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Application of crude enzyme extracts produced in solid-state fermentation by *Trichoderma harzianum* on coffee waste for the hydrolysis of pretreated waste

Aplicación de extractos enzimáticos crudos producidos en fermentación en estado sólido por *Trichoderma harzianum* sobre residuos de café para la hidrólisis de residuos pretratados

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Abstract
Residues from the coffee industry are an important source of organic matter to produce hydrolytic enzymes by solid-state fermentation (SSF) using filamentous fungi and *Trichoderma harzianum*. SSF was performed with sterilized (SR) and non-sterilized (nSR) residues with particle sizes between 1.68 mm and 2.37 mm. In the aqueous crude enzyme extracts from SSF-SR, a xylanase activity of 7.59 ± 1.18 IU/g dry of coffee residues mix was detected after 8 days, as well as CMCase activity, 3.4 ± 0.2 IU/g, after 6 days. In SSF-nSR, FPase activity, was 29.65 ± 3.14 IU/g, at 8 days of fermentation. Subsequently, SSF was carried out with pretreated residue with ultrasound-assisted aqueous extraction (SSF-pUAA) and with residues no-pretreatment; in both cases, a particle size between 1 mm-1.68 mm was used. The highest CMase activity with 9.4 ± 1.36 IU/g CRM was detected in the SSF-pUAA. Finally, the crude enzymatic extract obtained with buffer was used to hydrolyze residues treated by ultrasound-assisted organosolv testing different residue:enzyme extract ratios. In the best hydrolysis ratio (1:15), the highest presence of galactose was observed at 10.78 ± 0.84 mg/g, which can indicate hydrolysis, preferably of the hemicellulose present in the pretreated residues.
Keywords: Crude enzyme extract, CMCase, pretreatment coffee residues, solid-state fermentation, *Trichoderma harzianum*.

Resumen
Los residuos de la industria del café son una fuente importante de materia orgánica para producir enzimas hidrolíticas mediante fermentación en estado sólido (FES) utilizando hongos filamentosos y *Trichoderma harzianum*. Se evaluó FES de residuos esterilizados (RE) y no esterilizados (RnE) a tamaño de partícula entre 1.68-2.37 mm. En extractos enzimáticos crudos acuosos de FES-RE se detectó actividad xilanasa de 7.59 ± 1.18 UI/g secos de mezcla de residuos de café después de 8 días, así como, actividad CMCasa, 3.4 ± 0.2 UI/g, después de 6 días. En FES-RnE, actividad FPasa, 29.65 ± 3.14 UI/g, a los 8 días. Posteriormente, la FES se evaluó con residuos pretratados por extracción acuosa asistida por ultrasonido (FES-pAAU), y con residuos sin pretratamiento; en ambos casos se utilizó tamaño de partícula entre 1.00-1.68 mm. La actividad de CMasa más alta, 9.4 ± 1.36 UI/g, se detectó en FES-pAAU. Finalmente, el extracto enzimático crudo obtenido con tampón se utilizó para hidrolizar residuos tratados por organosolv asistido por ultrasonido probando diferentes proporciones de residuo:extracto. La mejor relación de hidrólisis (1:15) presentó la mayor concentración de galactosa (10.78 ± 0.84 mg/g), indicando una hidrólisis preferentemente de hemicelulosa residual presente en los residuos pretratados.
Palabras clave: Extracto enzimático crudo, CMCasa, residuos de café de pretratamiento, fermentación en estado sólido, *Trichoderma harzianum*.

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1 Introduction

On International Coffee Day in 2022, members and partners of the ICO (International Coffee Organization) announced the need for a shift in the coffee sector from a linear to a circular economy (International Coffee Organization 2022). This change of vision aims to reduce the environmental impact of the coffee industry in more than 50 producing-exporting countries that generate around 10,000 tons of coffee waste in the year 2020/22 (International Coffee Organization 2021) of which leaves, pulp, silver skin, and peel are considered agro-industrial waste highly polluting (Gallardo-Ignacio *et al*., 2022). On the other hand, the ICO reconsidered continuing to support projects focused on building a sustainable coffee economy, as it has been doing in the last 60 years with more than 38 projects (International Coffee Organization 2023).

Solid-State Fermentation (SSF) is considered a key technology for the development of processes focused on the circular economy due to its low technological requirements and multiple high-value bioproducts generation (Barrios-González 2012; Martínez *et al*., 2018; Leite *et al*., 2021). The SSF requires an organic matrix, such as coffee residues, capable of supporting the growth of microorganisms that have a low water requirement, such as filamentous fungi of the genus *Trichoderma*, *Penicillium,* and *Aspergillus* (Pandey 2003; Barrios-González 2012; Catalán *et al*., 2019).

The production of hydrolytic enzymes has been widely evaluated in SSF (Kapoor *et al*., 2016), considering it a reference for this type of bioprocesses and products since it provides the microorganism with conditions similar to its habitat, promoting high productivity (Lizardi-Jiménez and Hernández-Martínez 2017). The importance of producing hydrolytic enzymes such as cellulases lies in their great industrial demand and can impact the reduction of environmental damage during the hydrolysis of lignocellulosic waste (de Souza-Vandenberghe *et al*., 2016; Radenkovs *et al*., 2018). The coffee residues have been evaluated for the production of hydrolytic enzymes, such as xylanases, FPases, and CMCases, from coffee husk, silver skin, and pulp individually, using filamentous fungi such as *Rhizopus* or *Aspergillus* (Murthy and Naidu 2012; Navya and Pushpa 2013; Cerda *et al*., 2017).

On the other hand, crude enzymatic extracts have been evaluated to hydrolyze various lignocellulosic residues. Solarte-Toro *et al*., (2020) employed an enzyme cocktail to hydrolyze coffee cut-stems. Martínez-Avila et al. (2021) obtained crude enzymatic extracts with citrate buffer to hydrolyze leftover brewer's spent grain, grape pomace, and olive-mill solid waste, while Moran-Aguilar *et al*., (2021) used enzymatic extract obtained from the fermentation process using crude brewery spend grain to carry out the enzymatic hydrolysis in sugar cane bagasse. Finally, Otieno *et al*., (2022) prepared the crude enzyme by solid-state fermentation of pineapple peels to perform mushroom hydrolysis.

Among hydrolytic enzyme-producing microbes (e.g. *Aspergillus*, *Penicillium*, *Rhizopus*), the genus *Trichoderma* has shown the hyper-enzymatic activity of xylanase, CMCase, FPase (Park *et al*., 2017; Gómez-García *et al*., 2018; Lopez-Ramirez *et al*., 2018; Li *et al*., 2019). However, the production of hydrolytic enzymes by *T. harzianum* using mixtures of coffee waste as substrate has not been reported, nor has the possible application of crude enzyme extracts to complement the hydrolysis of coffee waste mixtures subjected to previous pretreatments to facilitate access to cellulose or hemicellulose (Hernández-Teyssier *et al*., 2023b; Ramos-Villacob *et al*., 2023). The present study focused on producing crude enzymatic extracts from SSF a mixture of coffee waste with *T. harziamun* to hydrolyze pretreated waste for ultrasound-assisted organosolv method and determine hydrolysis efficiency and sugar release.

2 Materials and methods

2.1 Sampling and conditioning of waste

The coffee residue mix was obtained from wet coffee processing. They were collected in the municipality of Amixtlan, located in the Sierra Norte of Puebla, Mexico (20° 02' 53.1" North, 97° 47' 56.1" West), between 400 and 1,700 meters above sea level with a warm subhumid climate and rain all year round. At the sampling site, coffee residue mix was taking from three regions of the residue pile (Figure 1A), avoiding waste with the presence of mycelium (Figure 1B). The coffee residue mix was transported in plastic bags to the experimental site and dried in a convection oven at 60 °C until a humidity percentage of less than 10 % (Hames *et al*., 2008). Subsequently, the coffee residue mix was manually classified to determine the percentages of bagasse, husk, and parchment. Finally, the dry residue was ground and sieved, collecting the material of two particle sizes, between 1.68 mm to 2.37 mm (Figure 1C) and 1.0 to 1.68 mm (Figure 1D).

2.2 Experimental arrangement

First, solid-state fermentation (SSF) was performed with sterilized residue (SR) and non-sterilized residue (nSR) at particle sizes 1.68 mm to 2.37 mm (PS1).

After, residues at particle size 1.0 to 1.68 mm (PS2) were treated in ultrasound-assisted aqueous extraction and extraction with ultrasound-assisted

organosolv and with ultrasound-assisted aqueous extraction.

Second, SSF was performed with PS2 pretreated in ultrasound-assisted aqueous extraction and compared with no-pretreated residues of PS2. Finally, PS2 treated by extraction with ultrasound-assisted organosolv was used to evaluate hydrolysis with crude enzymatic extracts.

2.3 *Pretreatments for particle size 2 residues*

The treatment of PS2 was performed two methods: ultrasound-assisted aqueous extraction and extraction with ultrasound-assisted organosolv according to Hernández-Teyssier *et al*., (2023b).

The first pretreated residues were used to generate SSF, identified as SSF-pUAA. The second residues were employed to generate hydrolysis conditions with crude enzymatic extracts, identified as rUAO.

The organosolv solution was prepared at 68 % ethanol and 1.5 % H_2SO_4. For extraction method, 1 g of PS2 was mixed with 10 mL of organosolv, then was incubated for 40 min at 40 °C in an AquaWave 9380 50/60 Hz ultrasonic bath (Ravindran *et al*., 2018); in ultrasound-assisted aqueous extraction changed organosolv by distilled water.

2.4 *Preparation of coffee waste mixture for SSF*

The firstly SSF was evaluated in PS1, sterilized and no-sterilized, denominated SSF-SR and SSF-nSR, respectively. To SSF-nSR, 300 g of PS1 were moistened with 300 mL of distilled water and inoculated. Instead for SSF-SR, 300 g were moistened with 300 mL of distilled water, sterilized at 15 lb of pressure for 20 min, and inoculated.

The second SSF was evaluated with pretreated PS2 and no-pretreated PS2, labeled as SSF-pUAA and SSF-npUAA, respectively. SSF-pUA was prepared with 300 g dry of pretreated PS2, moistened with 300 mL of distilled water, sterilized at 15 lb of pressure for 20 min, and inoculated. On the other hand, SSF-npUAA was prepared with 300 g dry of no-pretreated PS2, moistened with 300 mL of sterilized distilled water, and inoculated.

2.5 *Inoculum preparation*

The fungus *T. harzianum* was maintained in a stock prepared by growing it on barley straw at 28 ºC with 65 % humidity (Serna-Díaz *et al*., 2020). From this stock, fractions of barley straw invaded by the fungus were placed in 20 mL PDA agar contained in a 200 mL Erlenmeyer flask was incubated at 28 °C until the fungus completely invaded the surface and sporulated. The collection of conidiospores was carried out by adding 30 mL of sterilized distilled water to the flask; and with the help of a sterilized magnetic stirrer, the conidiospores were separated from the mycelium. The conidiospore suspension was preserved in 15 mL sterilized plastic tubes and refrigerated (4 °C) for up to 30 days. This stock prepared conidiospore suspensions that were subsequently used for SSF inoculation. The final volume of inoculum was 300 mL at 1 x 10^7 conidiospores/mL.

2.6 *Inoculation of SSF*

The SSF-SR, SSF-nSR, SSF-pUAA and SSF-npUAA were inoculated with 300 mL conidiospore suspension at 1 x 10^7 conidiospores/mL to obtain 67 ± 2 % relative humidity and 1 x 10^7 conidiospores/g from the initial conditions of each SSF (Lopez-Ramirez *et al*., 2018; Hernández-Teyssier *et al*., 2023a).

2.7 *Assembly of the solid-state fermentation system*

Fermentations were carried out in Raimbault-type columns (Raimbault and Alazard 1980), which were sterilized at 15 lb of pressure for 15 min. All columns were packed with residues, according to the treatment at a height of 5 cm with a packing density of 0.51 ± 0.01 g/cm^3 (Quintanar-Gómez *et al*., 2012). The columns were connected to a humidifier with sterilized distilled water, and air was passed through at a flow rate of 1 L/min. The columns were incubated at 25 ± 1 °C in a water bath. To estimate the production of CO_2 as a product of fungal respiration, the top of the columns was sealed with a rubber plug attached to a hose to conduct air and metabolic CO_2 into a solution (100 mL) of 1 M NaOH. Subsequently, CO_2 was quantified by titration as described by Hernández-Teyssier *et al*., (2023a).

2.8 *Biomass modeling and substrate in SSF*

After quantifying metabolic CO_2, the conversion factor of 3.53 g of CO_2 per gram of biomass was applied (Hernández-Teyssier *et al*., 2023a). Subsequently, the kinetic growth parameters (μ_{max} and λ) were estimated through approximations with the Excel Solver add-in. Finally, the growth was modeling by the equation-modified Gompertz (Equation 1) (Zwietering *et al.*, 1990; Rosero-Delgado and Dustet-Mendoza 2017; Chu 2020).

$$y = X_0 + (X_{max} - X_0) \left(\exp\left(-\exp\left(\left(\frac{\mu_{max} exp(1)}{X_{max} - X_0}\right)(\lambda - t) + 1\right)\right)\right) \quad (1)$$

Where:

y = estimated microbial population at time t;

X_o = experimental initial biomass (mg/g);
X_{max} = maximum experimental biomass (mg/g);
μ_{max} = maximum specific growth rate (d^{-1});
λ = latency period (d);
t = sampling time (d).

On the other hand, based on the stoichiometric growth equation solved by Hernández-Teyssier *et al*., (2023a) (Equation 2) and biological efficiency of 35 %, Equation 3 was applied to determine the dry weight of the residual substrate, since both studies were similar systems and the value of the dry weight of the fermented residue was quantified.

$$CH_{1.14}O_{0.62}N_{0.05} + 0.59\ O_2 + 0.02\ NH_3 \rightarrow 0.34\ CH_{1.8}O_{0.5}N_{0.2} + 0.66\ CO_2 + 0.3\ H_2 \quad (2)$$

$$S_r = S_0 - \frac{DWFR * CO_{2t} * 23.76}{44 * 0.33 * 1000} - \frac{DWFR * X_t * 23.76}{24.62 * 0.66} \quad (3)$$

Where:
S_r = Residual mass of fermented residue (g);
S_o = Initial substrate dry weight (g);
$DWFR$ = Dry weight of fermented residue (g)
CO_{2t} = Carbon dioxide concentration at time t (mg CO_2/g);
23.76 = Substrate molecular weight according to Eq. 2 (g);
44 = Molecular weight of carbon dioxide (g);
0.33 = Stoichiometric relationship between substrate and carbon dioxide with biological efficiency of 35 %;
1,000 = Conversion factor from mg to g;
X_t = Biomass obtained by the factor 3.53 g of CO_2 per gram of biomass at time t (g X/g);
24.62 = Molecular weight of biomass (g);
0.66 = Stoichiometric relationship between substrate and biomass with biological efficiency of 35 %.

2.9 Determination of humidity and total polyphenol content

The sampling was done by randomly taking three columns every two days during the fermentation time, 12 days for SSF-nSR and SSF-SR, and 7 days for SSF-pUAA and SSF-npUAA; to estimate the moisture percentage in each sample of fermented residue, the methodology described by Sluiter *et al*., (2008) was employed to calculated in g dry weigh for all parameters.

Besides, the total polyphenol content (TPC) was quantified according to Geremu *et al*., (2016) using the modified Folin-Ciocalteu method described by Hernández-Teyssier *et al*., (2023a), which consisted of placing 1 g of fermented residue with 3 mL of distilled water in a sterilized 15 mL plastic tube, then was incubated at 30°C for 30 min and stirred using an orbital shaker (SEV-PRENDO Mod. 6040) at 150 oscillations/min. Subsequently, it was centrifuged at 3,226 xg for 25 min at 4 ºC by Thermo Scientific^{TM} Sorvall^{TM} Legend^{TM} XFR Series Centrifuge. After, 200 μL the supernatant, 1 mL of commercial Folin-Ciocalteu solution diluted 10 times, 800 μL of 7.5 % Na_2CO_3 was placed in 2.5 mL amber Eppendorf tubes; the mixture was allowed to stand for 30 min in the dark at 25 ± 2.0 °C. Samples were read at 765 nm using a Spectrophotometer (Thermo Scientific Genesys 10S UV-Vis) and gallic acid as a standard. The latter was reported in milligrams of equivalents per gram of dry residue (mg GAE/g).

2.10 Obtaining crude enzyme extracts

Two types of crude enzyme extracts (CEE) were obtained, the first CEE was obtained from distilled water by mixing 1 g of fermented residue with 3 mL of distilled water in a sterilized 15 mL plastic tube, and incubated at room temperature (30° C) for 30 min and stirred using an orbital shaker (SEV-PRENDO Mod. 6040) at 150 oscillations/min. Subsequently, it was centrifuged at 3,226 xg for 25 min at 4 ºC. The supernatant was placed in sterilized plastic tubes of 2 mL and centrifuged at 9,500 xg for 12 min at 4 ºC (Beckman Coulter^{TM} Microfuge® 22R Centrifuge), the final supernatant was considered an aqueous crude enzyme extract (aqCEE). The second type of CEE was obtained with acetate buffer (50 mM, pH 5) (Díaz *et al*., 2007) as described above, labeled as bCEE. Both CEE were temporarily stored at 4 °C and used to conduct assays within 2 h to prevent enzymatic degradation (Ang *et al*., 2013).

2.11 Determination of enzymatic activities

Xylanase, CMCase, and FPase activities were determined for aqCEE and bCEE using birch xylan, carboxymethylcellulose (CMC), and Whatman filter paper No. 1 sterilized (Ang *et al*., 2013). In both cases, 0.1 % (w/v) solutions of birch xylan and 0.1 % (w/v) CMC were prepared in acetate buffer (0.05 M, pH 5).

Xylanase and CMCase were assayed by incubating 500 μL of substrate, 250 μL of buffer, and 250 μL of the CEE for 10 min at 50 ºC. FPase activity was assayed by incubating 25 mg of filter paper, 1 mL of buffer, and 500 μL of CEE for 30 min at 60 °C.

At the end of the incubation time, the reactions were stopped by adding 1 mL of DNS reagent (10 g of 3-5 dinitrosalicylic acid, 30 g of sodium potassium tartrate, and 16 g of sodium hydroxide per liter (Wood, *et al*., 2012); then mixed for 10 s in a vortex and this mixture was placed in a boiling water bath for 5 minutes. After, it was cooled quickly and read in a spectrophotometer at 514 nm.

The standard curves for the determination of xylanolytic activity or CMCase, D-xylose, and D-

glucose were used, respectively, which were diluted with acetate buffer in the range 0.2 to 0.6 mg/mL and each point of the curve was prepared with 500 μL of the substrate and 500 μL of standard sugar solution. One international unit of enzyme (IU) was defined as the amount of enzyme required to release one μmol of reducing sugar (xylose or glucose) per minute under the study conditions. Enzymatic activity was reported about one dry gram of fermented residue, IU/g.

2.12 Enzymatic hydrolysis of pretreated residue

The aqCEE and bCEE obtained by SSF-pUAA and SSF-npUAA were used to evaluate the hydrolysis efficiency of the crude enzymatic extract. The solids resulting from the extraction with ultrasound-assisted organosolv (rUAO) were treated with aqCEE and bCEE in a ratio (g:mL) 1:05, 1:10, and 1:15, incubated at 30 ºC for 15 min without stirring; Subsequently, the samples were centrifuged at 3,226 x*g* for 25 min at 4 ºC, the supernatant or hydrolysis liquor was used to determine reducing sugars according to Ávila-Núñez *et al*., (2012); then monosaccharides glucose, fructose, and galactose were quantified by UHPLC (UltiMate 3000, Thermo Scientific) following the methodology described by Hernández-Teyssier *et al*., (2023b), preparing the monosaccharide standards with distilled water or buffer depending on the type of extract.

2.13 Statistical analysis

All experiments and measurements were performed in triplicate. The statistical software used was the open access R software version 4.3.1 with the dplyr package and ggplot2, agricolae, and ggsci libraries (R Core Team 2023, Wickham 2016, Wickham *et al*., 2022).

The t-student test was applied in SSF-SR and SSF-nSR using $\alpha = 0.05$ on parameter μ_{max}. The validation of the kinetic model was through the correlation coefficient (R^2) and the root mean square error (RMSE) (Hyndman and Koehler 2006). The results of the hydrolysis ratio were statistically analyzed by one-way ANOVA and Duncan multiple range post-hoc tests ($p<0.05$).

3 Results

3.1 Coffee residue mix

The manual classification of the residues showed the following composition: 55 % bagasse, 40 % husk, and 5 % parchment.

3.2 Biomass modeling and substrate in SSF

Biomass modeling was just performed in SSF-SR and SSF-nSR. Table 1 shows the results of the kinetic parameters, μ_{max} and λ, in addition to the R^2 coefficient and the RMSE value for each fermentation. The T-Student test indicated that both fermentations were different from each other with a significance level of 95 % (p-value = $2.3x10^{-05}$) in the μ_{max}.

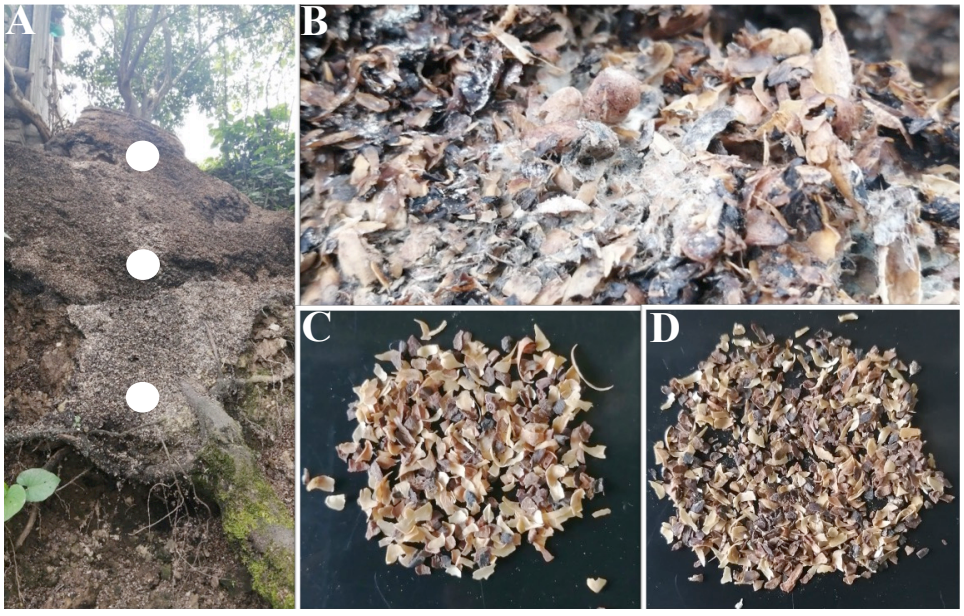

Figure 1. Appearance of the sampling location indicating (A) the collection points (B) waste with the presence of natural microbiota and appearance of the waste mixture after grinding and particle size separation (C) 2.38 mm > PS1 > 1.68 mm y (D) 1.68 mm > PS2 > 1.0 mm.

Table 1. SSF Kinetics parameters with sterilized waste and no-sterilized waste.

Parameter	SSF-SR	SSF-SnR
X_0 (mg/g CRM)	6.57 ± 3.20	10.97 ± 1.29
X_{max} (mg/g CRM)	31.25 ± 0.93	85.33 ± 3.20
μ_{max} (h^{-1})	0.22 ± 0.06 b	1.30 ± 0.05 a
λ (d)	0.00 ± 0.00	1.18 ± 0.16
R^2	0.97 ± 0.01 b	0.98 ± 0.01 a
RMSE	2.22 ± 0.68 b	5.98 ± 2.06 a

CRM: coffee residue mixture; RMSE: root mean square error; Different letter in the same row is significant.

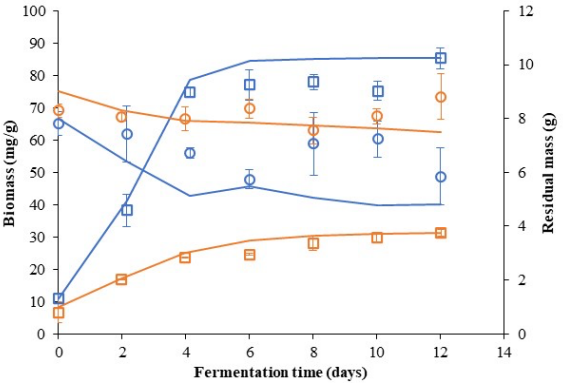

Figure 2. Experimental values of (□) biomass and (O) residual mass in (■) SSF-SR and (■) SSF-nSR, solid line are values obtained by modified Gompertz model and Equation 3.

On the other hand, greater growth was observed in the SSF-nSR treatment (Figure 2) and the greatest slope compared to the SSF-SR treatment; the biomass in SSF-nSR was 85.33 ± 3.20 mg/g while for the SSF-SR treatment was 31.25 ± 0.93 mg/g. Also, the adjustment of the model with the experimental data was better in SSF-SR because the modeling data reached an R^2 value close to 1.0 and a lower RMSE value compared with their counterpart (SSF-nSR).

3.3 *Enzymatic activities and TPC in SSF with PS1*

Figure 3A shows the TPC's behavior; after two days a concentration decrease is observed for both SSFs. In the case of SSF-nSR, an increase in TPC initial was observed at four days of fermentation, of 6.11 ± 0.59 mg GAE/g to 8.19 ± 0.39 mg GAE, but constantly decreased until day 10 to a minimum concentration of 3.26 ± 0.58 mg GAE/g. Contrary to SSF-nSR, SSF-SR showed a decrease in TPC for four days managed to reduce up it to 5.36 ± 0.81 mg GAE/g.

The xylanolytic activity (Figure 3B) in SSF-SR was higher than SSF-nSR, with 7.6 IU/g at eight days of fermentation, while for SSF-nSR, the highest extracellular activity was observed at two days of fermentation with 1.9 IU/g.

On the other hand, CMCase activities (Figure 3C) were higher in SSF-SR at four and six days of fermentation, while for SSF-nSR the highest activities were detected on days two and four. Finally, the FPase activity (Figure 3D) was more noticeable in SSF-nSR because it reached a maximum of 29.65 ± 3.14 IU/g at eight days of fermentation.

3.4 *Production of CMCase by SSF with pUAA and npUAA*

Figure 4 shows the enzymatic activities in aqCEE and bCEE from SSF-pUAA and SSF-npUAA. Both crude enzymatic extracts obtained by SSF-pUAA showed enzymatic activities similar throughout the seven days. In contrast, aqCEE and bCEE from SSF-npUAA were different from each other; the highest enzymatic activity was in bCEE at three and seven days of fermentation, however, the third day of fermentation presented the highest activity with 9.44 ± 1.36 IU/g.

3.5 *Hydrolysis with CEE of waste pretreated by UAO*

Table 2 presents the sugars released during the enzymatic hydrolysis of the residues pretreated with UAO to different dilution ratios. The highest concentration of released sugars was observed in the 1:10 ratio of rUAO hydrolyzed with both CEE generated by SSF-pUAA. While in the SSF-npUAA, the highest concentration of released sugars was observed in the 1:15 ratio of rUAO hydrolyzed with both CEE.

The equation of Otieno *et al*., (2022) was used to calculate the hydrolytic efficiency, and significant differences were observed in the hydrolysis ratios of each SSF and extract according to the Duncan test. Both aqCEE in the 1:05 ratio showed values lower than 1.00 %; whereas in, aqCEE in the 1:10 ratio the hydrolytic efficiencies were different; the highest efficiency with 3.2 ± 0.3 % was in SSF-pUAA (Table 2).

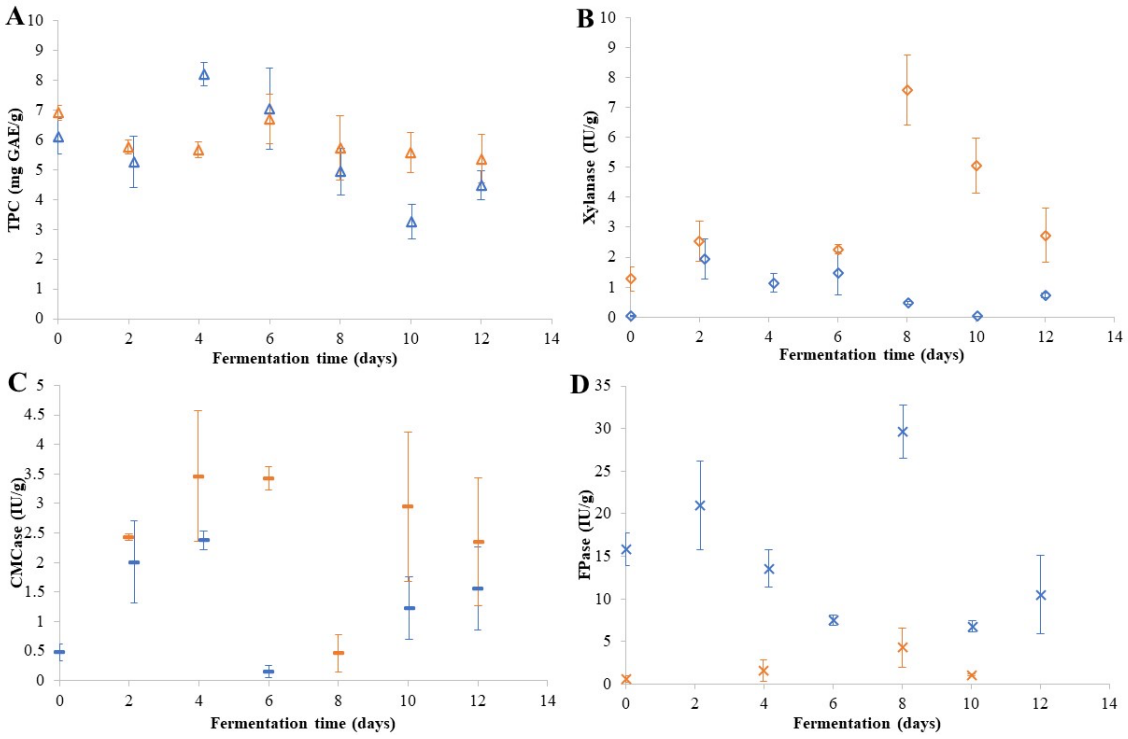

Figure 3. Consumption of A) TPC and enzyme activities B) xylanase, C) CMCase and D) FPase in (■) SSF-SR and (■) SSF-nSR.

Table 2. Concentration of reducing sugars released using the aqueous crude enzyme extracts (aq) and of buffer (b) resulting from the SSF on the pretreated and non-pretreated waste.

Hydrolysis ratio (g/mL)	SSF-PR (mg/g CRM)		SSF-PnR (mg/g CRM)	
	CEEaq	CEEb	CEEaq	CEEb
1:05	10.00 ± 0.95 b	15.35 ± 1.55 a	7.65 ± 0.93 b	12.45 ± 1.26 a
1:10	35.80 ± 3.60 a	21.20 ± 2.60 b	20.30 ± 1.96 a	9.40 ± 1.10 b
1:15	16.80 ± 2.70 a	12.90 ± 1.44 a	24.90 ± 2.09 a	28.05 ± 2.39 a

CRM: coffee residue mix; Different letter in the same row of the SSF is significant.

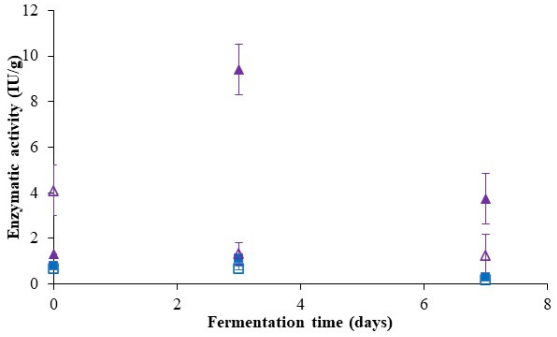

Figure 4. CMCase enzymatic activity present in CEE. (■) bCEE of SSF-pUAA; (□) aqCEE of SSF-pUAA; (▲) bCEE of SSF-npUAA; (Δ) aqCEE of SSF-npUAA.

In Figure 5 are shown the sugars released in hydrolysis liquor of rUAO obtained by bCEE of both SSF. Glucose was detected in the three hydrolysis ratios with both SSF (Figure 5A), highlighting the 1:15 ratio with 3.83 ± 0.61 mg of glucose/g.

On the other hand, fructose was only detected in three cases (Figure 5B), the highest concentration was 1.31 ± 0.16 mg/g in the 1:10 ratio with an extract from SSF-pUAA. Finally, galactose was detected in the three ratios (Figure 5C), but from hydrolysis with extracts from SSF-pUAA, quantifying the maximum concentration of 10.78 ± 0.84 mg/g in the 1:15 ratio.

4 Discussion

This study sets the standard for working with a mixture of coffee waste and taking advantage of all the waste present in producers' final disposal sites, since most of the studies carried out have focused on one type of coffee waste.

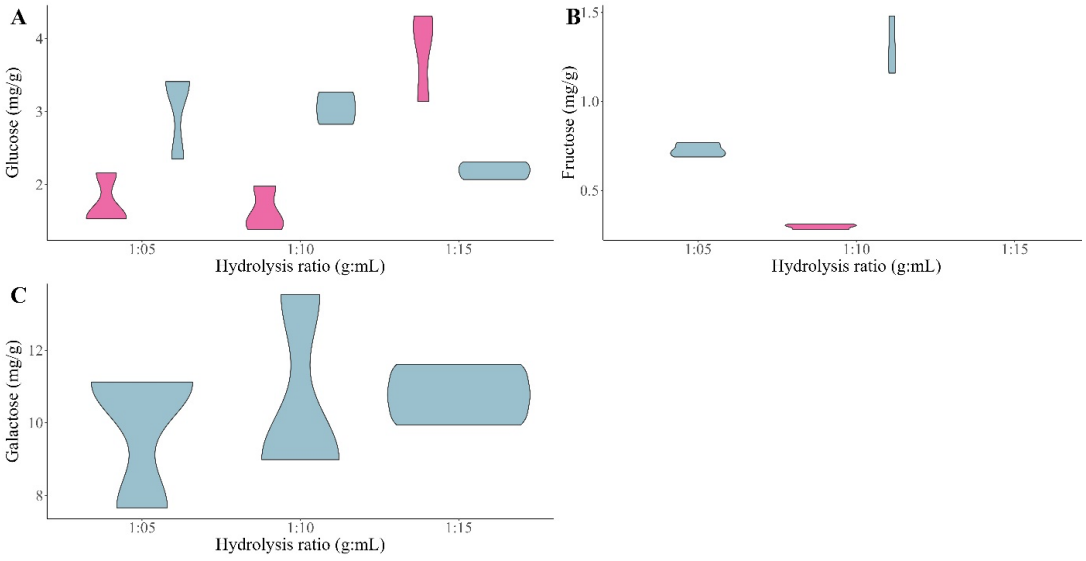

Figure 5. Monosaccharides obtained during the enzymatic hydrolysis of rUAO with bCEE produced in (●) SSF-pUAA and (●) SSF-npUAA.

Table 3. Hydrolytic efficiency of crude enzyme extracts.

Hydrolysis ratio (g/mL)	SSF-PR (%)^A		SSF-PnR (%)^B	
	CEEaq*	CEEb**	CEEaq*	CEEb**
1:5 b	0.90 ± 0.09	1.38 ± 0.14	0.69 ± 0.08	1.12 ± 0.11
1:10 AM	3.22 ± 0.32	1.91 ± 0.23	1.83 ± 0.18	0.85 ± 0.10
1:15 AM	1.51 ± 0.24	1.16 ± 0.13	2.24 ± 0.19	2.52 ± 0.21

Same letter o symbol between factors, SSF, ratio and extract, is not significant.

In SSF-nSR, *T. harzianum* growth was not observed, maybe because the coffee residues mix had latent microorganisms, due to microbial growth observed from to sampling place, making possible greater growth of the native microbiota of the waste. For this reason, it is possible to indicate that a higher μ_{max} and greater substrate consumption in SSF-nSR is due to the adaptability and quantity of microorganisms present in the waste microbiota (Brethauer and Studer 2014).

It is also worth noting that the kinetic parameters (μ_{max} and λ) and metric values (R^2 and RMSE) observed in SSF-SR are very similar to those shown by Hernández-Teyssier *et al*., (2023a). Therefore, the modeling of growth in SSF by the modified Gompertz model is acceptable according to the value of RMSE and R^2, since it is known that R^2 values closer to unity and, in combination with small RMSE, are indicative of a better fit and prediction of the real behavior of a mathematical model (Hyndman and Koehler 2006).

The TPC concentration was an indirect parameter to quantify the degradation of the waste and adaptability from microbiota and *T. harzianum* due to chemical composition of coffee residues mix; since some microorganisms not only detoxify phenols, but also metabolize these compounds (Ristinmaa *et al*., 2022; Hernández-Teyssier *et al*., 2023a; Iqbal and Kapoor 2012).

The production of specialized metabolites occurs depending on the growth conditions of the microorganisms and the metabolic needs or presence of specific molecules (Deborde *et al*., 2017; Mosunova *et al*., 2021). Hydrolytic enzymes are considered specialized metabolites because their expression is induced in the presence of polysaccharides (hemicellulose, cellulose) to hydrolyze them to more easily metabolizable compounds (de Souza-Vandenberghe *et al*., 2016; Kapoor *et al*., 2016).

Hydrolases are enzymatic complex from several enzymes from the same family, like the cellulase family (Escudero-Agudelo *et al*., 2013; Kapoor *et al*., 2016). Therefore, if the enzymatic complex is considered a dynamic system (Mosunova *et al*., 2021), hydrolases will be secreted according to metabolic needs and environmental (de Souza-Vandenberghe *et al*., 2016; Kapoor *et al*., 2016); for this reason, in SSF was observed increases and decreases in activities enzymatic. This behavior was also observed by Lopez-Ramirez *et al*. (2018) in the production of cellulolytic enzymes by *T. harzianum* in SSF of pine sawdust.

Table 4 shows enzymatic activities obtained by other studies using coffee residues as a substrate or

genus *Trichoderma* as the microorganism producing. The enzyme of most significant interest and popularity is CMCase because is considered very useful for the paper, textile, detergent, and food industries (Singhania *et al*., 2010); in comparison with Lopez-Ramirez *et al*., (2018), the enzymatic activity of CMCase in SSF-SR is similar but smaller than the value obtained by Navya and Pushpa (2013).

The second important enzyme was xylanase since has various applications (fruit and vegetable processing industry, beverages, animal feed, pastry, textiles, etc.) (Collins *et al*., 2005). The xylanase activity in the present study was similar to that reported by Gómez-García *et al*., (2018), Jampala *et al*., (2017), Lopez-Ramirez *et al*., (2018), and Cerda *et al*., (2019). Finally, the FPase activity was higher than the other two enzymes, approximately ten times more than that obtained by Cerda *et al*., (2017), but ten times lower than that reported by Li *et al*., (2019).

After identifying the first SSF, the day with the highest enzymatic activity, the CMCase was produced for seven days in SSF but with other types of residues and only for *T. harzianum*; because it was evaluated whether the change in particle size and substrate conditions (TPC) affected the low enzymatic activity as suggested by Zheng *et al*., (2017). However, in SSF-pUAA, CMCase activity was not observed better, until the extraction was obtained by buffer at a temperature of 4 ºC and long contact time as reported by Díaz *et al*., (2007).

The purification of enzyme extract increases the production costs of an enzyme and increases the environmental impact; therefore, it is recommended to apply the crude extract (de Souza-Vandenberghe *et al*., 2016; Catalán *et al*., 2019). To use hydrolytic enzyme extracts, the reaction must occur at specific pH, temperature, substrate concentration, and reaction time conditions which makes the improvement of hydrolysis and increase hydrolytic efficiency (Cheng *et al*., 2021; Otieno *et al*., 2022).

The present study was similar to that of Martínez-Avila *et al*., (2021) because obtained enzymatic extracts in SSF using brewery bagasse and *A. niger*, subsequently applying them to the hydrolysis of pretreated residues of brewery bagasse, grape pomace and residues of olives ground, concluding that according to the extraction method and type of waste, the reaction conditions change.

Likewise, Moran-Aguilar *et al*., (2021) carried out the optimization of enzymatic hydrolysis of pretreated cane sugar bagasse residues under alkaline conditions, but contrary to the conclusion reached by these authors; the present study showed that there is a significant difference between extract:residue ratio in hydrolytic efficiency.

On the other hand, Nguyen *et al*., (2017) hydrolyzed spent coffee beans pretreated with ethanol to obtain glucose, mannose, and galactose; while this study reported glucose, fructose, and galactose. This diversity of released monosaccharides between both studies is due to the composition of the matrix of each waste and pretreatment (Ravindran *et al*., 2018; Hernández-Teyssier *et al*., 2023b). According to these monosaccharides, the remnant composition of the matrix contains hemicellulose because the galactose concentration was higher than other monosaccharides, and pretreatment ultrasonic assisted organosolv created cavities on the surface of the matrix that facilitated the hydrolysis of cellulose (Al-Dhabi *et al*., 2017; Ravindran *et al*., 2017; Lin *et al*., 2021).

Conclusion

This study showed that application of the bCEE produced at three days by SSF-pUAA to hydrolyze rUAO allowed to obtain glucose, fructose, and mostly galactose as enzymatic hydrolysis product to in a ratio of 1:15 (w:v).

In addition, this study demonstrated the possibility of applying crude enzyme extracts to hydrolyze agroindustrial waste and reduce enzyme production costs. Furthermore, coffee residues could be used as a carbon source for SSF and a source of monosaccharides such as galactose.

The production of hydrolytic enzymes, CMCase, xylanase, and FPase, of *T. harzianum* in SSF from a coffee residue mix could be improved if the study is extended toward the optimization of production parameters, as well as the reaction conditions of the crude extract and residue to expand the hydrolytic efficiency.

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