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Isolation and evaluation of chlorogenate hydrolase producing fungi

Aislamiento y evaluación de hongos productores de clorogenato hidrolasa

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ABSTRACT

Chlorogenate hydrolase catalyzes the hydrolysis of chlorogenic acid, releasing caffeic and quinic acids. This enzyme can be used to extract caffeic acid from agro-industrial by-products (apple marc, coffee pulp, brewer's spent grain, among others), and to synthesize caffeic acid derivatives. This work aimed to isolate and identify fungal strains capable of producing enzymes with chlorogenate hydrolase activity. Twelve fungal strains were isolated from fresh coffee cherries, dry coffee cherries, and green coffee beans. The isolated fungi were analyzed for their morphology and ability to assimilate chlorogenic acid. Four fungal isolates were identified as *Aspergillus* sp., two as *Fusarium* sp., and one as *Cladosporium* sp. All the fungal isolates were able to grow using chlorogenic acid as the sole carbon source. The CR1 strain, identified as *Aspergillus niger* by sequencing of the ITS region, was selected due to its high radial growth rate (0.27 ± 0.01 mm h^{-1}). This strain grew and showed chlorogenate hydrolase activity in Submerged (SmF) and Solid-State Fermentation (SSF).

The maximum chlorogenate hydrolase activity in SSF (34.6 ± 11.8 U/L) was 2.1-fold higher than in SmF (16.2 ± 1.6 U/L). These results provide the basis for developing applications based on the use of enzymes with chlorogenate hydrolase activity.

Keywords: Chlorogenic acid, Chlorogenate hydrolase, Solid State Fermentation, Submerged fermentation.

RESUMEN

La clorogenato hidrolasa cataliza la hidrólisis del ácido clorogénico liberando ácidos cafeico y quínico. Esta enzima puede ser utilizada para extraer ácido cafeico a partir de subproductos agroindustriales (orujo de manzana, pulpa de café, granos gastados de cervecería, entre otros), y para la síntesis de derivados del ácido cafeico. El objetivo de este trabajo fue aislar e identificar cepas de hongos capaces de producir enzimas con actividad clorogenato hidrolasa. Se aislaron doce cepas de hongos de cerezas de café frescas, cerezas de café secas y granos de café verdes. Los hongos aislados se caracterizaron morfológicamente y por su capacidad de asimilar ácido clorogénico. Cuatro aislados se identificaron como *Aspergillus* sp., dos aislados como *Fusarium* sp. y un aislado como *Cladosporium* sp. Todos los aislados de hongos pudieron crecer con ácido clorogénico como única fuente de carbono. La cepa CR1, identificada como *Aspergillus niger* mediante secuenciación ITS, fue seleccionada debido a su alta tasa de crecimiento radial (0.27 ± 0.01 mm h^{-1}). Esta cepa pudo crecer y mostrar actividad clorogenato hidrolasa en fermentación sumergida (FS) y fermentación en estado sólido (FES). La máxima actividad clorogenato hidrolasa obtenida en FES (34.6 ± 11.8 U/L) fue 2.1 veces mayor que la obtenida en FS (16.2 ± 1.6 U/L). Estos resultados proporcionan la base para el desarrollo de procesos basados en el uso de enzimas con actividad clorogenato hidrolasa.

Palabras clave: Ácido clorogénico, clorogenato hidrolasa, fermentación en estado sólido, fermentación sumergida.

1. INTRODUCTION

Chlorogenic acid is a soluble phenolic compound formed by the esterification of caffeic and quinic acid. Chlorogenic acid is one of the most abundant phenolic acids in the human diet, as it is found in beverages such as coffee and tea (Naveed *et al*., 2018). This compound is also found in considerable amounts in sweet potatoes, potatoes, eggplants, artichokes, sunflower seeds, apples, pears, peaches, plums, cherries, and tomatoes, among others (Lu *et al.*, 2020). Chlorogenic acid is considered an antioxidant agent, inhibitor of lipid oxidation (Cao *et al*., 2020) and has hepatoprotective activity among other biological activities (Zhou *et al*., 2016).

Chlorogenate hydrolase is an enzyme (EC 3.1.1.42) that hydrolyzes chlorogenic acid to yield caffeic and quinic acids. This enzyme also acts on other caffeoyl-quinic acids but not on esters of ferulic, sinapinic, and *p*-coumaric acids (Mateos *et al*., 2009). Type B, C, and D feruloyl esterases can hydrolyze caffeic acid methyl esters. However, there are only a few

studies that focus on the hydrolysis of chlorogenic acid (Nieter *et al*., 2015). Asther *et al*. (2005) used sugar beet pulp as an inducer for the production of a chlorogenate hydrolase by *Aspergillus niger* BRFM 131 in SmF. Adachi *et al*., (2008) studied the production of a chlorogenate hydrolase by *Aspergillus sojae* AKU 3312 in Czapek broth using coffee powder and coffee pulp as inducers. Butiuk *et al.* (2015) studied the use of a yerba mate extract to induce the production of a chlorogenate hydrolase by four strains of *Aspergillus* spp in a two-stage SmF. On the other hand, Okamura and Watanabe (1982) purified and characterized a chlorogenate hydrolase from *Aspergillus japonicus* produced in solid-state fermentation using wheat bran as a support and substrate. More recently, Ramírez-Velasco *et al.* (2016) studied the production of enzymes with chlorogenate hydrolase activity by *Aspergillus ochraceus* in solid-state fermentation using sugarcane bagasse as a support and coffee husk as an inducer.

Enzymes with chlorogenate hydrolase activity can be used for the extraction of caffeic acid from agricultural by-products, such as apple marc (Benoit *et al*., 2006), coffee pulp (Ramírez-Velasco *et al*., 2016), brewer's spent grain (Szwajgier *et al.*, 2010), and yerba mate (Butiuk *et al*., 2018). Caffeic acid has an antioxidant and antimicrobial activity; therefore, it has potential medical and cosmetic applications (Magnani *et al.*, 2014). Furthermore, chlorogenate hydrolases can be used for the enzymatic synthesis of caffeic acid esters, such as caffeic acid phenethyl ester (Kishimoto *et al*., 2005), methyl caffeate, and 3-cyclohexyl propyl caffeate (Kurata *et al*., 2011). These compounds have antimicrobial, antioxidant, anti-inflammatory, and cytotoxic activities, so they have potential therapeutic applications (Murtaza *et al*., 2014). However, the development of these applications is complex due to the lack of commercial sources of chlorogenate hydrolases. Therefore, the aim of this work was to isolate and evaluate chlorogenate hydrolase-producing fungi.

2. MATERIALS AND METHODS

2.1. Sample collection

Samples of fresh coffee cherries (Costa Rica and Rojo Colombia Cultivars), dry coffee cherries, and defective green coffee beans (broken, too small, or black beans) were collected. The samples of fresh and dry coffee cherries were obtained from a coffee farm located in the state of San Luis Potosí (21°21'42.5 "N 98°57'07.2" W), while the sample of green beans was obtained from a store located in Xilitla, San Luis Potosí (21°23'42.3" N 98°59'35.2" W). All samples were transported to the laboratory in refrigerated containers where the isolations were performed.

2.2. Isolation and morphological characterization of fungi

The coffee samples were subjected to an enrichment culture; 10 g of each sample was placed in sterile 250 mL Erlenmeyer flasks, and 100 mL of sterile peptone water (BD Bioxon) was added. The flasks were incubated for 24 h at 30 °C with constant shaking (50 rpm). Subsequently, the enriched culture was diluted (10^{-1} to 10^{-6}) with a sterile Tween 80 solution (0.1% v/v). One mL of each dilution was inoculated by the spread plate technique on potato dextrose agar (PDA, BD Bioxon) plates, which were acidified with tartaric acid (0.14% w/v)

and added with ampicillin (500 mg/L) to avoid bacteria growth. The plates were incubated at 30 °C for 3 days. The stab technique was used to isolate fungal colonies with different morphologies, and they were periodically sub-cultured to maintain active cultures.

The isolated fungi were inoculated in Petri plates with PDA and incubated at 30 °C for 7 days. At the end of incubation, macroscopic characterization of the colony (shape, size, color, appearance of mycelium, pigment formation) was performed, and microscopic characterization was carried out using lactophenol blue staining. A preliminary identification was done using identification keys (Dugan, 2017). Observations were carried out with an Omax M837 optical microscope equipped with a 14 MP Omax A35140U3 digital camera.

2.3. Inoculum preparation

Fungi were inoculated in flasks with PDA and incubated for 7 days at 30 °C. Subsequently, they were subcultured and incubated for seven more days at 30 °C. Twenty mL of a sterile Tween 80 solution (0.1 % v/v) was added, and the spores were removed with magnetic stirring.

2.4. Strain selection

The fungal strains were evaluated for their ability to grow in a medium with chlorogenic acid as the sole carbon source. A solid medium composed of chlorogenic acid (15 g/L), ammonium tartrate (1.842 g/L), yeast extract (0.5 g/L), KH_2PO_4 (0.2 g/L), $CaCl_2{\cdot}2H_2O$ (0.0132 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L) and agar (15 g/L) was prepared as follows: salts, yeast extract and agar were sterilized by autoclave (15 psi, 15 min), chlorogenic acid was dissolved in distilled water, adjusted to a pH of 6.0 and sterilized by filtration through 0.22 µm membranes. Petri dishes with 25 mL of sterile medium were inoculated at the center with 10 µL of a spore suspension (1 x 10^7 spores/mL). The plates were incubated for 5 days at 30 °C. Radial growth was measured every 24 h. Growth rates were calculated using linear regression.

2.5. Molecular identification

The selected strain was identified by ITS sequencing. The fungus was inoculated in Erlenmeyer flasks with nutrient broth and incubated for 48 h at 30 °C and 150 rpm. Four pellets were transferred to a sterile microtube and centrifuged (2 min, 8,000 rpm). The supernatant was discarded, and 0.3 g of 0.5 mm glass microspheres (BioSpec) was added and vortexed for 20 seconds to disrupt the cells. DNA was extracted with the Wizard genomic DNA isolation kit (Promega) following the manufacturer's instructions.

The amplification of the ITS region was performed using the oligonucleotides ITS1 (5´-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5´-TCCTCCGTCTATTGATATGC-3') (Zarrin *et al*., 2016). The reaction mixture was composed of DNA (100 ng/µL), dNTPs (0.2 mM), $MgCl_2$ (1.5 mM), ITS1 primer (0.5 µM), ITS4 primer (0.5 µM), Taq polymerase (0.05 U/µL), in GoTaq reaction buffer (1X). The reaction was carried out in a MaxyGene II Thermal Cycler (Axygen). It consisted of an initial denaturation step (94 °C, 5 min), 30 cycles consisting of a denaturation step (94 °C, 30 s), an annealing step (57 °C, 30 s), and an extension step

(72 °C, 60 s). The reaction was terminated with a final extension step (72 °C, 7 min). The amplification product was separated by electrophoresis in a 1.5% agarose gel for 30 min at 100 V. This reaction resulted in an amplicon of approximately 550 bp. Sanger sequencing of the amplicon was performed by Eurofins Genomics (USA) following the Barcoded Tubes protocol, in which 20 ng/µL of the template and 10 pmol/µL of the ITS1 primer were mixed. The sequence obtained was subjected to a similarity analysis with the NCBI nr/nt database using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. Submerged fermentation

SmF was carried out in 250 mL Erlenmeyer flasks with three baffles containing 25 mL of culture medium. The culture medium proposed by Asther *et al*. (2005) with some modifications was used. The medium was composed of ammonium tartrate (1.842 g/L), yeast extract (0.5 g/L), KH_2PO_4 (0.2 g/L), $CaCl_2 \cdot 2H_2O$ (0.0132 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), and chlorogenic acid as carbon source and inducer (15.0 g/L). Medium without inducer was prepared at a 2X concentration and sterilized by autoclave (15 min, 15 psi). The medium was combined with a similar volume of a chlorogenic acid solution (30 g/L), adjusted to an initial pH of 6 with 2M NaOH, and filtered through sterile cellulose membranes with 0.22 µm pore size. The medium was inoculated with a suspension of spores to have a final concentration of 1 x 10^6 spores/mL. The flasks were incubated for 4 days at 30 °C with orbital shaking at 150 rpm.

During fermentation, three experimental units were withdrawn every 24 h. The content was filtered through Whatman No 1 filter paper. The filtrate was used to determine the concentration of caffeic acid, chlorogenic acid, and chlorogenate hydrolase activity. The retained mycelium was used to determine biomass by dry weight.

2.7. Solid state fermentation

SSF was carried out in 250 mL Erlenmeyer flasks using perlite as a solid support and the liquid culture medium described above. Perlite was sieved to select particles with a size of 0.84 to 2 mm, washed twice with hot water and then once again with cold water, and dried in a convection oven for 48 h at 60 °C. 7.5 g of support was weighed into each flask and autoclaved (15 min, 15 psi). Subsequently, 12.5 mL of inoculated culture medium was added, mixed well, and incubated at 30 °C for 4 days.

During the fermentation, three flasks were withdrawn every 24 h, and used to obtain the extracellular enzyme extract. 50 mL of distilled water was added to each flask and shaken for 10 min at 150 rpm on an orbital shaker. The contents were filtered through Whatman No. 1 filter paper. The filtrate was used to determine the content of caffeic acid, chlorogenic acid, and chlorogenate hydrolase activity, while the solid was used to determine biomass.

2.8. Analytical methods

The quantification of caffeic and chlorogenic acid was carried out by a chromatographic method. The analysis was conducted on a Waters 2695 HPLC instrument with a Waters 2996 diode array detector (Milford, USA). The separation was performed on a Restek C18

column (150 x 3.6 mm, 5 µm). An isocratic method was used with a mobile phase composed of methanol (25 %), acetic acid (0.9 %), and water (74.1 %) at a flow rate of 0.5 mL/min. Detection was performed at 320 nm. Standard curves of caffeic and chlorogenic acid were prepared at concentrations of 15.6 to 250 mg/L.

Chlorogenate hydrolase activity was measured by a chromatographic method using chlorogenic acid as substrate (Rodríguez-Durán, 2016); 900 µL of 5 mM chlorogenic acid in phosphate buffer (50 mM pH 6.5) were placed in glass tubes, 100 µL of enzyme extract was added and incubated for 60 min at 37 °C. The reaction was stopped by adding 1 mL of methanol. The reaction mixture was filtered through 0.2 µm pore nylon membranes and injected into HPLC equipment to quantify the release of caffeic acid during the enzymatic reaction. Control tubes were prepared in which the enzyme extract was added after adding methanol. The enzyme unit was defined as the amount of enzyme required to release 1 µmol of caffeic acid per minute.

Microbial growth was measured gravimetrically. The solids retained on the filter paper (see sections 2.6 and 2.7) were washed 3 times with 50 mL of distilled water. The solids were dried at 60 °C until constant weight. Biomass was determined by weight difference.

2.9. Data analysis

Radial growth rates were calculated by linear regression using Microsoft Excel 365 software (Redmond, USA). Analysis of variance and Tukey's HSD test (α = 0.05) were performed using JMP 18 software (Cary, USA). Growth in SmF and SSF were fitted to a logistic model using the following equation 1:

$$X = \frac{X_{max}}{1+\left(\frac{X_{max}}{X_0}-1\right)e^{-\mu_{max}t}} \qquad \text{(Eq.1)}$$

Where X = Biomass (g/L), X_0 = Initial biomass (g/L), X_{Max} = Maximum growth (g/L), μ_{Max} = Maximum specific growth rate (h^{-1}) and t = time (h). The data were fitted using the solver function of Microsoft Excel 365 (Redmond, USA).

3. RESULTS

3.1. Isolation and characterization of fungal strains

Four fungal isolates were obtained from fresh coffee cherries of Costa Rica cultivar (CR1, CR2, CR3, and CR4), one isolate from Rojo Colombia cultivar coffee cherries (RC1), four isolates from dried coffee cherries (CS1, CS2, CS3, and CS4) and three isolates from green coffee beans (CV1, CV2, and CV3). Fig. 1 shows the macroscopic morphology of fungal isolates, and Fig. 2 shows the microscopic morphology of isolated fungi.

Isolates CR1, CR2, CR3, and CS3 present similar microscopic morphology, but different macroscopic characteristics. All four isolates show rapid growth, white mycelium without pigment production. Isolates CR1 and CS3 have black spores while CR2 and CR3 isolates

have yellow to brown spores. The four isolates present conidia and conidial heads characteristic of fungi of the genus *Aspergillus*. According to the microscopic and macroscopic characteristics (slightly granular conidiophore, with a thick wall and abundant black conidia), it indicates that isolates CR1 and CS3 belong to the *Aspergillus niger* group, while isolates CR2 and CR3 belong to other groups of the genus *Aspergillus*. Colonies of isolate CR4 are small, white, with a velvety texture. The CR4 isolate has a microsiphonated mycelium with elongated conidiophores ending in grouped conidia simulating a head as in the genus *Acremonium*. The RC1 isolate forms small, dark green velvety colonies. The RC1 isolate has melanic septate mycelium, with ellipsoid conidia formed by successive budding, with characteristics that coincide with the genus *Cladosporium*.

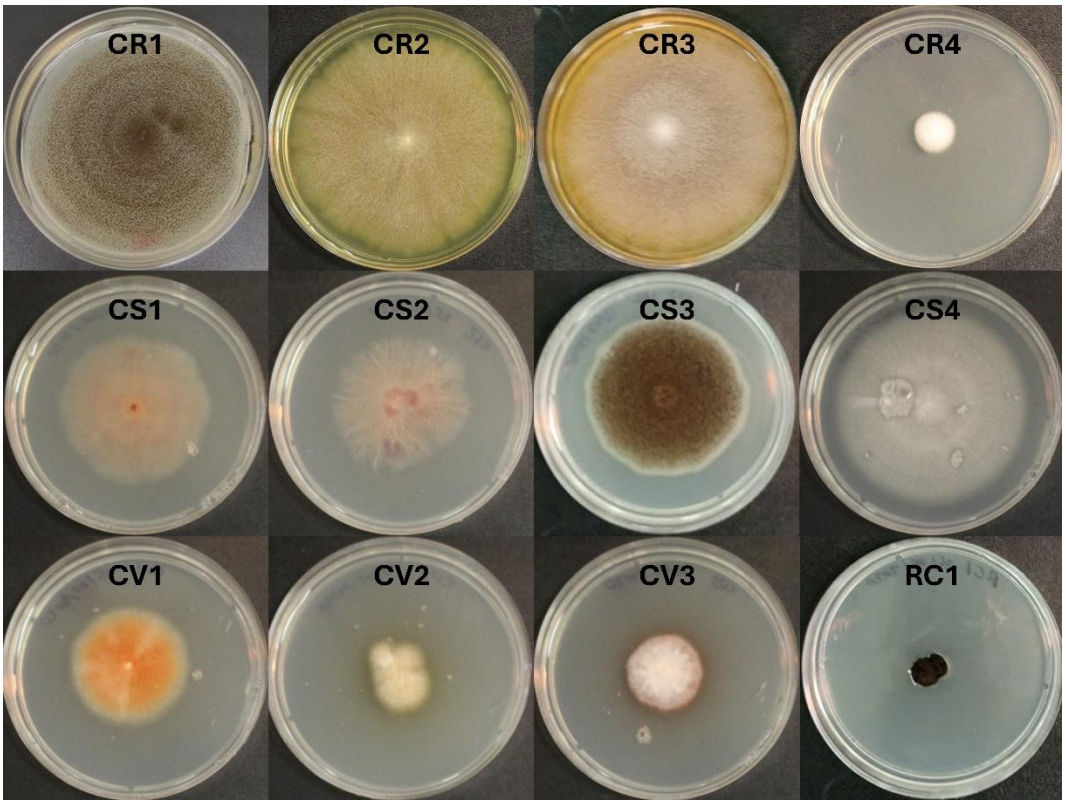

Fig. 1. Colony morphology of isolated fungi on PDA (7 d, 30 °C).

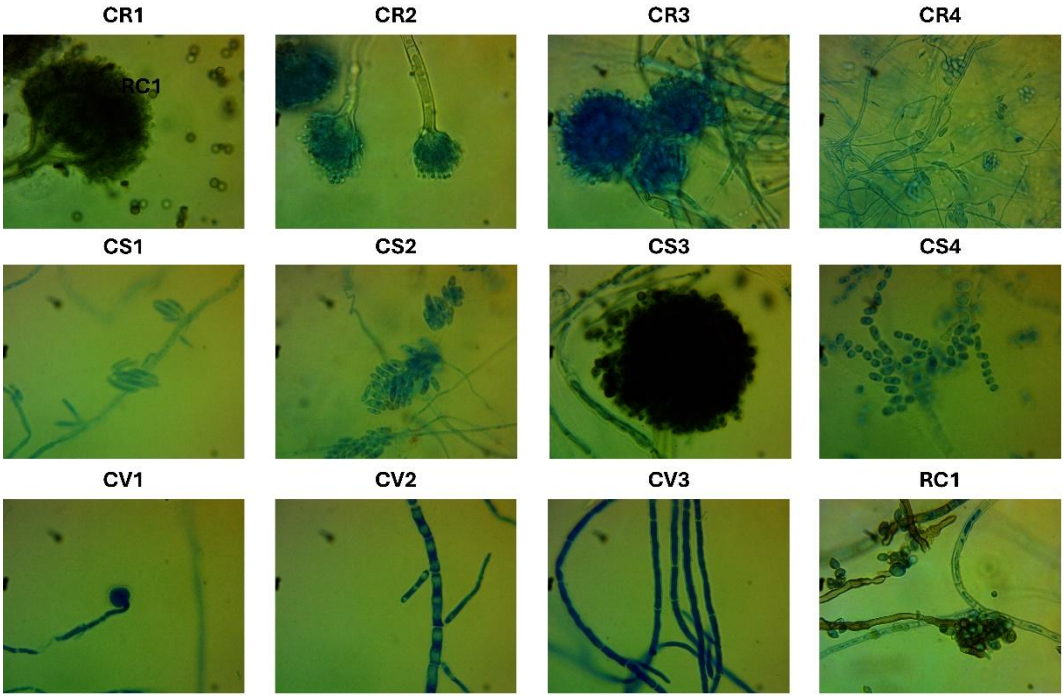

Fig. 2. Microscopic morphology (100 X) of fungi isolated from coffee products and by-products.

Isolates CS1 and CS2 grow as medium-sized, thin, pink to purple colonies. CS1 and CS2 isolates presented a thin septate, poorly branched hyphae, and septate canoe-shaped macroconidia, characteristic of the genus *Fusarium*. Isolate CS4 grows rapidly on PDA with white, flat, and thin colonies. The CS4 isolate has thick septate and poorly branched hyphae that divide into numerous arthroconidia. These characteristics suggest that it belongs to the genus *Geotrichum*. Isolate CV1 has microsiphonated septate mycelium. The conidiospores are solitary, ovoid, smooth, black, and opaque, like those of *Nigrospora* sp. Isolates CV2 and CV3 have a similar microscopic morphology, with microsiphonated septate mycelium. No reproductive structures were observed in either of the two isolates.

3.2. Selection of fungal strains

All fungal isolates evaluated were able to grow in the culture medium with chlorogenic acid as the sole carbon source, so they have the potential to produce enzymes with chlorogenate hydrolase activity. Fig. 3 shows the radial growth of fungal isolates in a culture medium with chlorogenic acid as the sole carbon source. The data obtained were analyzed by linear regression, and radial growth rates and confidence intervals were calculated (Table 1). Statistically significant differences were observed between the growth rates of the different isolates evaluated. The highest growth rate (0.270 ± 0.013 mm/h) was observed for the CR1 isolate. This growth rate is not statistically different from that obtained for strain CS3 (0.259 ± 0.018 mm/h). The strain CR1 was selected for the following experiments due to its higher growth in a selective culture medium. The ITS sequence was deposited in the Genebank (Accession number PV211186.1) and the comparison with the NCBI database indicates that the CR1 isolate belongs to the *Aspergillus niger* species with an identity percentage of 95%.

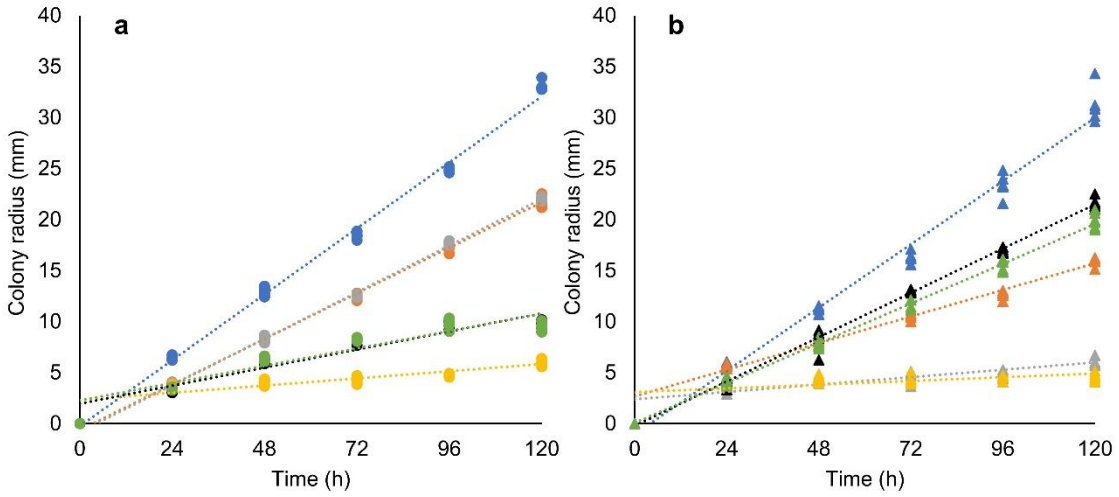

Fig. 3. Radial growth on agar with chlorogenic acid as the only carbon source of fungal strains isolated from coffee products. CR1 (blue circles), CR2 (orange circles), CR3 (grey circles), CR4 (yellow circles), CS1 (black circles) CS2 (green circles), CS3 (blue triangles), CS3 (orange triangles), CV1 (grey triangles), CV2 (yellow triangles), CV3 (black triangles), and RC1 (green triangles).

Table 1. Radial growth rates of fungal isolates in selective medium.

Microorganism	Code	Growth rate (mm/h)*
Aspergillus niger	CR1	0.270 ± 0.013 [a]
Aspergillus sp.	CR2	0.187 ± 0.004 [b]
Aspergillus sp	CR3	0.192 ± 0.002 [b]
Acremonium sp.	CR4	0.024 ± 0.003 [f]
Fusarium sp.	CS1	0.070 ± 0.005 [e]
Fusarium sp.	CS2	0.066 ± 0.005 [e]
Aspergillus sp.	CS3	0.259 ± 0.018 [a]
Geotrichum sp.	CS4	0.103 ± 0.005 [d]
Nigrospora sp.	CV1	0.025 ± 0.005 [f]
Unidentified	CV2	0.009 ± 0.002 [g]
Unidentified	CV3	0.181 ± 0.003 [b]
Cladosporium sp.	RC1	0.162 ± 0.008 [c]

*Growth rates expressed as mean ± standard deviation. Different letters in columns indicate significant statistical differences (Tukey HDS test, α =0.05).

3.3. Chlorogenate hydrolase production

The production of chlorogenate hydrolase by *A. niger* CR1 was evaluated in SmF and SSF using a culture medium with chlorogenic acid as a carbon source and inducer. Fig. 4 shows the kinetic profiles of chlorogenic acid consumption and caffeic acid accumulation during SmF and SSF. In SSF, chlorogenic acid was completely consumed after 24 h, while in SmF, only 44.7% of chlorogenic acid was consumed after 72 h. In both cases, an accumulation of

caffeic acid was observed, which indicates the presence of enzymes with chlorogenate hydrolase activity. In SSF, the highest accumulation of caffeic acid (3.10 ± 0.17 g/L) was obtained at 24 h, while in the SmF, the highest accumulation of caffeic acid (1.42 ± 0.22 g/L) was reached at the end of the culture.

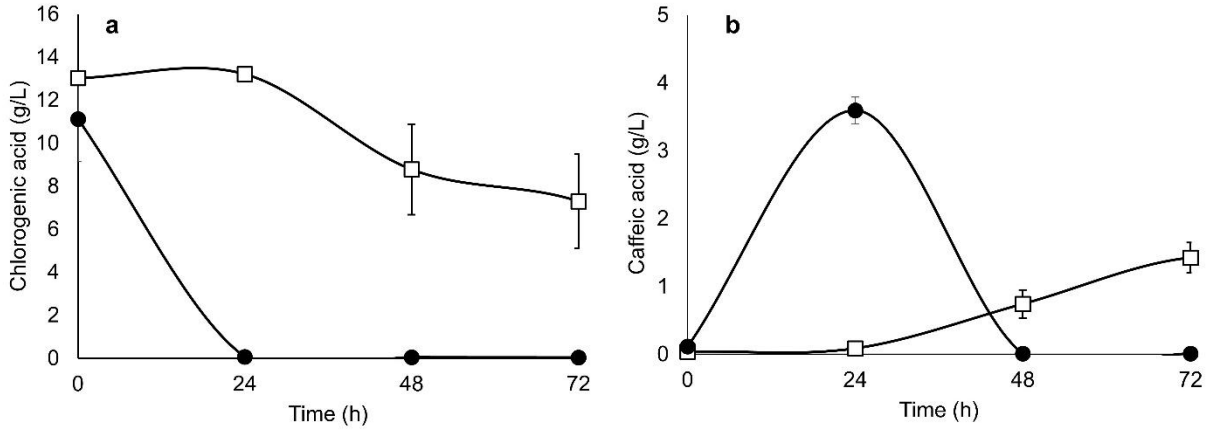

Fig. 4. Chlorogenic acid consumption (a) and caffeic acid accumulation (b) during growth of *A. niger* CR1 in SmF (white squares) and SSF (black circles).

Fig. 5 shows the growth of *A. niger* CR1 and chlorogenate hydrolase activity on SmF and SSF. The biomass data were adjusted to the logistic model with a coefficient of determination greater than 0.95. The highest X_{max} value was obtained in SSF (9.06 g/L) and was 2.3 fold higher than in SmF (3.96 g/L). The μ_{Max} value in SSF (0.09 h^{-1}) was 1.3 times higher than in SmF (0.07 h^{-1}).

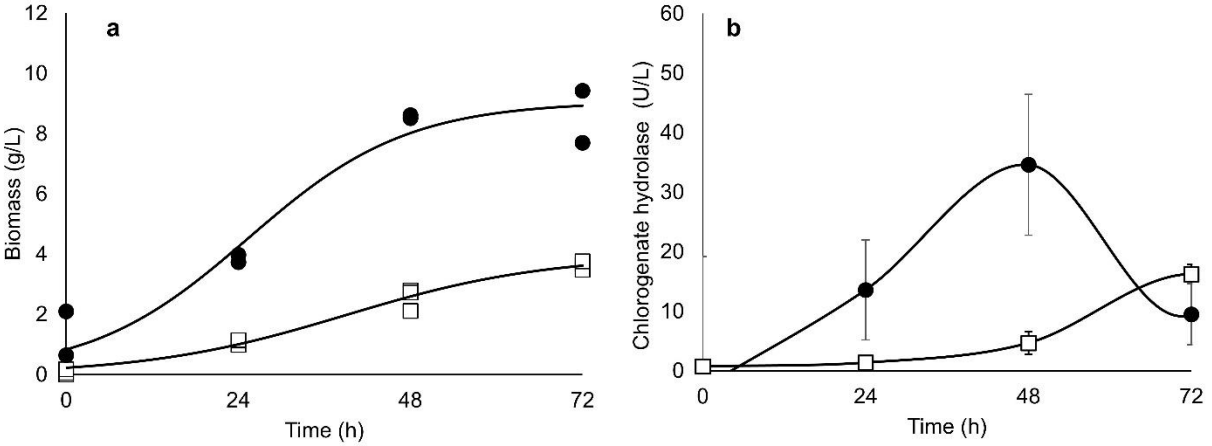

Fig. 5. Growth (a) and chlorogenate hydrolase activity (b) kinetic profiles of *A. niger* CR1 in SmF (white squares) and SSF (black circles).

The strain *A. niger* CR1 produced enzymes with chlorogenate hydrolase activity in SmF and SSF. In SSF, a peak of activity (34.6 ± 11.8 U/L) was observed at 48 h, followed by a decrease at 72 h. In SmF, the maximum chlorogenate hydrolase activity (16.2 ± 1.6 U/L)

was reached at 72 h. In both cases, the highest chlorogenate hydrolase activity coincides with the maximum accumulation of caffeic acid.

4. DISCUSSION

Coffee fungi have different modes of life: endophytes, pathogens, and saprobes. Pathogens can infect pre-harvest or post-harvest coffee (Lu *et al*., 2022). Some post-harvest pathogens produce mycotoxins, which reduce the quality of the drink and represent a risk to consumer health (Bozza *et al*., 2009). In this study, twelve fungal strains were isolated from coffee products. Based on the macroscopic and microscopic morphology, we identified four strains of the genus *Aspergillus*, two strains of *Fusarium* spp, one *Acremonium* sp, one *Cladosporium* sp., and one *Geotrichum* sp.

The fungi isolated from coffee products coincide with those found in similar studies (Alves da Silva *et al*., 2020; Lu *et al*., 2022; Salazar Téllez *et al*., 2023). *Aspergillus* is one of the most common fungal genera in coffee. *A. westerdijkiae*, *A. steynii*, *A. ochraceus*, *A. carbonarius*, and some isolates of *A. niger* have been reported to produce ochratoxin in coffee (Lu *et al*., 2022). On the other hand, other *Aspergillus* species do not produce ochratoxin and can even inhibit the growth of ochratoxin-producing fungi (de Almeida *et al*., 2019). *Fusarium* is one of the most common phytopathogenic fungi in coffee. Some *Fusarium* species cause coffee wilt disease and dry root rot (Al-Faifi *et al*., 2022). *Acremonium* spp. has been isolated from green coffee beans from Puerto Rico (Vega *et al*., 2008). *Cladosporium* spp has been isolated from coffee leaves infected by coffee leaf rust, and it is probably a natural antagonist to the causal agent of this disease (*Hemileia vastatrix*) (Pereira *et al*., 2023). *Geotrichum* sp has been isolated from coffee cherries in Colombia, but its ecological relationship with coffee plants is unclear (Salazar Téllez *et al*., 2023).

All the fungi isolated from coffee products could grow on a solid medium with chlorogenic acid as the sole carbon source, which indicates the ability to produce enzymes with chlorogenate hydrolase activity. The highest growth rates (0.259 to 0.270 mm/h) were obtained with the black *Aspergillus* isolates (CR1 and CS3). These growth rates are within the range of those calculated for nine strains of *Aspergillus* spp. (0.216 – 0.277 mm/h) in a medium with chlorogenic acid as the sole carbon source (Rodríguez-Durán, 2016). The radial growth rates obtained for strains CR1 and CS3 are close to those reported by De la Cruz *et al.* (2015) for four *A. niger* strains using Czapek Dox agar modified with pomegranate peel ellagitannins as the sole carbon source.

The growth and production of chlorogenate hydrolase activity by *A. niger* CR1 were evaluated in SmF and SSF. The highest growth (X_{Max}) was obtained in SSF, while the specific growth rate (μ_{Max}) was 25% higher in SSF than in SmF. The specific growth rates are lower than those obtained with *Aspergillus japonicus* with coffee extract as a carbon source (Marín Morales, 2019). They obtained a specific growth rate of 0.366 and 0.098 h^{-1} for SSF and SmF, respectively. These differences in specific growth rates may be related to the strains used, the analytical methods, and the increased mass transfer due to forced aeration. In both cases, a higher specific growth rate was observed in SSF compared to SmF. The higher specific growth rate in SSF may be related to a more efficient oxygen

transfer rate than in SmF (Salgado-Bautista *et al.*, 2020). Furthermore, the low water content in SSF affects the diffusion of nutrients and toxic metabolites, which influences the growth and metabolism of microorganisms (Torrado *et al*., 2011). SSF resembles the natural habitat of fungi. This generally results in higher yields, lower costs, and lower carbon repression than SmF (Canavati-Alatorre *et al*., 2016).

The highest chlorogenate hydrolase activity in SmF (16.2 U/L) is lower than that produced by *A. niger* using sugar beet pulp as a source of inducers (27.6 U/L). However, in that case, the maximum activity was reached after 11 days of culture, corresponding to a productivity of 2.5 U/L·d (Asther *et al*., 2005). In this study, maximum activity was obtained at 72 h, which corresponds to a productivity of 5.4 U/L·d.

In SSF, the maximum enzymatic activity (34.6 U/L) corresponds to 0.058 U/g of solid support. This value is higher than that obtained with *Aspergillus* sp. Café I (0.039 U/g of initial dry matter) but lower than that obtained by *A. ochraceus* AS III in SSF using coffee pulp and sugarcane bagasse as support (0.133 U/g of initial dry matter) (Ramírez *et al.*, 2008). However, *A. ochraceus* AS III, reached the highest enzymatic activity after 6 days of fermentation, corresponding to a productivity of 0.022 U/g·d. In this study, the highest activity was reached at 48 h, resulting in a productivity of 0.029 U/g·d. These results can be explained by differences between microorganisms, but also by the substrates used. Chlorogenate hydrolase is an inducible enzyme. Although its induction and repression mechanisms have not been described, it is considered that chlorogenic acid, its isomers, and dimers can act as inducers of this enzyme (Butiuk *et al*., 2015). For this reason, several authors have studied the production of chlorogenate hydrolase using agroindustrial by-products with high content of chlorogenic acid, such as coffee pulp (Adachi *et al*., 2008), coffee husk (Ramírez-Velasco *et al*., 2016), yerba mate extract (Butiuk *et al*., 2023).

The highest chlorogenate hydrolase activity obtained in SSF was 2.1 times higher than in SmF. Similar behavior has been observed for other enzymes, such as tannases, pectinases, and invertases (Viniegra-González *et al*., 2003). Most commercial enzymes are produced by SmF using inexpensive liquid culture media (Hernández-Sánchez *et al*., 2019). However, solid-state fermentation is a more robust process in terms of nutrients, which favors the growth of *Aspergillus* spp and leads to more productive processes (Mora-Pérez *et al*., 2023). There is strong evidence that SSF reduces catabolite repression and substrate inhibition, allowing higher protein and enzyme production than SmF (Mora-Pérez et al., 2024). For example, Aguilar *et al*. (2001) observed a greater resistance to catabolic repression during the production of tannase by *A. niger* Aa20 in SSF compared to SmF, and they also observed an enzymatic degradation process in SmF, which was absent in SSF. On the other hand, Viniegra-González *et al*. (2003) proposed that differences in mass transfer processes can explain the different productivity of both systems.

5. CONCLUSION

Twelve fungal strains were isolated from coffee products (fresh coffee cherries, dry coffee cherries, and green coffee beans). All strains were able to grow in a culture medium with chlorogenic acid as the sole carbon source. The strain *Aspergillus niger* CR1 was selected

due to its high radial growth rate in a selective culture medium. *A. niger* CR1 was able to grow and produce chlorogenate hydrolase activity in submerged fermentation (SmF) and solid-state fermentation (SSF). The maximum chlorogenate hydrolase activity obtained in SSF (34.6 ± 11.8 U/L) was 2.1 times higher than in SmF (16.2 ± 1.6 U/L). These results provide the basis for developing applications based on the use of enzymes with chlorogenate hydrolase activity.

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AUTHOR CONTRIBUTION

Laura Patricia Soria-Ibarra performed the experimental work and analyzed data. María Alejandra Pichardo-Sánchez and Luis Víctor Rodríguez-Durán planned the experiments, conceived the initial idea, supervised the research, analyzed data, and wrote the manuscript. Francisco Alejandro Paredes-Sánchez, and Ma. Guadalupe Bustos-Vázquez supervised the study and analyzed data. All authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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