.4–33.8% previously recorded for peels without the starch storage pith [9, 22, 50], the pulp [51], the stem [48], and some grasses [1]%. Similarly, hemicellulose content was only 6.00 ± 0.02%, compared with 24.3% reported for the stem [48] and 27% for peels without the starch storage pith [49] and 4.6% for the pulp [51]. Lignin and hemicelluloses, which are intimately associated with cellulose fibrils in the lignocellulosic matrix, are major factors contributing to the biomass recalcitrance to enzymatic digestion. Lignin reduces the effectiveness of enzymatic hydrolysis by limiting enzyme access to glycosidic bonds [52, 53], requiring pretreatment to open the structure and make carbohydrate more accessible to enzymes [54]. In addition, adsorption of cellulases onto lignin increases the amount of cellulase required for hydrolysis of high lignin content biomass [55–57]. The low levels of lignin and hemicellulose content of cassava peel make it an ideal low-cost substrate for enzymatic digestion without pretreatment. It has been estimated that pretreatment alone contributes 18–20% of the total production cost of bioalcohols from lignocellulose biomass [58, 59]. Hemicellulose which is a heterogenous carbohydrate is mainly made up of pentose and hexose sugars. The low hemicellulose content of the peel explains why the substrate could be digested to completion without xylanases and xyloglucanase [60, 61] and the low pentose sugar content of the cassava peel. These underscore the importance of cassava peel as an ideal substrate for bioprocess because, although pentose sugars, such as xylose, can be fermented to ethanol by many bacteria and yeasts, the slow conversion rate and by-product formation limits its economic suitability for ethanol production [62] and has remained a key problem in cellulolytic substrate ethanol fermentation. Equally, toxic by-products resulting from harsh chemical and physical pretreatment conditions, which hinder the viability of fermenting microbes to convert the sugar to the downstream bioprocess products, will be minimal in cassava peel hydrolysates.

3.2 Single enzyme hydrolysis of the feedstock

As a result of the carbohydrate composition, four hydrolytic enzymes (cellulase, β-glucosidase, α-amylase, and amyloglucosidase) targeting β-(1 → 4) glycosidic linkages in cellulose and α-(1 → 4) and α-(1 → 6) linkages in starch were selected for hydrolysis of cassava peel. In the single enzyme hydrolysis, glucose recovery was insignificant except in the hydrolysis with amyloglucosidase (Online Resource: Tables S2–S5). The maximum glucose recovery recorded for hydrolysis with cellulase was 6.9%, while hydrolysis with β-glucosidase and α-amylase yielded only 5.5% and 4.4% glucose, respectively. The substrate water ratio and time of hydrolysis were significant ($p < 0.00$) in all single enzyme hydrolysis except for β-glucosidase (Online Resources: Tables S7 (A–D)). Glucose recovery was generally low except in the hydrolysis with amyloglucosidase. This was anticipated because the individual enzymes have low capacity in recovery of glucose from complex polysaccharide matrixes in isolation. Glucose recovery following hydrolysis with either cellulase or β-glucosidase were particularly low but not surprising given that less than 10% w/w of the substrate composition was made up of cellulolytic material. It is also recognised that cellulases do not significantly catalyse the hydrolysis of cellulolytic substrates to produce monomeric glucose units, which is the measured response variable. In addition, cellobiose, the disaccharide product of cellulase hydrolysis, exerts an inhibitory action on exo- and endoglucanases [63, 64], thereby limiting the activity of the enzyme. Thus, the maximum 6.9% glucose concentration recorded in cellulase-catalysed hydrolysis of the cassava peel cannot be assumed to account for the total activity of the enzyme on the cellulolytic content of the peel. On the other hand, β-glucosidase which catalyses the hydrolysis of β-1,4 linkages of the terminal non-reducing residues in oligosaccharides or a cellulose polymer produces glucose. However, the rate of glucose recovery is low, and it is postulated to be due to the number of non-reducing cellulose chain ends that are available to be acted upon by the enzyme [65–67].

Unlike cellulose, which forms structural components of the cell wall and resistant to hydrolysis, starch is a temporary glucose storage, designed to be quickly and easily mobilised by amylases and amyloglucosidases. α-Amylases catalyse random hydrolysis of α-1,4-glycosidic linkages, yielding a mixture of dextrin, oligosaccharides, and a limited amount of glucose. Its capacity to yield high concentration of glucose from cassava peel is therefore limited. On the other hand, amyloglucosidase, which yielded the highest amount of glucose, successively catalyses the hydrolysis of terminal α-1,4- and α-1,6-glycosidic linkages from the non-reducing ends of oligo- or polysaccharides, with the release of glucose residues. Hydrolysis with amyloglucosidase resulted in the recovery of up to 41.5% in 48-h long hydrolysis (Online Resource: Table S4). The hydrolysis time had the most significant effect on the process. The high recovery is due to the ability of the enzyme to act directly on starch granules, hydrolysing both terminal α-(1 → 4) and α-(1 → 6) linkages, removing limit dextrin, and allowing starch digestion to go to completion [68]. Given that the substrate is 57% starch, the high glucose recovery from the peel with amyloglucosidase alone was not surprising. The high amyloglucosidase activity notwithstanding, the need for simultaneous hydrolysis of the substrate with the amylolytic enzymes, α-amylase and amyloglucosidase, and the cellulolytic enzymes for

Table 3 Analysis of variance for % glucose recovery in mixed enzyme reaction

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	*F*-value	*p*-value
Model	*13*	*49,980.1*	*94.83%*	*49,980.1*	*3844.6*	*107.32*	*0.000*
Linear	6	46,043.5	87.36%	46,043.5	7673.9	214.21	0.000
Cellulase	1	1.2	0.00%	1.2	1.2	0.03	0.854
β-Glucosidase	1	143.5	0.27%	143.5	143.5	4.01	0.049
Amyloglucosidase	1	263.5	0.50%	263.5	263.5	7.36	0.008
α-Amylase	1	162.1	0.31%	162.1	162.1	4.52	0.037
Temperature	1	1762.0	3.34%	1762.0	1762.0	49.19	0.000
pH	1	43,711.2	82.94%	43,711.2	43,711.2	1220.18	0.000
Square	1	2360.2	4.48%	2360.2	2360.2	65.88	0.000
Temperature*Temperature	1	2360.2	4.48%	2360.2	2360.2	65.88	0.000
2-Way interaction	6	1576.4	2.99%	1576.4	262.7	7.33	0.000
Cellulase*Temperature	1	75.6	0.14%	75.6	75.6	2.11	0.150
β-Glucosidase*α-Amylase	1	225.9	0.43%	225.9	225.9	6.31	0.014
β-Glucosidase*pH	1	159.9	0.30%	159.9	159.9	4.46	0.038
Amyloglucosidase*pH	1	253.3	0.48%	253.3	253.3	7.07	0.010
α-Amylase*Temperature	1	136.6	0.26%	136.6	136.6	3.81	0.055
Temperature*pH	1	725.0	1.38%	725.0	725.0	20.24	0.000
Error	*76*	*2722.6*	*5.17%*	*2722.6*	*35.8*		
Lack of fit	63	2325.4	4.41%	2325.4	36.9	1.21	0.370
Pure error	13	397.2	0.75%	397.2	30.6		
Total	*89*	*52,702.7*	*100.00%*				

maximum glucose recovery from the peel remained obvious. Virunanon et al. [12] have shown that simultaneous liquefaction and saccharification of cassava pulp and wastewater with α-amylase, glucoamylase and β-glucanase mixed enzymes at a single incubation temperature of 50 °C recovers more reducing sugar than in a step-by-step sequential hydrolysis with the same enzymes.

3.3 Optimisation of mixed enzyme hydrolysis by response surface methodology

To optimise the recovery of glucose from the substrate, all four enzymes were combined in a one-step mixed enzyme simultaneous hydrolysis. This resulted in a dramatic increase in glucose recovery compared with the low recoveries obtained with single enzyme-catalysed hydrolysis (Online Resource: Tables S2–S5). In the test results, % glucose recovery was calculated and recorded (data is presented in Online Resource: Table S6). The highest glucose recovery (97.96%) occurred at pH 4 and 60 °C incubation temperature. The concentrations of cellulolytic enzymes, cellulase and β-glucosidase, for the highest recovery were at their minimum concentrations (10 FPU/g and 0.5 U/g, respectively) in the mixture, while the amylolytic enzymes (α-amylase and amyloglucosidase) were at their highest concentration of 50 U/g each (Online Resource: Table S6—RunOrder 5). The lowest recovery of 3.01% occurred at 40 °C and pH 6 with the concentration of the cellulolytic enzymes set at their highest (cellulase at 50 FPU/g and β-glucosidase at 2.0 U/g) (Online Resource: Table S6—RunOrder 56).

3.4 Statistical analysis and model fitting

The % glucose recovery (GR%) were fitted with a second-order polynomial, following a stepwise selection of significant terms to fit a hierarchical model in uncoded units (Eq. 6). Analysis of variance (ANOVA) was used to analyse the model for significance and suitability. The results of ANOVA, goodness-of-fit, and the adequacy of the reduced model are summarised in Tables 3 and 4, after removing (see Online Resource: Table S8 for full model terms). The *t*-test and *p*-values were used to identify the effect of each factor on GR%. The analysis of the *p*-value of the factors suggests that all the linear terms, except cellulase concentration, had a significant effect ($p < 0.05$) on glucose recovery. The small *p*-value for the quadratic and the interaction terms ($p < 0.00$) suggests that there is curvature in the response surface and interactions between the factors were significant. A significant interaction between terms means that the relationship between a factor and the response depends on the other factors in the term. The most remarkable effects were the linear term of pH ($p < 0.00$), which accounts for 82.94% of the variation in the response variable, with the quadratic term of temperature ($p < 0.00$), followed by the linear term of

Table 4 Estimated regression coefficients (in coded units) of the second-order polynomial model for glucose recovery in mixed enzyme hydrolysis

Term	Effect	Coef[a]	SE Coef	*T*-value	*p*-value[b]
Constant		43.660	1.220	35.73	0.000
Cellulase	0.271	0.136	0.737	0.18	0.854
β-Glucosidase	− 2.949	− 1.475	0.737	− 2.00	0.049
Amyloglucosidase	3.996	1.998	0.737	2.71	0.008
α-Amylase	3.134	1.567	0.737	2.13	0.037
Temperature	10.334	5.167	0.737	7.01	0.000
pH	− 51.470	− 25.735	0.737	− 34.93	0.000
Temperature*Temperature	− 23.160	− 11.580	1.430	− 8.12	0.000
Cellulase*Temperature	− 2.174	− 1.087	0.748	− 1.45	0.150
β-Glucosidase*α-Amylase	− 3.758	− 1.879	0.748	− 2.51	0.014
β-Glucosidase*pH	3.162	1.581	0.748	2.11	0.038
Amyloglucosidase*pH	− 3.979	− 1.990	0.748	− 2.66	0.010
α-Amylase*Temperature	2.922	1.461	0.748	1.95	0.055
Temperature*pH	− 6.732	− 3.366	0.748	− 4.50	0.000

[a]The coefficient describes the size and direction of the relationship between a term in the model and the response variable. To minimise multicollinearity among the terms, the coefficients are all in coded units. The coefficient for a term represents the change in the mean response associated with an increase of one coded unit in that term, while the other terms are held constant. The sign of the coefficient indicates the direction of the relationship between the term and the response

[b]If the *p*-value is less than or equal to the significance level, it can be concluded that there is a statistically significant association between the response variable and the term:

Linear terms

If the coefficient for a linear term is statistically significant, it can be concluded that the coefficient does not equal to 0.

Quadratic terms

If the coefficient for a quadratic term is statistically significant, it can be concluded that the response surface contains curvature

Interactions among factors

If the coefficient for an interaction is statistically significant, it can be concluded that the relationship between a factor and the response depends on the other factors in the term

temperature ($p < 0.00$) and the interaction term of temperature and pH ($p < 0.00$) contributing 4.48, 3.34, and 1.38%, respectively. Although significant, the contribution of the enzyme concentration in the mixture to glucose recovery was not as dramatic as the effects of pH and temperature. Similarly, the significant interactions between the enzymes, β-glucosidase and α-amylase ($p = 0.014$), and between pH and β-glucosidase or amyloglucosidase had less remarkable effect on glucose recovery compared with the effects of pH and temperature (Table 4). In a substrate unlimited enzyme assay, reaction rate will normally be expected to increase as enzyme concentration is increased unless significant interactions occur between enzyme and substrate or products. β-Glucosidase exerted a negative effect (Coef = − 1.475; $p = 0.049$) in the mixed enzyme hydrolysis. This negative effect was most significant in the interacting with α-amylase (Coef = − 1.879; $p = 0.014$), lowering glucose recovery at increasing concentrations of β-glucosidase in the enzyme mix. Although the analysis of the linear terms showed that cellulase concentration was not significant ($p > 0.05$), it was added to the model, considering that the substrate contained 6% cellulose, and the interaction terms, cellulase and temperature, met the criteria ($p = 0.150$) to be added to the hierarchical model.

$$\begin{aligned} GR(\%) = & -233.9 + 0.279\,Cellulase - 8.75\,\beta\text{-}Glucosidase \\ & + 0.597\,Amyloglucosidase - 0.130\,\alpha\text{-}Amylase \\ & + 13.72\,Temperature - 8.56\,pH - 0.1158\,Temperature^2 - 0.00544\,Cellulase \\ & \times Temperature - 0.1253\,\beta\text{-}Glucosidase \times \alpha\text{-}Amylase + 2.108\,\beta \\ & \text{-}Glucosidase \times pH - 0.0995\,Amyloglucosidase \\ & \times pH + 0.00730\,\alpha\text{-}Amylase \times Temperature \\ & - 0.3366\,Temperature \times pH \end{aligned} \quad (6)$$

The parameters of the equation were obtained by multiple regression analysis of the test results. The coefficient of regression (R^2) and adjusted regression coefficient (R^2_{adj}), which determined the adequacy of the regression model to describe the response surface, were respectively 94.83% and 93.95%. The adequacy of the

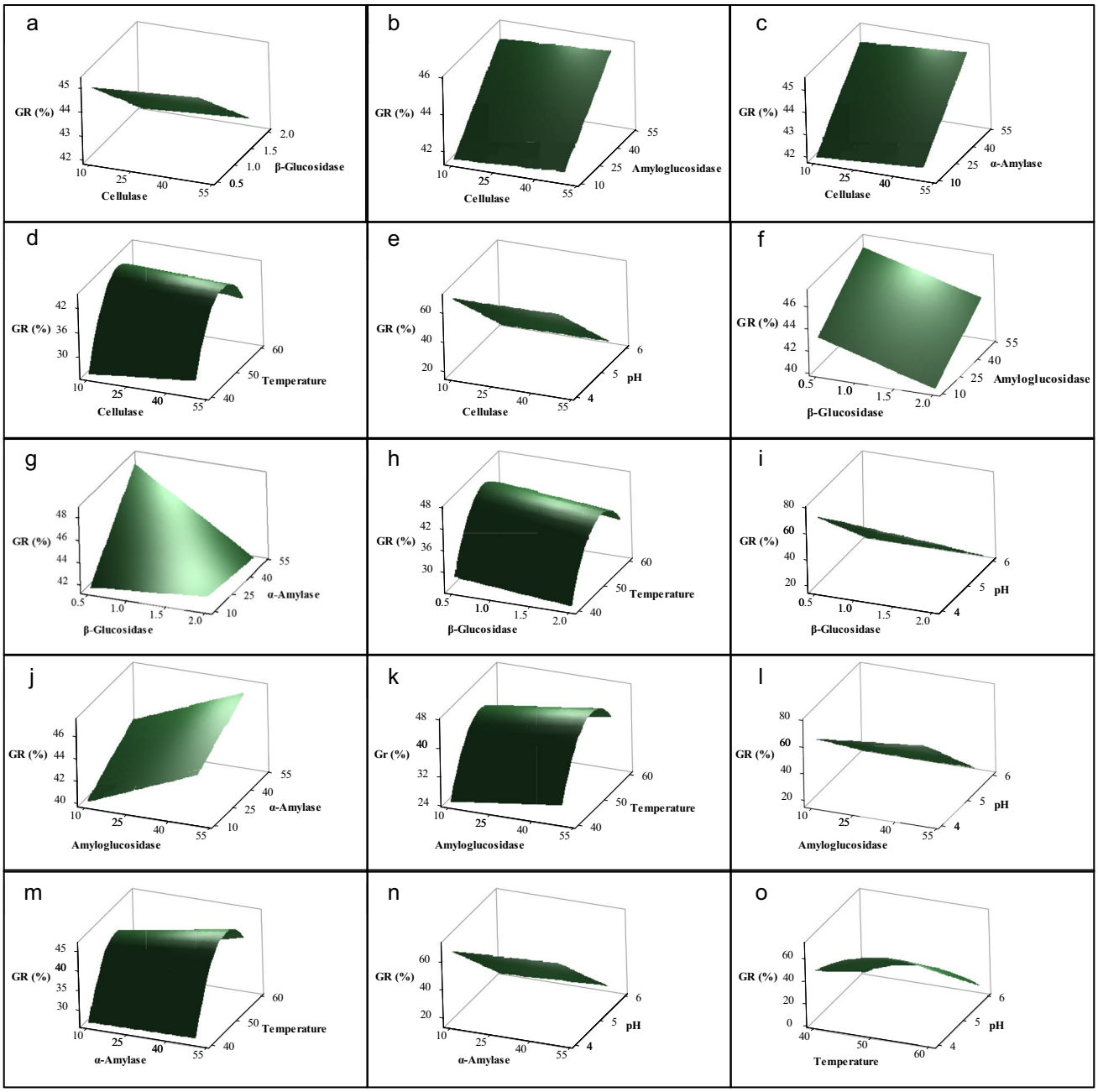

Fig. 1 Response surface curves for the effects of mixed enzymes, temperature, and pH process variables on percentage glucose recovery. Percentage glucose recovery (GR%) was plotted against any two of the six independent variables, while other variables were fixed at their respective midpoint settings. **a** Cellulase and β-glucosidase, **b** cellulase and amyloglucosidase, **c** cellulase and α-amylase, **d** cellulase and temperature, **e** cellulase and pH, **f** β-glucosidase and amyloglucosidase, **g** β-glucosidase and α-amylase, **h** β-glucosidase and temperature, **i** β-glucosidase and pH, **j** amyloglucosidase and α-amylase, **k** amyloglucosidase and temperature, **l** amyloglucosidase and pH, **m** α-amylase and temperature, **n** α-amylase and pH, and **o** temperature and pH

fitted model to predict the response for new observations was determined by the coefficient of prediction (R^2_{pred}) as 92.50%. All the coefficients are greater than 90% and in reasonable agreement, indicating the adequacy of the model. The ANOVA results also showed that there was a non-significant ($p > 0.05$) lack of fit, with the model p-value < 0.000, further validating the statistically significant association between the response variable and the model terms.

Table 5 Experimental and model predicted values for glucose recovery at different substrate water ratio and hydrolysis time

StdOrder	RunOrder	PtType	Blocks	Substrate water ratio (g/ml)	Time (hrs)	Glucose (g/L)	Glucose recovery (%)		
							Observed	Predicted	SE
11	1	0	1	0.13	24	110.773	80.4	76.43	1.32
5	2	−1	1	0.06	24	53.553	84.2	82.58	1.95
7	3	−1	1	0.13	0	6.523	4.7	4.17	2.02
3	4	1	1	0.06	48	61.134	96.1	99.95	3.03
2	5	1	1	0.20	0	7.490	3.5	2.02	3.03
1	6	1	1	0.06	0	2.752	4.3	6.32	3.03
6	7	−1	1	0.20	24	137.544	64.9	70.28	1.95
4	8	1	1	0.20	48	168.137	79.3	79.65	3.03
8	9	−1	1	0.13	48	129.501	94.0	89.8	2.02
13	10	0	1	0.13	24	106.940	77.6	76.43	1.32
9	11	0	1	0.13	24	105.677	76.7	76.43	1.32
10	12	0	1	0.13	24	101.223	73.5	76.43	1.32
12	13	0	1	0.13	24	107.050	77.7	76.43	1.32

3.5 Validation of the optimisation model

To determine the optimal setting at which each factor contributes to maximum glucose recovery, 3D response surface plots (Fig. 1) were constructed by plotting GR% against any two independent variables, while other variables were fixed at their respective midpoint settings. The effects of temperature, pH, and enzyme concentration on GR% are presented in Fig. 1a–o. Changes in cellulase concentration did not significantly change GR% (Fig. 1a–e). β-Glucosidase had a negative effect, reducing GR% as the concentration in enzyme mixture increases (Fig. 1f–i). The highest GR% occurred at the lowest concentration of β-glucosidase. For the amylolytic enzymes, amyloglucosidase and α-amylase, however, both had a positive effect on GR% with increase in either enzyme concentration resulting in increased GR% (Fig. 1j). The highest recovery occurred at the maximum concentration of both enzymes. pH had a linear effect on GR%. The highest GR% was achieved at pH 4, decreasing linearly with increase in pH (Fig. 1 e, i, l, n, and o). However, with temperature, GR% increased steadily with increase in temperature to a maximum at the midpoint temperature of 50 °C. GR% then decreased with further increase in temperature (Fig. 1 d, h, k, m, and o). This phenomenon is typical of all enzyme-catalysed reactions, where enzymatic activity picks at a characteristic optimum pH or temperature beyond which the activity declines. However, within the pH range from 4 to 6, spanning the minimum and maximum recommended pH optima for the enzymes in the mixture, no curvature was observed in the response surface.

In theory, a maximum of $82.39 \pm 2.4\%$ glucose can be recovered from 0.06 g/ml cassava peel in 24 h of hydrolysis with an enzyme mixture containing 10 FPU/g cellulase, 0.5 U/g β-glucosidase, 50 U/g amyloglucosidase, and 50 U/g α-amylase at pH 4 and 54.75 °C. This was determined by the canonical analysis of the ridge maximum

Table 6 Analysis of variance table for optimum substrate water ratio and hydrolysis time

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	*F*-value	*p*-value
Model	4	14,091.7	99.31%	14,091.7	3522.9	287.27	0.000
Linear	2	11,226.5	79.12%	11,226.5	5613.3	457.73	0.000
Substrate water ratio	1	226.9	1.60%	226.9	226.9	18.51	0.003
Time (h)	1	10,999.6	77.52%	10,999.6	10,999.6	896.96	0.000
Square	1	2801.1	19.74%	2801.1	2801.1	228.42	0.000
Time (h)*Time (h)	1	2801.1	19.74%	2801.1	2801.1	228.42	0.000
2-Way interaction	1	64.0	0.45%	64.0	64.0	5.22	0.052
Substrate water ratio*Time	1	64.0	0.45%	64.0	64.0	5.22	0.052
Error	8	98.1	0.69%	98.1	12.3		
Lack of fit	4	73.5	0.52%	73.5	18.4	2.99	0.157
Pure error	4	24.6	0.17%	24.6	6.1		
Total	12	14,189.8	100.00%				

Table 7 Estimated regression coefficients (in coded units) of the second-order polynomial model for glucose recovery in mixed enzyme hydrolysis at varied substrate water ratio and hydrolysis time

Term	Effect	Coef	SE Coef	*T*-value	*p*-value
Constant		76.43	1.32	57.74	0.000
Substrate concentration	−12.30	−6.15	1.43	−4.30	0.003
Time	85.63	42.82	1.43	29.95	0.000
Time*Time	−58.89	−29.45	1.95	−15.11	0.000
Substrate concentration*Time	−8.00	−4.00	1.75	−2.28	0.052

using the response optimiser tool of MINITAB v17 software. To validate the adequacy of Eq. 6 and determine the optimum substrate water ratio and hydrolysis time, 13 verification experiments were conducted within the range of 0.06–0.20 g/ml and 0–48 h. The pH and enzyme concentration were maintained at their respective theoretical optimum, while temperature was maintained at 55 °C for operational expediency. The GR% was determined and compared with the predicted value (Table 5: RunOrder 2). At 24 h of hydrolysis, 84.2% glucose was recovered from 0.06 g/ml substrate water ratio sample. This agrees with the predicted 82.39 ± 2.4% glucose recovery at the same substrate water ratio and time. A new reduced regression model (Eq. 7) based on the 13 verification experiments was fitted (see Online Resources Table S9 for full model terms). The R^2 (99.31%) and R^2_{adj} (98.96%) of the new model were in good agreement suggesting that the data adequately describe the model. The R^2_{pred} value suggests that the model is 96.59% accurate in predicting the GR%.

$$GR(\%) = 8.16 - 30.7\ \text{Substrate water ratio} + 4.547\ \text{Time} - 0.05112\ \text{Time} \times \text{Time} - 2.38\ \text{Substrate water ratio} \times \text{Time} \quad (7)$$

The ANOVA results showed a non-significant ($p > 0.05$) lack of fit, with the model *p*-value < 0.000. Based on these, GR% were predicted at each factor setting and compared with the observed values. There was good agreement between the predicted and observed values (Table 5). All linear model terms were significant at $p < 0.05$. The most significant effect was the hydrolysis time, which accounted for 77.52% of the observed response with substrate water ratio accounting for just 1.6% of the effect (Table 6). The quadratic term Time x Time showed a significant ($p < 0.00$) curvature in the response surface with interaction between the two factors also being significant ($p = 0.041$). The coefficients table (Table 7) showed that the substrate water ratio exerted a negative effect on GR% indicating that glucose recovery decreased with increase in substrate water ratio, with no curvature in the response surface (Substrate water ratio x Substrate water ratio; $p > 0.05$).

Response surface and contour plots (Fig. 2 a and b) were constructed and evaluated. Maximum GR% was obtained at the lowest substrate water ratio of 0.06 g/ml and decreased slightly with increase in substrate concentration ($p = 0.003$). This is thought to be due to mass transfer problems resulting from increased viscosity and unspecific binding of enzymes to substrate molecules, often associated with high substrate loading in enzyme assays [25–27]. GR% peaked between 38 and 48 h of hydrolysis at the lowest substrate water ratio, with curvature in the response surface (Fig. 2b).

The optimum conditions for maximum GR% were determined using the response optimiser tool of Minitab. Maximum GR% (101.19 ± 2.52%) was predicted to occur at 0.06 g/ml in 43.15 h on hydrolysis with 10 FPU/g cellulase, 0.5 U/g β-glucosidase, 50 U/g amyloglucosidase, and 50 U/g α-amylase at pH 4 and 55 °C. Three additional hydrolysis experiments were conducted at these factor settings to validate the optimum hydrolysis condition. GR% of 95.48 ± 0.80 which is > 95% of the predicted value (101.19 ± 2.52%) was recorded. The regression model was therefore

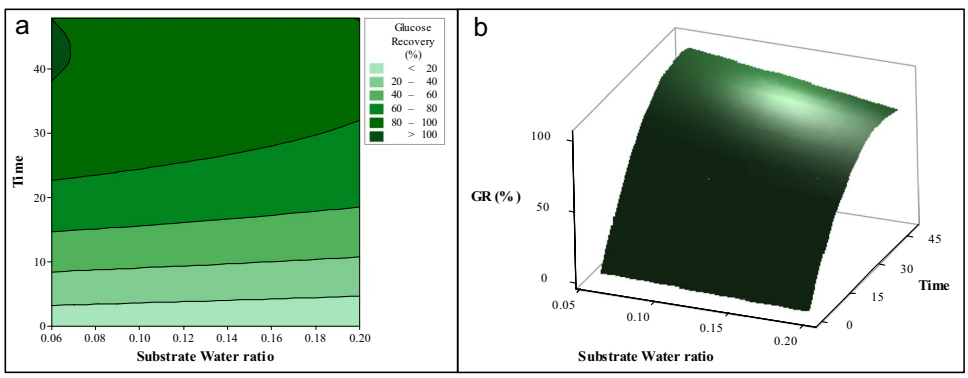

Fig. 2 Two- and 3D plots for the determination of effects of process variables on percentage glucose recovery. **a** Contour plot of GR% against substrate water ratio and hydrolysis time. **b** Response surface plot of GR% against substrate water ratio and hydrolysis time

considered adequate to predict GR% from cassava peel. Overall, the empirical models (Eqs. 6 and 7) developed were reasonably accurate and present an efficient tool to optimise the conditions for enzymatic hydrolysis of cassava peel.

4 Conclusion

Simultaneous hydrolysis of lignocellulosic biomass in a one-step mixed enzyme bioprocess may lead to shorter process time but risks suboptimal GR% due to the wide differences in the optimal pH and temperature of enzyme in the mixture. It is therefore critical to find an optimum pH, temperature, and substrate concentration at which each enzyme in the mixture maintains an optimum level of activity for efficient release of glucose from the biomass. Cassava peel is a biomass low in lignin from which high concentrations of glucose can be recovered using a combination of cellulase, β-lucosidase, α-amylase, and amyloglucosidase. The central composite design statistical optimisation was applied to evaluate the effect of enzyme concentration, substrate water ratio, hydrolysis time, pH, and temperature on percentage of glucose recovered from the peel. Second-order polynomial models were fitted with significant terms only and the reduced equations used to calculate the optimal conditions for maximising GR% from the milled cassava peel. Maximum GR% occurred at 0.06 g/ml substrate water ratio in a 43.15-h hydrolysis using an enzyme mixture containing 10 FPU/g cellulase, 0.5 U/g β-glucosidase, 50 U/g amyloglucosidase, and 50 U/g α-amylase at pH 4 and 55 °C. This simultaneous liquefaction and saccharification process does not require chemical or hydrothermal pretreatment which is estimated to account for ~ 35% of the total bioethanol production cost [15, 16]. In addition, the chance of the hydrolysate containing inhibitors of microbial fermentation process is minimised. Compared with most agricultural wastes used as feedstock, cassava peel offers a competitive advantage and requires more attention than it has received. Due to its low lignin and cellulose content, the peel can also be digested more rapidly with microorganisms than most lignocellulosic biomass. It is therefore important to determine the effects of the residual cyanogenic glycoside content, the low protein, lipid, and mineral contents of the peel [69] on microbial growth and sugar metabolism. These are also important considerations for the application of microorganisms in the liquefaction, saccharification, or fermentation of cassava peel feedstock without significant requirement for additional industrial enzymes.

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Author contribution The study was conceptualised and designed by RB, FKS, W-PD, and A-BB. RB and ACHH performed the experiments. W-PD, RB, and FKS analysed the data. W-PD and RB prepared the manuscript. All authors reviewed and approved the final manuscript.

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Declarations

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Conflict of interest The authors declare no competing interests.

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