# Enzymatic hydrolysis of cassava stalks pretreated with the alkaline method

## Hidrólisis enzimática de tallos de yuca pretratados por el método alcalino

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### ABSTRACT

This study was developed with the aim of evaluating the pH, enzymatic complex load and temperature effects on the saccharification of pretreated cassava stalks (CS) using the response surface methodology (RSM). The factor levels evaluated were temperature 35 - 40°C, pH 4.0 - 5.0 and dose of enzymatic complex Accellerase 1500™ 2.9 - 14.5 FPU/g of substrate. The reducing sugar (RS) response was used. The pH was controlled through the use of hydrochloric acid and sodium hydroxide solutions and the system was shaken orbitally at 120 rpm with a solids loading of 10% w/v. The fitted model showed that the optimal operating conditions were: pH 4.0, 38°C and enzyme dose of 14.5 FPU/g substrate, reaching a sugar concentration of 18.4 g L$^{-1}$.

**Key words:** starch digestion, *Manihot esculenta* Crantz, enzymolysis, lignocellulose.

### RESUMEN

Este estudio fue desarrollado con el objetivo de evaluar el efecto del pH, la carga del complejo enzimático y la temperatura sobre la sacarificación de tallos de yuca pretratados (CS) utilizando la metodología de superficie de respuesta (RSM). Los niveles de los factores evaluados fueron para la temperatura 35 - 40°C, pH 4,0-5,0 y la dosis del complejo enzimático Accellerase 1500™ 2,9 - 14,5 FPU/g de sustrato. Se utilizó el azúcar reductor (RS) como respuesta. El pH se controló a través del uso de soluciones de ácido clorhídrico e hidróxido de sodio y el sistema se agitó orbitalmente a 120 rpm con una carga de sólidos de 10% w/v. El modelo ajustado mostró que las condiciones óptimas de operación fueron: pH 4,0, 38°C y la dosis de enzima de 14,5 FPU/g de sustrato, alcanzando una concentración de azúcar de 18,4 g L$^{-1}$.

**Palabras clave:** digestión del almidón, *Manihot esculenta* Crantz, enzimólisis, lignocelulosa.

## Introduction

Lignocellulosic biomass, such as cassava stalk (CS), is one of the most abundant renewable cellulose resources around the world (Martín *et al*., 2006; Niño *et al*., 2013). Composed of 35-40% cellulose, 10-20% hemicellulose and a relatively low content of lignin (12%), it is an attractive hydrolysis feedstock for the production of biofuels, in particular ethanol (Ferreira-Leitão *et al*., 2010). It is estimated that Colombia generates 320,000 t of this crop's residue per year (Castaño, 2008). These materials can be hydrolyzed to produce monomeric sugars such as glucose and xylose, which can be further used as substrates for the fermentative production of useful products (Lawford *et al*., 1997). If carried out in a technical and economical way, the productivity of the production of ethanol from cassava as an agribusiness could be increased. In Colombia, the cassava tuber is used, but the plant has further potential in terms of the use of the stems and foliage as biomass energy and animal feed, given their chemical composition and nutritional factors. The biomass productivity of cassava has a CRR rate (ratio of harvest residue) of 0.162, which is seen in a crop with a yield of 3 kg m$^{-2}$ per year. This results in 0.48 kg m$^{-2}$ per year of biomass (CS) (FAO, 1996).

The bioconversion of biomass-derived products to produce value-added fuels and chemicals offers potential economic, environmental, and strategic advantages over traditional fossil-based products. In recent decades, research efforts have been devoted to converting lignocellulosic materials to bioethanol (Mielenz, 2001; Sun and Cheng, 2002). The conversion of lignocellulosic biomass to monomeric sugars can be achieved with dilute acid or cellulases. The enzymatic process is believed to be the most favorable method available because enzymatic hydrolysis is milder, more specific and does not produce by-products (Alkasrawi *et al*., 2003). However, currently, the bioconversion process is not economically viable because enzymatic hydrolysis is slow and requires high enzyme loading to achieve reasonable rates and yields. Furthermore, the process is affected

by a wide variety of factors. These factors can be divided into two categories: structural substrate factors and process parameters. The structural substrate factors include degree of polymerization, degree of crystallinity, structural composition and available surface area, and enzymatic interaction and mechanistic factors, *e.g.*, thermal inactivation, cellulose adsorption, and synergism (Zhu *et al.*, 2005; Vásquez *et al.*, 2007). Process parameters contain enzyme loading, substrate concentration, substrate type, reaction time, addition of surfactant and others (Hahn-Hägerdal *et al.*, 2006).

In this study, the hydrolysis of CS pretreated with the alkaline method was investigated using an enzymatic complex with focus on evaluating the pH, enzymatic complex load and temperature effects on the saccharification of pretreated CS using the response surface methodology (RSM).

## Materials and methods

### Cassava stalk (CS)
The cassava stalk was obtained from local farmers in the Uraba region of Colombia. Cassava variety ICA Copiblanca was used. Before the alkaline pretreatment, the stalks were cut to 1-2 cm in length, dried at 60°C in an oven and then milled to a length of 1 mm.

### Enzyme
An enzyme complex was used that was derived from a genetically modified strain of *Trichoderma reesei*, Accellerase 1500™ (Genencor®, DuPont™ Science, Palo Alto, CA), mainly composed of exoglucanase, endoglucanase, hemicellulase and β-glucosidase at a pH of 4.6 to 5.0.

Other chemicals (*e.g.*, NaOH, citric acid, citrate sodium, etc.) were of an analytical grade and purchased from a local supplier.

### Pretreatment of the CS with the alkaline method
Before the enzymatic hydrolysis, the CS was dried, ground, and delignified with the alkaline method with NaOH at 2% (w/v) in a shaker at 120 rpm and 60°C for 11 h at a solid-liquid ratio of 10% (w/v). These operational conditions were selected for evaluation in the first step of the research. The solid residue was collected through filtration under a vacuum with a 400 mesh filter cloth, washed thoroughly with tap water until the filtrate had a neutral pH, and then dried at 60°C in an oven until a constant weight was obtained. The effectiveness of the pretreatment was determined according to equation (1).

$$\% \text{ ligning removed} = \frac{F_1 y_1 - F_2 y_2}{F_1 y_1} \times 100 \qquad (1)$$

Where: $F_1$, mass of material fed; $F_2$ mass of material recovered; $Y_1$, mass lignin fraction in the feed material; $Y_2$, mass lignin fraction of recovered material.

### Enzymatic hydrolysis
Hydrolysis experiments were performed in 200 mL stoppered conical flasks containing 7 g of pretreated CS at a solid-liquid ratio of 10% (w/v). The dose of the Accellerase 1500™ enzymatic complex, temperature and pH were set according to the experimental matrix. The flasks were incubated for 60 h at in a rotary shaker (VWR, Darmstadt, Germany) at 120 rpm. A sample taken from the hydrolysis solution was immediately heated to 90°C for 3 min to denature the enzymes, cooled to room temperature, and then centrifuged for 5 min at 5,000 rpm. The supernatant was used for the reducing sugar (RS) analysis. The enzymatic convertibility was calculated using the following equation:

$$\text{Enzymatic convertibility} = \frac{RS\ (g) \times 0,9 \times 100}{\text{Cellulose in the substrate}} \qquad (2)$$

### Analytical methods
The content of cellulose, hemicellulose and lignin in the raw, dried CS and pretreated CS were estimated according to the methods described by (Van Soest, 1963). The filter paper activity was determined according to the standard procedures recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 2009). One unit of filter paper activity (FPU) is defined as the amount of enzyme that forms 1 μmol of glucose (RS as glucose) per minute under the assay conditions (Ghose, 2009).

### Central composite design (CCD)
The central composite design is a class of rotatable or nearly rotatable second-order designs. In the present study, the two-level three-factorial CCD was applied to investigate and validate process parameters affecting the sugar yield from the enzymatic saccharification of CS. The enzymatic complex loading (2.9-14.5 FPU/g substrate), pH (4.0-5.0) and temperature (35-40°C) were the parameters and levels that were evaluated. The ranges of the above three examined factors were based on evaluating the formation of RS with fermentation conditions mediated by Ethanol Red™ yeast (Lesaffre Yeast Corporation,

Milwaukee, WI) and defining the posterior simultaneous saccharification - fermentation (SSF) process conditions of the CS. The experimental data analyses were performed using the Statgraphics® Centurion XVI (Versión 16.0.07, Statpoint Technologies, Warrenton, VA) statistical software package to fit the regression coefficients of the second order multiple regression model (equation 3).

$$RS = \beta_0*pH + \beta_1*T + \beta_2pH^2 + \beta_4T^2 + \beta_5E^2 + \beta_6pH*T + \beta_7pH*E + \beta_8T*E \tag{3}$$

Where: $\beta_0....\beta_8$ are coeffcient of fit; T, temperatura; E, enzymatic complex loading.

The statistical significance of the model coefficients was determined by analysis of the variance (ANOVA). To find the maximum value, a genetic algorithm software with the help of MatlabR2008b (The Mathworks, Inc., Natick, MA) was used, crossing a factor of 0.8, 100 generations, an initial population of 20 and a 1e-6 error stop criterion.

## Results and discussion

### Pretreatment of CS with the alkaline method

The CS was analyzed for chemical components after pretreatment with the alkaline method. The cellulose, hemicellulose, and lignin contents of the CS before and after the pretreatment are shown in Tab. 1. As can be seen in Tab. 1, the percentage of the cellulose, hemicellulose and lignin content of the pretreated CS increased from 38.4 to 56.5%, from 7.2 to 12.6%, and from 11.8 to 12.2%, respectively. According to equation (1), 44.24% of the lignin was removed and 78.54% of the cellulose and 89.5% of the hemicellulose in the CS were conserved after the alkaline pretreatment (Segura *et al.*, 2007).

**TABLE 1.** Compositions percentage of pretreatment in cassava stalks with the alkaline method*.

|        | Cellulose | Hemicellulose | Lignin |
|--------|-----------|---------------|--------|
| Before | 38.8      | 7.2           | 11.8   |
| After  | 56.5      | 12.6          | 12.2   |

* Percent dry bases.

It should be noted that the results of this research are within the range of results obtained by other authors despite the fact that the grade of lignin solubilization depends on the substrate and operational conditions of the process. Generally, an increase in the cellulose and hemicellulose content of pretreated CS would increase the level of fermentable glucose obtained from enzymatic saccharification, while a decrease in the lignin content could improve the efficiency of the enzymatic hydrolysis of ligno cellulosic materials

(Mussatto *et al.*, 2008; Alvira *et al.*, 2010). In the literature, there are reports of higher percentages of lignin removal than those achieved in this study, which can be attributed to the operating conditions, such as the use of higher concentrations of NaOH, lower solids loading and particle size, the latter implies greater energy expenditure (Varga *et al.*, 2002; Gutiérrez and Arias, 2009; Niño *et al.*, 2013). For instance, Niño *et al.* (2013) reported a 74% removal of lignin when operating with a solids loading of 1%, NaOH concentration of 5% and particle size of 0.6 mm at 90°C for 15 min.

### Saccharification

The activity of the enzyme complex was 57.9 FPU/mL and the RS obtained for the saccharification of the CS pretreatment are shown in Tab. 2. In this table, the parameters of each experiment evaluated in this study can be found.

**TABLE 2.** Central composite design matrix for the three independent variables for the reducing sugar concentration results after 60 h of process time of pretreated cassava stems.

| pH | Temperature (°C) | Enzyme loading (FPU/g substrate) | Reducing sugar (g L⁻¹) |
|------|-------|-------|-------|
| 3.66 | 37.5 | 8.7 | 16.70 |
| 4.00 | 35.0 | 14.5 | 20.30 |
| 4.00 | 35.0 | 2.9 | 11.31 |
| 4.00 | 40.0 | 2.9 | 13.92 |
| 4.00 | 40.0 | 14.5 | 18.27 |
| 4.50 | 37.5 | 8.7 | 14.82 |
| 4.50 | 37.5 | 8.7 | 16.05 |
| 4.50 | 37.5 | 8.7 | 15.06 |
| 4.50 | 37.5 | 8.7 | 13.03 |
| 4.50 | 37.5 | 18.5 | 13.71 |
| 4.50 | 37.5 | 8.7 | 9.61 |
| 4.50 | 37.5 | 0.0 | 0.00 |
| 4.50 | 37.5 | 8.7 | 11.80 |
| 4.50 | 33.3 | 8.7 | 9.80 |
| 4.50 | 41.7 | 8.7 | 7.18 |
| 5.00 | 35.0 | 2.9 | 8.50 |
| 5.00 | 40.0 | 14.5 | 13.03 |
| 5.00 | 35.0 | 14.5 | 13.06 |
| 5.00 | 40.0 | 2.9 | 10.26 |
| 5.34 | 37.5 | 8.7 | 14.26 |

### Regression models of response

Table 2 shows the statistical treatment combinations of the test variables along with the measured response values, expressed as the RS concentration.

$$RS = -64.7295*pH + 8.61487*T + 0.547076*E + 6.81598*pH^2 - 0.115715*T^2 \tag{4}$$

The statistical significance of the Eq. 4 was checked with a *F* test and the analysis of variance (ANOVA) for the response surface quadratic model is shown in Tab. 3.

A model was obtained with an $R^2$ of 95.9%, indicating that the statistical model explained 95.9% of the variability in the response. Table 3 shows that all of the factors, enzymatic complex loading, pH and temperature, had a statically significant effect on the RS response. Figure 1 shows a good fit of the data between the data predicted by the model and the data observed experimentally in the range of the operating variables.

**TABLE 3.** Analysis of variance for the regression model of response of the reducing sugar of pretreated cassava stems.

| Parameter | Estimation | Standard error | Statistical T | P-value |
|---|---|---|---|---|
| pH | -64.7295 | 26.6434 | -2.42948 | 0.0282 |
| T | 8.61487 | 3.20401 | 2.68878 | 0.0168 |
| E | 0.547076 | 0.146984 | 3.72201 | 0.0020 |
| pH$^2$ | 6.81598 | 2.9577 | 2.30448 | 0.0359 |
| T$^2$ | -0.115715 | 0.0432874 | -2.67317 | 0.0174 |
| **Analysis of variance** | | | | |

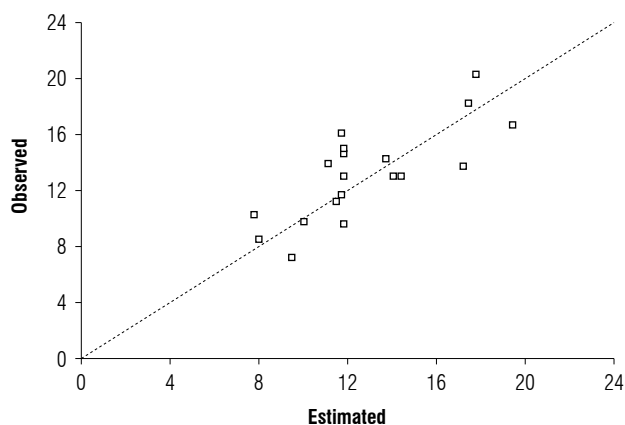| Source | Sum of squares | DF | Mean square | F-ratio | P-value |
|---|---|---|---|---|---|
| Model | 3365.81 | 5 | 673.162 | 70.70 | 0.0000 |
| Residue | 142.822 | 15 | 9.5215 | | |
| Total | 3508.63 | 20 | | | |



**FIGURE 1.** Estimated *vs.* observed reducing sugar of pretreated cassava stems.

In Fig. 2, randomness can be observed in the residue, showing that the model is adjusted so appropriate.

In Fig. 3, it should be noted that when the pH was 5.0, the RS concentration was low. A maximum was observed near the central point of the temperature.

A significant improvement in the concentration of sugars can be obtained at pH 4.0, this level is suitable for the metabolism of yeast and is recommended as the pH value for future processed of simultaneous saccharification and fermentation. By keeping the pH at its central point, as shown in Fig. 4, a maximum was observed at the central
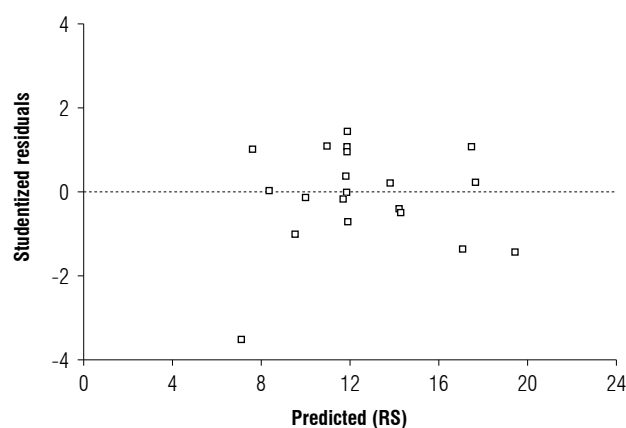


**FIGURE 2.** Studentized *vs.* predicted reducing sugar (RS) of pretreated cassava stems.
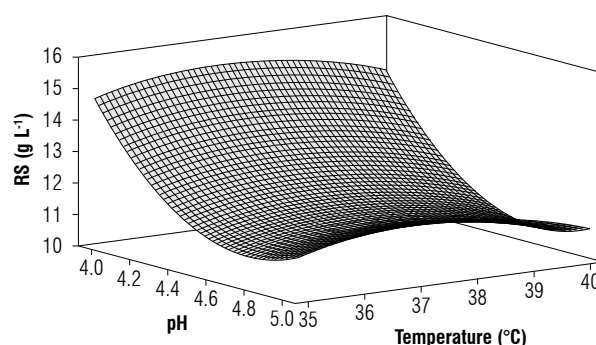


**FIGURE 3.** Response surface of the combined effects of the pH and temperature on the reducing sugar (RS) concentration at a constant enzyme load of 8.7 FPU/g of pretreated cassava stems.
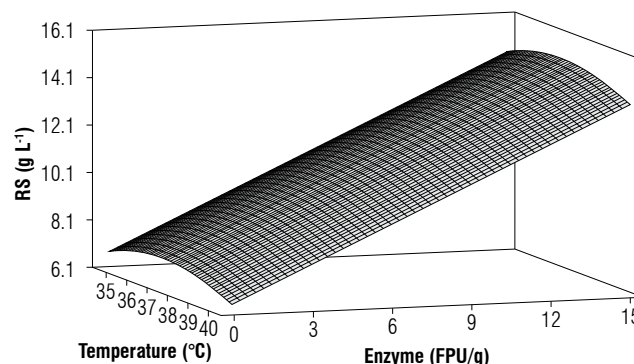


**FIGURE 4.** Response surface of the combined effects of the enzyme load and temperature on the reducing sugar concentration at a constant pH of 4.5 of pretreated cassava stems.

point of the temperature, as seen in Fig. 3; furthermore, increasing the enzyme dose increased the release of RS.

The effects of the enzyme dose and the pH on the release of RS, when the temperature was kept constant at the central point, are shown in Fig. 5. The analysis of the effect of the enzyme on the production of RS showed that increasing the enzyme dose promoted the release of sugars.
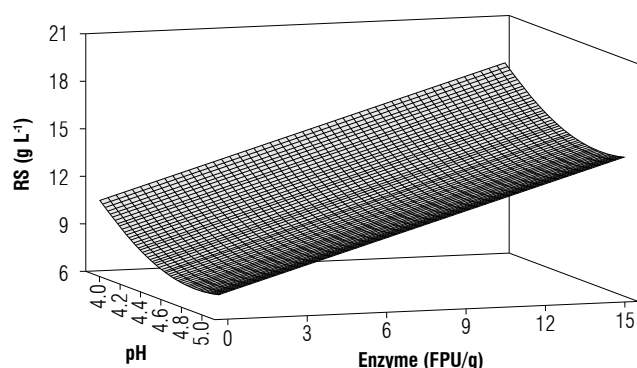
**FIGURE 5.** Response surface of the combined effects of the pH and enzyme loading on the reducing sugar (RS) concentration at a constant temperature of 37.5°C of pretreated cassava stems.

When estimating the maximum RS concentration with equation (4), the pH ranges were limited to conditions where the enzyme was more stable, *i.e.* pH 4-5. The highest value of this function was outside the experimental region. Therefore, the estimated maximum within the experimental region showed that the highest RS concentration was 18.4 g L$^{-1}$ (pH 4.0, 14.5 FPU/g 38°C), a value representing a cellulose enzyme convertibility of 29.3% from the cellulose content present in the cassava stems pretreated with alkali. The obtained RS concentration was greater than that reported by Niño *et al.* (2013), who reached a concentration of 3.7 g L$^{-1}$.

The enzymatic convertibility found in this study was much lower when compared with Han *et al.* (2011), who obtained a saccharification yield of 70% using a solvent solid ratio of 5% and 30 CBU / g of β-glucosidase (NS50010) together with 20 FPU/g cellulose of a mixture of cellulases (NS50013). Martín and Thomsen (2007), who worked with a solvent solid ratio of 2%, a dose of the enzyme Celluclast 1.5 L of 25 FPU/g of dry matter and 0.46 CBU/mL of the enzyme Novozyme 188 at 50°C and 150 rpm for 24 h, reported an enzymatic cellulose convertibility 43.2% of cassava stems pretreated by wet oxidation (195 °C, 10 min, 2 g Na$_2$CO$_3$, 12 bar O$_2$). Vásquez *et al.* (2007) found that the conditions of high glucose conversion are achieved with low-solvent solid relationships, which could explain why Martín and Thomsen (2007) and Han *et al.* (2011) obtained a higher conversion than that reported in their study. The solids loading used in this study favored the presence of hydrolysis products at inhibitory concentrations for the process (Peng and Chen, 2011; Guo *et al.*, 2009). Still, in this study, the best conversion results were obtained using a lower enzyme concentration and a higher ratio of solids than those reached by Martin *et al.* (2007), who obtained a enzymatic convertibility of 14.8% of cassava

stems pretreated with dilute sulfuric acid and a solvent solid ratio of 2% with a dose of the enzyme Celluclast 1.5 L of 25 FPU/g of dry matter and 0.46 CBU/mL of the enzyme Novozyme 188 at 50°C and 150 rpm for 24 h. This result was attributed to a more efficient delignification.

## Conclusion

The pretreatment of CS with the alkaline delignification method produced a substrate consisting of 56.5% cellulose, 12.6% hemicelluloses, and 12.2% lignin.

RSM is a powerful tool to optimize the enzymatic hydrolysis of pretreated CS for the production of RS. The highest RS concentration was obtained at a pH of 4.0, 38°C and 14.5 FPU/g of substrate.

According to the results, 29.3% of the theoretical hydrolysis yield of the cellulose was obtained, which is low compared with results seen in better known substrates, but this provides opportunities to initiate studies on cassava stalk as ethanol resources.

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