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Hydrolysis of pineapple crown biomass by cellulolytic enzymes produced by *Fusarium oxysporum*

Hidrólise da biomassa da coroa do abacaxi por enzimas celulolíticas produzidas por *Fusarium oxysporum*

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The development of microbial enzymes is of great importance in biochemical process industries. Among the enzymes of industrial importance, cellulolytic and xylanolytic enzymes stand out, which are responsible for the degradation of lignocellulosic material that is usually discarded by the agro-industrial sector. In order to produce the enzymes CMCase, β-glycosidase, xylanase and β-xylosidase, the fungus *Fusarium oxysporum* was used in liquid fermentation using pre-treated pineapple crown biomass as a carbon source. In order to optimize the production of these enzymes, cultures were carried out for 168 hours, 30 °C at 180 rpm. The biomass underwent a pre-treatment in two stages, the first being submitted to 121 °C for 7 min, while the second was added diluted sulfuric acid to 1%, in the proportion of 1:5 (m/v), at 121 °C for 27 minutes. The partial characterization of the crude extract showed a temperature of 50 ºC as the most satisfactory for the action of these enzymes, and the ideal pH range ranged from 3.5 to 5.5. The enzymatic hydrolysis of pineapple crown, with and without pretreatment, was performed using lyophilized crude extract (CMCase = 9.78 U/mL, β-glucosidase = 10.86 U/mL, xylanase = 27.90 U/mL and β-xylosidase = 0.018 U/ml). An experimental design of the Central Composite Rotational Design was carried out. It was possible to evaluate the effect and influence of pH, biomass concentration and incubation time on the release of glucose in the medium (R^2>0.85); pH and biomass (%) had a significant effect on glucose release.
Keywords: *Ananas comosus*, celullolytics enzymes, experimental planning.

O desenvolvimento de enzimas de origem microbiana é de grande importância nas indústrias de processos bioquímicos. Dentre as enzimas de importância industrial, destacam-se as enzimas celulolíticas e xilanolíticas grandes responsáveis pela degradação do material lignocelulósico que costuma ser descartado pelo setor agroindustrial. Com objetivo de produção das enzimas CMCase, β-glicosidase, xilanase e β-xilosidase, foi utilizado o fungo *Fusarium oxysporum* em fermentação líquida usando biomassa pré-tratada de coroa de abacaxi como fonte de carbono. Visando otimizar a produção dessas enzimas os cultivos foram realizados por 168 horas, 30 °C a 180 rpm. A biomassa passou por um pré-tratamento em duas etapas, sendo a primeira submetida à 121 °C por 7 minutos, já à segunda foi acrescentado ácido sulfúrico diluído a 1%, na proporção de 1:5 (m/v), a 121 °C por 27 min. A caracterização parcial do extrato bruto apontou temperatura de 50 ºC como a mais satisfatória para ação dessas enzimas, e a faixa de pH ideal variou de 3.5 a 5.5. As hidrólises enzimáticas de coroa de abacaxi com e sem pré-tratamento, foram realizadas usando extrato bruto liofilizado (CMCase = 9,78 U/mL, β-glicosidase = 10,86 U/mL, xilanase = 27,90 U/mL e β-xilosidase = 0,018 U/mL). Para tal, foi realizado um planejamento experimental de Delineamento Composto Central Rotacional (DCCR). O qual foi possível avaliar o efeito e influência do pH, da concentração de biomassa e tempo de incubação na liberação de glicose no meio (R^2>0,85); o pH e a biomassa (%) apresentaram efeito significativo sobre a liberação de glicose.
Palavras-chave: *Ananas comosus*, enzimas celulolíticas, planejamento experimental.

1. INTRODUCTION

The use of agro-industrial residues is a trend in the development of research aimed at the production of biofuels and enzymes of commercial interest. These residues basically consist of lignocellulosic biomass, which favors the growth of microorganisms that produce

lignocellulolytic enzymes [1, 2]. Currently, these enzymes represent about 20% of commercially available enzymes [3].

Pineapple residue is rich in simple sugars and carbohydrates, such as cellulose and hemicellulose, which can be hydrolyzed into fermentable sugars. However, it is necessary that the residue is pre-treated and saccharified before fermentation [4].

The pre-treatment aims to prevent the loss of these carbohydrates, prevent the formation of fermentation inhibiting agents and facilitate the formation of fermentable sugars by hydrolysis reactions. In addition to the elimination of lignin, which has an affinity to form bonds with lignocellulolytic enzymes, causing an inhibitory effect [5, 6].

Enzymes are a safe and effective alternative for agro-industrial waste management. The efficiency of the enzymatic action depends on several factors such as temperature, extraction time, pH conditions and substrate availability [7, 8].

Microbial cellulases are mainly obtained by filamentous fungi, such as *Trichoderma reesei* and *Aspergillus niger*. In the search for new microorganisms, *Fusarium oxysporum*, which has the ability to degrade cellulose, has been studied for its high rate of production of endoglycosidases [9, 10].

Fusarium oxysporum is one of the few microbial species with recognized ability to carry out saccharification under aerobic conditions and fermentation under anaerobic conditions. [11]. In addition to the high production of endoglycosidase, *F. oxysporum* is also capable of producing β-glucosity with sufficient activity to prevent a drastic inhibition by excess cellobiose, which directly affects enzymatic hydrolysis [12].

Thus, this work aimed to evaluate the potential of cellulolytic and hemicellulolytic enzymes produced by *F. oxysporum* under submerged conditions to hydrolyze the pineapple crown for the release of glucose.

2. MATERIALS AND METHODS

The experiments were carried out at the Laboratory of Biotechnology/Analysis of Food and Product Purification (LABAP), Federal University of Tocantins, Gurupi, Tocantins, Brazil. *Fusarium oxysporum* strain was isolated from decaying pequi fruits [13], preserved by the Castellani method [14]. The reactivation of the strain was carried out in PDA medium (agar-dextrose-potato) for 5 days at 28 °C.

2.1 Submerged cultivation

Cultivations of *F. oxysporum* were carried out in 125 mL Erlenmeyer flasks containing 20 mL of culture medium with the composition: H_2PO_4 2.0 g/L, (NH_4) 0.4 g/L, urea 0.3 g/L, $MgSO_4$+$7H_2O$ 0.3 g/L, $CaCl_2$ 0.3 g/L, $FeSO_4$+$7H_2O$ 5.0 mg/L, $MnSO_4$+H_2O 1.56 mg/L, $ZnSO_4$+$7H_2O$ 1.4 mg/L, $CoCl_2$ 2.0 mg/L, meat peptone 0.75 mg/L, yeast extract 0.25 g/L and Tween 80 0.2 mg/L. The culture medium was supplemented with 1% pretreated or fresh pineapple crown as a carbon source. The medium was sterilized in an autoclave at 121 °C for 20 min. The cultures were inoculated with 1 mL of spore suspension at a concentration of 10^6 spores per mL at 30 °C under constant agitation of 180 rpm for 120 hours. Samples were taken every 24 hours up to 240 hours to determine the best cultivation time for enzyme production.

2.2 Enzymatic cellulase activities

To determine the optimum pH of the enzymes produced by *F. oxysporum* different pH ranges were used using glycine-sodium hydroxide buffer (pH 8 to 13) and McIlvaine buffer (pH 2.0 to 8.0).

2.2.1 CMCase activity

The determination of endoglucanase enzymatic activity was performed using CMC 1% (w/v) solubilized in McIlvaine pH 5.0 buffer as reaction medium. In test tubes, 400 µl of this medium was added. The tubes were kept at 50 °C for 5 min. Tubes were prepared with 200 µL of DNS reagent, composed of double sodium potassium tartrate tetrahydrate, sodium hydroxide and 3,5-dinitrosalicylic acid [15]. Experiments were taken in duplicate at 540 nm. A unit is defined as the amount of enzyme that releases 1 µmol of glucose per milliliter per minute of reaction.

2.2.2 β-glycosidase activity

Enzyme activity was performed using 1% (w/v) salicin solution in McIlvaine pH 5.0 buffer as reaction medium. In test tubes, 400 µL of this medium were added, the tubes were kept at 37 °C for 5 minutes. Tubes were prepared with 200 µL of DNS reagent, composed of double sodium and potassium tartrate tetrahydrate, sodium hydroxide and 3,5-dinitrosalicylic acid [15]. Experiments were taken in duplicate at an absorbance of 540 nm. A unit is defined as the amount of enzyme that releases 1 µmol of glucose per milliliter per minute of reaction.

2.2.3 Xylanse activity

Enzyme activity was performed using 1% (w/v) xylan in McIlvaine pH 5.0. In test tubes, 400 µL of the reaction medium were added, which were kept at 50 °C for 5 minutes. Tubes were prepared with 200 µL of DNS reagent, composed of double sodium and potassium tartrate tetrahydrate, sodium hydroxide and 3,5-dinitrosalicylic acid [15]. Experiments were taken in duplicate at an absorbance of 540 nm. A unit is defined as the amount of enzyme that releases 1 µmol of xylose per milliliter per minute of reaction.

2.2.4 β-xylosidase activity

β-xylosidase activity was performed using a 0.25% (w/v) solution of p-nitrophenyl β-D-xylopyranoside (pNP-xyl) solubilized in McIlvaine pH 5.0 buffer. In test tubes, 50 µL of the reaction medium was placed and then 150 µL of McIlvaine pH 5.0 buffer was added. Tubes were prepared with 1 mL of saturated sodium tetraborate solution. Tubes with reaction medium and buffer were incubated at 50 °C for 5 minutes. Experiments were taken in duplicate at an absorbance of 410 nm. A unit is defined as the amount of enzyme that releases 1 µmol of p-nitrophenol per milliliter per minute of reaction.

3. ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC BIOMASS

3.1 Acid pre-treatment of pineapple crown

Pineapple crown was dried in an oven at 60 °C for 48 hours. After drying, the biomass was milled in a knife mill in the particle size range between 16 and 60 mesh.

Biomass pretreatment was divided into 2 steps. First a physical pre-treatment at a temperature of 121 °C for 7 min. Then, a chemical pre-treatment that consisted of adding sulfuric acid (H_2SO_4) diluted to 1% to the biomass, in the proportion of 1:5 biomass to acid and heating at 121 °C for 27 minutes according to the methodology of De Araújo et al. (2002) [13].

After pretreatment, the biomass was washed in order to stabilize the pH to neutrality. This biomass was oven dried at 60 °C to constant weight. Four repetitions of this process were performed to determine the biomass yield obtained after this pre-treatment.

3.1.1 Freeze drying of the gross extract

The crude extract obtained from the filtration of cultures was frozen at -20 °C. After this freezing, the crude extract was lyophilized at -47 °C under vaccum.

3.1.2 Enzymatic hydrolysis and hydrolysis analysis

The hydrolysis reactions were carried out in flasks containing 2 mL of buffered medium, biomass with and without pre-treatment and lyophilized crude enzymatic extract (2.5 U/mL). Flasks were incubated with refrigerated orbital shaking at 50 °C under 180 rpm shaking.

To analyze the hydrolysis of the pineapple crown biomass, the enzymatic colorimetric method of the Glucose Liquiform Labtest® kit was used, and a standard curve was performed with a glucose solution (0.1 - 1.0 mg/mL). Samples were taken in a spectrophotometer in the absorbance range of 505 nm, according to the Labtest methodology (2011) [16].

3.2 Experimental planning

Enzymatic hydrolysis experiments were carried out with biomass pretreated or without pretreatment with Central Composite Rotational Design (CCRD) with 3 variables: pH, biomass concentration and incubation time, as shown in Table 1. The program used to carry out the design was STATISTICA Trial®.

Table 1: Actual values of the variables studied in planning the study of pH, biomass and reaction time for biomass with and without pretreatment.

Variables	-1.68	-1	0	+1	+1.68
pH	2.48	3.5	5.0	6.5	7.52
Biomass (%)	1.96	4.0	7.0	10	12.04
Time (hours)	31.7	48	72	96	112.3

4. RESULTS AND DISCUSSION

4.1 Pre-treatment of pineapple crown

After the physical and chemical pre-treatment steps it was obtained 6.37 g, 5.63g, 5.88 g and 6.05 g of treated biomass. The mean pretreatment yield between repetitions in percentage was 29.6% (Table 2). The significant drop in weight is justified by the loss of most of the lignin and hemicellulose that was present in the biomass and by the loss of other compounds that were not directly linked to the cellulose [17].

Table 2: Determination of initial and final weight and biomass yield of pineapple crown after heat and acid pre-treatment.

Repetition	Initial Weight (g)	Final Weight (g)	Performance (%)
1	20.0	6.3	31.5
2	20.0	5.6	28.0
3	20.0	5.8	29.0
4	20.0	6.0	30.0

4.2 Determination of cultivation time

The best time for the production of CMCase and β-glucosidase enzymes was 168 hours, obtaining an activity of 0.90 U/mL for CMCase and an activity of 1.12 U/mL for β-glucosidase, results similar to those found by Santos et al. (2021) [18], when analyzing the production of cellulases by *Penicillium* sp. FSDE15 reaching maximum production for CMCase (1.19 ± 0.06 U/mL) in cultivation using corn bran in 168 hours. In tests with combination of cob and corn bran, the highest activity (1.41 ± 0.05 U / mL) was obtained in 216 hours (Figure 1).

For the xylanase enzyme, the best times were between 144 hours and 240 hours, reaching an activity of 2.50 U/mL. The β-xylosidase enzyme had the best fermentation time with 192 hours, with an activity of 0.002 U/mL (Figure 1).

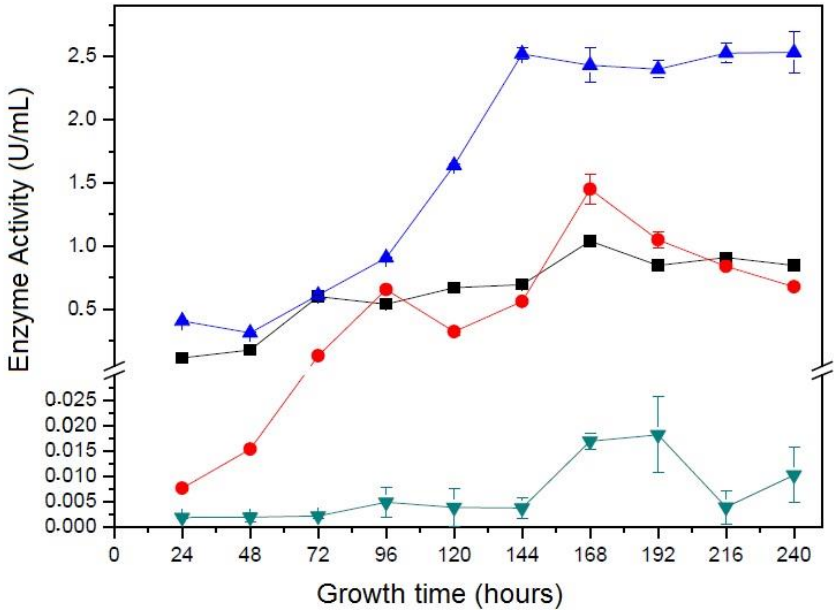

Figure 1: Cellulase (●), β-glucosidase (■), xylanase (▲) and β-xylosidase (▼) production by Fusarium oxysporum *in submerged cultures using pretreated pineapple crown.*

Different results were reported by Kumar et al. (2017) [19], in their work on xylanase production from *Thermomyces lanuginosus* VAPS-24, in which the optimal production of the xylanase enzyme occurred within 120 hours. After 144 hours the activity has decreased, this decline in production may be due to nutrient depletion or production of toxic metabolites.

4.3 Partial characterization of the gross statement

4.3.1 Effect of temperature on the activity of cellulolytic enzymes

The best temperature for the activity of cellulolytic and xylanolytic enzymes produced by *F. oxysporum* was determined by activity tests varying the temperature from 15 to 70 °C. The best temperature of the enzyme CMCase and β-xylosidase was 50 °C, and for the enzymes β-glucosidase and xylanase the optimum temperature was 50 to 60 °C (Figure 2).

Xu et al. (2016) [20], obtained an optimal temperature for cellulase activity at 45 °C, but when the temperature increased to 55 °C, the enzyme lost more than half of its activity. The recombinant *Aspergillus nidulans* xylanase had an optimal temperature of 50 °C [21].

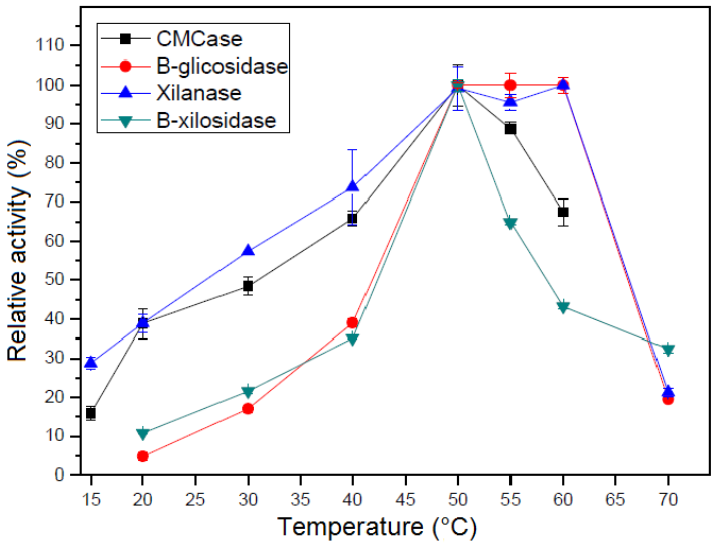

Figure 2: Optimum temperature for enzymatic activity of cellulase, β-glucosidase, xylanase and β-xylosidase enzymes produced by Fusarium oxysporum *under submerged conditions.*

4.3.2 Effect of pH on cellulolytic enzyme activity

For CMCase enzyme, the best activity pH was 3.5, with an activity of 1.53 U/mL; for β-glucosidase the best pH was 5.0, with an activity of 0.58 U/mL. For xylanolytic enzymes more than one optimum pH value was obtained; xylanase showed activity peaks at pH 4.5, 6.5 and 8.5, respectively with activities of 3.22 U/mL, 2.76 U/mL and 2.88 U/mL; for the β-xylosidase enzyme, the best pH range was 4.5 to 5.5 with an average activity of 0.004 U/mL (Figure 3).

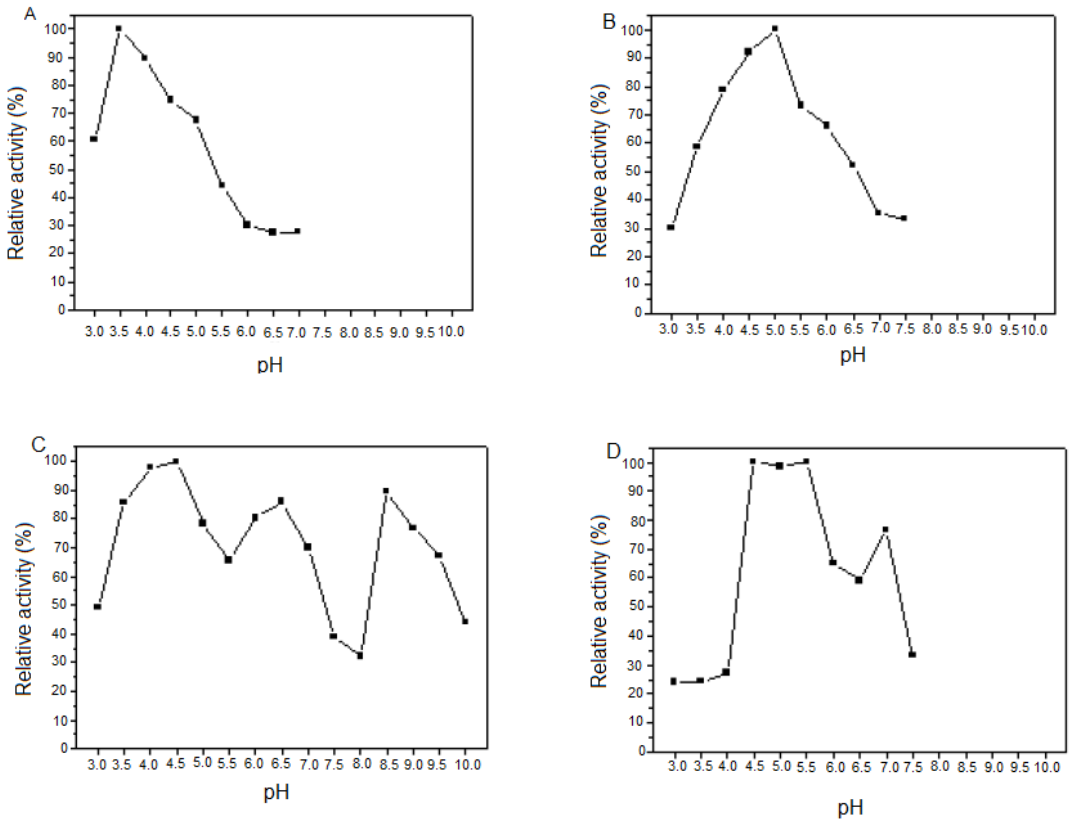

Figure 3: Optimal pH of enzyme activity for CMCase (A), β-glycosidase (B), xylanase (C) and β-xylosidase (D) produced by Fusarium oxysporum *under submerged conditions.*

The pH for enzyme activity is mainly related to the isoelectric point of proteins. With the balance of positive and negative charges of the ionic groups, it is concluded that the active sites of the molecule are free to bind to the target molecules (substrates). Therefore, at the pH where the molecule has a net electrical charge, the greatest formation of the substrate-enzyme complex occurs, with the greatest release of product, maximizing the enzymatic activity.

The CMCase and β-glucosidase enzymes showed, respectively, an acidic pH range, the xylanase enzyme showed an optimal pH for acidic media, neutral and basic, and can be applied to several industrial biochemical processes due to its wide pH range. The β-xylosidase enzyme showed greater activity in the pH range between 4.5 and 5.5.

Singh et al. (2021) [22], when evaluating the production of cellulase and xylanase by *Aspergillus flavus* using agro-industrial residues as a carbon source, obtained optimal conditions of initial pH of 5.5.

4.4 Hydrolysis

4.4.1 Compound central rotational design (DCCR)

The results of glucose release are shown in Table 3.

Table 3: Values of glucose concentrations released into biomass with pre-treatment (PT) and without treatment (ST) using the DCCR experimental design.

Assay	Glucose PT (mg/mL)	Glucose ST (mg/mL)
12	11.18	9.40
5	8.40	0.01
7	14.27	0.31
15 (C)	10.06	10.15
2	0.63	1.70
10	7.66	0.02
17 (C)	10.45	9.09
8	19.75	10.51
4	4.48	1.93
9	0.12	0.30
14	10.36	7.84
16 (C)	10.03	10.30
1	1.09	1.12
11	2.70	1.33
3	1.89	3.09
13	4.27	10.48
6	14.63	2.00

Experimental conditions: 250 µL of lyophilized enzymatic extract, 50 °C and 180 rpm.

The highest values of glucose release for biomass PT occurred in tests 6, 7 and 8; all at pH 6.5 and outside the endpoints for biomass and incubation time. For ST biomass, the best glucose release values were recorded in tests 8, 13 and 16. Test 8 at pH 6.5 and the other two at pH 5.0. Untreated biomass has lignin, which justifies the relatively lower values, as lignin forms irreversible bonds with the enzymes responsible for hydrolysis [23, 24].

The lowest values of glucose release from the PT biomass occurred in tests 1,2 and 9; where 1 and 2 had a pH of 3.5 and test 9 consisted of the minimum pH endpoint, with a value of 2.48. For ST biomass, the lowest values of glucose release were observed in tests 5, 9 and 10; where test 5 had a pH of 6.5 and tests 9 and 10 corresponded to the minimum (2.48) and maximum (7.52) pH endpoints.

The use of biomass without any type of treatment is not common in works that aim to release glucose by the hydrolysis process. In the literature, the main lines of research seek to optimize the conditions of hydrolysis, mainly facilitating the access of enzymes to crystalline cellulose.

Saini et al. (2016) [6] showed that the pretreatment steps are important to increase the accessibility of glucose and xylan to hydrolytic enzymes, and the yield of this hydrolysis is related to the type of pretreatment adopted.

4.4.2 Statistical data analysis

The estimate of the effect of pH, biomass concentration and time for biomass with pretreatment (PT) is shown in Table 4 and for biomass without treatment (ST) in Table 5.

Table 4: Effects of independent variables on biomass with pretreatment (PT) for glucose release using the DCCR experimental design.

Factors	R^2	R^2 adj.	Effect	Error	T	P
(1)pH (L)			9.029	1.727	5.225	0.001
pH (Q)			-2.951	1.901	-1.551	0.164
(2)Biomass (L)			4.379	1.727	2.534	0.038
Biomass (Q)			-0.798	1.901	-0.419	0.687
(3)Time (L)	0.857	0.673	3.532	1.727	2.044	0.080
Time (Q)			-0.530	1.901	-0.279	0.788
1 (L) by 2 (L)			1.590	2.257	0.704	0.503
1 (L) by 3 (L)			2.393	2.257	1.060	0.324
2 (L) by 3 (L)			0.575	2.257	0.255	0.806

Table 5: Effects of independent variables on untreated biomass (ST) for glucose release using the DCCR experimental design.

Factors	R^2	R^2 adj.	Effect	Error	T	P
(1)pH (L)			0.665	1.593	0.417	0.688
pH (Q)			**-7.515**	**1.753**	**-4.286**	**0.003**
(2) Biomass (L)			3.600	1.593	2.260	0.058
Biomassa (Q)			-3.836	1.753	-2.188	0.064
(3) Time (L)	0,807	0,560	1.050	1.593	0.659	0.530
Time (Q)			-1.147	1.753	-0.654	0.533
1 (L) by 2 (L)			1.651	2.081	0.793	0.453
1 (L) by 3 (L)			3.189	2.081	1.532	0.169
2 (L) by 3 (L)			1.621	2.081	0.778	0.461

The regression R^2 values obtained (0.857 for PT biomass and 0.807 for ST biomass) were considered good, indicating that the experimental points fit the model. For the values of R^2 adj. of regression (0.673 for PT biomass and 0.560 for ST biomass) it is considered that the coefficient of determination of the model does not adequately adjust to the experimental points [25, 26].

For biomass PT (Table 3), it can be seen that the linear effect of pH had a positive response on the release of glucose, in parallel, the linear effect of biomass concentration also had a positive response. For the ST biomass (Table 4), it was observed that only the quadratic effect of pH showed a significant response, which was negative on hydrolysis. The evaluated effects were significant at the level of 5% in the induction of glucose release.

4.4.3 Reparameterized equation and response surface

A reparametrized regression model was developed taking into account only the significant variables, to predict the glucose concentration released by hydrolysis as a function of the linear effects of pH (LX1) and biomass concentration (LX2) and the quadratic effect of pH (QX4) for biomass PT (Equation 1) and for biomass ST (Equation 2).

$$Glucose\ \left(\frac{mg}{mL}\right) = 9{,}363 + 4{,}514X1 + 2{,}189X2 \quad \text{(Eq. 1)}$$

$$Glucose\ \left(\frac{mg}{mL}\right) = 9{,}642 - 3{,}757X4 \quad \text{(Eq. 2)}$$

Quadratic models act as a three-dimensional function to generate a response surface. Figure 4 represents the response surface for biomass hydrolysis PT taking into account the pH and biomass (%) parameters, Figure 5 represents the response surface for the biomass hydrolysis ST considering the pH and biomass parameters (%).

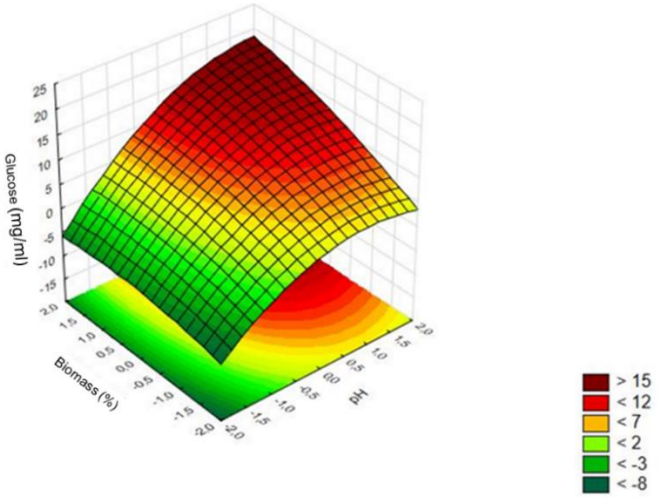

Figure 4: Response surface graph of the influence of pH and biomass on glucose release using pretreated biomass. Values according to the design.

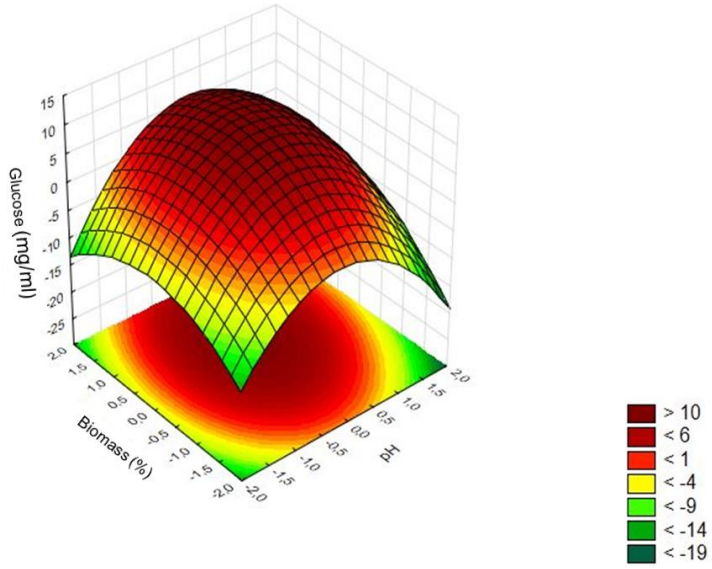

Figure 5: Response surface graph of the influence of pH and biomass on glucose release using biomass without pretreatment. Values according to the design.

From the response surface graph, it can be concluded that for the PT biomass, the best value of released glucose concentration is reached with the increase in pH to the central point; the biomass concentration had a progressive effect between the minimum and maximum points.

For biomass ST, the quadratic effect of pH was high in relation to the other variables, thus forming a parabola, indicating that an intermediate pH is ideal for hydrolysis, noting that biomass (%) also had a parabolic effect, but not as expressive as the pH.

5. CONCLUSION

Pineapple crown proved to be a good source of carbon for the growth of *F. oxysporum* and for the production of cellulolytic and xylanolytic enzymes, mainly xylanase.

The pre-treatment, with an average yield of almost 30%, favored the saccharification of cellulose into glucose, when compared to the hydrolysis of biomass without treatment.

Statistical planning was efficient in determining the effect and response of variables (pH, biomass concentration and incubation time) in relation to glucose release, since pH is the main factor that has an effect on this process.

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