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Environment

Evaluation of the hydrolysis yield of banana tree pseudostem using *Penicillium oxalicum* enzyme extract

Avaliação do rendimento de hidrólise do pseudocaule da bananeira utilizando extrato enzimático de *Penicillium oxalicum*

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ABSTRACT

The objective of this research was to perform, under different conditions, the hydrolysis of banana pseudostem using enzymes of the species *Penicillium oxalicum*. A hydrothermal pretreatment (121 °C/30 min) was performed, and subsequently the characterization of the biomass *in natura* and pretreated was used as a substrate in the production of enzymatic extracts. The enzymatic activities were determined (CMCase, Avicelase, and FPase), showing better results for the extract produced in biomass *in natura*. Biomass hydrolysis was performed under different conditions, using the crude enzyme extract produced in pretreated biomass. Through a Rotational Central Composite Design (RCCD), the best hydrolysis condition (20 mg of biomass, 2 mL of enzyme, and pH 5) occurred in assay 9, obtaining 26.36% of hydrolysis yield. Under the same conditions, another hydrolysis was performed, using the enzyme extract produced from *in natura* biomass, resulting in a 42% hydrolysis yield. It was found that the pre-heat treatment used did not positively influence the hydrolysis process of this biomass. The *P. oxalicum* strain (F-3380) showed potential to produce cellulolytic enzymes, and the banana pseudostem *in natura* is an attractive biomass for ethanol production.

Keywords: Banana pseudostem; Crude enzyme extract; Hydrolysis; Ethanol

RESUMO

O objetivo desta pesquisa foi realizar, em diferentes condições, a hidrólise do pseudocaule da bananeira, utilizando enzimas da espécie *Penicillium oxalicum*. Foi realizado um pré-tratamento

hidrotérmico (121 °C/30 min) e posteriormente a caracterização da biomassa *in natura* e pré-tratada, estas que foram utilizadas como substrato na produção de extratos enzimáticos. Foram determinadas as atividades enzimáticas (CMCase, Avicelase e FPase), apresentando melhores resultados o extrato produzido em biomassa *in natura*. Foi realizada a hidrólise da biomassa, em diferentes condições, utilizando o extrato enzimático bruto produzido em biomassa pré-tratada. Através de um Delineamento Composto Central Rotacional (DCCR), verificou-se a melhor condição de hidrólise (20 mg de biomassa, 2 mL de enzima e pH 5) que ocorreu no ensaio 9, obtendo 26,36% de rendimento de hidrólise. Nas mesmas condições, foi realizada outra hidrólise, utilizando o extrato enzimático produzido de biomassa *in natura,* tendo como resultado 42% de rendimento de hidrólise. Verificou-se que o pré-tratamento hidrotérmico utilizado não influenciou positivamente no processo de hidrólise dessa biomassa. A Cepa *P. Oxalicum* (F-3380) demonstrou potencial para produzir enzimas celulolíticas e o pseoducaule da bananeira *in natura* é uma biomassa atrativa na produção de etanol.

Palavras-chaves: Pseudocaule da bananeira; Extrato enzimático bruto; Hidrólise; Etanol

1 INTRODUTION

Brazil is one of the largest agricultural producers in the world, being the third largest producer of fruit, behind only China and India (Food and Agriculture Organization [FAO], 2021; Associação Brasileira dos Produtores e Exportadores de Frutas e Derivados [ABRAFRUTAS], 2023). Banana is the second most produced fruit in the country. According to IBGE data (Instituto Brasileiro de Geografia e Estatística, 2024), the 2023 harvest of this fruit was 6,862,774 tons.

With the large banana production, there is also a large generation of lignocellulosic residues. For each ton of banana harvested, about four tons of residues are generated, 75% of which consist of the banana pseudostem (Linzmeyer et al., 2019). These residues are usually spread on the planting soil, providing nutrients. However, according to Gupta et al. (2022), the accumulation of these residues is considered a problem by rural producers. According to Uchôa et al (2021), there have been searches for the use of this residue, thus avoiding waste and reducing environmental impacts.

The lignocellulosic biomass has been attracting interest for the production of ethanol, because it is a means of waste disposal, contribute to the reduction of

production costs of the generating source (Bhushan et al., 2019) and be a renewable resource, serving as an alternative to oil, which has limited availability and causes negative impacts to the environment (Hernández et al., 2019).

The use of lignocellulosic biomass for this type of exploitation faces some barriers. The first is the need for a pre-treatment to break the recalcitrance of biomass, caused by the structural properties of its compounds and make the material accessible for conversion into sugar. The second is the subsequent step, hydrolysis, in which cellulose is converted into fermentable sugars (Baksi et al., 2019; Rabelo et al, 2019, Qiao et al., 2022; Sawarkar et al., 2022).

One of the means of performing hydrolysis is to make the use of enzymes, which have high costs, representing around 25% of the total costs in the production of cellulosic ethanol (Qiao et al., 2022). Inaddition, for many lignocellulosic wastes, such as banana residues, there is still no precise information about the optimal conditions for enzymatic conversion.

Many studies related to the improvement of the production process of E2G have been developed in several countries, including Brazil, which, being a large agricultural producer, has a good potential for the generation of this biofuel. However, according to Lorenzi and Andrade (2019), with regard to enzyme development, investment by Brazilian companies is low, thus causing dependence on international suppliers, such as the companies: Beta Renewables e Novozymes.

The creation of new enzymes can contribute both to minimize dependence on the international market, as well as reduce costs and improve the process of biomass degradation. For this, a crucial step is the selection of a good enzyme producer. Currently, filamentous fungi are the most exploited microorganisms for this purpose, especially the genus Trichoderma (Bischof; Ramoni & Seiboth, 2016; Sperandio & Ferreira, 2021; Zhang et al., 2023). There are also several other genera under study, which have potential for production of lignocellulosic enzymes, such as

Penicillium (Bomtempo et al., 2017; Espinoza-Abundis et al. 2023; Chavan et al., 2024; Gaizauskaite et al., 2024).

Bomtempo et al. (2017), in their study on the production of *Penicillium oxalicum* cellulases in agroindustrial substrates used the *Penicillium oxalicum* F-3380 fungic strain, obtained from a colony of leaf-cutting ants, *Acromyrmex Balzani* (Hymenoptera: Formidae). The results of the study were considered satisfactory. The Strain was chosen for presenting, among isolates of *Acromyrmex Balzani*, previously tested, the highest productivity.

Given the high production of agricultural waste from banana cultivation and the search for viable techniques for ethanol production, this study aimed to perform, under different conditions, the hydrolysis of banana pseudostem using enzymes produced by the fungal strain *Penicillium oxalicum* F-3380, in order to obtain a higher yield of hydrolysis.

2 MATERIALS AND MATHODS

2.1 Raw Material

The raw material used for the present study, the pseudostem of the banana tree *Musa cavendishii*, was collected in the city of Dianópolis -TO, in the Manuel Alves project area, one of the largest fruit production centers in the state, which is located 347 km from the capital Palmas. The pseudostem was harvested manually and cut into pieces around 30 cm long. The leaf sheaths were detached to facilitate the drying process, which was initially performed at room temperature over a period of two days and then in an oven at 60 °C for 72 h. After drying the material, the grinding process occurred in order to increase the surface area and decrease the degree of polymerization of the residue, using a knife mill coupled with a 20 mesh sieve. Then the material was stored in plastic bags in order to remain conserved for laboratory analysis.

2.2 Chemical characterization of the banana pseudostem

The following chemical analyzes were performed: moisture content, extractive content, ash content, lignin content (soluble and insoluble), holocellulose content, hemicellulose content and cellulose content. The determination of lignin was performed according to the methodology of Sluiter et al. (2008); cellulose according to Sun et al. (2004) and the other analyzes according to Morais, Rosa e Marconcini (2010).

2.3 Pre-treatment

In 500 mL erlenmeyer flask, 16g of the dried and ground pseudostem were added in 240 mL of water (ratio 1:15). The erlenmeyer flask was shaken manually for a few seconds and forwarded to the autoclave in which it remained for 30 min after reaching a temperature of 121 °C. After this period, the samples were cooled at room temperature, filtered, and the solid fraction was dried in an oven at 50 °C until constant weight. Then cooled and stored at room temperature.

2.4 Microorganism and culture conditions

The microorganism used for the production of cellulosic enzymes was the filamentous fungus *Penicillium oxalicum* F-3380, which was chosen because it is considered a good producer of cellulases, as pointed out in the study of Bomtempo et al. (2017), being promising for industrial uses. The strain was obtained from a colony of leaf-cutting ants, *Acromyrmex Balzani* (Hymenoptera: Formidae). This strain belongs to the collection of microorganism culture Carlos Rosa Culture Collection, Laboratory of Environmental Microbiology and Biotechnology, Federal University of Tocantins. The cultivation occurred, as carried out by Bomtempo et al. (2017), in Petri dishes in Potato Dextrose Agar (PDA) medium, in a period of 7 days, at a temperature of 25 °C. With the addition of distilled water, were obtained the spore suspensions whose counting was performed in the Neubauer chamber.

2.5 Production of enzymes

The production of enzymes occurred by Solid State Fermentation (SSF) using as substrate the pseudostem of pretreated and banana *in natura*. In 250 mL Erlenmeyer flasks, 5g of substrate (banana pseudostem) and a mineral salt solution described by Mandels and Weber (1969) were added. The flasks with the samples were sterilized in an autoclave at 120 °C for 20 min, cooled, inoculated with spore suspension of the fungus *P. oxallicum* F-3380 and incubated (Aguiar e Lucena, 2011). The enzymatic extraction was performed by the addition of 50 mL of citrate buffer (0.05 M, pH 4.8), followed by agitation at 150 rpm for 1 h, simple filtration, thus obtaining the Crude Enzymatic Extracts (CEE's), these stored at 20 °C for further analysis. The enzymatic extract, from the fermentation of the pre-treated biomass, was entitled CEE1, and the extract obtained from the *in natura* biomass as CEE2.

The incubation time, temperature, inoculum concentration, humidity and pH were performed, according to Bomtempo et al. (2017), which obtained higher yields of *P. oxalicum* F-3380 enzymes, using sorghum as substrate, under the following conditions: incubation time of 4 days, temperature of 40 °C, inoculum concentration of 10^6 spores mL^{-1} initial humidity of 60% and pH 4.

2.6 Enzymatic activities

The methodology of Ghose (1987) was used to determine the enzymatic activity of endoglucanase (CMCase), (Avicelase) and total cellulase (FPase). The step by step of the whole process of determination of CMCase and Avicelase are the same, changing only the substrate. The substrate of CMCase was carboxymethylcellulose diluted to 2% in citrate buffer (0.05 M, pH 4.8), while the substrate of Avicelase was microcrystalline cellulose diluted to 1% in citrate buffer (0.05 M, pH 4.8). The incubation time for both assays was 30 min. Already in the determination of FPase, which used as substrate a strip of Whatman n. 1 (1.0 x 6 cm), incubation occurred for 60 min.

The enzymatic activities were quantified by the dosage of the reducing sugars produced, using the 3,5-dinitrosalysyl acid method (DNS) (Miller,1959). The method consisted in the application of 3 mL of DNS in the samples, after the incubation period. Then the samples were boiled in a water bath for 5 min. The reaction was stopped by placing the tubes in cold water and adding 20 mL of distilled water. The reading was performed in spectrophotometer, Gehaka Uv 380 G, at a wavelength of 540 nm.

A calibration curve was constructed, with concentrations between 0.12 and 1 mg. 0.5 mL^{-1} (Y axis) and absorbance between 0.024 and 0.278 nm (X axis), generating the following equation of the line: y= 3.2959x + 0.056, with R^2 = 0.9921. Based on this equation, the concentrations of reducing sugars were calculated. The calculations of the activities of CMCase, Avicelase and FPase were performed by the following equation (Equation 1):

$$\text{Enzyme activity } (\text{U.mL}^{-1}) = \frac{AR.\text{V}_\text{T}}{0{,}18 * \text{V}_\text{C} * \text{T}_\text{H}} \quad (1)$$

Were:
AR = reducing sugars produced in the hydrolysis stage ($mg.mL^{-1}$);
V_T = total volume used in hydrolysis (buffer volume + extract volume crude enzymatic) (mL);
V_C = volume of crude enzymatic extract in hydrolysis (mL);
T_H = hydrolysis time (min);
0.18 = 1µmol of glucose (mg)

A unit of cellulosic activity ($U.mL^{-1}$) is considered as the amount of enzyme capable of releasing 1µmol of glucose per mL per min of reaction (Ghose (1987). The conversion in $U.mL^{-1}$ to $U.g^{-1}$ was made by the following equation (Equation 2):

$$\text{Enzyme activity } (\text{U.g}^{-1}) = \frac{\text{enzymatic activity } (\text{U.mL}^{-1}) * \text{ TP}}{\text{MA}} \quad (2)$$

Were:
TP = citrate buffer used in SSF (mL);
MA = mass of the sample used in SSF (g)

2.7 Enzymatic hydrolysis of the banana pseudostem

2.7.1 Hydrolysis of biomass *in natura* with CEE1

The *in natura* substrate with 8 mL sodium citrate buffer solution and the CEE1 were added in 250 mL Erlenmeyer flasks. The container with the mixture was incubated under agitation of 150 rpm and 50 °C. An aliquot of the hydrolysate was collected at 24, 48 and 72 h. The samples were stored in freezer until they were submitted to glucose quantification ($g.L^{-1}$), performed by means of High Performance Liquid Chromatography (HPLC). The equipment of the Agilent brand, model 1260 Infinity II, equipped with refractive index detector at 40 °C, using a chromatographic column Supelcogel C-610H (30cm x 7.8mm) - Sigma-Aldrich, pre-column Supelguard C610H (5cm x 4.6mm) - Sigma Rich. The mobile phase used was 0.1% H_3PO_4 (deionized water and phosphoric acid), with flow (flow rate of the mobile phase) of 0, 5$mL.min^{-1}$, and the total running time of 18 min and oven temperature of 40 °C.

After obtaining the glucose concentration, the hydrolysis yield was calculated, according to Andrade et al. (2019) and Siqueira et al. (2016) (Equation 3).

$$\text{HY}\ (\%) = \left(\frac{\text{Gl}}{\text{Cel}} \times 0.9\right) \times 100 \quad (3)$$

Were:
HY: hydrolysis yield (%)
Gl.: glucose concentration ($g.L^{-1}$);
Cel.: concentration of cellulose from biomass ($g.L^{-1}$);
0.9: cellulose conversion factor

2.7.1.1 Statistical analysis

A Rotational Central Composite Design (RCCD) 2^3+ 2*3 axial point + 4 central points, totaling 18 trials, was used to determine the best condition for obtaining HY. The levels used in the RCCD are presented in Table 1.

Table 1 – Values used in RCCD

Variables	Levels				
	-1.68	-1	0	1	1.68
Biomass (mg)	20	28	40	52	60
Enzyme (mL)	1	1.4	2	2.6	3
pH	4	4.4	5	5.6	6

Source: Authors (2023)

An Analysis of Variance (ANOVA) was generated by the Statistics software to verify the significance, at the level of 5% of the glucose concentration in relation to the times of 24, 48 and 72h. After the choice of time, which obtained the highest concentration of glucose, an estimate of the effects of the variables and their interactions on HY % was performed, considering the significance level of 5%. ANOVA was used to test the validity of the mathematical model obtained by the F test and the calculation of the coefficient of determination of the model (R2). This model was represented by response surface graphics and contour curves. With the exception of ANOVA of glucose concentration, in relation to the periods evaluated, all other experimental data were analyzed using the software Protimiza Experimental Design.

2.7.2 Hydrolysis of biomass *in natura* with CEE2

After performing the enzymatic hydrolysis of the biomass *in natura*, using CEE1, it was verified which of the conditions of the variables evaluated obtained higher hydrolysis yield. From this, a new hydrolysis of pseudostem *in natura* was performed, but this time, using CEE2. The experiment was carried out in triplicate.

3 RESULT AND DISCUSSION

3.1 Chemical characterization of the banana pseudostem

The data of the characterization of the pseudostem of banana *in natura* and pre-treated are presented in Table 2.

Table 2 – Chemical composition of pseudostem in natura and after hydrothermal pretreatment

Parameters	Biomass *in natura*	Pre-treated biomass (120 °C/30 min)
Humidity (%)	90.98 ± 0.80	-
Ashes (% DB)	6.97 ± 0.013	-
Extractive (% DB)	24.35 ± 0.94	-
Lignin (% DB)	8.58 ± 0.64	12.08 ± 1.22
Hemicellulose (% DB)	24.02 ± 1.18	19.05 ± 0.99
Cellulose (% DB)	35.86 ± 0.70	40.58 ± 1.40

DB: Dehydrated Biomass. Source: Authors (2023)

The percentage found in the biomass of the present study was 90.98%, close to the value obtained by Brissac et al. (2018) (95.2%), who also evaluated the banana pseudostem. This high percentage of humidity shows that the percentage of dry biomass is low, and this directly affects the yield of second generation ethanol, which will also be reduced.

The percentage of ash (6.97%), which represents the inorganic biomass material, was much lower than those found in the pseudostems analyzed by Rosa et al. (2021) (12.5%) and Fiorese et al. (2020) (14.45%). The smaller the amount of ash, the greater the use of biomass, which results in the reduction of waste generated after hydrolysis (Fiorese et al. 2020).

The extractives content (24.35%) was close to that found in the pseudostem studies of Rosa et al (2021) (24.6%) and Díaz et al. (2021) (21.11%). It is considered a high percentage, comparing, for example, with bagasse in sugarcane (12.80%) (Rodrigues et al., 2020), which is one of the main raw materials used in the production of second generation ethanol.

The lignin found in the biomass (8.58%) was close to the results of Rosa et al (2021) (8.4%), who also evaluated the banana pseudostem. This percentage is lower than that of sugarcane bagasse (22.78%) (Andrade et al., 2019), and this is a positive factor, since it facilitates access to cellulose.

Hemicellulose (24%) was close to the results of Legodi et al. (2021) (22,56%) and was higher than that of Rosa et al. (2021) (11.3%) and Díaz et al. (2021) (19.11%). This high percentage indicates that the biomass needs a pre-treatment that solubilizes this compound, because, according to Ogeda and Petri (2010), the hydrolysis of hemicellulose can generate by-products (mainly, diphenols, derivatives of Fenilpropane, ketones, furfural and acetic acid which often inhibit microbial fermentation.

The cellulose fraction presented (35.86%) is considered satisfactory in the use for ethanol, being higher than the values found in the analysis of pseudostems of Guerrero et al. (2018) (20.1%) e Díaz et al. (2021) (26.29%).

Regarding the pre-treated biomass, the lignin content increased 28.9% after pretreatment. Maione et al. (2019), in their study of the effect of hydrothermal pretreatment on malt bagasse, obtained an increase in lignin content in all pretreatment conditions (160 °C/20 min 200 °C/20 min, 160 °C/60 min, 200 °C/60 min). According to the same author, it is expected that biomasses submitted to this type of pretreatment have a slightly higher percentage of lignin in relation to *in natura* biomass. Hemicellulose content decreased 20%, considered low compared to the study by.

Dos Santos Rocha et al. (2017), which obtained 70.7% hemicellulose removal at 170°C/15 min and 89.7% at 195°C/10 min in sugarcane straw and 58.7% at 170 °C/15 min and 67.8% to 195 °C/10 min in corn cob. It is worth mentioning that these results were obtained at much higher temperatures than the current study (121°C) and with agitation (200 rpm), which may explain the greater removal of hemicellulose.

The cellulose content increased by 11.63%. Rosa and Pinheiro (2021), evaluating the effect of hydrothermal pretreatment on orange bagasse, under conditions similar to that of the present study (121 °C/60 min), obtained a 3.66% increase in cellulose. The increase in cellulose in the present study, despite the increase in lignin, can be explained by the disruption of cellulosic fiber after pretreatment and also by the solubilization of other components such as hemicellulose, which decreased after pretreatment.

3.2 Enzymatic activities of cellulases

The data of the enzymatic activities evaluated (CMCase, Avicelase and FPase) are presented in Table 3. Some studies, using different microorganisms and cellulase-inducing substrates, were selected to compare with the results of the present study.

Table 3 – Comparison of cellulase activities produced by fungal strains cellulolytic

		Enzymatic Activity (U.g $^{-1}$)			
Microorganism	**Substrate**	**CMCase**	**Avicelase**	**FPase**	**Reference**
P. oxallicum (F-3380)	PB (*in natura*)	1.54	1.31	2.33	Current study
P. oxallicum (F-3380)	PB (Pré-tratada)	0.52	0.94	1.29	
P. oxallicum (F-3380)	S	9.2	8.4	4.2	Bomtempo et al. (2017)
P. oxallicum (F-3380)	BC	1.3	1.6	3.3	
Aspergillus niger	BC	2.4	-	1.09	Cavalcante et al. (2018)
	SB	3.37	-	1.06	
Trichoderma reesei	BC	-	-	25.6	Mekala et al. (2008)

Source: Authors (2023). PB - Pseudostem of banana, S - Sorghum, BC- Bagasse of sugar cane and SB - Corn cob

According to the data of Table 3, it is noticed that the cellulases produced *in natura* biomass, in this study, presented higher enzymatic activities than those produced in pre-treated biomass. Fungi need celluloses to produce cellulases. The cellulose contents of the biomass *in natura* and pretreated were close, and the biomass pretreated (40.58%) higher than *in natura* (35.86%). Therefore, the difference in the results of the enzymatic activities in the two evaluated biomasses could not be explained by the percentage of this component. It is assumed that the lower enzymatic activity of cellulases, produced in the pre-treated biomass, was caused by inhibitors originated in the pre-treatment.

Bomtempo et al. (2017) presented the highest enzymatic activities obtained in their study of optimization of enzyme production conditions, of the same strain of this study (P. oxallicum F-3380), using sorghum and sugarcane bagasse as substrate. The results of CMC (9.2 U.g^{-1}) and Avicelase (8.4 U.g^{-1}) in sorghum were considerably higher

than those of the present study. Already the sorghum FPase (4.2 $U.g^{-1}$), as well as all enzymatic activities evaluated (CMC, Avicelase and FPase) in sugarcane, was close to the results obtained.

Data from the enzymatic activities of Cavalcante et al. (2018) of the enzymes obtained by the fungus Asperrgillus Niger, show that both CMC (2.4 $U.g^{-1}$) and FPase (1.09 $U.g^{-1}$) in sugarcane bagasse, as CMC (3.37 $U.g^{-1}$) and FPase (1.1 $U.g^{-1}$).maize cob were also similar to the data of the present study.

All cellulases analyzed in this study, both those produced *in natura* biomass and pre-treated biomass, showed FPase considerably lower than the result obtained by Mekala et al. (2008), using Trichoderma reesei and sugarcane bagasse (25.6 $U.g^{-1}$). This is to be expected, since this same fungus is considered a reference in the commercial production of cellulases. However, it is still considered that the strain analyzed (P. oxallicum F-3380) obtained attractive results.

Bomtempo et al. (2017) and Cavalcante et al. (2018), using the same substrate (sugarcane bagasse) and different microorganisms (*Penicillium oxalicum* and *Aspergillus Niger*, respectively) obtained results close to CMCase (3.5 and 2.4 $U.g^{-1}$) and FPase (3.3 and 1.09 $U.g^{-1}$). However, the greatest enzymatic activities were the cellulases produced by the fungus Penicillium oxalicum. Both the results of Bomtempo et al. (2017) and the current study prove the potential of the fungus *Penicillium oxalicum* (F-3380) as cellulase producer.

3.3 Enzymatic hydrolysisa

3.3.1 Hydrolysis of biomass *in natura* with CEE1

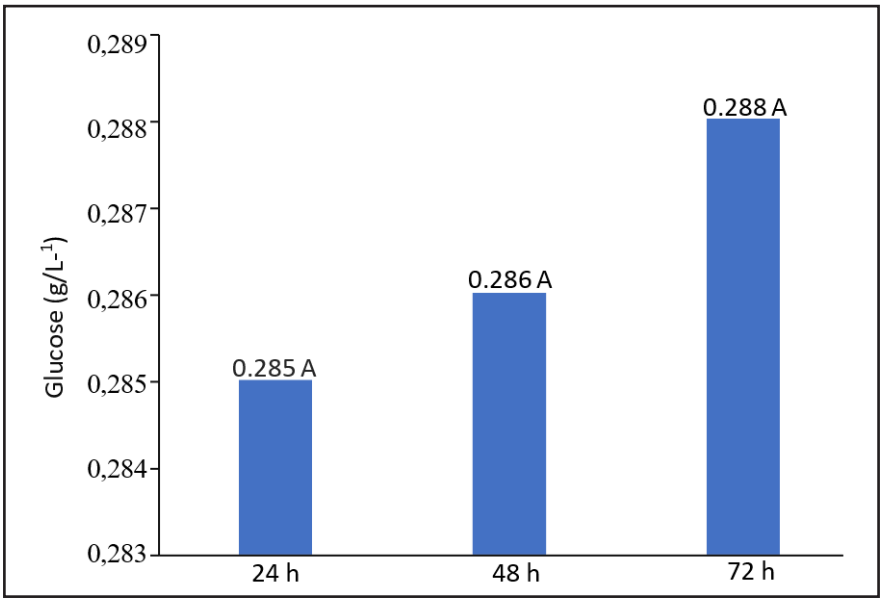
Table 4 shows the ANOVA used to verify, at 5% level, the significance of the periods evaluated (24, 48 and 72h) in relation to glucose release ($g.L^{-1}$). There was no significant difference in glucose release between these periods. Graph 1 shows the amount of glucose ($g.L^{-1}$) released in each of the evaluated periods.

Table 4 – ANOVA of the release of glucose ($g.L^{-1}$) in times of 24, 48 e 72 h

Source of variation	Degree of Freedom	Sum of Squares	Mean Squared	Fcalc	Ftab	p-value
Hours	2	0.000104	0.000052	0.015	5.78	0.9848
Error	51	0.172328	0.003379			
Total Corrected	53	0.17431				
CV (%)	20.29					
Overall average	0.28648					
Number of observations	54					

Source: Authors (2023). CV = Coefficient of Variation, Fcalc = Calculated F, Ftab = Tabulated F

Graph 1 – Glucose release ($g.L^{-1}$) in the periods of 24, 48 and 72 h



Source: Authors (2023)

Graph 1 above shows that glucose released ($g.L^{-1}$) between 24, 48 and 72 h was similar (p 0.05), ranging from 0.285 - 0.288 $g.L^{-1}$. According to Medeiros (2014), this glucose release behavior, after 24 h, can be attributed to the inhibition generated by the enzyme itself, which occurs naturally, when the reaction medium already meets a certain concentration of glucose. Another hypothesis is the possible inhibition generated in the pretreatment.

As statistically, the results showed no difference, it is observed that the reaction time of 24 h seems to be more viable than maintaining the hydrolytic process up to 48

or 72 h, because of the lower energy expenditure and less time in the process. Thus, the glucose obtained in 24h was used to calculate the hydrolysis yield (HY), as shown in Table 5.

Table 5 – Experimental design, using three variables (biomass, enzyme and pH), for the concentration and yield of glucose in the enzymatic hydrolysis step

	Codified Variables			Real Variables			Answers 24 h	
Assay	Biomass (mg)	Enzymes (mL)	pH	Biomass (mg)	Enzymes (mL)	pH	Conc. of glucose (g/L)	HY (%)
1	-1	-1	-1	28	1	4	0.21	17.62
2	1	-1	-1	52	1	4	0.34	15.22
3	-1	1	-1	28	3	4	0.28	26.17
4	1	1	-1	52	3	4	0.37	18.95
5	-1	-1	1	28	1	6	0.22	18.35
6	1	-1	1	52	1	6	0.34	15.30
7	-1	1	1	28	3	6	0.25	24.19
8	1	1	1	52	3	6	0.33	16.72
9	-1.68	0	0	20	2	5	0.21	26.36
10	1.68	0	0	60	2	5	0.39	16.20
11	0	-1.68	0	40	1	5	0.25	14.12
12	0	1.68	0	40	3	5	0.33	22.53
13	0	0	-1.68	40	2	4	0.25	15.46
14	0	0	1.68	40	2	6	0.29	18.37
15	0	0	0	40	2	5	0.28	17.37
16	0	0	0	40	2	5	0.24	14.97
17	0	0	0	40	2	5	0.30	18.51
18	0	0	0	40	2	5	0.30	18.94

Source: Authors (2023). mg = milligrams, mL = milliliters, pH = hydrogen potential, $g.L^{-1}$ = grams/liter, HY = Hydrolysis Yield

Observing the results of Table 5, it is noted that the assay 9 presented the highest hydrolysis yield (26.36%). The conditions of this assay were: 20 mg of biomass and 2 mL of enzyme and pH 5, since the last variable was not statistically significant.

Evaluating the trials 9 and 10, which represent the axial points, in which varied only the quantity of biomass, it is noticed that the first, having less amount of lignocellulosic material, obtained HY of 26.36%, being superior to the second test that obtained 16.20% HY.

In relation to axial points, in which only the amount of enzyme varied, assays 11 and 12, it is noted that the assay 11, which had less amount of enzyme, presented RH of 14.12%, lower than assay 12, which obtained HY of 22.53%.

Alves (2017), in his study on optimization of enzymatic hydrolysis in rice husk, obtained in the axial points related to the amount of biomass, higher HY in the assay that contained less biomass. The same study, in the axial points referring to the amount of enzyme, obtained higher HY in the assay that contained greater amount of enzyme.

As seen, the highest HY among the assays evaluated occurred by the lower amount of biomass and greater enzyme, which facilitates the action of enzymes in biomass degradation.

In order to verify the effect of the variables and their interactions, at a significance level of 5%, the calculations of regression coefficients of linear, quadratic and interaction factors were generated, as shown in Table 6.

The data presented in Table 6 show that the significant variables, at 5% level, were Biomass (mg), Enzyme (mL) and the interaction between them. The pH had no significant interference in the results. It is noteworthy that the pH was adjusted only at the beginning of the reaction, so there may have been variation during the process.

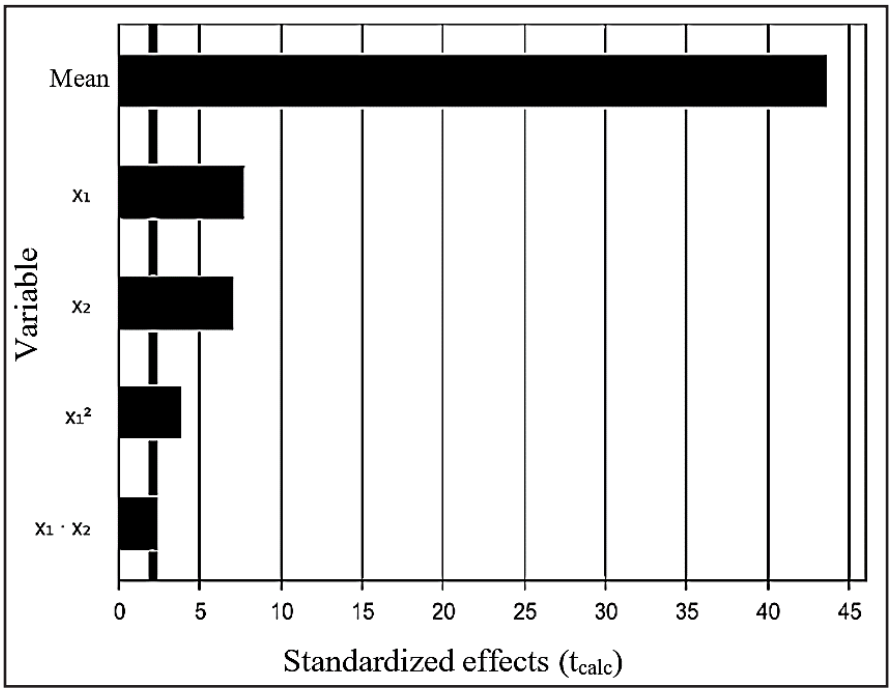
Table 6 – Regression coefficients of yield at 24 h

Name	Coefficient	Standard Error	T calculate	p-value
Mean	17.44	0.70	24.74	0.0000
X_1	-2.73	0.38	-7.13	0.0001
X_1^2	1.39	0.40	3.49	0.0082
X_2	2.47	0.38	6.46	0.0002
X_2^2	0.34	0.40	0.86	0.4158
X_3	0.11	0.38	0.29	0.7819
X_3^2	-0.16	0.40	-0.40	0.7013
$X_1.X_2$	-1.16	0.50	-2.31	0.0494
$X_1.X_3$	-0.11	0.50	-0.23	0.8273
$X_2.X_3$	-0.63	0.50	-1.26	0.2442

Source: Authors (2023). X_1= Biomass (mg), X_2 = enzyme (mL) e X_3= pH

The Pareto Diagram (Graph 2) shows the variables in order of importance for the regression model. The vertical line shows that the coefficients are significant at a significance level of 5% ($p<0.05$)

Graph 2 – Pareto diagram of significant variables of standardized effects



Source: Authors (2023). X_1= biomass (mg), X_2 = enzyme (mL) e X_3 = pH

Using the experimental data indicated in Table 6, a mathematical model (Equation 4) was obtained that relates the HY with the significant variables at the level of 5% significance, Biomass (X_1) and enzyme (X_2).

$$Y = 17.60 - 2.73\ x_1 + 1.35\ x_1^2 + 2.47\ x_2 - 1.16\ x_1\ x_2 \quad (4)$$

To evaluate the fit quality of the Equation 4 model, Table 7 shows the results of the Analysis of Variance (ANOVA), at a 5% level of significance.

Table 7 – ANOVA of Hydrolysis Yield in 24h

Source of variation	Sum of Squares	Degree of Freedom	Mean Squared	Fcalc	Ftab	p-value
Regression	220.2	4	55.1	33.2	3.179	0.00000
Residue	21.5	13	1.7			
Lack of Fit	12.0	10	1.2	0.4	8.785	0.89258
Pure Error	9.5	3	3.2			
Total	241.7	17				

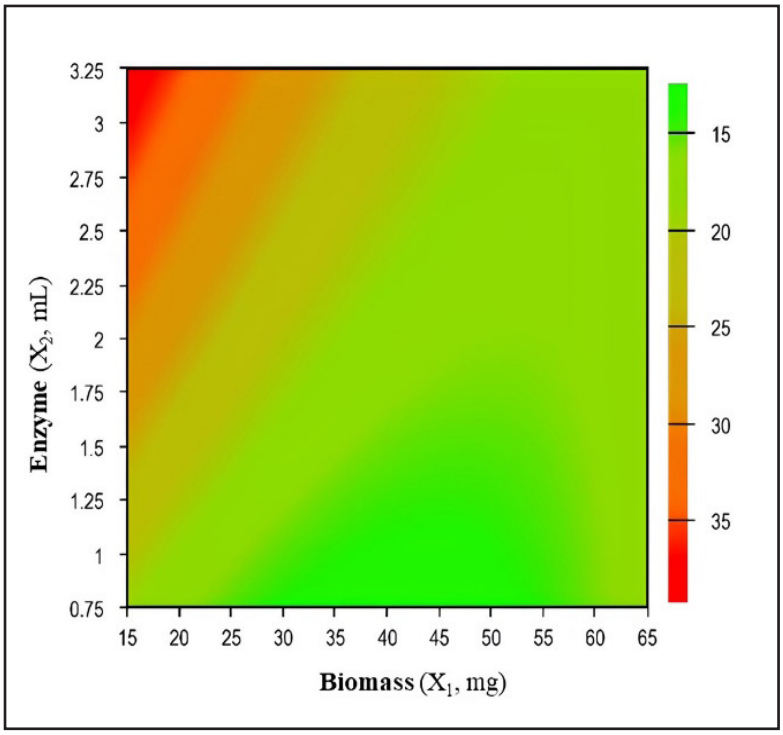
Source: Authors (2023). F_{calc} = F Calculate, F_{tab} = F Tabelated, R^2 = 91,10%

According to Protimiza Experimental Desing software, a mathematical model is considered statistically significant when the Fcal (regression/residues) ≥ Ftab, the Fcal (lack of fit/pure error) ≤ Ftab and the R^2 is sufficient. Thus, analyzing the results of the table above, it is verified that the model generated in the regression is significant. The R^2 of this model was 91.10% which means that 91.10% of the data were well represented.

The response surface and contour curves, considering only the significant effects, are presented, respectively, in Graph 3 and Graph 4, in order to demonstrate the process trend estimated by the model found. Both graphs show that the highest hydrolysis yields are in the orange-tinted region, where such yields are directly related to the lower amount of biomass and higher amount of enzyme.

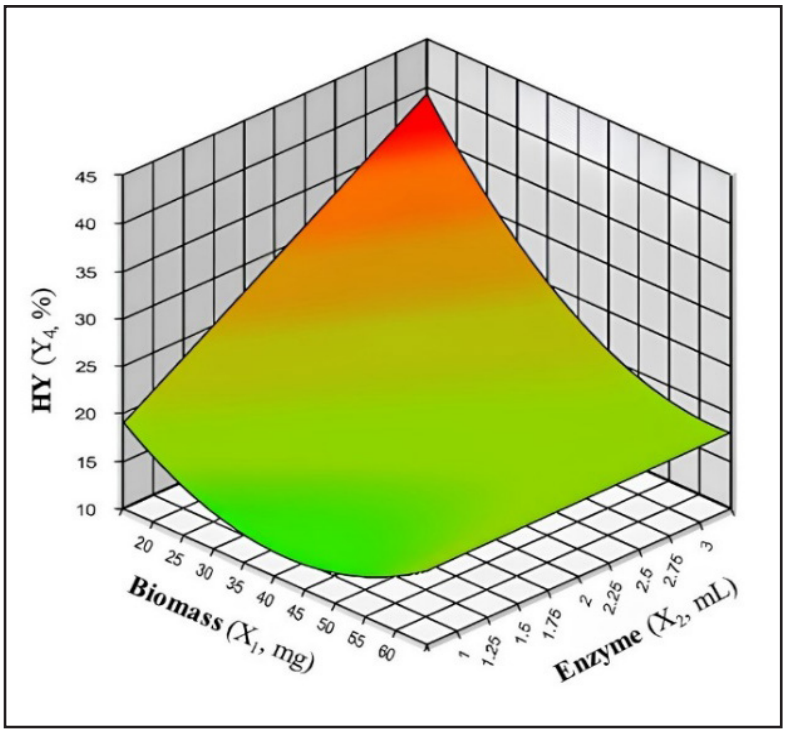
It is emphasized that the response surface methodology, technique used in this research, is a highly used tool to analyze the interference of variables in the response of a given process, thus identifying the best conditions of a study (Goswami et al., 2022). This technique can promote positive prospects in the use of plant waste, contributed both to processes developed on a small and large scale (Da Silva et al., 2019).

Graph 3 – Contour curve for or HY in 24 h



Source: Authors (2023)

Graph 4 – Contour curve for or HY in 24 h



Source: Authors (2023)

3.3.2 Hydrolysis of biomass in natura with CEE2

As the assay 9 showed the highest HY (26.36%) among the 18 assays using CEE1, the same hydrolysis conditions of this assay (20 mg of biomass, 2 ml of enzyme and pH 5) were chosen for the hydrolysis of biomass *in natura* with CEE2. It is worth mentioning that pH was not a significant variable in hydrolysis optimization. However, the same pH of the test 9 was used for the present hydrolysis, in order to compare with the previous hydrolysis data.

The RH obtained was in hydrolysis with CEE2 of 42% ± 1.71. Comparing with the obtained yield, using the CEE1, it is noticed that there was an increase of 37.24%. This can be explained by the fact that CEE2 enzymatic activities are higher than CEE1 (Table 3). CEE1, because it was produced in pre-treated biomass, may contain inhibitors that have limited glucose production. In this sense, it is more feasible to hydrolysis in biomass *in natura*, for reducing costs in relation to pretreatment and obtaining higher yield compared to pre-treated.

HY, using CEE2 (42%), was relatively close to that found in other biomasses, such as elephant grass (53.9%) (Siqueira et al., 2016) and sugarcane bagasse (57.7%) (Andrade et al., 2019), which is one of the main raw materials currently used, for the production of lignocellulosic ethanol. Therefore, it is noted that the pseudostem of banana *in natura* has potential for the use of lignocellulosic ethanol.

4 CONCLUSION

The hydrothermal pretreatment, under the conditions used (121 °C/30min), did not promote large variations in the percentage of cellulose, lignin and hemicellulose. In addition, it may have been the limiting factor in enzymatic activities and, consequently, in the hydrolysis yield.

The *P. oxalicum* F -3380 strain showed potential to produce cellulolytic enzymes. The evaluated enzymatic activities (CMCase, Avicelase and FPase) of the extract

produced by the fungus *P. oxalicum* F -3380, in fresh biomass, obtained superior results compared to the enzymatic extract produced in pretreated biomass, which promotes cost reduction for its production.

The enzymatic hydrolysis of pseudostem *in natura* with CEE1, under the conditions evaluated, considered only the quantity of biomass and enzyme as significant variables. The proposed model was considered significant, which demonstrates to be adequate for the desired responses. The enzymatic hydrolysis of pseudostem *in natura* with CEE2 obtained HY superior to hydrolysis with CEE1, thus demonstrating to be more effective and economical.

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