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CELLULASES AND XYLANASES PRODUCTION BY *Penicillium citrinum* CGETCR USING COFFEE PULP IN SOLID STATE FERMENTATION

PRODUCCIÓN DE CELULASAS Y XILANASAS POR *Penicillium citrinum* CGETCR USANDO PULPA DE CAFÉ EN FERMENTACIÓN EN ESTADO SÓLIDO

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Abstract
This work reports, for the first time, the use of coffee pulp residue to the expression of cellulolytic and xylanolytic enzymes by the native fungus *Penicillium citrinum* CGETCR by solid-state fermentation (SSF). Coffee is one of the most important drinks in the world producing several residues such as coffee pulp, which is considered a contaminant if not properly disposed. This residue causes damage to the fauna from rivers due to its composition including tannins, pH 3.5, (1-4%) caffeine, polyphenolic compound, ochratoxin A, the presence of the latter three have toxic or nephrotoxic effects on higher animals. For these reasons, a study on the use of these residues to obtain high-added value products as enzymes of biotechnological interest, was performed. The enzyme production was optimized using the response surface methodology in a composite central design to study pH, moisture and fermentation time using endoglucanase activity as the response variable. The enzymatic activities found were β-glucosidases, endoglucanases, exoglucanases and xylanases which were able to function under acidic (4.0-5.5) and basic (7.5-8.0) conditions. Additionally, protein extract was used on agroindustrial residues as substrate to evaluate reducing sugar production.
Keywords: surface response, enzymatic hydrolysis, phenolic compounds, solid state fermentation, saccharification of agroindustrial wastes.

Resumen
Este trabajo reporta, por primera vez, el uso de residuos de pulpa de café para la expresión de enzimas celulolíticas y xilanolíticas por el hongo nativo *Penicillium citrinum* cepa CGETCR por fermentación en estado sólido. El café es una de las bebidas más importantes a nivel mundial produciendo diversos residuos como la pulpa de café, la cual es considerada un contaminante, si no se trata adecuadamente. Se ha demostrado que este residuo causa daños en la fauna de los ríos ya que contiene taninos, cafeína (1-4%), alto contenido de polifenoles, presencia de ocratoxina A y una alta acidez (pH 3.5). Por estos motivos, se realizó un estudio sobre el aprovechamiento de estos residuos para obtener productos de alto valor agregado como son enzimas de interés biotecnológico. La expresión enzimática fue optimizada usando la metodología de superficie de respuesta mediante un diseño central compuesto para estudiar el pH, humedad y tiempo de fermentación teniendo como variable de respuesta la actividad endoglucanasa. Las actividades enzimáticas encontradas fueron β-glucosidasas, endoglucanasas, exoglucanasas y xilanasas las cuales fueron capaces de funcionar bajo condiciones de acidez (4.0-5.5) y basicidad (7.5-8.0). Adicionalmente, el extracto enzimático se usó sobre residuos agroindustriales para evaluar la producción de azúcares simples.
Palabras clave: superficie de respuesta, hidrólisis enzimática, compuestos fenólicos, fermentación en estado sólido, sacarificación de residuos agroindustriales.

1 Introduction

The main activity of the coffee industry is the processing of fruits to obtain coffee beans. These processes are typically dry or wet processes, which result in differing coffee quality, solid waste generated and coffee species used (Murthy and Naidu 2012a; Mussatto *et al*. 2011). "Arabica" (*Coffea arabica*) and "robusta" (*Coffea canephora*) are the most commonly grown varieties, particularly the former. Arabica coffee is wet processed, and each ton of processed coffee cherries is known to generate a half-ton of solid residues, which mainly consist of coffee pulp.

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Conversely, in dry processing, each ton of processed coffee cherries generates 0.18 tons of coffee husk (Mussatto *et al*. 2011; Roussos *et al*. 1995). Both processes have certain degrees of pollution because the residues are not adequately treated and are deposited in soil or bodies of water (Murthy and Naidu 2012a), causing damage to nearby flora and fauna. The presence of caffeine in water bodies may cause damage to organisms inhabiting these ecosystems, as has been demonstrated with African frog embryos (*Xenopus laevis*), which are killed when exposed to caffeine concentrations of 0.48 ng/L and higher (Fort *et al*., 1998). On the other hand, the composition of the coffee pulp is quite interesting because it consists of carbohydrates 33.7-42.9%, cellulose 20.7-63.0±2.5%, hemicellulose 2.3-3.6%, lignin 14.3-17.5±2.2, proteins, minerals (Na, Ca, Zn, Mn, Mg, Cu, Fe y K), tannins, polyphenols and caffeine (Bonilla-Hermosa *et al*., 2014; Murthy and Naidu, 2012; Ramírez-Velasco *et al*., 2016), which makes it an ideal substrate to be applied in enzyme production processes such as solid state fermentation (SSF). Therefore, this study aimed to assess the coffee pulp potential using a native strain of *Penicillium citrinum* CGETCR isolated from coffee pulp vermicompost (Coutiño-Gutiérrez, 2014) to establish a cellulases and xylanases production system by optimizing the growth conditions of the microorganism at laboratory scale using SSF, characterizing qualitatively and quantitatively both enzymatic activity. Also, the total polyphenols (which are present in the residues of this beverage industry) were measured because they are considered the main sources of contaminants in aquifers and soils. By last, enzymes expressed in this system were evaluated in a saccharification assay of two agroindustrial residues (sugarcanne and coffee pulp) which are used in industrial boilers as a source of energy or simply discarded in soil or aquifer systems.

2 Materials and methods

2.1 Substrate conditioning and characterization

The coffee pulp used for this study was provided by a coffee plantation located in Carrillo Puerto Ejido in the municipality of Tapachula, Chiapas, Mexico. The sample was collected immediately after pulping and transported to the laboratory for conditioning and analysis. A physical treatment was performed, which consisted of the following steps: tap water washing, sun drying, particle size reduction by grinding, and sieving through a mesh 18. The coffee pulp was characterised by bromatological analysis, which consisted of assessing the crude protein (NMX-F-608-NORMEX-2011), fat (NMX-F-615-NORMEX-2004), moisture (NOM-116-SSA1-1994) and ash (NMX-F-607-NOMRMEX-2013) content. Crude fibre was determined by neutral and acid detergent digestion. The contents of cellulose, hemicellulose and lignin were determined using the method described by Van Soest (1994), the carbohydrates were determined by calculation. Calorific energy was determined by closed combustion in the presence of oxygen, according to Analytical Methods for Oxygen Bombs (1987).

2.2 Microorganism and inoculum preparation

A native strain of *P. citrinum* CGETCR, isolated from a vermicompost sample, composed of cattle waste and coffee pulp residues and transformed by *Eisenia foetida*, was used in this study (Coutiño-Gutierrez, 2014). The filamentous fungus was cultivated on potato dextrose agar (PDA) medium at 28 °C. Spore recovery was performed in the PDA culture after 6 days of sporulation in 10 mL of a sterile 0.9% NaCl solution. The solution was then centrifuged at 3,000 g for 10 min and used as inoculum in SSF. To store the fungus the spores were preserved in 20% glycerol at -20 ° C.

2.3 Scanning electron microscopy

Penicillium citrinum CGETCR colonization on the coffee pulp was observed using scanning electron microscopy (TOPCON, model SM-510, Japan). The following two samples were processed: uninoculated coffee pulp and coffee pulp with fungus growth. Samples were fixed in 3% glutaraldehyde and 0.1 M sodium cacodylate buffer solution during 1 h. Samples were dehydrated with increasing ethanol concentrations, changing from 30, 50, 70, and 90 to 100% every 15 min. Drying was performed with two changes of hexamethyl disilasan at 10- and 5-min intervals. The samples were mounted in aluminium cylinders, covered with a gold-palladium layer approximately 20 mm thick. Observations were made under high vacuum at a 10-kV acceleration.

2.4 *Solid-State Fermentation*

Five grams of coffee pulp with a particle size of 1 mm were placed in 125-mL Erlenmeyer flasks. Impregnation medium (0.5% urea, 2 g/L K_2HPO_4 and 0.3 g/L $MgSO_4 \cdot 7H_2O$) was added to adjust the substrate moisture according to the experimental design outlined in Table 1. Subsequently, the solid medium was sterilised at 121 °C for 20 min, inoculated with 1×10^8 spores/g of substrate and incubated at 28 °C for 144 h. Then, 30 mL of 0.1 M sodium acetate buffer pH 5.5 was added to prepare the enzymatic extract. The extract was centrifuged at 1,000 g for 10 min at room temperature.

2.4.1 *SSF Optimization by response surface method*

A 2^3 experimental design was used to optimize cellulase production from *P. citrinum* CGETCR and coffee pulp by SSF, the selected variables were pH, moisture and culture time, and endoglucanase activity was the response variable used to determine the mathematical optimum. Then, six central points and six axial points, as outlined in Table 1, were added to the experimental design. The response surface method, which comprises adjusting data provided to a second-order regression model, was applied to optimise the process, according to the following equation:

$$Y = \beta_0 + \Sigma_{i=1}^{3}\beta_i x_i + \Sigma_{i=1}^{3}\beta_{ii} x_i^2 + \Sigma_{i=1}^{2}\Sigma_{j=i+1}^{3}\beta_{ij} x_i x_j \quad (1)$$

where Y is the response variable, β_0 the intersection, β_i the coefficient of linear terms, β_{ii} the coefficient of quadratic terms and β_{ij} the coefficient of interactions between two factors. Data analysis was performed using the software R (R Core Team, 2014) by response surface methodology "RSM" (Lenth 2009).

2.5 *Enzymatic activity*

One unit of enzyme activity (U) was defined as the amount of enzyme that produced 1 μmol of glucose, xylose or 4-nitrophenol per minute under following assay conditions. Endoglucanase, exoglucanase and xylanase activity were determined by incubation with carboxymethylcellulose (CMC), crystalline cellulose and xylan from birch wood substrates, all at 1% concentrations, by assessing the reducing sugars using the method of Miller (1959). The assay was performed by using 0.1 M sodium acetate buffer pH 5.5, incubating at 50 °C for 30 min, and measuring the absorbance of the samples at 540 nm.

Table 1. Factors and coded levels used in central composite design for optimization of SSF.

Factors	Label	Coded levels				
		(-α)	(-1)	(0)	(+1)	(+α)
pH	A	5.318	6	7	8	8.682
Moisture (%)	B	29.77	40	55	70	80.23
Time (h)	C	71.44	96	132	168	192.55

The amount of released sugars was quantified using glucose and xylose standards respectively. β-glucosidase activity was determined by incubation with 10 mM 4-nitrophenyl β-D-glucopyranoside (pNPG) in 0.1 M sodium acetate buffer pH 5.5 at 50 °C for 15 min. The reaction was stopped by adding 2 M Na_2CO_3. Sample absorbance was measured at 400 nm, a standard curve of 4-nitrophenol was prepared. Each experiment was performed in triplicate.

2.5.1 *Cellulase and xylanase production kinetics*

Enzyme production kinetics was assessed according to the previously optimized conditions (pH 7.11 and 61.79% moisture). Samples were collected at 24, 48, 72, 96, 120, 144, 168, 192 and 216 h, assessing endoglucanase, exoglucanase, β-glucosidase and xylanase activity according to the previous section. The amount of proteins was determined using the method of Bradford (1976). Each experiment was performed in triplicate.

2.5.2 *pH and temperature effects on endoglucanase, β-glucosidase and xylanase activity*

The optimal pH values for endoglucanase, β-glucosidase and xylanase activity were determined by incubation with the corresponding substrate, according to section enzymatic activity, in 0.1 M sodium acetate buffer at pH 4.0-5.5, 0.1 M sodium phosphate buffer at pH 6.0-8.0 and 0.1 M glycine-NaOH buffer at pH 8.5-9.0. The optimal temperature was determined for each activity assessed at 40, 45, 50, 55, 60, 65, 70 and 75 °C, with incubation in the buffer with the optimal pH. Each experiment was performed in triplicate.

2.5.3 *Assessment of hydrolytic enzyme activity*

Raw sugarcane bagasse and coffee pulp were used in both with alkaline pretreatment and without pretreatment, with a particle size of 250 μm. Alkaline pretreatment consisted of dilute sodium hydroxide solution of 1% (w/v) to achieve solid loading of 10%

on a dry basis. Pressure cooking was carried out in a vertical sterilizer for 30 min at 121 °C at 15 psi. (Brijwani *et al*., 2010). One gram of each dry substrate was placed in 20 mL of 0.1 M sodium acetate buffer pH 5.5 with 0.01% sodium azide and 10, 11 y 1 U of endoglucanase, xylanase and β-glucosidase activities, respectively, using the enzymatic extract obtained by SSF under the previously optimised conditions (pH 7.11, 61.79% moisture and a 145.51 h fermentation time) and incubated at 50 °C/150 rpm. A sample was collected from each hydrolysate at 24, 48, 72, 96, 120, 144, 168 and 192 h to assess the released reducing sugars using the method of Miller (1959). Each experiment was performed in triplicate.

2.6 Determination of phenolic compounds

The content of total phenolic compounds present in the crude enzymatic extract was determined by the Folin-Ciocalteu method, according to the procedure described by Singleton *et al*. (1999), with some modifications proposed by Zuorro and Lavecchia (2012). The content of total phenols was expressed as milligrams of gallic acid equivalents per grams of sample (mg GAE/ g sample) using a gallic acid calibration curve. Each experiment was performed in triplicate.

3 Results and discussion

3.1 Physicochemical analysis of coffee pulp

The results from the bromatological analysis performed on wet-processed coffee pulp showed that the coffee pulp had a similar composition to that reported by other authors with respect to certain parameters (moisture, protein, fat, carbohydrates, neutral detergent fiber and cellulose) as shown in Table 2. However, the contents of cellulose, lignin and tannins differed from the values reported by Bonilla-Hermosa *et al*. (2014) and Murthy and Naidu (2012b). This was likely due to the varying chemical compositions of the coffee residues as a function of specific factors, including altitude, variety and growth conditions, as previously indicated by Elías (1979) and Pandey *et al*. (2000).

Table 2. Chemical composition of coffee pulp

Parameters %	**This work**	**Bonilla-Hermosa *et al*. (2014)**	**Murthy and Naidu (2012)**	**Ramírez-Velasco *et al*. (2016)**
Moisture	86.09	82.44	-	6.9
Ashes	6.36	7.33	-	9.6
Protein	10.48	14.79	11.5±2.0	15.3
Fiber	32.55	14.1	-	23.9
Fat	1.32	1.2	2.0±2.6	1.2
Carbohydrates	49.31	-	-	42.9
NDF*	69.79	-	60.5±2.9	-
ADF**	65.59	-	-	-
Hemicellulose	4.19	3.6	2.3±1.0	-
Lignin	44.98	14.3	17.5±2.2	-
Cellulose	18.99	20.7	63.0±2.5	-
Silica	0.79	-	-	-
Condensable Tannins (mg/kg)	0.007	-	3.0±5.0	-
Calorific energy (kcal/g)	0.32	-	-	-

*NDF: neutral detergent fiber
**ADF: acid detergent fiber

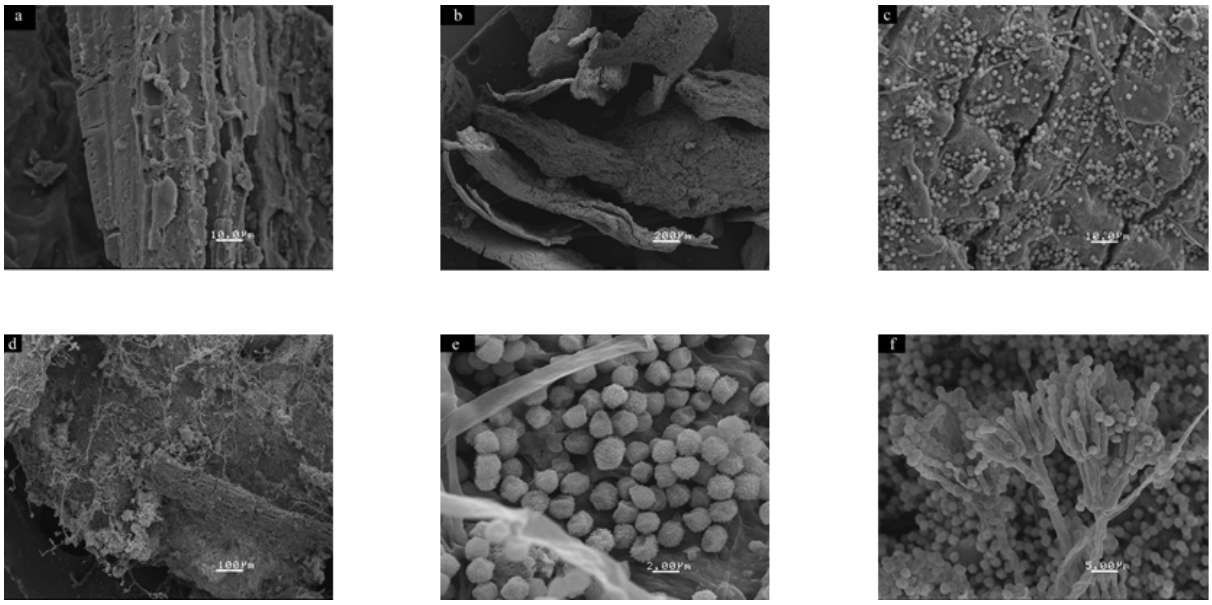

Fig. 1. Microphotographs by scanning electron microscopy, a) and b): surface of coffee pulp; c) and d): colonization of coffee pulp by *P. citrinum* CGETCR; and e) and f): morphology of *P. citrinum* CGETCR.

The presence of carbohydrates, lipids, and proteins make the crude coffee pulp a suitable substrate to use as a solid support and as a source of carbon and energy to grow microorganisms in SSF (Pandey *et al*. 2000).

3.2 Substrate colonization in SSF

The structure of the materials used in SSF is very important because it markedly affects how easily microorganisms may affect substrate colonization through mycelial growth. Figures 1a and 1b show that coffee pulp has a structure with micropores, clefts and capillaries on the surface. These structures were spacious enough to allow substrate colonization via penetration by *P. citrinum* CGETCR vegetative hyphae, as shown in Figures 1c and 1d. Figures 1e and 1f show that the only microorganism growing on the coffee pulp was *P. citrinum* CGETCR, i.e., no other contaminants, such as organisms from the *Penicillium* genus with septate hyphae, conidiospores and spherical conidia with rough walls, were observed (Figure 1e). The microphotographs in Figures 1c and 1d clearly showed fungal growth on the coffee pulp, despite the presence of anti-physiological and anti-nutritional factors (caffeine, chlorogenic acid, tannins, etc.) in this residue. This residue is considered a non-suitable substrate for bioconversion processes (Pandey *et al*. 2000). However, *P. citrinum* CGETCR should have assimilated part of the compounds embedded in coffee pulp for proliferation and as sources of carbon, nitrogen and minerals because the impregnation medium contained minimum nutrients.

3.3 SSF optimization by the response surface method

The SSF process was optimized to maximize cellulase production under the studied system using the response surface method by applying a composite central design (see Table 3). The data obtained were adjusted to a mathematical model that correlated the studied factors (i.e., pH, percentage of moisture and time) to the response variables (i.e., endoglucanase activity) by second-order regression analysis, resulting in the following equation: $y_i = 0.527 - 0.011A + 0.142B + 0.023C - 0.052AB + 0.104AC - 0.057BC - 0.018A^2 - 0.127B^2 - 0.012C^2$. Data relevance was assessed using the coefficient of determination (R^2), which was 0.916, indicating that 91.60% of the response variability was explained by the model (see Table 4). The R^2 value also showed that the equation obtained sufficiently predicted the response of theoretical values and corroborated the experimental values. These results were also confirmed by the lack of model fit, which was not significant (5 and 10 degrees of freedom, 3.494 F value and a p value of 0.097). The analysis of variance outlined in Table 5 indicated that the studied factors and their

interactions were significant. Thus, the pH, moisture content, fermentation time and interactions affected cellulase production in SSF, as shown in Figure 2. The substrate moisture content was the variable that most affected the solid-state fermentation process. Experiment 11 (see Table 3) showed that cellulase expression was repressed for moisture percentages lower than 30%. This moisture was insufficient for *P. citrinum* CGETCR proliferation because low moisture content results in reduced substrate nutrient solubility. Conversely, endoglucanase activity began to decrease at moisture percentages higher than 80% because high moisture content decreases porosity, enhances tackiness and decreases oxygen transfer (Bansal *et al*., 2012). Figure 2a shows that the highest enzymatic activities were obtained at moisture values ranging from 50 to 70%. The contributions of pH and enzyme production time were smaller than that of the moisture effect, as shown in Figures 2b and 2c. The optimization of the SSF process using *P. citrinum* CGETCR and coffee pulp substrate resulted in optimal values of pH 7.11, 61.79% moisture and a 145.51 h fermentation time. This provided a theoretical maximum endoglucanase activity of 0.563 U/g. It is noteworthy that no pretreatment for the removal of phenolic compounds from the crude enzymatic extracts was performed in this study. Therefore, phenols may have likely affected the enzymatic activity studied because reports have shown that these compounds absorb proteins and deactivate cellulolytic enzymes (Ximenes *et al*., 2011).

Table 3. Central composite design for the optimization of cellulases production

Run	pH	% Moisture	Time, h	Endoglucanase activity, Y (U/g)	
	(A)	(B)	(C)	Y exp	Y pred
1	6(-1)	40(-1)	96(-1)	0.145	0.21
2	8(+1)	40(-1)	96(-1)	0.036	0.082
3	6(-1)	40(-1)	168(+1)	0.083	0.161
4	8(+1)	40(-1)	168(+1)	0.491	0.453
5	6(-1)	70(+1)	96(-1)	0.638	0.715
6	8(+1)	70(+1)	96(-1)	0.416	0.377
7	6(-1)	70(+1)	168(+1)	0.444	0.437
8	8(+1)	70(+1)	168(+1)	0.544	0.519
9	5.318(-α)	55(0)	132(0)	0.602	0.494
10	8.682(+α)	55(0)	132(0)	0.405	0.456
11	7(0)	29.77(-α)	132(0)	0	0
12	7(0)	80.23(+α)	132(0)	0.393	0.408
13	7(0)	55(0)	71.44(-α)	0.524	0.453
14	7(0)	55(0)	192.55(+α)	0.518	0.531
15	7(0)	55(0)	132(0)	0.462	0.527
16	7(0)	55(0)	132(0)	0.473	0.527
17	7(0)	55(0)	132(0)	0.536	0.527
18	7(0)	55(0)	132(0)	0.533	0.527
19	7(0)	55(0)	132(0)	0.55	0.527
20	7(0)	55(0)	132(0)	0.602	0.527

Y: Endoglucanase activity; Y exp: Experimental Endoglucanase activity; Y pred: Predicted Endoglucanase activity

Table 4. Coefficients for the second-order regression model

Factor	Coefficient	Standard Error	t-value	p-value Prob > t*
Intercept	0.527	0.031	16.667	1.27E-08
A	-0.011	0.021	-0.538	0.602
B	0.142	0.021	6.791	4.79E-05
C	0.023	0.021	1.104	0.295
AB	-0.052	0.027	-1.917	0.084
AC	0.104	0.027	3.821	0.003
BC	-0.057	0.027	-2.09	0.063
A^2	-0.018	0.02	-0.906	0.385
B^2	-0.127	0.02	-6.215	9.95E-05
C^2	-0.012	0.02	-0.604	0.559

*The values of Prob>t less than 0.05 indicate that the terms of the model are significant.; R^2 0.916, adj R^2 0.840.

Table 5. Analysis of variance for composite central design

Source	Squares of squares	Df1	Mean square	*F*-value	*p*-value Prob > F*
A,B,C	0.286	3	0.095	15.876	3.94E-04
AB,AC,BC	0.136	3	0.045	7.551	0.006
A^2,B^2,C^2	0.233	3	0.077	12.905	8.98E-04
Residuals	0.06	10	0.006		
Lack of fit	0.046	5	0.009	3.493	0.097
Pure error	0.013	5	0.002		

*The values of Prob>F less than 0.05 indicate that the terms of the model are significant; [1]Degrees of freedom.

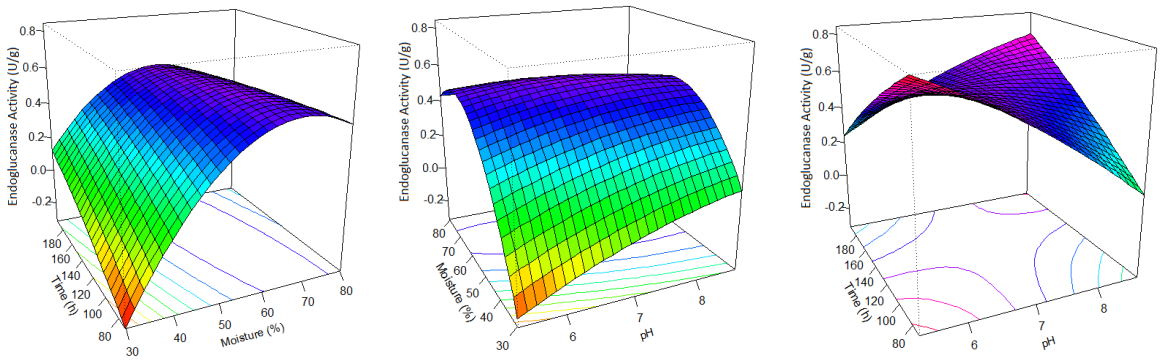

Fig. 2. Response surfaces that show: a) effect of time and moisture, b) effect of moisture and pH and c) effect of time and pH, on the production of cellulases by *P. citrinum* CGETCR cultivated in coffee pulp by SSF at 28 °C.

Conversely, it is noteworthy that no previous studies have reported the use of crude coffee pulp to produce cellulases and xylanases using *P. citrinum* CGETCR, thereby highlighting the importance of analysing the cellulases expressed under this environment.

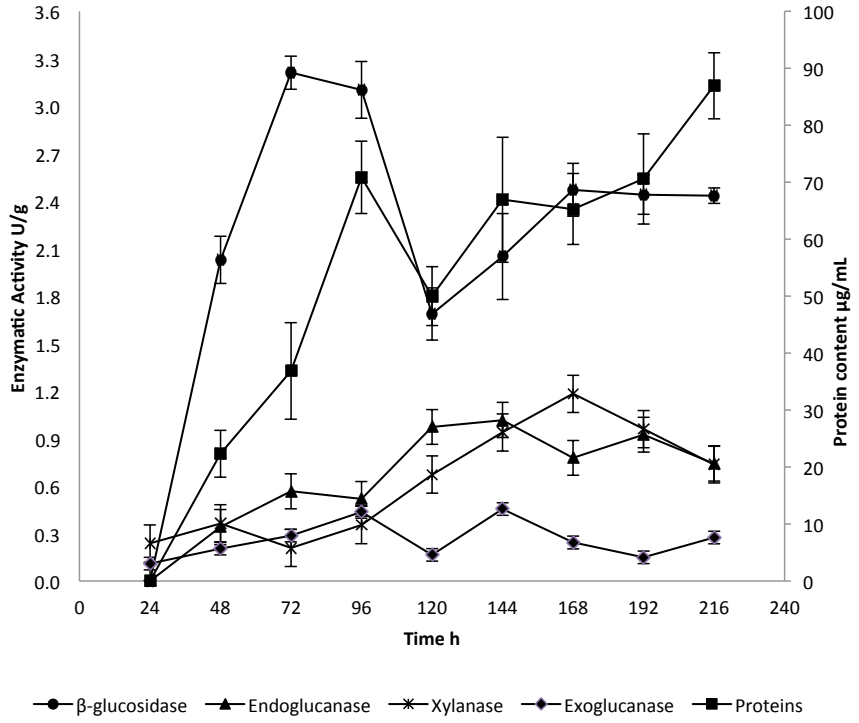

Fig. 3. Kinetics of cellulases and xylanases expression by SSF from *P. citrinum* CGETCR and coffee pulp at pH 7.11, moisture 61.79% and 28 °C. All the results were expressed in mean ± SD from three separated experiments.

3.4 Enzyme production kinetics

The optimal conditions found in the optimization of the SSF fermentation were used for the enzymes production. Various hydrolytic enzymes are produced during *P. citrinum* CGETCR growth on coffee pulp, including cellulases and xylanases, which are essential for cellulose and hemicellulose hydrolyses. Therefore, the expressions of these enzymes and amount of protein were assessed with respect to the SSF process time. Figure 3 shows that the maximum activities of β-glucosidase occurred at 72 and 168 h with activities of 3.212 and 2.472 U/g, respectively; endoglucanase activity peaked at 72, 144, and 192 h with activities of 0.567, 1.018 and 0.924 U/g; exoglucanases peaked at 96 and 144 h with activities of 0.436 and 0.454 U/g; and xylanases peaked at 48 and 168 h with activities of 0.364 and 1.183 U/g. Each enzymatic activity studied showed the presence of two or three peaks of enzymatic activity, which may be related to the presence of isoenzymes. β-glucosidase had the highest enzymatic activity among the enzymes studied. It is well known that *Penicillium* species extensively produce these enzymes, even at higher quantities than those produced by the most studied microorganism *Trichoderma reesei* (Un *et al*., 2014). The values of the enzymatic activity determined per gram of substrate were not as high as those reported in other studies using by-products of the coffee industry. For example, Bhoite *et al*., 2013, reported a maximum production of β-glucosidases of 1,991.17 U/g from coffee pulp (with pretreatment) and *Penicillium verrucosum*. Murthy and Naidu (2012b), reported a maximum production of xylanases of 14,765 U/g substrate using crude coffee pulp and *P. citrinum*. Navya and Murthy (2012) reported values of endoglucanase production of 22,109 U/g substrate using coffee husk (with pretreatment) and *Rhizopus stolonifer*. Although the values of enzymatic activity determined in this study were not similar to those reported in the afore mentioned studies, it is noteworthy that the protein concentrations assessed in this study were also low. Furthermore, Bhoite *et al*. (2013) performed substrate pretreatment with vapor to remove lignin, whereas only the crude substrate was used in this study. Although *P. citrinum* CGETCR proliferated under the previously determined conditions, as shown by the microphotographs in Figure 1, the presence of

polyphenolic compounds in the enzymatic extract and residual coffee pulp could have had a significant effect on the protein concentrations reported and on the values of enzymatic activity measured. Reports have shown that the hydroxyl phenolic groups associated with tannins and lignin absorb proteins and deactivate cellulolytic enzymes and β-glucosidases during the hydrolysis of microcrystalline cellulose (Ximenes *et al*., 2011). Finally, it is also noteworthy that cellulases with cellulose-binding domains could have remained attached to the cellulose fibers or lignin; reports have shown that non-specific bonds with the latter polymer may occur (Rahikainen *et al*., 2013).

These results show that the enzyme production by *P. citrinum* CGETCR is according with the results found in the SSF optimization, and the maximum endoglucanase activity was obtained at 145.51 h.

3.4.1 Characterization of crude enzymatic extract

The crude enzymatic extract showed more than one optimal pH for the assessed enzymatic activities (Figure 4). β-glucosidase showed optimal pH at 5.0 and 6.5, endoglucanase at 5.5 and 8 and xylanase at 7.5 and 4.0, maintaining more than 50% of their catalytic activity. The different enzymes were expressed at different times based on cellulase and xylanase production kinetics. Furthermore, these enzymes each had more than one optimal pH where they show catalytic activity. These results suggested the presence of isoenzymes for the measured activities. Therefore, these results indicate that *P. citrinum* CGETCR expressed an enzymatic complex consisting of various types of cellulases and xylanases required for substrate hydrolysis when grown on coffee pulp without pretreatment. These enzymes may function under both acidic and basic conditions, making this enzymatic extract attractive for various potential applications, including the hydrolysis of lignocellulosic residues, which requires various hydrolytic enzymes to perform the hydrolysis of cellulose and xylan. The optimal temperatures for cellulases and xylanases in the crude enzymatic extract are shown in Figure 5. *P. citrinum* CGETCR β-glucosidase showed a higher enzymatic activity from 65 to 70 °C and showed more than 60% of its catalytic activity at 55 °C. Endoglucanases and xylanases had the highest enzymatic activity at 60 °C and maintained more than 50% of their catalytic activity at 40 °C. The β-glucosidase of *P. citrinum* CGETCR was acidophilic and thermophilic because it had a higher enzymatic activity at low pH (4.0-5.5) and high temperature (65-70 °C) ranges. These results corroborated data reported by Ng *et al*. (2010), who found that *P. citrinum* YS40-5 β-glucosidase activity had optimal pH and temperatures of 5.0 and 70 °C, respectively. Endoglucanase at an optimal pH of 5.5 had an optimal temperature of 60 °C, which corroborated the results of a study by Dutta *et al*. (2008), who reported endoglucanases with similar characteristics.

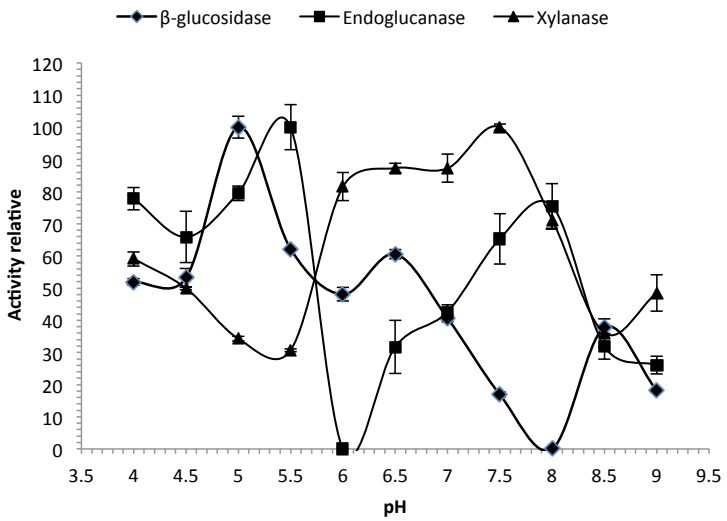

Fig. 4. Effect of pH on the enzymatic activity of the cellulases and xylanases of the crude extract obtained by solid state fermentation from coffee pulp and *P. citrinum* CGETCR. All the results were expressed in mean ± SD from three separated experiments.

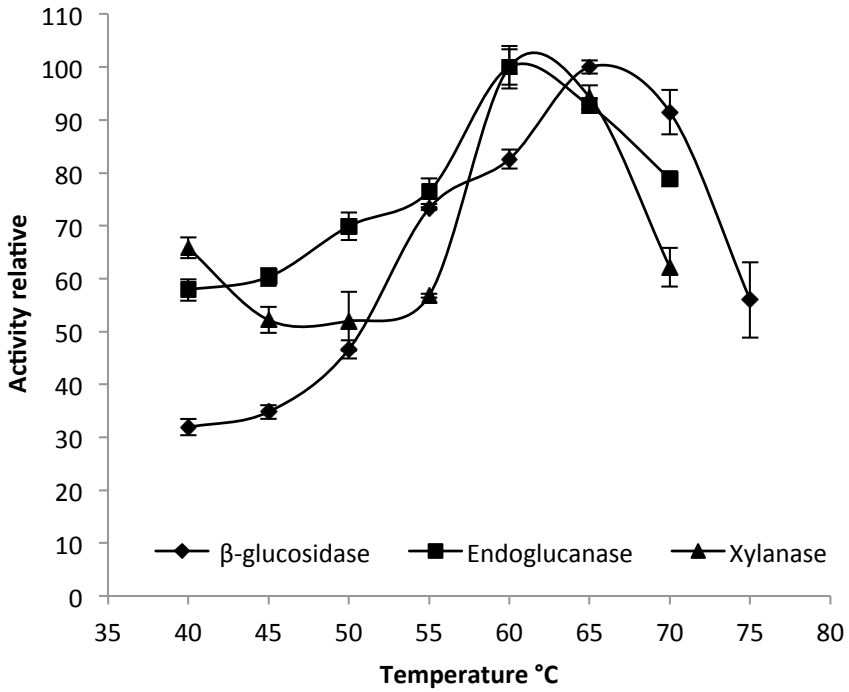

Fig. 5. Effect of temperature on the enzymatic activity of the cellulases and xylanases of the crude extract obtained by solid-state fermentation from coffee pulp and *P. citrinum* CGETCR. All the results were expressed in mean ± SD from three separated experiments.

3.5 Determination of phenols in the crude enzymatic extract

Given the nature of the substrate used in this study for enzyme production, the concentrations of phenolic compounds present in the crude enzymatic extract were measured to determine their toxicity levels. It has been shown that the presence of these compounds may have cellulase-inhibiting or inactivating effects (Ximenes *et al*. 2011). The content of phenolic compounds in the crude extract at 24 h of fermentation was 0.318 mg/mL GAE, which decreased after 48 h and reached 0.15-0.16 mg/mL GAE during the period of 120-216 h. This was a 48.74% decrease in phenolic compounds levels, which indicated that the *P. citrinum* CGETCR fungus could be used to remove phenolic compounds from coffee pulp and decrease the impact on soils and bodies of water otherwise affected if these contaminants were untreated. The extracts analyzed in this study showed enzymatic activity even in the presence of phenolic compounds. However, the enzyme activities could have also been affected by the presence of these substances. Reports have shown that the hydroxyl phenolic groups associated with tannins and lignin absorb proteins and deactivate cellulolytic enzymes, especially *β*-glucosidases, during the hydrolysis of microcrystalline cellulose (Ximenes *et al*., 2011).

3.6 Saccharification of agroindustrial residues by cellulases and xylanases

The enzymatic hydrolysis of crude coffee pulp and sugarcane bagasse was performed with alkaline pretreatment using the enzymatic extracts obtained by SSF under the previously optimised conditions (pH 7.11, 61.79% moisture and a 145.51 h fermentation time). The maximum concentrations of reducing sugars assessed when using crude sugarcane bagasse and coffee pulp were 14 and 23 mg/g of substrate within 192 h, respectively, in contrast to 100 and 105 mg/g within 168 and 192 h for sugarcane bagasse and coffee pulp subjected to alkaline pretreatment, respectively. The results clearly showed that pretreatment with sodium hydroxide had a significant effect on the release of reducing sugars because the accessibility of enzymes to cellulose and hemicellulose increased (Menon and Rao, 2012).

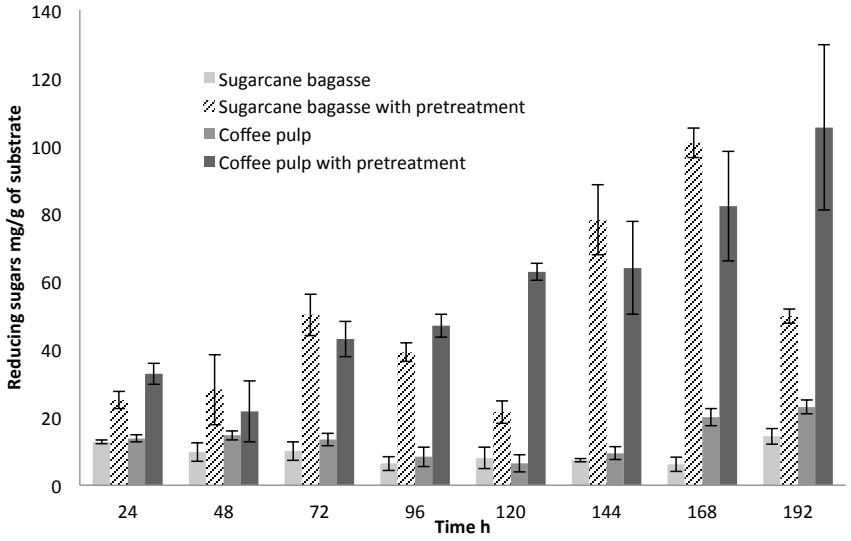

Fig. 6. Hydrolysis of crude and hydrolyzed lignocellulosic residues at pH 5.5, 60 °C and 150 rpm with crude enzymatic extracts obtained by solid state fermentation from *P. citrinum* CGETCR and coffee pulp. All the results were expressed in mean ± SD from three separated experiments.

The alkaline pretreatment breaks the bonds between hemicellulose and lignin causing the contents of these compounds to be reduced in the pretreated substrates (González-Rentería *et al*., 2011). The results of the present study suggest that the crude enzymatic extract obtained from *P. citrinum* CGETCR and coffee pulp has potential for applications in the hydrolysis of lignocellulosic residues. However, these results could be improved by optimising the conditions of the hydrolysis reaction.

Conclusions

The optimized SSF of *P. citrinum* CGETCR in coffee pulp, which has a high content of phenolic compounds, achieved the expression of active cellulolytic and xylanolytic enzymes, although, phenolic compounds are reported as an inhibitor of cellulases. Therefore, it is presumed that this kind of enzymes from this native strain could have a better performance against those coming from species that have not been isolated from environments rich in polyphenols. Also, the extract of enzymes could hydrolyse sugarcane bagasse and coffee pulp residues, making these extracts attractive for their subsequent use in reducing sugars production, which could be utilized in the beverage industry, human and animal food or biofuels. Especially, these enzymes can be used in juice clarification from fruits with higher content in polyphenols, like berries.

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