
# El Colegio de la Frontera Sur

## Producción de celulasas y xilanasas a partir de *Penicillium citrinum* CGETCR y pulpa de café por fermentación en estado sólido

TESIS

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Con orientación en Agroecología y Manejo de Plagas

Por

Miriam Peña Maravilla

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# El Colegio de la Frontera Sur

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Las personas abajo firmantes, miembros del jurado examinador de:

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Para obtener el grado de **Doctora en Ciencias en Ecología y Desarrollo Sustentable**

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## Tabla de contenido

**Resumen**

El objetivo de esta investigación fue obtener celulasas y xilanasas a partir de *Penicillium citrinum* CGETCR y pulpa de café cruda por fermentación en estado sólido, para la degradación de residuos lignocelulósicos de la región. Se efectuó la optimización del proceso por metodología de superficie de respuesta aplicando un diseño central compuesto. Luego se realizó la purificación del extracto por isoelectroenfoque en rotofor. Las fracciones con mayor actividad enzimática y cantidad de proteínas fueron seleccionadas para efectuar una caracterización bioquímica e identificación por secuenciación De Novo. Por último, se realizaron ensayos de hidrólisis de residuos celulósicos (con y sin pretratamiento alcalino) aplicando extractos enzimáticos crudos y extractos enzimáticos de alta pureza evaluando la liberación de azúcares reductores. Se obtuvieron valores óptimos de pH 7.1, humedad 61.7 % y tiempo de fermentación 145.5 h, con una actividad endoglucanasa máxima teórica de 0.563 U/g. Se logró la separación de compuestos fenólicos por medio de isoelectroenfoque, resultando en extractos enzimáticos de alta pureza con actividad celulolítica y xilanolítica. Los extractos de alta pureza tuvieron un pH óptimo de 4.0, 5.0 y 4-6 para actividad endoglucanasa, β-glucosidasa y xilanasa, respectivamente. La temperatura óptima de la β-glucosidasa y endoglucanasa fue de 65-70 °C, y la xilanasa entre 60-70 °C. La β-glucosidasa presento termoestabilidad a 40 °C, manteniendo más del 70% de su actividad enzimática durante 7 h. La endoglucanasa y xilanasa mostraron termoestabilidad a 40 y 50 °C, manteniendo más del 80 y 70 % de su actividad respectivamente durante 7 h. Las secuencias de péptidos de las dos proteínas (49.1 y 40 kDa) secuenciadas mostraron gran similitud con celobiohidrolasas de especies de *Penicillium.* Por último, el extracto enzimático de alta pureza aplicado en la hidrólisis de residuos celulósicos con pretratamiento alcalino resultó en una mayor liberación de azúcares reductores que el extracto enzimático crudo.

Palabras clave: fermentación sólida, optimización, isoelectroenfoque, hidrólisis, azúcares reductores

## Capítulo I. Introducción

La celulosa es un homopolímero de glucosa unidas por enlaces β-1,4-glucosídicos, donde las cadenas de celulosa son empacadas dentro de microfibrillas las cuales son estabilizadas por puentes de hidrógeno (Menon y Rao 2012). Estas fibrillas a su vez se unen a biopolímeros estructurales, principalmente hemicelulosa (20-35 %) y lignina (5-30 %), y están asociadas en forma de paquetes o macrofibrillas (Lynd et al. 2002; Menon y Rao 2012; Mussatto et al. 2011). Una característica de la celulosa es su cristalinidad que va desde pura a amorfa (estructura no cristalina), y esta se adquiere en el empaquetamiento de las microfibrillas que incluso moléculas tan pequeñas como el agua no la penetran, haciéndola insoluble (Lynd et al. 2002; Menon y Rao 2012).

Dentro de los microorganismos capaces de utilizar la celulosa como fuente de carbono se encuentran las bacterias y los hongos, mediante la producción de celulasas extracelulares, enzimas que son inducibles y se producen o sintetizan cuando los microorganismos crecen sobre materiales celulósicos (Lee et al. 2008), estas enzimas trabajan de forma individual o colectiva. En este último caso se tiene un mejor desempeño y es un fenómeno conocido como sinergismo (Lynd et al., 2002).

En la hidrólisis enzimática al menos tres tipos de celulasas están implicadas: 1) endoglucanasas (EG: endo-1,4-β-D-glucanasa, 1,4-β-D-glucano-4-glucano hidrolasas, EC 3.2.1.4), que atacan las regiones amorfas y rompen al azar los enlaces internos de las cadenas de glicanos; esto proporciona extremos reducidos y no reducidos de celooligosacáridos para las exoglucanasas; 2) exoglucanasas (CBH: o celobiohidrolasas, 1,4-β-D-glucano-celobiohidrolasa (celobiohidrolasas), EC 3.2.1.91, 1,4-β-D-glucano-hidrolasas (celodextrinasas), EC 3.2.1.74) las cuales actúan sobre los celooligosacáridos. Las exoglucanasas hidrolizan los extremos de las cadenas de manera procesiva, teniendo como productos principales celobiosa o glucosa; 3) β-glucosidasas (BG; celobiasa, β-D-glucósido glucohidrolasas, EC 3.2.1.21) este tipo de enzimas hidrolizan celobiosa a glucosa y también liberan glucosa de los extremos no reducidos de los celooligosacáridos solubles (Lynd et al. 2002; Menon y Rao 2012; Phitsuwan et al. 2012).

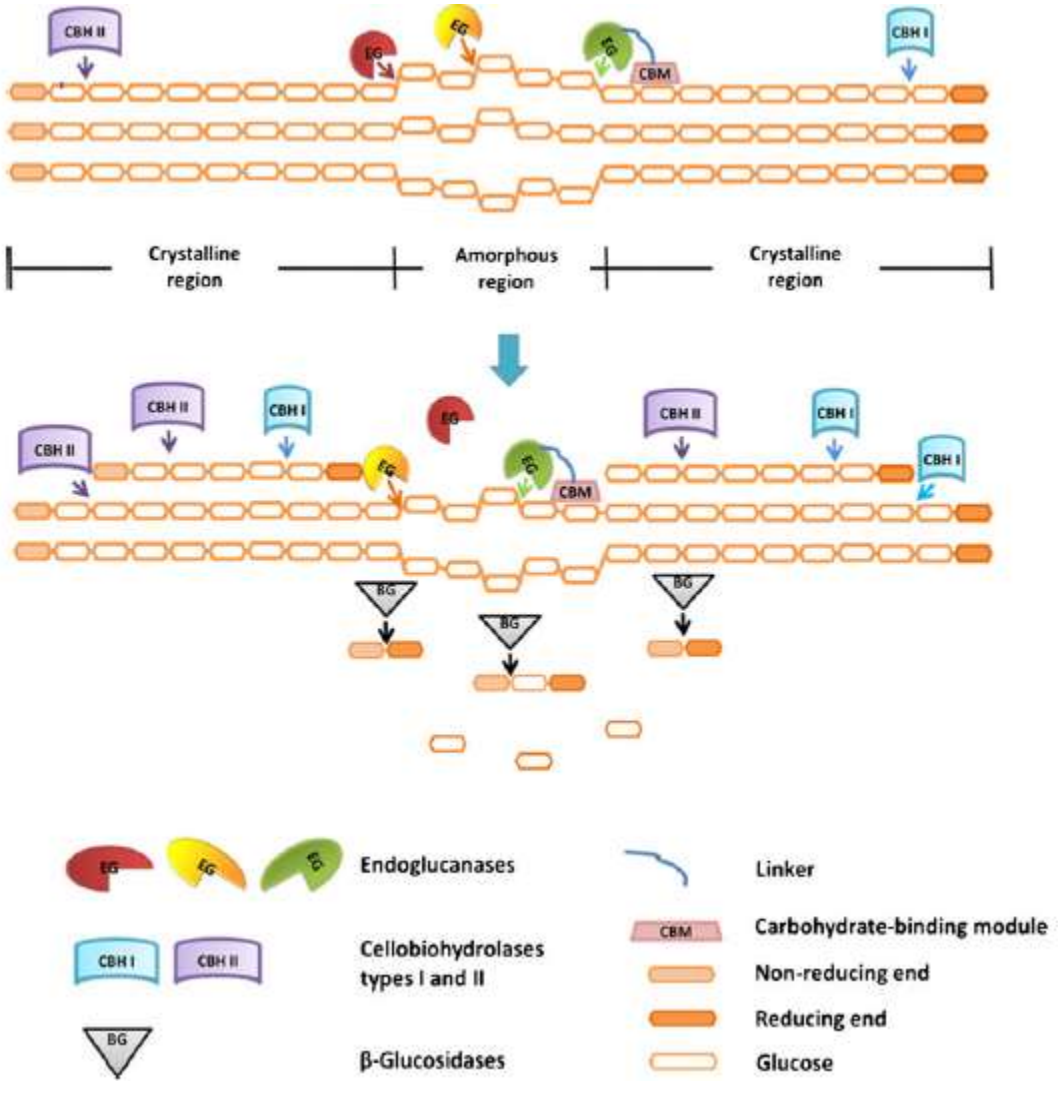


Fig. 1. Modelo simplificado de la hidrólisis enzimática de la celulosa (Phitsuwan et al. 2012)

Aproximadamente 90% de las enzimas empleadas a nivel industrial son obtenidas por fermentación sumergida, aunque la fermentación en estado sólido está tomando gran interés dadas las ventajas que ofrece, como son: requerimientos bajos de energía (para aireación, agitación y control de temperatura), menor producción de aguas residuales, empleo de materiales sólidos de bajo costo (residuos agrícolas, agroindustriales, residuos urbanos, materiales sintéticos, etc.), producción de enzimas específicas, similitud de hábitat para algunos microorganismos de interés, resistencia a la contaminación bacterial, mayor concentración del producto y el empleo de equipos simples de fermentación (Dutta et al. 2008; Graminha et al. 2008; Murthy y Naidu 2012a; Singhania et al. 2009; Zhang y Sang 2012).

“La fermentación en estado sólido se define como el cultivo de microorganismos sobre un soporte sólido húmedo, ya sea en soportes inertes o sustratos insolubles que pueden además ser usados como fuente de carbono y energía”, en ausencia o casi ausencia de agua (Muhanty et al. 2009; Singhania et al. 2009). Los subproductos de la industria cafetalera en especial la pulpa de café tiene potencial para funcionar como sustrato para procesos de fermentación sólida debido a sus características físicas (estado sólido) y químicas; puede ser el soporte para el crecimiento de hongos celulolíticos proporcionando la fuente de carbono y nitrógeno así como también en el aporte de minerales como el potasio que podrían favorecer la proliferación de microorganismos celulolíticos en especial de hongos aerobios.

Dentro de los microorganismos que se han aplicado en el proceso de fermentación en estado sólido para la producción de celulasas se encuentran los hongos filamentosos como *Trichoderma* sp y *Aspergillus* sp., cultivados en diversos sustratos celulósicos como rastrojo de maíz, salvado de arroz, salvado de trigo, bagazo de caña, etc., sin embargo existen pocos estudios empleando pulpa de café dada la complejidad del sustrato (carbohidratos 33.7-42.9%, celulosa 20.7-63.0±2.5%, hemicelulosa 2.3-3.6%, lignina 14.3-17.5±2.2, proteínas, minerales (Na, Ca, Zn, Mn, Mg, Cu, Fe y K), taninos, polifenoles, ácido clorogénico 2.6%, ácido cafeíco 1.6% y cafeína 1.3% (Bonilla-Hermosa et al. 2014; Murthy y Naidu, 2012a; Ramírez-Velasco et al. 2016), pues no sólo se encuentra material lignocelulósico sino también diversos compuestos fenólicos que pueden intervenir y afectar el desempeño de las celulasas y/o xilanasas expresadas.

El café es el producto agrícola más importante y el segundo más comercializado a nivel mundial después del petróleo (Zuorro y Lavecchia, 2012). En México el café cereza contó con una superficie sembrada y cosechada de 732,036 y 664,963 ha respectivamente, donde el estado de Chiapas ocupó el primer lugar en producción con 361,850 ton durante el ciclo cafetalero 2015/2016 (SIAP-SAGARPA). Durante su procesamiento (húmedo o seco) se generan grandes cantidades de subproductos y/o residuos (Esquivel y Jiménez, 2012; Murthy y Naidu, 2012a; Mussatto et al. 2011), los cuales representan una fuente de contaminación para el agua y suelo que se

encuentran cerca del beneficio de café (Roussos et al. 1995). El procesamiento húmedo es el que genera una mayor cantidad de subproductos, donde la pulpa de café es el principal subproducto producido, por cada dos toneladas de café procesado se obtiene una tonelada de pulpa (Roussos et al. 1995).

Dentro de los estudios realizados para la aplicación de los subproductos del café en la producción de enzimas se encuentra el de Bhoite et al. (2013) quienes estudiaron la producción y optimización de β-glucosidasas a partir de *Penicillium verrucosum* por fermentación sólida empleando como sustrato pulpa de café (tratamiento con vapor a 85 °C/20 min) con una máxima actividad enzimática de 1,991.17 U/g de sustrato. Navya y Murthy (2012) realizaron la optimización estadística para la producción de endoglucanasas por fermentación en estado sólido a partir de *Rhizopus stolonifer* de la pulpa de café obtenida por el beneficio seco (pretratamiento con explosión de vapor a 121 °C y 1.1 kg/cm$^2$ por 30 min), con una actividad enzimática de 22,109 U/g de sustrato. Murthy y Naidu (2012b), estudiaron la producción de xilanasas a partir de *Penicillium* sp., empleando subproductos del café, obteniendo actividades enzimáticas en un rango de 19,560–20,388 U/g de sustrato, la producción de enzimas fue mejorada con un pretratamiento con vapor (23,494 U/g de sustrato).

De acuerdo a los resultados presentados en estos estudios, la pulpa de café sometida a un pretratamiento es un buen inductor de celulasas; sin embargo, faltan más estudios acerca de la caracterización e identificación de las enzimas expresadas utilizando pulpa de café cruda. En este trabajo de investigación se empleó una cepa nativa de *P. citrinum* CGETCR, la cual fue aislada a partir de una muestra de lombricomposta generada a partir de desechos de ganado vacuno y residuos de pulpa de café y transformados por *Eisenia fetida* (Coutiño-Gutiérrez, 2014). Debido a su procedencia se espera que este microorganismo pueda ser adecuado y eficiente en la producción de celulasas a partir de pulpa de café.

## Pregunta de investigación

- ¿La pulpa de café sin pretratamiento y con presencia de compuestos fenólicos es un sustrato adecuado para la producción de celulasas?

- ¿El extracto enzimático crudo con presencia de compuestos fenólicos provenientes de la pulpa cruda de café es adecuado para su empleo en la obtención de azúcares reductores a partir de bagazo de caña y pulpa de café?

## Hipótesis

- El empleo de un pretratamiento a la pulpa de café para la eliminación de compuestos fenólicos es innecesario cuando se utiliza como sustrato para la producción de celulasas.

- Los compuestos fenólicos presentes en el extracto enzimático crudo provenientes de la pulpa de café no intervienen en la reacción de hidrólisis de residuos celulósicos.

## Justificación

Uno de los principales subproductos generados en la región del Soconusco es la pulpa de café obtenida por el beneficio húmedo, la cual ha tenido limitadas aplicaciones debido a su compleja composición y grandes cantidades generadas. Sin embargo, representa una fuente importante para la obtención de enzimas de interés biotecnológico como las celulasas y xilanasas, las cuales pueden ser aplicadas para la obtención de biocombustibles y compuestos químicos con interés comercial.

## Objetivo general

Producir celulasas y xilanasas a partir de *Penicillium citrinum* CGETCR para la degradación de residuos celulósicos.

## Objetivos específicos

- Optimizar condiciones de operación de fermentación en estado sólido para expresar enzimas celulolíticas.
- Aislar extractos de celulasas y xilanasas de alta pureza por Isoelectroenfoque.
- Evaluar la actividad celulolítica y xilanolítica de extractos enzimáticos crudos y de las enzimas aisladas en la degradación de residuos lignocelulósicos.

## Capítulo II. Producción de celulasas y xilanasas por *P. citrinum* CGETCR usando pulpa de café en fermentación en estado sólido

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*Revista Mexicana de Ingeniería Química*

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

# CELLULASES AND XYLANASES PRODUCTION BY *P. citrinum* CGETCR USING COFFEE PULP IN SOLID STATE FERMENTATION

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**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 *P. 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.

**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 *P. 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 cause 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.

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# 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 variety 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. 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 *P. 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 (sugarcane 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 characterized 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 fetida*, 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 $1x10^8$ 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:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii}\, x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij}\, x_i x_j$$

where $Y$ is the response variable, $\beta_0$ the intersection, $\beta_i$ the coefficient of linear terms, $\beta_{ii}$ the coefficient of quadratic terms and $\beta_{ij}$ the coefficient of interactions between two factors. Data analysis was performed using the R software (R Core Team, 2014) by response surface methodology "RSM" (Lenth 2009).

**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 |

## *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 beechwood 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. 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 optimized 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 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). 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).

**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

## *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.

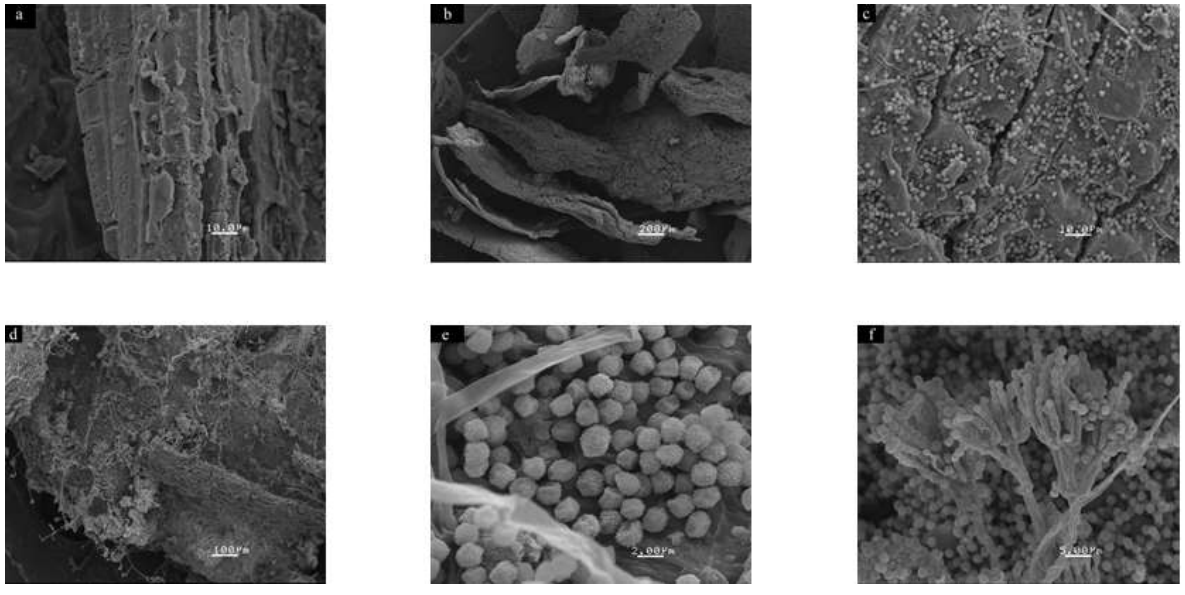



**Fig. 1.** Microphotographs by scanning electron microscopy, a) and b): surface of coffee pulp; c) and d): colonization of coffee pulp by *P. citrinum* CGETCR; and e) y f): morphology of *P. citrinum* CGETCR.

## *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 (*df* = 5, 10; *F* = 3.494; *p* = 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.

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

| Run | pH (A) | % Moisture (B) | Time, h (C) | Endoglucanase activity, Y (U/g) | |
|---|---|---|---|---|---|
| | | | | Y exp | Y pred |
| 1 | 6(-1) | 40(-1) | 96(-1) | 0.145 | 0.210 |
| 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) | 0.000 | 0.000 |
| 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) | 0.550 | 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**

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).

**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 | -2.090 | 0.063 |
| $A^2$ | -0.018 | 0.020 | -0.906 | 0.385 |
| $B^2$ | -0.127 | 0.020 | -6.215 | 9.95E-05 |
| $C^2$ | -0.012 | 0.020 | -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.

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). 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.

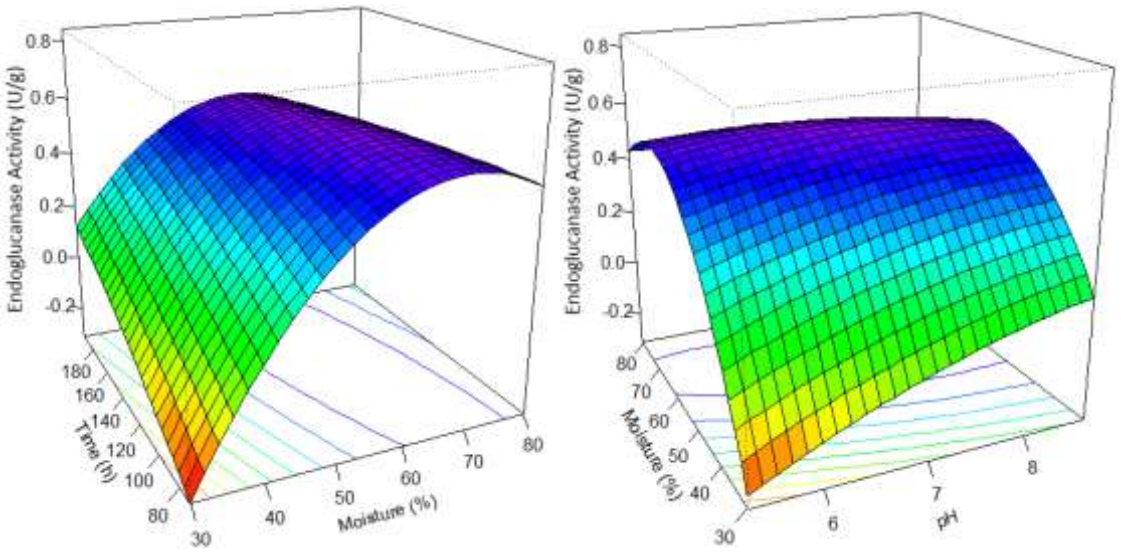
**Table 5**. Analysis of variance for composite central design

| Source | Squares of squares | Df[1] | 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 | 0.060 | 10 | 0.006 | | |
| Lack of fit | 0.046 | 5 | 0.009 | 3.493 | 0.097 |
| Pure error | 0.013 | 5 | 0.002 | | |

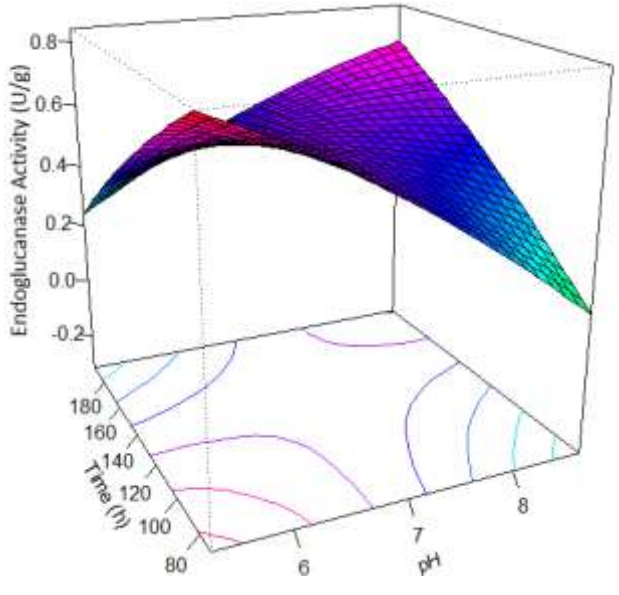
*The values of Prob>*F* less than 0.05 indicate that the terms of the model are significant; [1]Degrees of freedom.

## *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).




A) B)




C)

**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.

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 aforementioned 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.

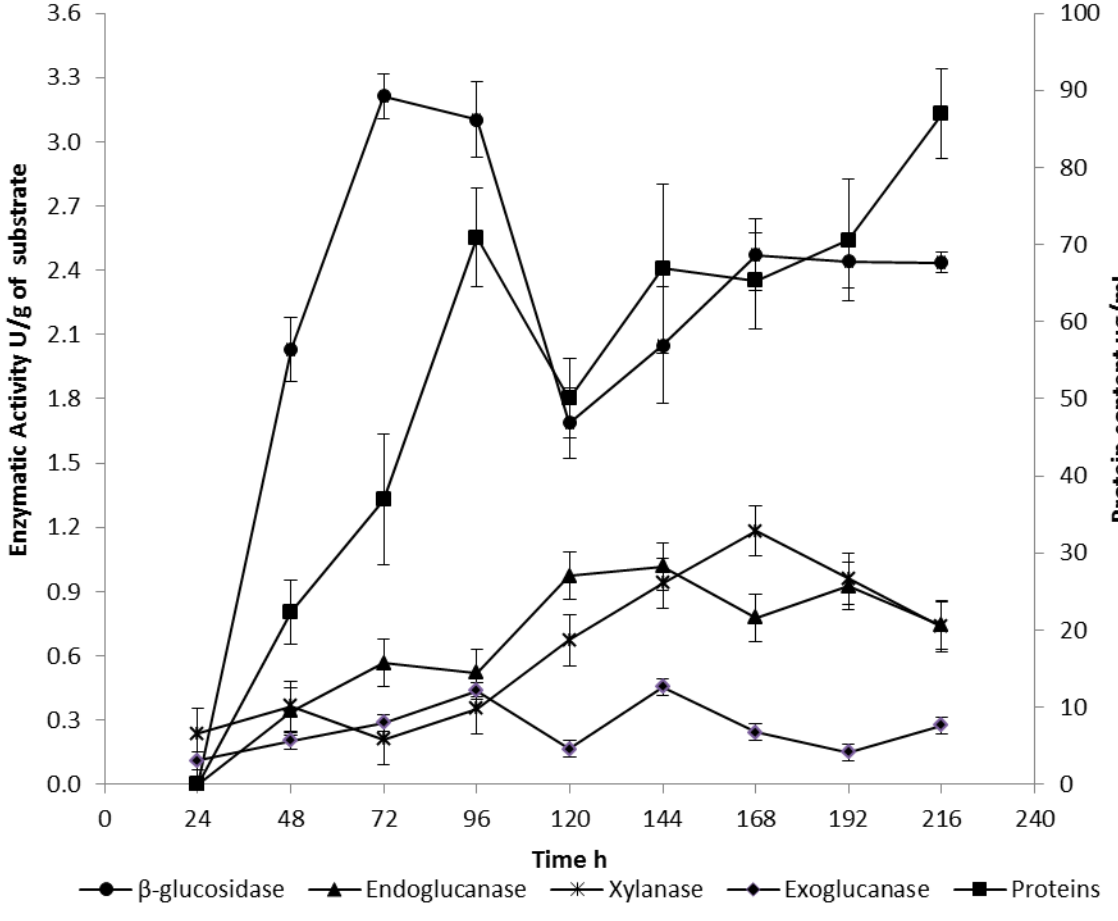



**Fig. 3.** Kinetics of cellulases and xylanases expression from *P. citrinum* CGETCR by SSF and coffee pulp at pH 7.11, moisture 61.79% and 28 °C. All the results were expressed in mean ± SD from three separated experiments.

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.

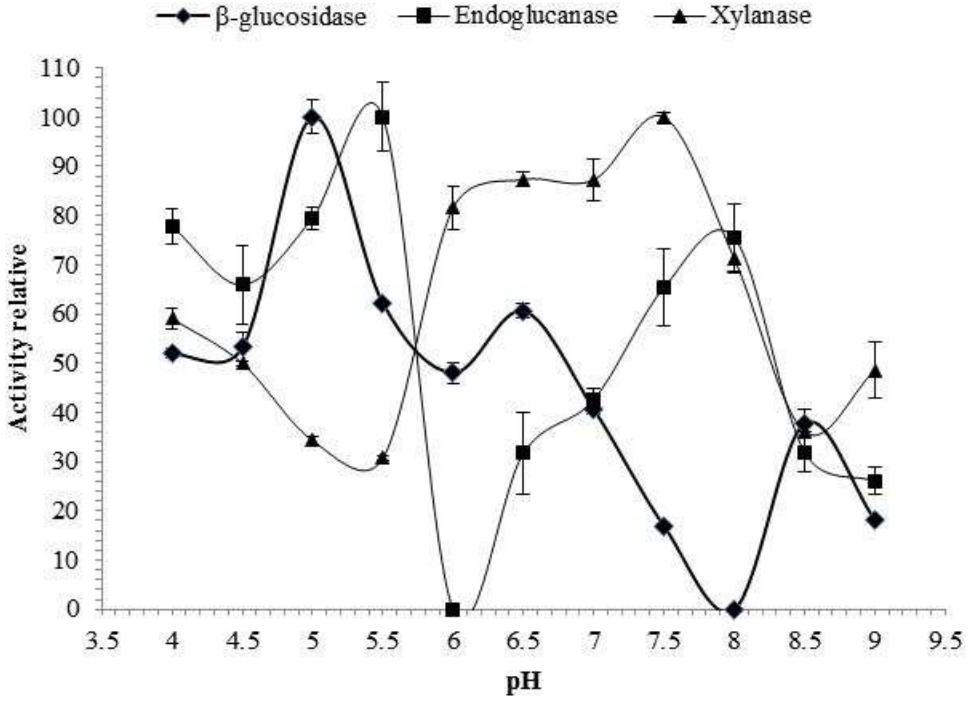



**Fig. 4.** Effect of pH on the enzymatic activity of cellulases and xylanases of the crude extract obtained by SSF from coffee pulp and *P. citrinum* CGETCR. All the results were expressed in mean ± SD from three separated experiments.

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.

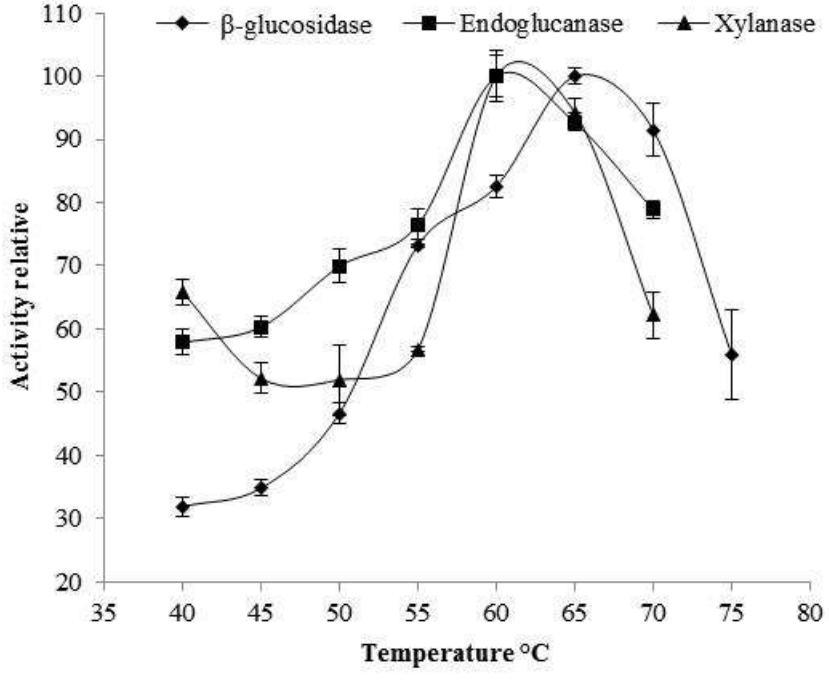



**Fig. 5.** Effect of temperature on the enzymatic activity of cellulases and xylanases of the crude extract obtained by SSF from coffee pulp and *P. citrinum* CGETCR. All the results were expressed in mean ± SD from three separated experiments.

## *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 optimized 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.

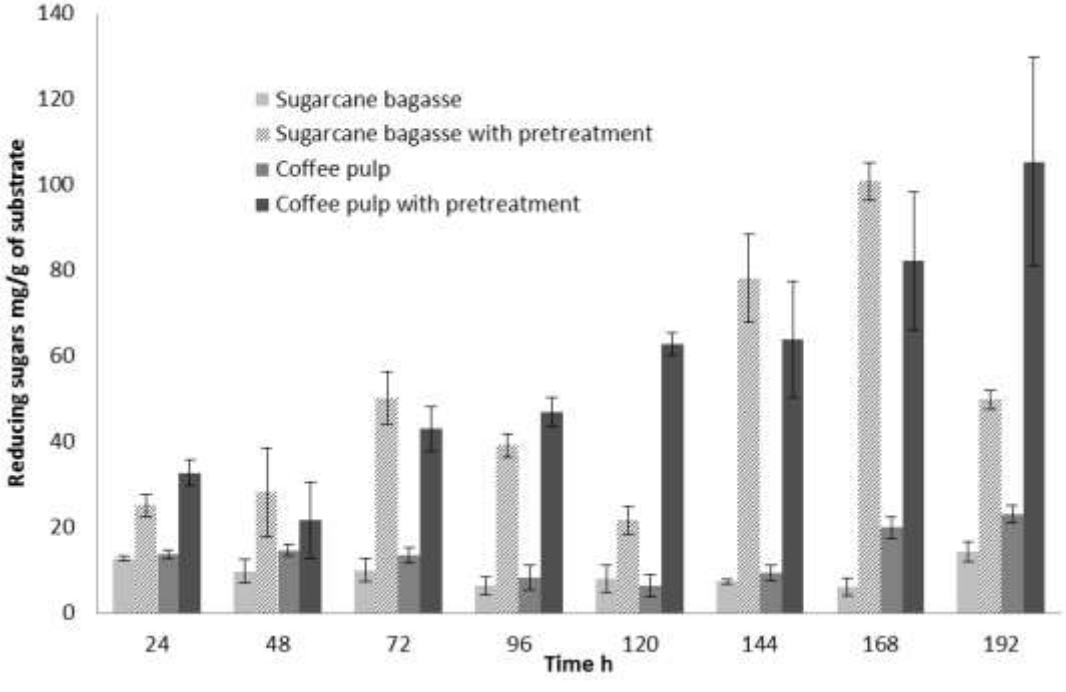



**Fig. 6**. Hydrolysis of no-pretreatment and pretrateatment lignocellulosic residues at pH 5.5, 60 ° C and 150 rpm with crude enzymatic extracts obtained by SSF from *P. citrinum* CGETCR and coffee pulp. All the results were expressed in mean ± SD from three separated experiments.

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, 2012b). 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.

## Acknowledgements

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## Capítulo III. Producción de extractos de celulasas y xilanasas de alta pureza por IEF provenientes de *Penicillium citrinum* CGETCR cultivado en pulpa de café y su aplicación en la degradación de residuos agroindustriales

Artículo sometido para su publicación a la Revista Mexicana de Ingeniería Química

*Revista Mexicana de Ingeniería Química*

# PRODUCCIÓN DE EXTRACTOS DE CELULASAS Y XILANASAS DE ALTA PUREZA POR IEF PROVENIENTES DE *Penicillium citrinum* CGETCR CRECIDO EN PULPA DE CAFÉ Y SU APLICACIÓN EN LA DEGRADACIÓN DE RESIDUOS AGROINDUSTRIALES

# HIGH PURITY CELLULASE AND XYLANASE EXTRACTS PRODUCTION BY IEF FROM *Penicillium citrinum* CGETCR GROWN ON COFFEE PULP AND ITS APPLICATION IN THE DEGRADATION OF AGROINDUSTRIAL RESIDUES

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**Resumen**:

Este trabajo reporta por primera vez la obtención de extractos de alta pureza de celulasas y xilanasas aisladas por isoelectroenfoque a partir de *Penicillium citrinum* CGETCR cultivado sobre pulpa de café por fermentación en estado sólido, para su aplicación en la hidrólisis de residuos celulósicos. El pH óptimo de la actividad endoglucanasa y β-glucosidasa fueron 4.0 y 5.0, respectivamente y la actividad xilanasa mostró estabilidad en un rango de 4-6. La temperatura donde se obtuvo la máxima actividad β-glucosidasa y endoglucanasa fue 65-70 °C y xilanasas 60-70 °C. Las endoglucanasas fueron más termoestables que las xilanasas o las *β*-glucosidasas conservando más del 80% de actividad residual durante siete horas de incubación a 40 y 50 °C. Los extractos de celulasas y xilanasas de alta pureza obtenidos lograron hidrolizar pulpa de café y bagazo de caña con pre-tratamiento alcalino generando azúcares reductores 81.05 y 126 mg/g en 144 h, respectivamente. Además, se reporta por primera vez la secuenciación *de novo* de celulasas de *Penicillium citrinum* CGETCR las cuales mostraron similitud con celobiohidrolasas reportadas para el género de *Penicillium sp.*

*Palabras clave*: isoelectroenfoque, fermentación en estado sólido, endoglucanasa, β-glucosidasa, xilanasa

**Abstract:**

This work reports, for the first time, the extraction of high purity extracts of cellulases and xylanases isolated by isoelectric focusing from *Penicillium citrinum* CGETCR grown on coffee pulp by solid-state fermentation (SSF), for its application in the hydrolysis of cellulosic residues. The optimal pH of endoglucanase and β-glucosidase activities in the high purity extracts were 4.0, 5.0, respectively and xylanase activity showed stability at the pH range from 4-6. The temperature at which maximum β-glucosidase and endoglucanase activity was determined was 65-70 ° C and xylanases at 60-70 ° C. Endoglucanases were the most thermos stable than

xylanases or *β*-glucosidases, conserving more than 80% of residual activity at 40 and 50 °C during 7 hours. The extracts of cellulases and xylanases high purity were able to hydrolyze alkaline-treated pulp of coffee and bagasse of cane generating reducing sugars 81.05 and 126 mg/g in 144 h, respectively. In addition de novo sequencing of cellulases from *Penicillium citrinum* CGETCR was reported for the first time, which showed similarity with cellobiohydrolases reported for the genus *Penicillium*.

Keywords: isoelectrofocusing, solid state fermentation, cellulase, endoglucanase, β-glucosidase, xylanase.

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# 1. Introduction

Cellulases are enzymes of great commercial importance because of their diverse applications, such as the formulation of laundry powders in the textile industry (Mawadza et al., 2000; Zhou et al., 2008), in the processing of animal feed and in the clarification of fruit juices (Mawadza et al., 2000). Although, recently, the use of these enzymes has gained more interest in the field of biofuels because vegetable biomass used as substrate represents an essential source of fermentable sugars for the production of second-generation bioethanol (Wilson, 2009). The application of commercial enzymes increases the production costs of biofuels (Singh et al., 2011); for this reason, investigations are focused on the implementation of efficient processes for the production of cellulases, which involves: searching for native microorganisms, using low-cost materials, optimizing the process of solid or submerged fermentation, among others. In México, a large amount of agroindustrial wastes are generated each year, which is underutilized, due to the lack of specific applications to create products of high commercial value, especially those wastes coming from the coffee industry, such as coffee pulp that is produced in large quantities. This cellulosic residue has the potential to function as a substrate for SSF process due to its physical and chemical characteristics, being the support for the growth of cellulolytic fungi providing the carbon and nitrogen sources as well as of some minerals (Pandey et al. 2000). Microorganisms applied in SSF for production of cellulases are filamentous fungi like *Trichoderma*, *Penicillium* and *Aspergillus* (Singh et al., 2011), grown on various cellulosic substrates such as corn stubble, rice bran, wheat bran, sugar cane bagasse, etc. There are few studies using coffee pulp given the complexity of the substrate, since not only lignocellulosic material but also various phenolic compounds (carbohydrates 33.7-36.9 %, cellulose 44.2-47.5 %, hemicellulose 15.6-19.1 %, lignin, proteins, minerals, tannins, polyphenols and caffeine) (Menon and Rao, 2012; Murthy and Naidu, 2012). Among the strategies that have been adopted to use this substrate in SSF is the physico-chemical pretreatment to eliminate the amount of lignin and to make cellulose and hemicellulose more available for the growth of cellulase-producing microorganisms (Bhoite et al., 2013; Murthy and Naidu, 2012; Navya and Murthy, 2012).

However, it is necessary to perform characterization and identification of enzymes expressed using this raw material. The hydroxyl groups of the phenolic compounds from tannins and lignin are bound to the cellulolytic enzymes and β-glucosidases during the hydrolysis of microcrystalline cellulose (Ximenes et al., 2011), causing an inhibitory effect. For this reason, it is necessary the elimination of the phenolic groups of the enzymatic extract. It is also worth mentioning that cellulases have cellulose binding domains which may cause non-specific binding with lignin because of the aromatic compounds from polyphenols (Rahikainen et al., 2013), which would decrease the catalytic power of the enzymes in the extract. Therefore, an option for the removal of phenolic compounds present in the crude extract would be purification by isoelectric focusing (IEF) obtaining extracts of high purity. It is known that cellulases exhibit higher collective activity than the sum of the activities of individual enzymes (Lynd et al., 2002), so it is more valuable to obtain extracts of high purity formed by several hydrolases (cellulases or xylanases between other kinds of hydrolases) rather than individual enzymes. So the objective of this work was to obtain a high purity enzyme extract by IEF from *P. citrinum* CGETCR grown on coffee pulp by SSF, for its enzymatic characterization and application in the hydrolysis of lignocellulosic residues.

# 2. Materials and methods

*2.1 Separation of proteins by IEF*

1.9 L crude enzyme extract was obtained by SSF from *P. citrinum* CGETCR and coffee pulp by SSF according to the methodology described by Peña-Maravilla et al. (2017). The SSF was performed at pH 7.1, moisture content 61.79% and fermentation time 145.5 h. Next, protein precipitation was carried out by salting out with 90% ammonium sulfate at 4 °C and separated by centrifugation at 10,000 g for 10 min. Protein pellets were resuspended in 0.1 M sodium acetate buffer, pH 5.5 and 0.01% sodium azide. The resuspension was ultrafiltered with 10 kDa polyethersulfone membrane (Millipore) with nitrogen at 20 psi to remove excess salts. Then, the first fractionation of crude extract of protein was performed in the Rotofor equipment (Bio-Rad, USA) using ampholytes 3/10. Once the fractions with the highest cellulolytic activity were identified, a second fractionation was performed with ampholytes 4/6. For each obtained fractions, enzymatic activity, amount of proteins (Bradford method), zymograms, SDS-PAGE and silver staining were determined.

*2.2 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 and xylanase activities were determined by separated incubations with 1% carboxymethylcellulose (CMC) and 1% xylan from beechwood substrates, respectively. Endoglucanase and xylanase activities were determined by incubating 6 µL of enzyme with 194 µL of the corresponding substrate in 0.1 M sodium acetate buffer pH 5.5, incubating at 50 °C, and measuring the absorbance of the samples at 540 nm. The amount of released reducing sugars was quantified measuring the reducing sugars with the dinitrosalicylic acid (DNS) method using glucose and xylose as standards, respectively. β-glucosidase activity was determined by incubating 4 µL enzyme with 196 µL of 4-nitrophenyl β-D-glucopyranoside (pNPG) in 0.1 M

sodium acetate buffer pH 5.0 at 50 °C. The reaction was stopped by adding 600 µL 2 M $Na_2CO_3$. Absorbance was measured at 400 nm, a standard curve of 4-nitrophenol was prepared. Each experiment was performed in triplicate.

*2.3 SDS-PAGE, zymograms and staining with silver*

One unit of enzyme activity (U) was defined as the amount of enzyme that produced one µmol of glucose, xylose or 4-nitrophenol per minute under following assay conditions. Endoglucanase and xylanase activities were determined by separated incubations with 1% carboxymethylcellulose (CMC) and 1% xylan from beechwood substrates, respectively. Endoglucanase and xylanase activity was determined by incubating 6 µL of the enzyme with 194 µL of the corresponding substrate in 0.1 M sodium acetate buffer pH 5.5, incubating at 50 °C, and measuring the absorbance of the samples at 540 nm. The amount of released reducing sugars was quantified measuring the reducing sugars with the dinitrosalicylic acid (DNS) method using glucose and xylose as standards, respectively. β-glucosidase activity was determined by incubating 4 µL enzyme with 196 µL of 4-nitrophenyl β-D-glucopyranoside (pNPG) in 0.1 M sodium acetate buffer pH 5.0 at 50 °C. The reaction was stopped by adding 600 µL 2 M Na2CO3. Absorbance was measured at 400 nm, a standard curve of 4-nitrophenol was prepared. Each experiment was performed in triplicate.

*2.4 Characterization of enzymatic extracts of high purity*

The optimal pH for the endoglucanase, xylanase, and β-glucosidase activities was determined by incubating 6 µL of the enzyme with 194 µL of the corresponding substrate with the corresponding substrate in 0.1 M sodium acetate buffer pH 4.0-5.5; 0.1 M sodium phosphate pH 6.0-8.0 and 0.1 M glycine-NaOH pH 8.5-9.0. The effect of temperature was determined for each activity in a range from 40 to 75 °C. The thermostability was determined by incubating the high purity extract at 40, 50 and 60 °C by measuring the residual activity of the enzyme at optimum pH and temperature. The effect of metals on the cellulases and xylanases activities in the high purity enzyme extract was determined by preincubating each enzyme with various metal ions 10 mM (Na1+, Mg2+, Ni2+, Zn2+, Mn2+, NH41+, K1+, Ca2+, Cu2+ y Fe3+) for 1 h to 30 °C followed by measuring the enzyme activity under the assay conditions. Each experiment was performed in triplicate

*2.5. De Novo sequencing*

Samples were previously reduced with dithiothreitol (Sigma-Aldrich), alkylated with iodoacetamide (Sigma-Aldrich) and digested “in gel” with trypsin (Promega Sequencing Grade Modified Trypsin). The peptides produced by enzymatic cleavage were desalted with Zip Tip C18 (Millipore) and applied in a system LC-MS (Liquid chromatography-Mass spectrometry) composed of a nanoflow pump EASY-nLC II (Thermo-Fisher Co. San Jose, CA) with nano-electrospray ionization source (ESI). Peptide fragmentation was performed using the Collision-Induced Dissociation (CID) and HCD (High-energy Collision Dissociation) methods. All spectra were acquired in positive detection mode. All sequences determined by manual analysis of the

MS / MS spectra (CID and HCD fragmentations) describe only one of the isobaric amino acids I (Isoleucine) and L (Leucine) since both have exactly the same mole mass (113.08406 Da). Except in the sequences that gave a high percentage of identity in the search in the Blast-nr /NCBI were discriminated as I (isoleucine).

*2.6. Evaluation of the capacity of enzymatic hydrolysis*

Sugarcane bagasse and coffee pulp were used in both with alkaline pretreatment and without pretreatment, with a particle size of 250 μm. Sodium hydroxide solution of 1% (w/v) was used for alkaline pretreatment until achieving a solid loading of 10% on a dry basis. Substrates were sterilized for 30 min at 121 °C at 15 psi. (Brijwani et al., 2010). One gram of each dried substrate was placed in 20 mL of 0.1 M sodium acetate buffer, pH 5.5 with 0.01 % sodium azide and one mL of the enzyme, the hydrolysis was carried out at 50 °C/150 rpm. A sample was collected from each hydrolysate at 24, 48, 72, 96, 120, 144, 168 and 192 h to measure the reducing sugars released by DNS method. Each experiment was performed in triplicate.

# 3. Results and discussion

*3.1. Fractionation by* IEF

As a result of the first focused with ampholytes from 3 to 10 in the ROTOFOR device, it was found that the cellulolytic and xylanolytic activities were located in the pH range from 4 to 5. Therefore, all these fractions were collected to perform the second approach with ampholytes from 4 to 6, finding that activities of either β-glucosidase, endoglucanase, and xylanases were mainly located in the fractions from 9 to 15 after the focusing carried out with ROTOFOR equipment (Fig. 1) with ampholytes 4/6. It is important to mention that the crude extract of proteins from coffee pulp contains a high content of polyphenols, and therefore could affect the separation of proteins during the IEF procedure. However, the polyphenols from crude extracts used for this experiment were almost eliminated during a first IEF run using ampholytes from 3 to 10 (data not shown), and during the second IEF with ampholytes 4-6 the polyphenols were removed entirely, resulting in high purity enzymatic extracts free of phenolic compounds. Table 1 shows results obtained during the extraction of high purity extracts (second IEF). The specific activities of β-glucosidases in the high purity extract obtained by IEF was lower than those reported by Krogh et al. (2010), Jeya et al. (2010), and Joo et al. (2010); while the specific activities of endoglucanases are higher than reported by Wei et al. (2010), and Krogh et al. (2009). The specific xylanase activity was lower than reported by Bagewadi et al. (2016).

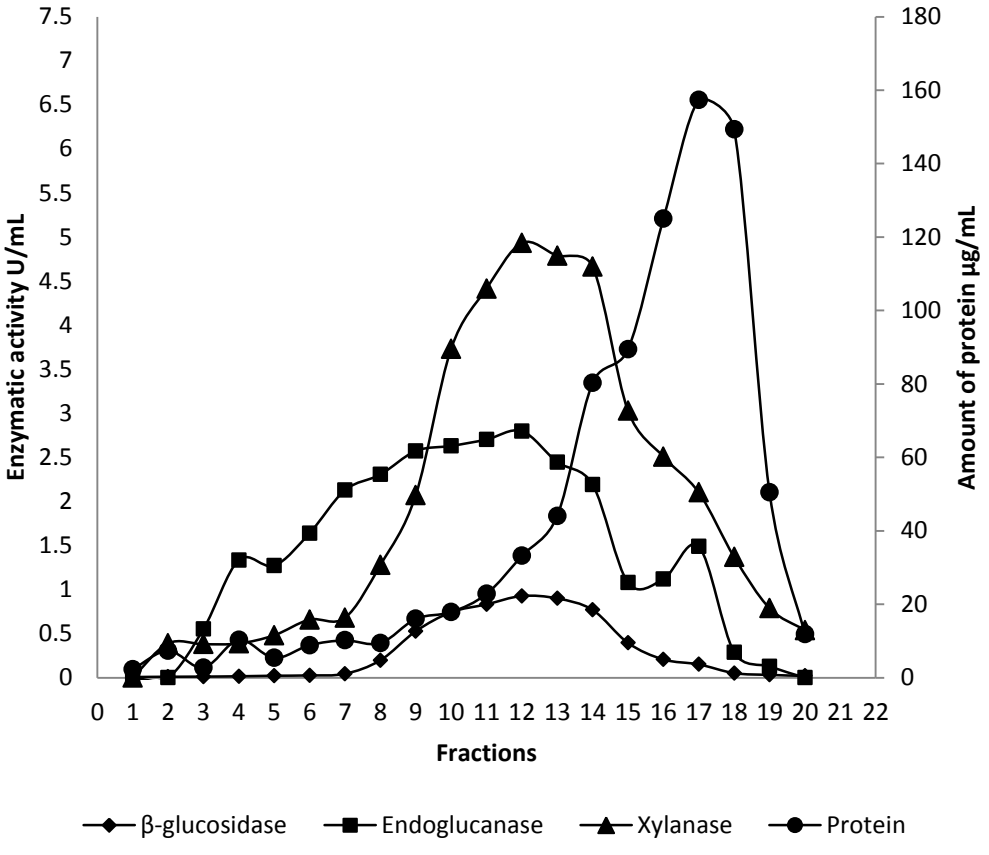



**Fig. 1**. Fractionation by isoelectrofocusing in rotofor with ampholytes of 4/6 of the crude enzymatic extract obtained from *P. citrinum* CGETCR and coffee pulp by SSF.

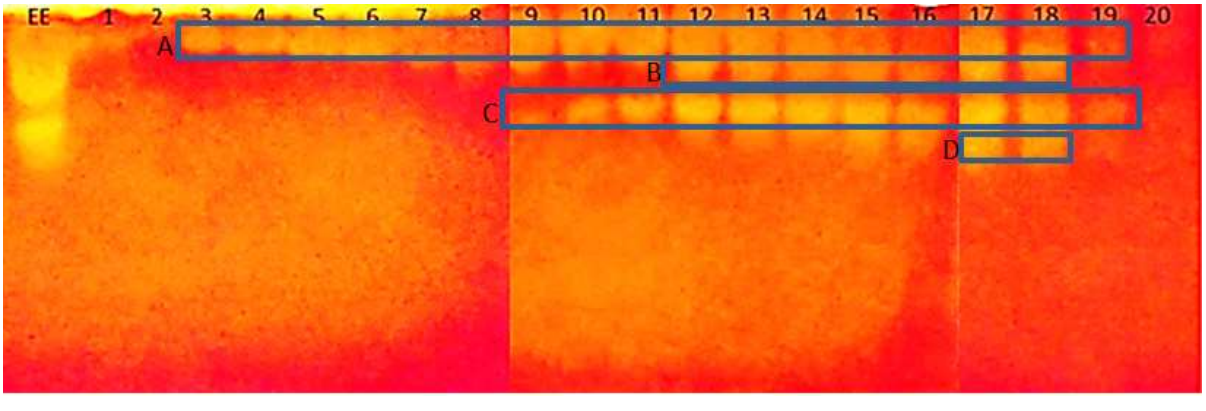
*3.2. Zymograms of the fractions obtained by isoelectric focusing*

As cellulases and xylanases are inducible enzymes, because they are overexpressed when microorganisms are cultured on lignocellulosic substrates (Lee et al., 2008), we could observe a multiple system of extracellular enzymes produced necessarily to perform the depolymerization of cellulose and hemicellulose, to obtain cellooligosaccharides or xylooligosaccharides easily assimilated during the growth of *P. citrinum* CGETCR on coffee pulp. The evidence of the multienzymatic expression was shown with the functional assays carried out on zymograms and with the pH effect study on the extracts of high purity obtained by IEF. The enzymatic activity qualitatively evaluated by zymograms showed a different migration pattern for each enzymatic activity (cellulases or xylanases) divided into four zones of hydrolysis, which showed a subtle migration in the gels and that we indicated with rectangles (Fig. 2a). These zones could be related to several isoforms for endoglucanase as has been stated for some species of *Penicillium spp*. that contain several isoforms as reported by Jogersen et al. (2003) who have found three different endoglucanases and two cellobiohydrolases of *Penicillium brasilianum*; Dutta et al. (2008) has reported two isoforms for endoglucanase produced from *Penicillium citrinum* MTCC 6489.

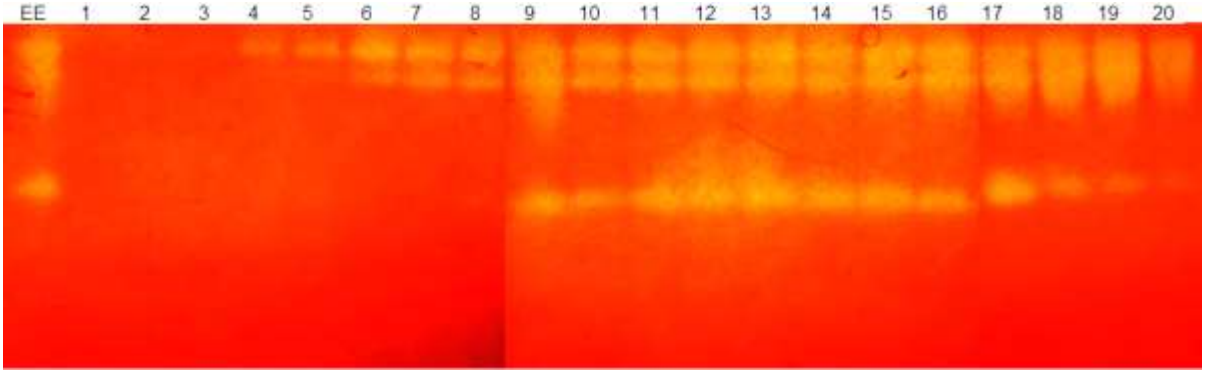
Table 1. Summary of the purification stages by isoelectric focusing of β-glucosidase, endoglucanase and xylanase activity from *P. citrinum* CGETCR and coffee pulp by SSF.

| Purification steps | Volume (mL) | Total Activity (U) | | | Total protein (mg) | Specific activity (U/mg) | | | Yield (%) | | | Purification factor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| Crude extract | 1,900 | 429.4 | 2859.5 | 1748 | 123.5 | 3.476 | 23.15 | 14.15 | 100 | 100 | 100 | 1 | 1 | 1 |
| $(NH_4)_2SO_4$ – precipitation 90% | 120 | 117 | 339.72 | 473.28 | 45.96 | 2.54 | 7.39 | 10.29 | 27.24 | 11.88 | 27.07 | 0.73 | 0.31 | 0.72 |
| Ultrafiltration | 50 | 49.35 | 287.1 | 235 | 20.05 | 2.46 | 14.32 | 11.72 | 11.49 | 10.04 | 13.44 | 0.70 | 0.62 | 0.83 |
| Isoelectrofocus Fractions 9-18 | 8 | 6.856 | 21.032 | 37.60 | 0.36 | 19.04 | 58.42 | 104.44 | 1.60 | 0.74 | 2.15 | 5.47 | 2.52 | 7.38 |

On the other hand, xylanase activity showed three bands of hydrolysis, related to three isoenzymes (Fig. 2b). Multiple cellulases have been reported for some species of *Penicillium* (Jogersen et al., 2003). The presence of isoforms in the extract of high purity was also reflected in the biochemical characterization, finding enzymatic activity for the same type of enzyme at different pH, obtaining endoglucanases working at 4, 6 and 7.5; three β-glucosidases at 4, 6 and 7 and three xylanases at 4, 6 and 7.5 (Fig. 4).




**a)**

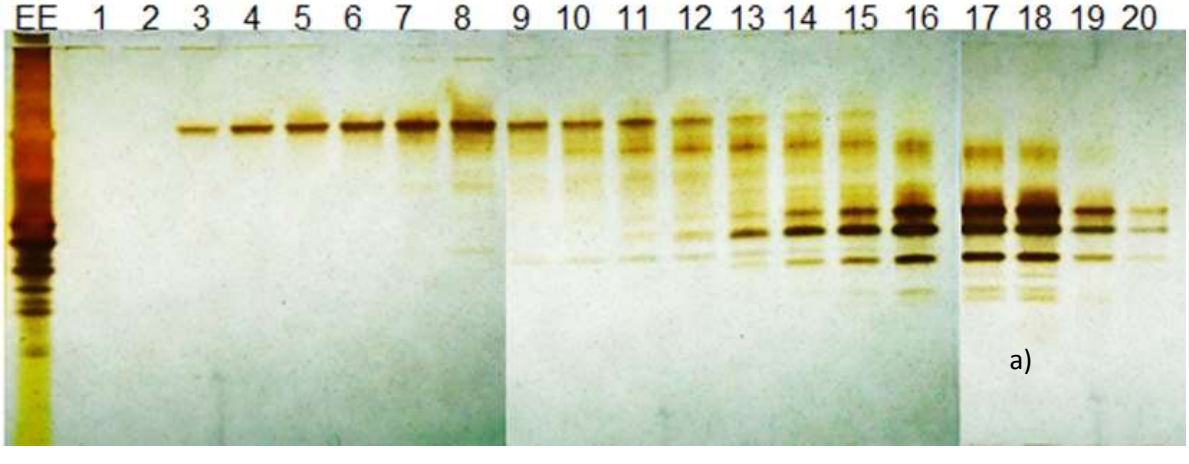



**b)**

**Fig. 2**. Zymograms of the fractions (1-20) obtained by isoelectrofocusing from *P. citrinum* CGETCR and coffee pulp by SSF; **a**) cellulases, **b**) xylanases. A, B, C and D represent hydrolysis zones. EE: crude enzymatic extract.

*3.3. Native-PAGE and SDS-PAGE of fractions obtained by isoelectric focusing*

The crude protein extract from *P. citrinum* CGETCR expressed in different fractions was observed in the lines from native-PAGEs (Fig. 3a) showing a differential pattern of bands, probably due to the hydrolysis of cellulose and its metabolites as oligocellulosides or dimers as cellobiose, obtained during the enzymatic action of the several isoforms of cellulases as endoglucanases and cellobiohydrolases. It is expected that due to the multiplicity of cellulases produced by *Penicillium* species, several bands that were visualized in the native-PAGE corresponded to another cellulases isoforms as β-glucosidases and cellobiohydrolases. The bands revealed in the silver-stained SDS-PAGE gel suggest that there were nine proteins in total with different molecular weight: 23.87, 26.73, 31.62, 40, 47.75, 49.1, 62.66 and 73.62 kDa (Fig. 3b), distributed in different fractions of the IEF, whose result may be due to the presence of several isoforms of endoglucanases, β-glucosidases and exoglucanases, as has been reported for other microorganisms, where more than 10 cellulases have been found (Lynd et al., 2002). β-glucosidases have a monomeric structure whose proteins posses a molecular weight less than 120 kDa (Bahia and Ali 2006; Galas and Romanowska, 1996; Jeya et al., 2010; Joo et al., 2009; Karnchanatat et al., 2007; Lahjouji et al., 2007), as well as some cellobiohydrolases that also present a simple structure with molecular weights between 36-66 kDa (Lahjouji et al., 2007; Lee et al., 2011; Rouau and Odier, 1986; Tuohy et al., 2002).



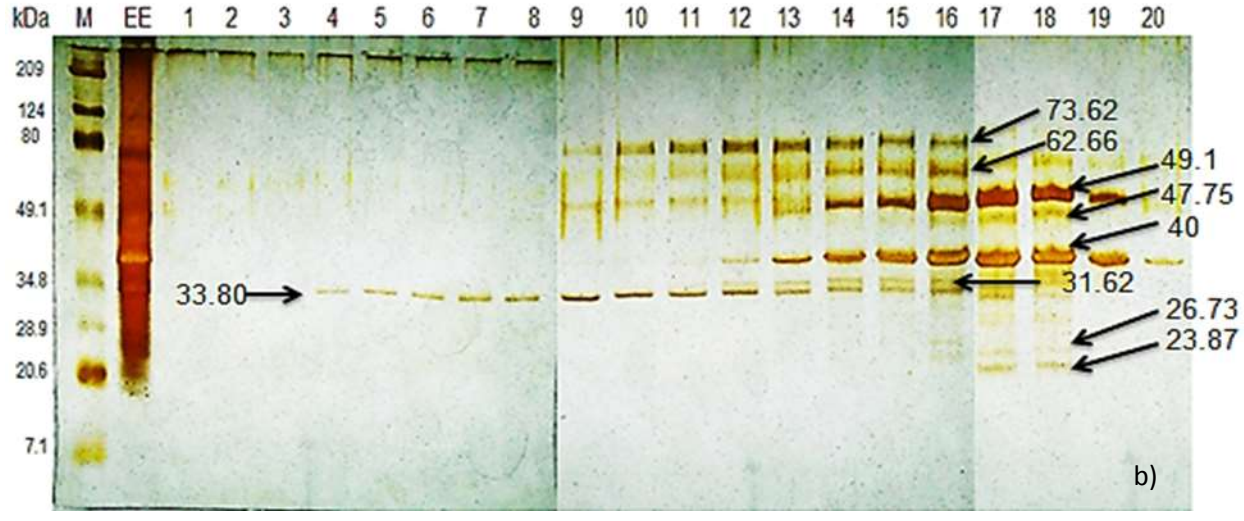
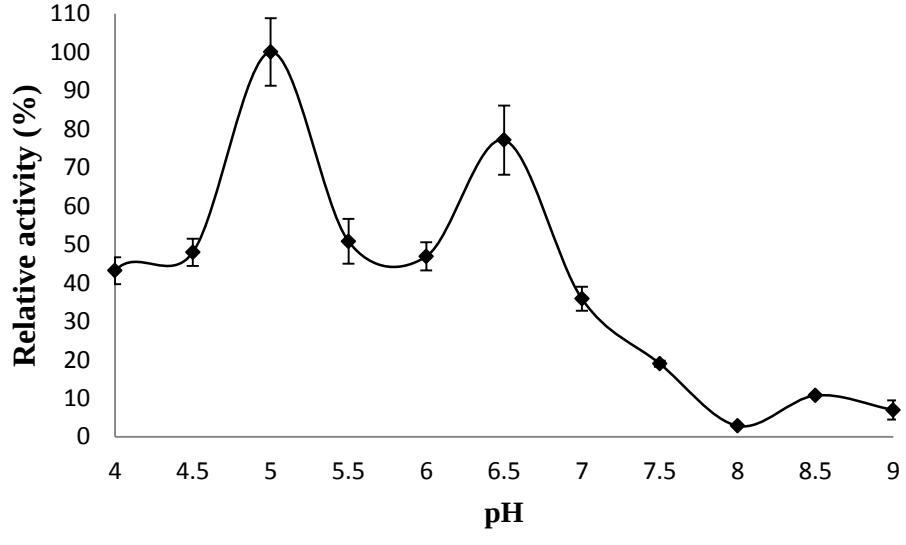


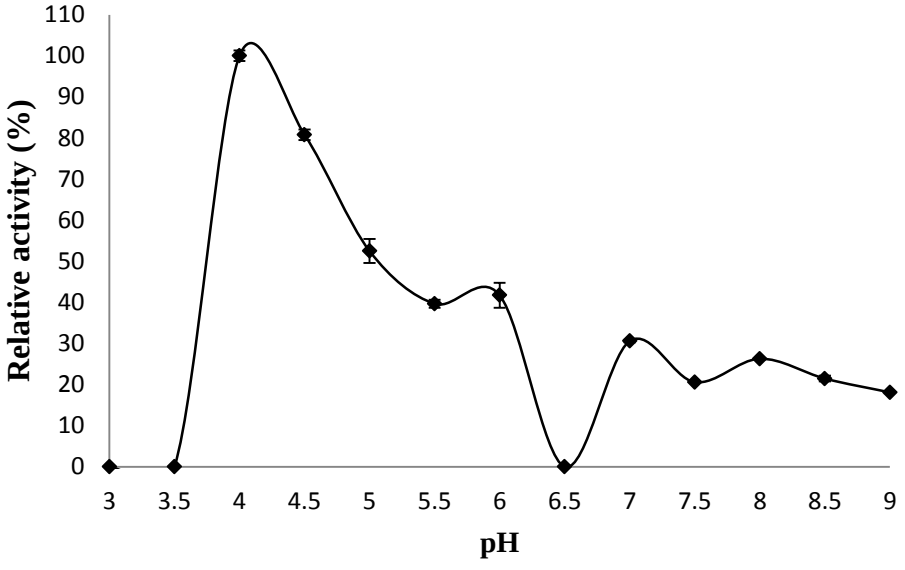

**Fig. 3**. a) Native-PAGE and b) SDS-PAGE with silver staining of the fractions obtained by isoelectric focusing from *P. citrinum* CGETCR and coffee pulp by SSF. EE: crude enzymatic extract; M: molecular weight marker; black arrows indicate the proteins of 49.1 and 40 kDa employed for *De novo* sequencing.

*3.4. Characterization of enzymatic extracts of high purity*

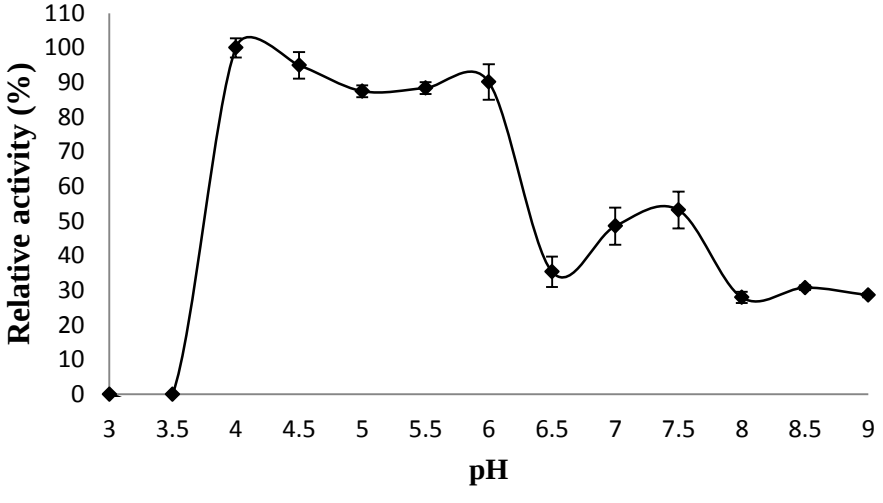
The fractions that had the highest enzymatic activity and higher protein content in the high purity enzymatic extracts were the fractions from 9 to 18. Therefore these fractions were used to carry out the characterization of cellulolytic and xylanolytic activity. The pH effect on β-glucosidase activity from enzymatic extract of high purity showed that the optimum pH was 4.0; also activity increments were showed at 6.0 and 7.0 (Fig. 4a).




a)




**b)**




c)

**Fig. 4**. Effect of pH on a) β-glucosidase, b) endoglucanase and c) xylanase activity in extracts enzymatic of high purity obtained by isoelectrofocusing from *P. citrinum* CGETCR and coffee pulp by SSF.

Endoglucanases activity showed an optimum pH at 4; however, two other optimum pH values were found (6.0 and 7.0), whose activity values did not exceed 50% (Fig. 4b). On the other hand, xylanase activity was stable at pH 4-6 retaining more than 90% of relative enzymatic activity, even showed another peak at 7.5 maintaining xylanase activity higher than 50% (Fig. 4c). This kind of behavior may be due to the presence of more than one isomer of cellulases or xylanases in the crude extract of enzymes as can be observed in zymograms (Fig. 2a and 2b). The characteristics of working at different pH could potentially be exploited for its subsequent production and commercialization of extracts of cellulolytic and xylanolytic enzymes that work

both at acid pH and neutral pH. The effect of temperature over cellulose or xylanase activities studied, indicated an optimal range between 65-70 °C for cellulases, while for xylanases its maximum activity was at a temperature of 60 °C (Fig. 5). The optimal pH and temperature obtained in this study are similar to those reported in the literature for *Penicillium* species as shown in Table 2. According to the results, the enzymes from *P. citrinum* CGETCR and coffee pulp by SSF can be applied in processes that require to be carried out under conditions of acidity as in the biofuels industry (Ng et al., 2010).

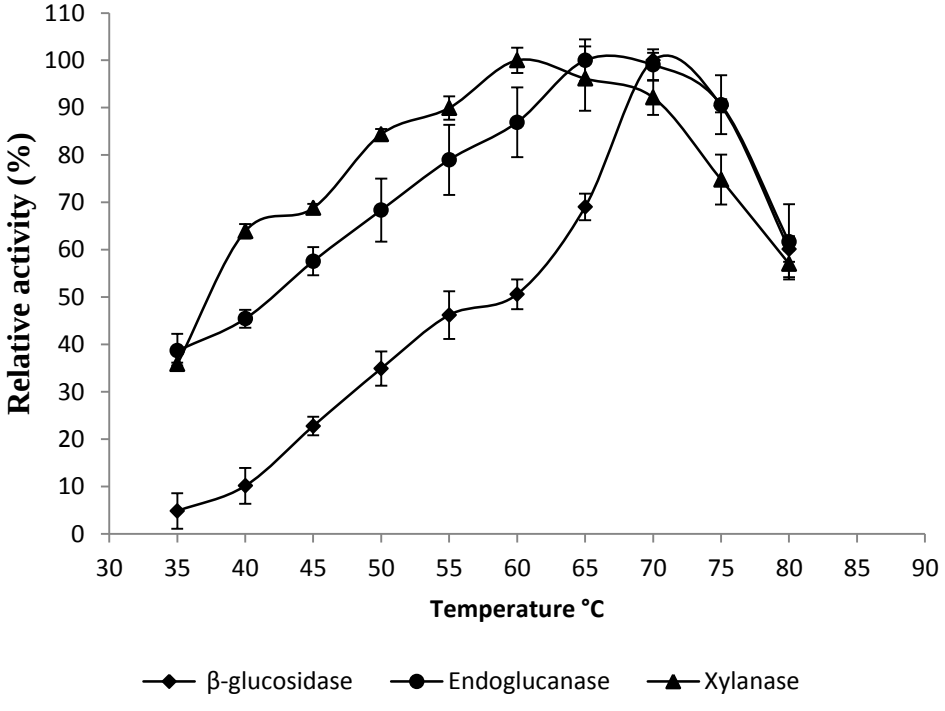


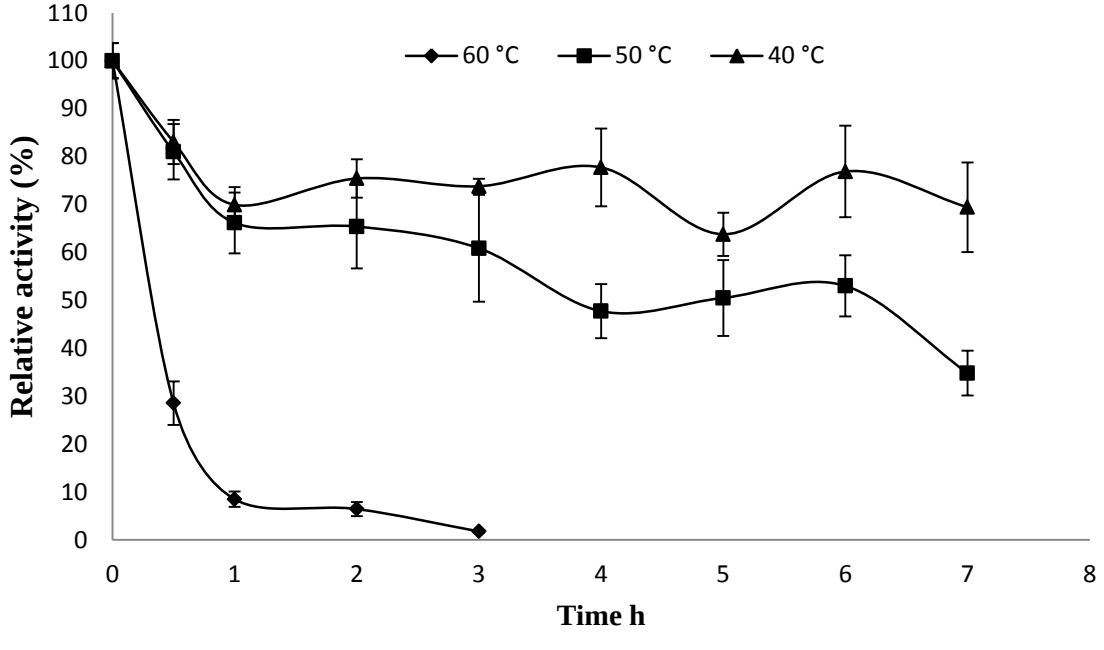

**Fig. 5.** Effect of temperature on the β-glucosidase, endoglucanase and xylanase activity in extracts enzymatic of high purity obtained by isoelectrofocusing from *P. citrinum* CGETCR and coffee pulp by SSF.

The behavior of cellulases or xylanases at several temperatures revealed that *β*-glucosidase retained almost 70% of the activity when treated at 40 °C, 50% at 50 °C after 6 h, and lost more than 50% of its activity before 0.5 h at 60 ºC (Fig. 6a). Endoglucanase activity retained 80% of activity after the treatment at 40 and 50 °C for 7 h; even retained more than 75% of activity after 6 h at 60 ºC (Fig. 6b). The xylanase activity retained above 70% of the activity after treatment at 40 and 50 °C for 6 h and retained 50% of activity after 1 h at 60 ºC (Fig. 6c). Thus, the enzyme that retained 70% of its relative activity at elevated temperatures was endoglucanase.

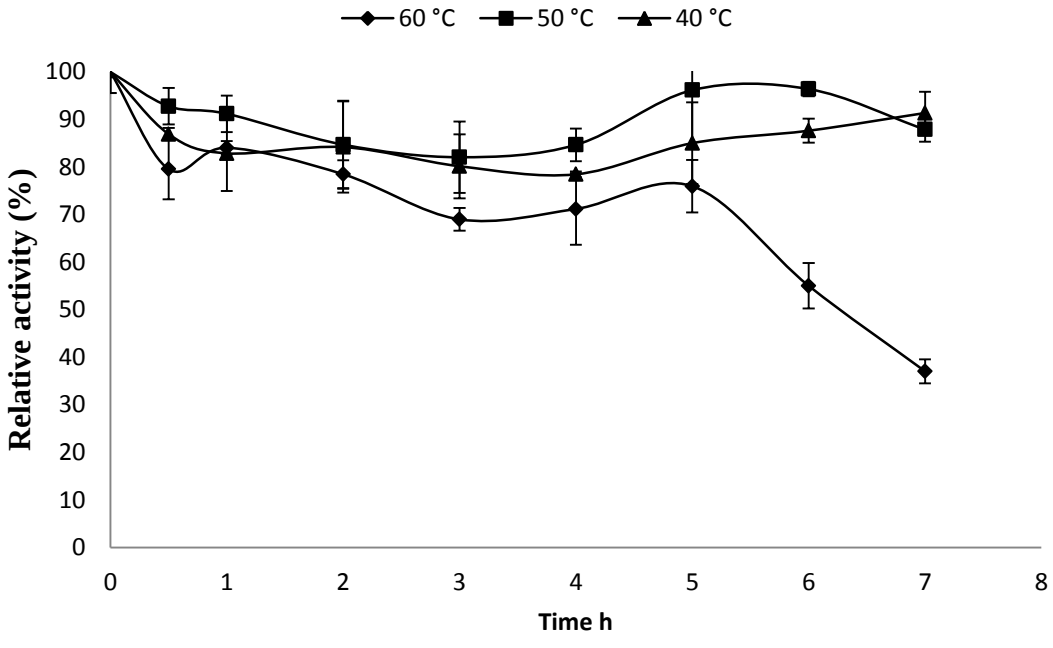
**Table 2**. Biochemical characteristics of *Penicillium* cellulases

| Species | MW (kDa) | pH | T (°C) | Specific enzymatic activity UI/mg | Enzyme | Reference |
|---|---|---|---|---|---|---|
| *P. brasilianum* | NR | 4-6 | 60 | 76 | β-glucosidase | Krogh et al., 2010 |
| *P. purpurogenum* | 110 | 5 | 65 | 875 | β-glucosidase | Jeya et al., 2010 |
| *P. decumbens* | 120 | 4.5-5.5 | 65-70 | 432.8 | β-glucosidase | Chen et al., 2010 |
| *P. pinophilum* | 120 | 5 | 65 | 83 | β-glucosidase | Joo et al., 2010 |
| *P. verruculosum* | 95 | 5 | 70 | NR | β-glucosidase | Chun et al., 1991 |
| *P. occitanis* | 98 | 4.5 | 60 | NR | β-glucosidase | Bhiri et al., 2008 |
| ***P. citrinum CGETCR*** | | **5** | **65-70** | **19.04** | **β-glucosidase** | **This work** |
| *P. decumbens* | 79 | 4 | 60 | 411.3 | Endoglucanase Cel 7B | Wei et al., 2010 |
| *P. decumbens* | 70 | 4 | 60 | 49 | Endoglucanase Cel 5A | Wei et al., 2010 |
| *P. brasilianum* | NR | 4 | 70 | 51 | Endoglucanase | Krogh et al., 2009 |
| *P. echinulatum* | 41 | 5-9 | 60 | NR | Endoglucanase | Rubini et al., 2009 |
| ***P. citrinum CGETCR*** | | **4** | **65-70** | **98.49** | **Endoglucanase** | **This work** |
| *P. occitanis* | 60 | 4-5 | 60 | 0.42 | Cellobiohydrolase CBHI | Liman et al., 1995 |
| *P. occitanis* | 55 | 4-5 | 65 | 0.13 | Cellobiohydrolase CBHII | Liman et al., 1995 |
| *P. purpurogenum* | 60 | 5 | 60 | 2.6 | Cellobiohyidrolase | Lee et al., 2011 |
| *P. decumbens* | NR | 5 | 50 | 1.9 | Cellobiohydrolase CBHII | Gao et al., 2011 |
| ***P. citrinum CGETCR*** | | **4-6** | **60-70** | **104.44** | **Xylanase** | **This work** |

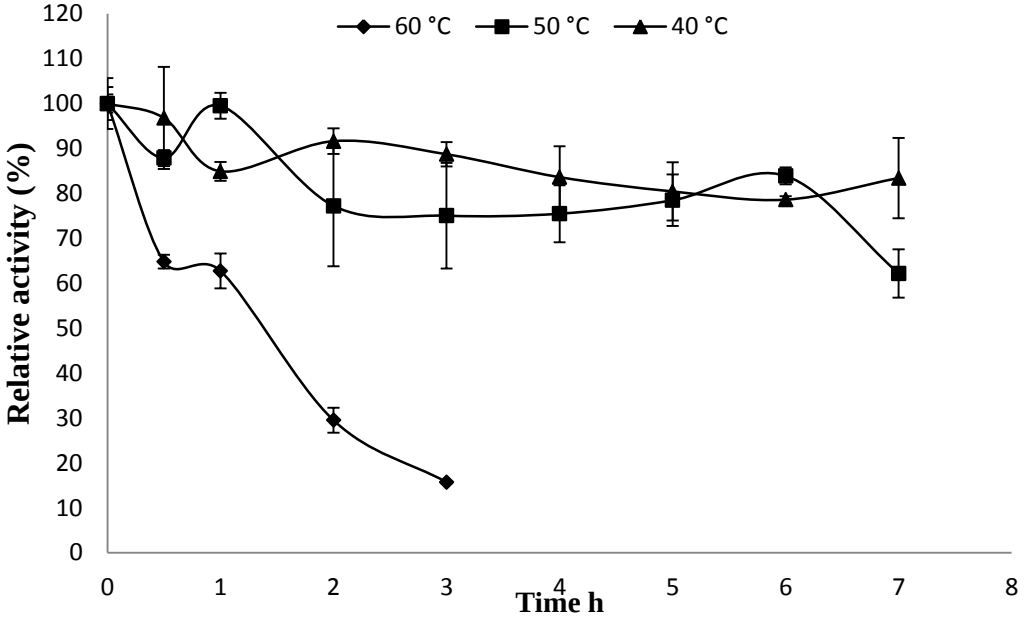
NR: Not reported




a)




b)




**c)**

**Fig. 6.** Thermostability on a) β-glucosidase, b) endoglucanase and c) xylanase activity in extracts enzymatic of high purity obtained by isoelectrofocusing from *P. citrinum* CGETCR and coffee pulp by SSF.

*3.5. Effect of metal ions*

Enzymatic activities evaluated in the presence of several ions, showed positive and negative effects: xylanolytic activity increased in the presence of Ca (211%), Na (172%), K (162%), $NH_4$ (151%), Mg (121%), and Ni (120%); while activity decreased in the presence of Mn (94%), Zn (85%), and Cu (40%) ions (Fig. 7). For the β-glucosidase activity Ca (123%) and K (112%) slightly increased catalytic activity; while Mn (68%), Zn (75%), Na (74%), Fe (38%), and Cu (30%) ions decreased its catalytic activity. Bhoite et al. (2013) reported a decrease of the activity β-glucosidase from *P. verrucosum* in the presence of Fe (75%), probably because this metal interacts with a cysteine that is required for activity and forms insoluble complexes with sulfur. Ng et al. (2010) reported decreased activity β-glucosidase purified from *P. citrinum* in the presence of Zn (78%) and Cu (33%), similar results to those found in this study. Endoglucanase activity increased in the presence of Ca (137%), $NH_4$ (122%), Na (111%), Mg (106%) and K (107%), while Zn decreased catalytic activity (83%). Dutta et al. (2008) reported decrease endoglucanase activity of *P. citrinum* in the presence of Zn (94%), Mg (87%) and Mn (37%), the latter has a greater effect on catalysis. This differs slightly from the results found in this study where the endoglucanase activity was stable in the presence of Mg (106 %) and Mn (97%). The fact that some metal ions increase the catalytic activity of the enzyme is related to the fact to that these can act as cofactors (Deswal et al., 2011).

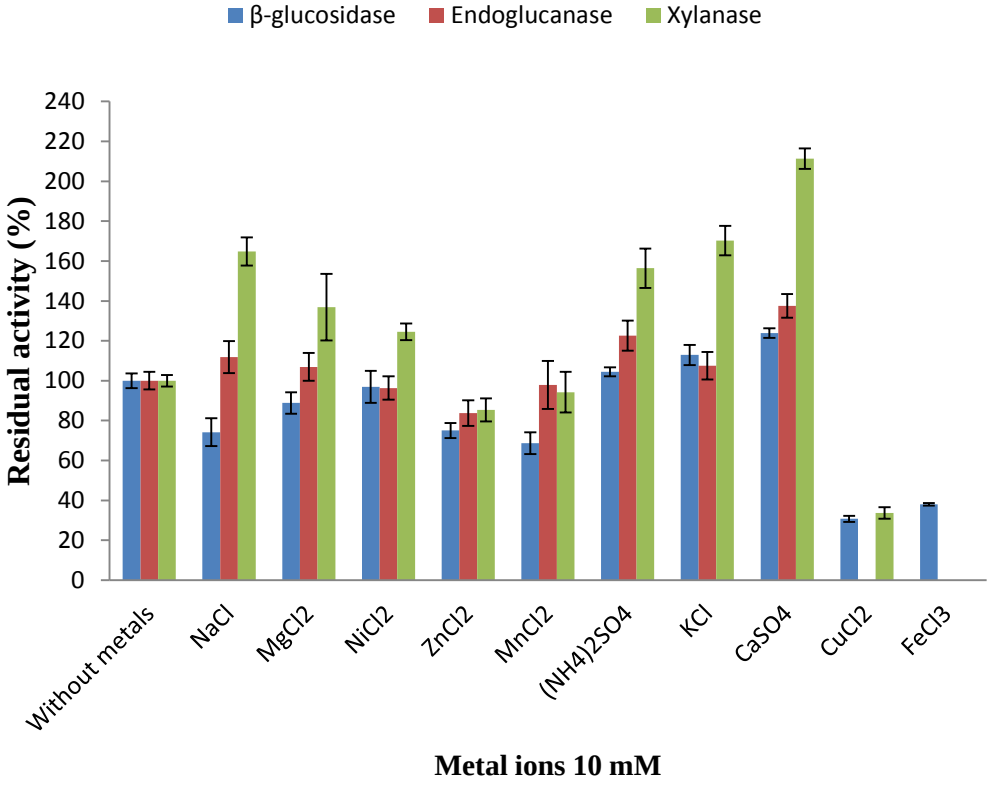



**Fig. 7**. Effect of metal ions on the β-glucosidase, endoglucanase and xylanase activity in extracts enzymatic of high purity obtained by isoelectrofocusing from *P. citrinum* CGETCR and coffee pulp by SSF.

*3.7. De Novo sequencing*

The proteins of 49.1 and 40 kDa were selected to carry out their identification by de novo sequencing, finding peptides with high similarity for cellobiohydrolases of the genus *Penicillium* (Table 3). On the other hand, we found two different enzymes among themselves, which demonstrates the presence of more than one cellulase expressed by *P. citrinum* CGETCR emphasizing that this is the first report of cellulases from *Penicillium citrinum*.

Table 3. Peptides identified by sequencing de novo

| Peptide | Ion | Protein |
|---|---|---|
| **Protein 49.1 kDa** | | |
| VQDGNVLSQS | 826.42 $2^+$ | Glycoside hydrolase family 7a |
| SGNSITSDFCSAQK | 923.93 $2^+$ | cellobiohydrolase/ hydrocellulase/ 1,4-beta-D-glucan cellobiohydrolase A |
| DTSICTDDATP | 857.86 $2^+$ | Cellobiohydrolase |
| DQSYSSTYGVTTSGNELR | 1155.02 $2^+$ | Hydrocellulase |
| SSGDPDFVESTYPNAYVK | 1262.06 2+ | cellobiohydrolase I |
| SQTETHPSLTWS | 999.98 2+ | 1,4-beta-D-glucan cellobiohydrolase B/Exoglucanase 1/glycoside hydrolase family 7 protein |
| YSGTCDPDGCDFNPYR | 962.36 $2^+$ | 1,4-beta-D-glucan cellobiohydrolase B |
| LNFVTTASQK | 554.80 $2^+$ | 1,4-beta-D-glucan cellobiohydrolase b |
| **Protein 40 kDa** | | |
| ATNVANYDGWSLSS | $2^+$ | 1,4 cellobiohydrolase |
| NKPPNAGIFVVYDL | $2^+$ | exoglucanase 2/ Cellulase/ cellobiohydrolase |
| TTDTGDALADAFVWVK | | 1, 4-beta cellobiohydrolase |

*3.8 Enzymatic hydrolysis*

Hydrolysis of raw and pre-treated coffee and bagasse pulp were carried out to evaluate the potential of the enzymatic extracts to obtain reducing sugars. Fig. 9 shows that the coffee pulp and sugar cane bagasse subject to alkaline pretreatment resulted in an increased release of sugars. It was evident that dehydration with sodium hydroxide had a significant effect on the release of reducing sugars, since the accessibility of enzymes towards cellulose and hemicellulose increased (Menon and Rao, 2012; Peña-Maravilla et al., 2017). The increase of the reducing sugars obtained from the substrate under alkaline treatment could be due to the increase of the surface area of the cellulose and the hemicellulose at the moment of eliminating the lignin, as well as by reducing the crystallinity of the cellulose fibers (Yoon et al., 2013). On the other hand, the high purity extracts achieved a higher release of reducing sugars during the hydrolysis process of the coffee pulp (126 mg / g) in 144 h than in crude protein extracts (105 mg / g) in 192 h. This increase in the release of reducing sugars may be due to compounds present in the crude extracts that could inhibit the action of hydrolytic enzymes such as polyphenols. The results of this study

suggest that the high purity enzyme extract obtained by isoelectric focusing from *P. citrinum* CGETCR and coffee pulp has the potential to be applied in the hydrolysis of lignocellulosic residues. Different cellulases and xylanases are found in these extracts, therefore a better performance in the hydrolysis reaction is expected since cellulases have a better performance when they work collectively than individually due to the synergism reported for these enzymes (Lynd et al., 2002). However, the results can be improved by performing the optimization of the hydrolysis reaction.

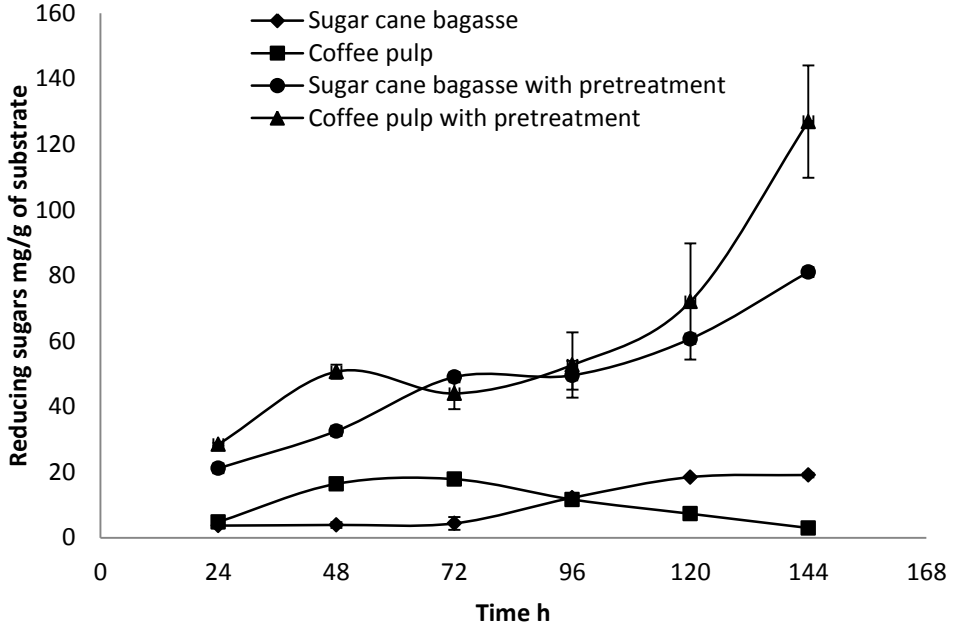



**Fig. 8.** Reducing sugars released from coffee pulp and sugarcane bagasse by using the high purity extracts obtained by isoelectric focusing from *P. citrinum* CGETCR and coffee pulp by SSF

## Conclusions

Solid-state fermentation of the fungus *Penicillium citrinum* CGETCR yielded extracts of robust cellulolytic and xylanolytic enzymes that work in a wide pH range of 4.0-7.5 and are thermostable at 50 and 60 °C after six or seven hours. The characteristics of working at different pH could potentially be exploited for its subsequent production and commercialization of extracts of cellulolytic and xylanolytic enzymes that work both at acid pH and neutral pH. The biochemical and functional characterization showed the presence of at least six different enzymes when the pH effect and zymograms studies were carried out, showing six bands of cellulase and xylanase hydrolysis. On the other hand, the molecular analysis by *de novo* sequencing allowed us to identify two cellobiohydrolases of 40.0 and 49.1 kDa. Finally, the extracts of high purity obtained by IEF were able to carry out the hydrolysis of agroindustrial residues in a better performance than crude protein extracts.

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## Capítulo IV. Conclusiones

La pulpa de café proveniente del beneficio húmedo y con un elevado contenido de compuestos fenólicos resultó ser un sustrato adecuado para la producción de celulasas y xilanasas a partir de *P. citrinum* CGETCR y fermentación en estado sólido. La producción de celulasas no se vio afectada en presencia de compuestos fenólicos por lo que la hipótesis fue validada. Debido a la complejidad del sustrato este puede proporcionar todos los nutrientes que necesita el microorganismo para su crecimiento, lo cual representa una disminución de costos en la producción de celulasas.

Aunque existen estudios donde se ha reportado que la pulpa de café también contiene micronutrientes como Na, Ca, Zn, Mn, Mg, Cu, Fe y K (Ramírez-Velasco et al. 2016), en trabajos posteriores se podría investigar si la pulpa de café, sin la adición de ningún nutriente, sea suficiente para la expresión de celulasas y/o xilanasas. Al emplear la pulpa de café cruda sin ningún pretratamiento podría resultar en la obtención de otras enzimas de interés, como las tanasas que pueden efectuar la degradación de taninos y tienen gran importancia en la industria alimenticia.

Se logró maximizar la producción de celulasas por medio de la optimización de superficie de respuesta, encontrando un punto de silla lo cual no es un óptimo matemático, pero desde un punto de vista práctico se consideró como un punto óptimo. Debido a que los experimentos realizados dependen de microorganismos y condiciones del sustrato utilizado, aunque se efectúen bajo condiciones controladas pueden existir variaciones en el ambiente que influyan en su respuesta. Por lo que un punto de silla puede ser considerado como óptimo para este tipo de experimentos.

Los extractos enzimáticos crudos mostraron actividad catalítica tanto en pH básicos como ácidos. Esta característica resulta de interés para la industria de los biocombustibles, puesto que antes de efectuar la reacción de hidrólisis de residuos celulósicos es necesaria la implementación de pretratamientos químicos que involucran el empleo de ácidos o bases. Así que el empleo de estos extractos sin importar que tipo de pretratamiento se realice a la biomasa lignocelulósica resulta de gran importancia, debido a que este extracto enzimático puede aplicarse en una amplia gama de pH´s.

Los extractos enzimáticos crudos tienen potencial para ser aplicados en la reacción de hidrólisis de pulpa de café y bagazo de caña para la obtención de azúcares reductores para su aplicación en la industria de los biocombustibles. A pesar que en los extractos crudos se encontraban presentes compuestos fenólicos provenientes de la pulpa de café estos no fueron suficientes para causar la inhibición de las celulasas y xilanasas en la reacción de hidrólisis de residuos celulósicos; comprobando así otra de las hipótesis planteadas. Debido a que las enzimas de interés fueron expresadas a partir de un sustrato con un elevado contenido de fenoles, es probable que estás tengan cierta tolerancia a la presencia de estos compuestos. Aunque en este estudio no se determinó cual es la máxima concentración de fenoles que se requieren para causar la inhibición de estas enzimas, este podría ser objeto para estudios posteriores. Debido a que provienen de un ambiente tóxico es de esperarse que su tolerancia hacia estos compuestos sea mayor, lo que permitirá hacer uso de estas enzimas en diferentes industrias.

La purificación de los extractos enzimáticos crudos obtenidos a partir de pulpa de café y *P. citrinum* CGETCR por fermentación sólida mediante isoelectroenfoque resultó en la obtención de extractos de alta pureza con actividad celulolítica y xilanolítica. Ya que durante el enfoque de las proteínas éstas se separaron de los compuestos fenólicos, ocasionaron la precipitación de dichos compuestos durante la corrida. Aunque no se logró la obtención de celulasas individuales durante el proceso de purificación, sí se obtuvieron extractos de alta pureza con distintas celulasas y xilanasas. De acuerdo a los zimogramas realizados, se observan distintas bandas que podrían asociarse a distintos tipos de enzimas y resulta de gran interés tener extractos de alta pureza con distintas hidrolasas, debido a que estás tienen un mejor desempeño cuando trabajan de manera colectiva (Lynd et al. 2002). La suma de la acción catalítica de cada una de estas enzimas puede resultar en una mayor liberación de azúcares reductores que la acción catalítica de una sola enzima. En el caso de las celulasas, existen diferentes tipos debido a que la celulosa presenta una configuración heterogénea formada por una estructura cristalina y otra amorfa, por lo que un mismo organismo puede expresar distintos tipos de estas enzimas y realizar una hidrólisis eficiente de la celulosa.

Aunque no se obtuvieron enzimas individuales durante el fraccionamiento del extracto enzimático mediante isoelectroenfoque, la separación de las proteínas en los geles teñidos con plata fue suficiente para realizar la secuenciación de dos proteínas para su identificación. Se obtuvieron secuencias de péptidos de dos posibles celobiohidrolasas de *P. citrinum* CGETCR, contribuyendo así a la identificación de celulasas de esta especie, información de gran importancia puesto que en las bases de datos de proteínas no existen reportes de celulasas para esta especie de *Penicillium*.

Los extractos de alta pureza resultaron adecuados para su aplicación en la hidrólisis de residuos celulósicos con pretratamiento alcalino para la obtención de azúcares reductores. La efectividad de este extracto resultó mayor al extracto crudo, haciéndolos atractivos para su aplicación en la industria de los biocombustibles. Los resultados obtenidos pueden mejorarse al efectuar un estudio más exhaustivo que permita identificar las condiciones óptimas para realizar la reacción de hidrólisis.

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