Appl Biochem Biotechnol (2008) 151:711–723
DOI 10.1007/s12010-008-8319-8

A New Microplate Screening Method for the Simultaneous Activity Quantification of Feruloyl Esterases, Tannases, and Chlorogenate Esterases

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Received: 17 December 2007 / Accepted: 11 July 2008 /
Published online: 2 October 2008

Abstract Feruloyl, chlorogenate esterases, and tannases are enzymes useful in phenolic modifications of pharmaceutical relevance as protectors against several degenerative human diseases. Therefore, there is a growing interest in discovering new sources of these enzymes. However, traditional methods for their activity measurements are time-consuming and poorly adapted for high-throughput screening. In this study, a successful new microplate high-throughput screening method for the simultaneous quantification of all mentioned activities is demonstrated. This method allows the detection of activities as low as 1.7 mU ml^{-1}. Furthermore, reaction rates increased proportionally with the amount of enzyme added, and no interferences with the other commercial hydrolases tested were found. The utility of the method was demonstrated after simultaneously screening feruloyl, chlorogenate esterase, and tannase activities in solid state fermentation extracts obtained during the kinetics of production of 20 fungal strains. Among these, seven strains were positive for at least one of the esterase activities tested. This result shows the potential for the rapid routine screening assays for multiple samples of moderate low to high enzymatic levels.

Keywords High-throughput screening · Esterases · Coffee wastes · Solid state fermentation

Introduction

Phenolic compounds are ubiquitous constituents of higher plants, including fruits, vegetables, cereals, and legumes, and in beverages of plant origin, such as wine, tea, and coffee [1]. These compounds are secondary metabolites of plants generally involved in defense against ultraviolet radiation or aggression by pathogens. Pulp and skin of coffee beans are a great source

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of different phenolic compounds such as chlorogenic acids, ferulic acid, and tannins, among others. Most of these compounds and its derivatives have received considerable attention as potentially protective factors against human chronic degenerative diseases such as diabetes mellitus, cancer, AIDS, and cardiovascular disease [2]. As a consequence, enzymes that allow the release or transformation of these aromatic compounds offer special interest.

Lipases (triacylglycerol ester hydrolases EC 3.1.1.3) are well-known hydrolases, with natural function to catalyze the hydrolysis of long-chain triacylglycerols. Contrary to many other enzymes, they show remarkable levels of activity and stability in non-aqueous environments which facilitates the catalysis of several unnatural reactions such as esterification and transesterification [3, 4]. Feruloyl esterases (FE; E.C. 3.1.1.73; also known as ferulic acid esterases, cinnamoyl esterases, cinnamic acid hydrolases) are able to hydrolyse the ester bond between hydroxycinnamic acids and sugars present in the plant cell walls [5]. These types of esterases have been characterized and divided into four subclasses (types A, B, C, and D), based on substrate utilization [5]. Feruloyl esterases are used as a tool for the release of phenolic compounds from agro-industrial by-products or as a key enzyme allowing a better hydrolysis of lignocellulosic substrates by polysaccharide hydrolases [6]. Tannin acyl hydrolase (E.C. 3.1.1.20) or tannase catalyses the hydrolysis of the ester bonds present in the gallotannins, complex tannins, and gallic acid esters. This enzyme is used in food and beverage processing, and some of the major commercial applications are the preparation of instant tea, acorn liquor and production of gallic acid [7]. Chlorogenic acid hydrolase (E.C. 3.1.1.42) or chlorogenate esterase catalyses the hydrolysis of the ester bond present in chlorogenic acid. It controls bitterness and prevents browning in potatoes, leaves, and dicotyledonous fruits. This esterase can also be used for the production of caffeic acid from natural substrates rich in chlorogenic acid like apple marc and coffee pulp [8].

These types of carboxyl ester hydrolases can also be very useful biocatalysts to modify the structure of many phenolic compounds by esterification, hence increasing their biological activity. Lipases are by far the most employed biocatalyst in this sense [9]. However, there are limited data in the literature concerning the utilization of tannases, feruloyl esterases, or chlorogenate esterases for this kind of reactions [6]. Giuliani et al. [10] successfully adopted feruloyl esterase (EC 3.1.1.73) from *Aspergillus niger* to synthesize pentylferulate in a cetyltrimethylammonium bromide (CTAB)/hexane/pentanol water-in-oil microemulsion system with 60% yield. The use of a ferulic acid specific esterase solubilized in a water-in-oil microemulsion appears to be a good way for bioconversion involving a hydrophilic substrate (ferulic acid) and a hydrophobic product (pentylferulate) [10]. Yu et al. [11] synthesized esters of gallic acid and alcohols in organic solvents using a chitosan–alginate complex microencapsulated tannase (EC 3.1.1.20) from *A. niger*. They obtained propyl gallate at the level of 37.3% yield in 8 h of reaction, using hexane as solvent. There is only one report on the synthesis of hydroxycinnamic acid ester derivatives catalyzed by a chlorogenate esterase. Kishimoto et al. [12] synthesized 2-phenethyl caffeate by transesterification of chlorogenic acid and 2-phenylethyl alcohol with a molar yield of 50%. The lack of specific enzymes may result in difficulty in obtaining value-added esters at low cost. Therefore, further research on the screening of useful specific enzymes for the synthesis of phenolic acid esters should be conducted to reduce the prolonged reaction times and to improve yields. Contrary to the availability of lipase activity screening methods [13, 14], there are few reports concerning rapid and continuous methods for the screening of feruloyl esterases, tannases, and chlorogenate esterases. Several methods for measuring their activities have been reported [15–17]. These methods are largely based on high-performance liquid chromatography (HPLC) techniques, using enzymatic hydrolysis of hydroxycinnamic esters. Unfortunately, these HPLC methods require expensive equipment, are time-consuming, and are not suitable for

rapid analysis of large numbers of samples. Capillary zone electrophoresis [18] and gas chromatography [19] have also been applied using natural substrates, their analogues, and hydroxycinnamic methyl esters, but these methods demonstrated similar disadvantages. Spectrophotometric analyses for this kind of esterases described in the literature rely on the use of differences in spectral properties of free hydroxycinnamic acid and its natural esters [20] or their analogues [21]. Such methods measure relatively low changes of absorbance and have not been generally adopted. There are some methods appropriate for high throughput screening of feruloyl esterases [16, 22, 23] and tannases [24]. For example, chromogenic mono- and diferuloyl-butanetriol analogs were prepared by chemical syntheses as substrates for feruloyl esterases [22]. Mastihuba et al. [23] reported a simple spectrophotometric method for determining feruloyl esterase activity using 4-nitrophenyl ferulate, and Haslam and Tanner [24] developed a spectrophotometric method for tannases, which used *p*-nitrophenyl esters of gallic acid as substrates. These methods are based on the measurement of 4-nitrophenol released upon enzyme action. Unfortunately, such methods are only useful as a first approach because they can be misleading since *p*-nitrophenyl esters are not natural substrates. Furthermore, in many cases, this type of molecules must be synthesized.

The objective of this study was to implement a new, rapid, and continuous screening method for chlorogenate esterases, tannases, and feruloyl esterases produced by a fungi collection isolated from coffee wastes. This method also demonstrated the ability of simultaneous quantification of the three different enzymatic activities present in solid state fermentation (SSF) extracts.

Materials and Methods

Reagents and Media

Chlorogenic acid, ethyl ferulate, methyl gallate, *p*-nitrophenyl palmitate, polyvinyl alcohol (PVA) 30,000–70 000 Wt, Tween 80, chloramphenicol, and potato dextrose agar (PDA) were purchased from Sigma-Aldrich-Fluka (Mexico). Coffee residues (coffee pulp and coffee husk) were provided by Diversificados Argovia S.A de C.V, Tapachula municipality, Chiapas. All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers.

Fungi Isolation, Identification, and Conservation

Samples (2–10 g) of coffee pulp (Tapachula municipality, Chiapas, Mexico) were taken in situ with sterile material and dissolved in 10 ml of distilled water. Aliquots of 50 μl were grooved in solid medium with agar (20 g l^{-1}), yeast extract (5 g l^{-1}), and commercial coffee (20 g l^{-1}) and incubated for 96 h at 30 °C. Fungal strains were first separated by macro and microscopic morphological characteristics and then transferred to potato dextrose agar slants (Sigma, México) containing 50 ppm of chloramphenicol (Sigma, Mexico) by an enrichment culture at 30 °C. Pure strains were maintained on PDA slants at 4 °C. From the 20 strains isolated, seven fungi isolates showing esterase activity were identified by culture morphological characteristics to genus level [25].

Aspergillus Identification

The identification of the *Aspergillus* AS III isolate was performed by cultural, morphological, and molecular approaches. The strain was grown on Czapek-Dox-agar (Merck, Darmstadtt, Germany) and malt-extract agar (Difco, Detroit, USA) to confirm purity and species identity

based on macro and micro-morphological criteria [26]. Identification was subsequently confirmed by sequence analysis of the ITS1-5.8-ITS2 region.

Culture Conditions and DNA Isolation

The *Aspergillus* strain was grown in Czapek Dox broth (Oxoid, Basingstoke, England) at 27 °C for 4 days at 100 rpm. Mycelia pellets were removed, frozen with liquid nitrogen, and ground to dust. Total genomic DNA was extracted by a modification of the hexadecyl–trimethyl–ammonium bromide method (Gilberto Ballesteros-Rodea, personal communication). Briefly, 50–100 mg of dust were placed in a 1.5-ml microcentrifuge tube; 30 μl of 10% sodium dodecyl sulfate, 6 μl proteinase K (20 mg ml^{-1}; Fermentas, Ontario, Canada), and 6 μl RNAse (Amresco, Ohio, USA) were added. The mixture was incubated in a water bath for 1 h at 37 °C. After cooling, 100 μl of NaCl 5 M and 80 μl CTAB NaCl were added and incubated for 10 min at 65 °C. The tube was mixed gently and centrifuged at 14,000×*g* for 5 min. The upper phase was transferred to a new microcentrifuge tube. An equal volume of a phenol/cloroform/isoamilic alcohol (25:24:1) was added, mixed, and centrifuged at 14,000×*g* for 5 min. The upper phase was transferred to a new tube, 60% of the volume of isopropanol (−20 °C) was added, and centrifuged again. Supernatant was discarded. DNA pellet was washed twice in 70% ethanol (−20 °C) and centrifuged at maximum speed for 5 min. Supernatant was discarded, the DNA pellet was dried at room temperature in a vacuofuge (Eppendorff, Hamburg, Germany) and resuspended in 50 μl of 10 mM Tris–HCl (pH 8), 1 mM ethylenediamine tetraacetic acid (EDTA) buffer. DNA concentration of stock solution was determined in a spectrophotometer (GeneQuant pro, Amersham Biosiences, Buckinghamshire, UK) at 260 nm.

Polymerase Chain Reaction Amplification and Sequence Reaction

The ITS1-5.8-ITS2 region was amplified with primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′; Accesolab, Mexico City, Mexico). Polymerase chain reaction (PCR) reactions were performed in 50 μl reaction volume containing 20 ng of genomic DNA, 1× PCR (10× 200 mM Tris–HCl, 500 mM KCl, pH 8.4) buffer (Invitrogen, San Diego, CA, USA), 0.20 mM of each of dNTPs (Invitrogen, San Diego, CA, USA), 1.5 mM $MgCl_2$, 1 μM primers, and 1 U *Taq* polymerase (Invitrogen, San Diego, CA, USA). DNA amplification program was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) consisting of an initial denaturing step at 94 °C for 1 min, followed by 30 cycles of 60 s at 94 °C, 2 min at 58 °C, and 60 s at 72 °C; and a final extension step of 5 min at 72 °C [27]. PCR products were analyzed on 1.5% ethidium bromide-stained agarose gel (Onbio, Ontario, Canada) in 0.5× Tris–borate EDTA buffer at 100 V, 500 mA for 25 min, and visualized under UV transilluminator (UVP, California, USA). Sequence reaction was performed by M. Sc L. Marquez Valdelamar of the Laboratory of Molecular Biology of the Institute of Biology, National University of Mexico. Alignment and edition were done with the BioEdit Program v 7.0.5 and visually corrected. Sequence obtained was compared with those available in the GenBank database and deposited in the same bank.

SSF Culture Media

Enzyme Production

The SSF impregnation medium had the following composition (g l^{-1}): glucose, 5; urea, 4; K_2HPO_4, 5; $MgSO_4$, 1. Initial pH was adjusted to 6.5. Sugarcane bagasse and coffee pulp were

used as SSF support and inducer, respectively. These agricultural residues were sifted to a particle size between 0.8 and 1.7 mm, washed with distilled water and then dried at 80 °C for 2 days. SSF and the impregnation media were adopted from a previous work [28]. The support (sugarcane bagasse) was mixed with the inducer (coffee pulp) at a 50:50 (*w*/*w*) ratio, impregnated with a concentrated (1.5 times) culture medium in the ratio of 1 g per 2 ml, and sterilized at 121 °C for 15 min. An inoculum was prepared by inoculating the fungi in 250 ml Erlenmeyer flasks containing 50 ml of PDA at 30 °C for 1 week. Spores were harvested with 50 ml of a Tween 80 solution (0.01%) adjusted to a final concentration of 3×10^7 spores per gram of dry matter, and the humidity was adjusted to 75%. Glass columns (2.5 cm×30 cm) were packed with 40 g of solid culture medium and incubated in a water bath at 30 °C. A constant stream of water-saturated air was injected into the bottom of the column at a rate of 50 ml min^{-1}. Two columns were sampled at 0, 24, 48, 72, 96, 120, and 144 h of fermentation for further analysis.

Enzyme Extraction from SSF Medium

Enzymes were extracted from the solid fermentation medium by passing 70 ml of 2.5 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (pH 7.2) three times at 4 °C through each glass column. A stream of air was then injected into the top of each glass column to pressurize and recover the liquid (50 ml). The contents of the columns were then manually removed and squeezed gently to recover the rest of the liquid (20 ml). Both liquid fractions were mixed.

Sample Preparation

After enzyme production, the extract obtained from the SSF columns were collected at different time intervals (0, 24, 48, 72, 96, 120, and 144 h) and conserved in aliquots at −20 °C. Prior to screening, these aliquots were thawed and diluted five- to tenfold in a MOPS 2.5 mM pH 7.2 buffer in order to prepare the different enzyme solutions. Enzyme solutions obtained during fermentations were used to measure the kinetics of enzyme production as follows.

Feruloyl Esterase, Tannase, and Chlorogenate Esterase Activity Quantification

Feruloyl esterase, tannase, and chlorogenate esterase activities were determined according to a high-throughput screening method implemented in our laboratory, adapted from previous work [29, 13, 30] by measuring the hydrolysis of ethyl ferulate, methyl gallate, and chlorogenic acid, respectively. Each substrate was prepared as follows: One volume of the substrate (50 mM) dissolved in isopropanol, also containing 5 mM of *p*-nitrophenol (a pH indicator), was mixed with nine volumes of 2.5 mM MOPS (pH 7.2). Next, 20 μl of each enzyme solution at an appropriate dilution in MOPS 2.5 mM (pH 7.2) were placed in each microplate well, and 100 μl of substrate was quickly added using an eight-channel pipette. Subsequently, the plate was positioned in the microplate reader and shaken for 5 s after each reading. The decrease in absorbance at 415 nm was recorded every 5 min at 30 °C. Blanks without enzyme were carried, and data were collected in triplicate for 60 min. The reaction rates were calculated by means of Eq. 1.

$$\text{rate}(\mu\text{mol/min}) = U = \frac{\text{dA}_{415}/dt}{\Delta\varepsilon_{415}\times l}\times\left(\frac{[\text{buffer}]}{[\text{indicator}]}+1\right)\times V\times 10^6 \qquad (1)$$

Where dA_{415}/dt is the absorbance decrease at 415 nm per minute determined by linear regression of absorbance vs time data, $\Delta\varepsilon_{415}$ is the difference in extinction coefficients for the protonated and unprotonated structures of the pH indicator (17,500 M^{-1} cm^{-1}), l is the path length (0.35 cm for a 120 μl reaction volume; in microplates), and V is the reaction volume in liters.

Lipase Activity Quantification

Lipase activity was determined on a 96-well polystyrene microplate by measuring the hydrolysis of emulsified *p*-nitrophenyl palmitate according to Mateos et al. [29]. The emulsion was prepared by mixing vigorously in a vortex one volume of a 0.010 mol l^{-1} substrate solution in 2-propanol with nine volumes of a 0.050 mol l^{-1} Tris–HCl buffer (pH 7.5) containing PVA 0.25% (*w*/*v*). Fifty microliters of the different enzyme solutions was added to each well of a 96-well microplate at an appropriate dilution in 0.010 mol l^{-1} Tris–HCl buffer (pH 7.5). Then, 100 μl of the substrate emulsion was added quickly using an eight-channel pipette, and the plate was placed at the microplate reader (Biorad instruments, Model 680 XR) and shaken for 5 s after each reading. The increase in absorbance at 415 nm was monitored at 30 °C every 25 s against a blank without enzyme. Data were collected for 20 min, in triplicate and averaged. Reaction rate was calculated from the slope of the curve absorbance versus time by using a molar extinction coefficient of 8,300 cm^{-1} M^{-1} for *p*-nitrophenol. This value was determined from the absorbance of standard solutions of *p*-nitrophenol in the reaction mixture. One enzyme unit was the amount of protein liberating 1 μl mole of *p*-nitrophenol per minute in the above conditions.

Results and Discussion

Fungi Identification

From the 20 fungi strains isolated from coffee wastes, only seven (Table 1) were found to be capable of producing at least one esterase of interest and were identified by morphological characteristics into genera *Aspergillus* (strains AI-II, ASII, Café I, PCS6) and *Rhizopus* (strains C-6, C3-I). Due to technological interest, only the *Aspergillus* strain ASIII was identified to species (Table 1) by cultural and micro- and macro-morphological features [26] and sequence analysis of the ITS1-I5.8-ITS2 region (GenBank accession number; this isolate was identified as *Aspergillus ochraceus*).

Assay Validation

The principle of the activity assay implemented here is based on the decrease in yellow coloration of a pH indicator caused by the release of acid during ester hydrolysis, monitored at 415 nm as reaction proceeds. It was already demonstrated by the group of Kazlauzkas [30] that the activity assay used in this work for screening is quantitative. They observed that when different known amounts of HCl were added, the absorbance decreased linearly owing to protonation of the pH indicator and that theoretical and experimental slopes were in agreement to within 5%. In addition, the Kazlauskas group focused mainly on enantioselectivity [30] and substrate selectivity [13] using enantiomeric substrates like solketal butyrate and different acyl chain length esters, respectively. It is very important to carefully choose the substrates and assay conditions to be used after implementing the screening for other

Table 1 Identification of fungal strains producing esterases.

Strain	Identified as	Macroscopic morphology[a]	Origin	Activity detected
AI-II	*Aspergillus* sp.	Compact white basal felt covered by a yellow to pale green layer with a rough reverse	Interior coffee pulp monticules	Lipase-feruloyl esterase
C-6	*Rhizopus* sp.	Woolly and initially white, becoming pale gray and developing black dots in the mycelium	Wastes of roasted coffee	Lipase-feruloyl esterase
Café-I	*Aspergillus* sp.	Compact white basal felt covered by a gray-green with a slight yellow reverse	Wastes of roasted coffee	Lipase-chlorogenate esterase
AS-II	*Aspergillus* sp.	White basal felt covered by a lime green woolly, cottony and granular layer	Superficial coffee pulp monticules	Lipase
AS-III	*A. ochraceus*[b]	Compact white basal felt covered by a finely granular cream-orange layer with a rough reverse	Superficial coffee pulp monticules	Lipase-chlorogenate esterase
PCS6	*Aspergillus* sp.	Compact white-yellow basal felt covered by a dense layer of black conidial heads	Superficial coffee pulp monticules	Feruloyl esterase
C3-I	*Rhizopus* sp.	Woolly and initially white, becoming pale gray and developing brown dots in the mycelium	Wastes of roasted coffee	Lipase-tannase

[a] Strains on potato dextrose agar at 30 °C for 5 days

[b] ASIII strain was identified by molecular means as *A. ochraceus*

enzymatic activities. Since the fungal hydrolases to be screened in this work (i.e., feruloyl esterases, tannases, and chlorogenate esterases) have maximal activity near neutral pH, we implemented an assay for pH 7.2. As a pH indicator, 4-nitrophenol was used because the similarity of its pKa (7.15) to the pH of the reaction mixture ensures that changes in pH give a large and linear color change, as stated previously by the group of Kazlauzkas [30]. Furthermore, the large difference in the extinction coefficients of the protonated and deprotonated forms of *p*-nitrophenol gives good sensitivity [30]. MOPS was used as buffer which has a pKa of 7.2, a value very close to that of 4-nitrophenol at our experimental conditions, ensuring that changes in proton concentration during the reaction give linear changes in absorbance [30]. Since chlorogenate, feruloyl esterases, and tannases are typically obtained in small amounts [8], a final MOPS concentration of 2.5 mM, which allows a good sensitivity without compromising accuracy, was chosen. As seen in Table 2, the assay allowed the detection of activities as low as ≥1.7 mU ml^{-1}. As a cosolvent, we used 10% (*v*/*v*) isopropanol, which is less of a denaturant than acetonitrile and allowed the solubility of all substrates without adding any surfactant (e.g., Triton X-100).

As a test reaction, we monitored the hydrolysis of chlorogenic acid catalyzed by the commercial preparation (Rapidase UF) originally obtained from *A. niger*, a known chlorogenate esterase producer [8]. The decrease of the indicator absorbance was linear (Fig. 1a). Control experiments without esterase showed only slight changes in absorbance caused by the spontaneous hydrolysis of the substrates. Reaction rates increased linearly with the amount of enzyme added (Rapidase UF) indicating that the enzyme-catalyzed reaction rates determined with this assay are proportional to the total enzyme concentration (Fig. 1b). Similar results (data not shown) were found for tannase and feruloyl esterase commercial preparations using

Table 2 Hydrolysis of *p*-nitrophenyl palmitate and chlorogenic acid using different commercial enzymatic preparations.

Name	Kind of enzyme	*p*-NPP hydrolysis (mU/ml)	CA hydrolysis (mU/ml)
Celluzyme	Cellulases	N.D	N.D
Puradax	Cellulases	N.D	N.D.
Texamil	Amylase	N.D	N.D.
Termamyl	Amylase	N.D	N.D.
Deterzyme	Protease	N.D	N.D.
Papaya Latex	Proteases + lipase	6	N.D.
Lecitase	Phospholipase	114	32.3
Lipozyme	Lipase	345	N.D.
Lipex	Lipase	35 000	54
8a	Lipase	28 500	1.7
Rapidase TF	*A. niger* pectinase	N.D	79
Rapidase UF	*A. niger* pectinase	< 1	320
Rapidase ADEX	*A. niger* pectinase	N.D	16.4
ASIII	SSF extract	70	122

Values are the average of three independent assays with SD less than 5%.

N.D. No activity detected.

methyl gallate and ethyl ferulate as substrates, respectively. We also monitored the chlorogenic acid hydrolysis by a classical method which measures the disappearance of chlorogenic acid spectrophotometrically at 350 nm [20]. A higher activity was obtained by Okamura's method, and we attribute the difference to the lower solvent concentration (1% vs 10%) present in the experiment. Nevertheless, we decided to measure the activity of the ASIII

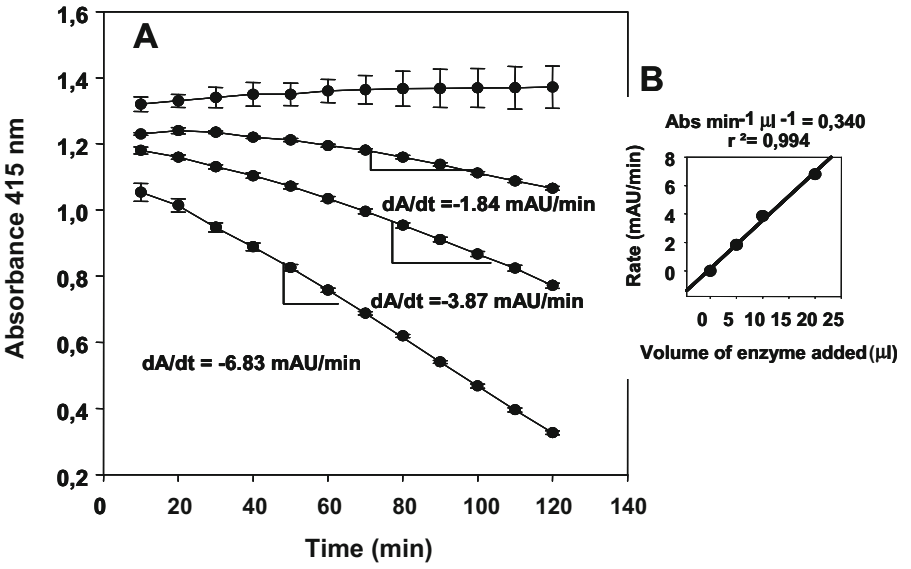

Fig. 1 Hydrolysis of chlorogenic acid catalyzed by the commercial preparation (Rapidase UF). **a** Initial rate measurement showing the absorbance change in the Rapidase UF catalyzed hydrolysis of chlorogenic acid using 5, 10, and 20 ml of enzyme solution. **b** Increased rate of hydrolysis of chlorogenic acid with increased amounts of enzyme. Values are the average of three independent assays with SD less than 5%

SSF extract and Rapidase UF as a reference by both methods. The ratio of activities (Rapidase UF/ASIII) obtained by our method was 2.62 while for Okamura's method was of 2.76. This result indicated that both methods measured very close relative activities, suggesting that our screening method can be implemented for the objective of this study.

On the other hand, it is important to run a parallel lipase activity assay. Some lipases that are currently used in phenolics modification (ester synthesis or hydrolysis) [9] can potentially hydrolyze feruloyl esterase, tannase, or chlorogenate esterase substrates, thus interfering with our method. Since the goal of this work was to implement a new screening method for the simultaneous detection of feruloyl esterases, tannases, and chlorogenate esterases using crude SSF extracts, it is very important to verify that there are no important interferences caused by the presence of lipases or other activities normally present in broth extracts (cellulases, amylases, and proteases). Table 2 shows the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP; lipase activity) and chlorogenic acid (CA; chlorogenate esterase activity) using different commercial enzymatic preparations. As expected, cellulases (Celluzyme and Puradax), amylases (Texamil and Termamyl), and proteases (Deterzyme) were not able to hydrolyze either *p*-NPP or CA. Mastihuba et al. [23] found a protease interference in their feruloyl esterases assay which caused the hydrolysis of substrate (*p*-nitrophenyl ferulate); however, the rate of hydrolysis was negligible when using ethyl ferulate, the substrate used in our screening method. Papaya latex which is a mixture of cystein proteases and lipase presented only a low activity (6 mU ml^{-1}) towards *p*-NPP, and no activity was detected on CA. Lecitase, a phospholipase, hydrolyzed both substrates, *p*-NPP (114 mU ml^{-1}) and CA (32.3 mU ml^{-1}), indicating that phospholipases could eventually interfere with our method or that Lecitase preparation contains a chlorogenate esterase activity. As expected, lipases (Lipozyme, Lipex, and 8a) hydrolysed *p*-NPP at high rates (345, 35000, 28500 mU ml^{-1}), but they had little effect (even at the very high concentrations) on CA, Lipozyme (N.D.), Lipex (54 mU ml^{-1}), and 8a (1.7 mU ml^{-1}). Finally, all commercial pectinase preparations (Rapidase TF, Rapidase UF, and Rapidase ADEX) showed a chlorogenate esterase but not a lipase activity. The presence of chlorogenate esterase in all preparations is not surprising because the source strain is *A. niger*, a chlorogenate esterase producer [8]. It was shown that lipases are only able to hydrolyze chlorogenic acid at very high concentrations as in commercial lipase formulations. This result suggests that hydrolysis of chlorogenic acid detected at our experimental conditions is not caused by the presence of lipases in the SSF extracts (Table 2).

Feruloyl Esterase, Tannase, and Chlorogenate Esterase Activity Screening

To show the utility of the screening method implemented for the simultaneous detection of feruloyl esterases, tannases, and chlorogenate esterases, their production in SSF was monitored for 144 h of culture in different SSF extracts. These activities were measured with our microplate assay by monitoring in the same plate the release of protons using a pH indicator, 4-nitrophenol, and specific substrates (ethyl ferulate, methyl gallate, and chlorogenic acid). Since many fungi are lipase producers and this kind of enzymes can potentially perform the hydrolysis of the phenolic esters employed, its activity was also monitored by a classical assay based on the hydrolysis of *p*-nitrophenyl esters (see "Materials and Methods") in the SSF cultures.

The enzymatic profile and maximal enzymatic activities varied depending on the fungal strain employed in the SSF (Fig. 2). Lipase activity was present in six (AI-II, C-6, Café-I, AS-II, AS-III, and C3-I) of the seven strains evaluated; highest [1,505 mU g^{-1} dry matter (DM)] and lowest (42.77 mU g^{-1} DM) lipase activities were observed for Café I and ASII after 24 and 120 h of culture, respectively (Fig. 2). Lipase activities were very low when compared to other reports in the literature [3]. As an example, Mateos et al. [3] found a maximum lipase

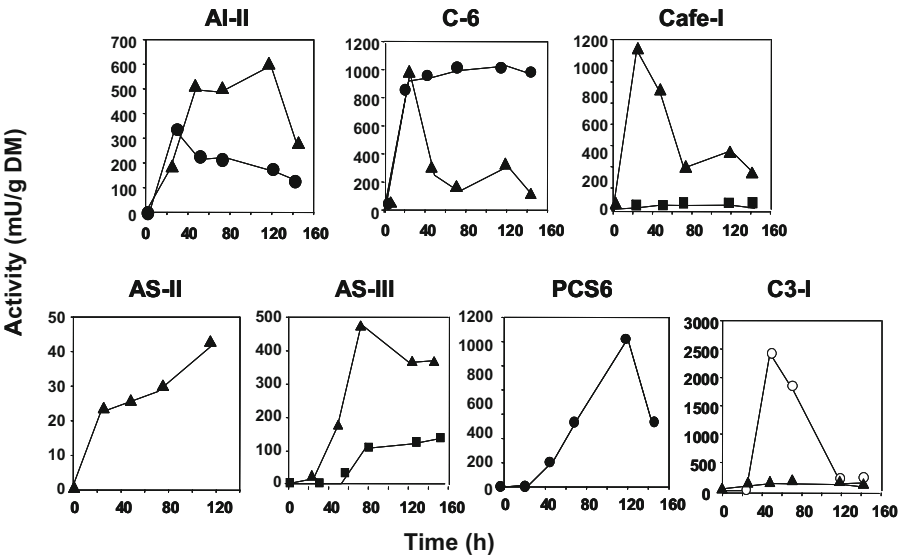

Fig. 2 Kinetics of lipase (*closed triangles*), chlorogenate esterase (*closed squares*), feruloyl esterase (*closed circles*), and tannase (*open circles*) production in solid state fermentation by different fungal strains

activity (1,500 U g^{-1}) after 10 h of *Rhizopus homothallicus* culture in SSF by using an impregnation media containing 40 g/g DM of olive oil. This activity is 1,000-fold higher than the maximal lipase activity found in this work (1,505 mU g^{-1}). This behavior could be explained by the high sugar concentration present in coffee pulp (6.8% to 12.4% dry matter basis) compared with the low quantities of lipids (2.5% to 5.02% dry matter basis) [31]. Lotti et al. [32] found that easily assimilable sugars can repress synthesis of lipases contrary to lipids that are well-known lipase inducers as reported by Rodriguez et al. [28]. Even if lipase activities found in this work do not seem to interfere with the screening of the other esterases, it is highly recommended that lipase activity be monitored in any further application of the current method in order to avoid any misleading result (i.e., hydrolysis of an esterase substrate by lipases).

AI-II, C-6, and PCS6 strains showed a feruloyl esterase activity; the lowest activity (335.6 mU g^{-1} DM) was observed for AI II, while similar feruloyl esterase activities (1,025 mU g^{-1} DM and 1,225 mU g^{-1} DM) were found for C-6 and PCS6, respectively (Fig. 2). Interestingly, even if maximal FE activities for C-6 and PCS6 were similar, the activity kinetics profile between both differs substantially. FE activity produced by C-6 appeared after only 24 h of culture (980 mU g^{-1} DM) and remained relatively constant until 144 h of fermentation (1,000 mU g^{-1} DM), while for the PCS6 strain, a slight FE activity (200 mU g^{-1} DM) appeared only after 48 h of fermentation and increased progressively until reaching its maximum (1,225 mU g^{-1} DM) after 122 h of culture before decreasing (<600 mU g^{-1} DM after 144 h). This behavior can probably be explained by the kind of strain employed. Feruloyl esterase activities were comparable with those reported in the literature for *A. niger* I-1472 (300 mU g^{-1} DM) and *Penicillium brasilianum* IBT 20888 (1,542 mU g^{-1} DM), both using methyl ferulate as substrate for the esterase activity determination and cultured in solid state fermentation [6]. Conversely, tannase activity was only produced by the C3-I strain. Its maximum activity (2,387 mU g^{-1} DM) was reached after 48 h of culture and decreased progressively; no activity was detected after 120 h of culture.

Tannase activity was the highest among the four activities evaluated (Fig. 2); however, this activity is moderate when compared with tannases produced by other authors: Mukherjee et al. [33] reached 31.8 U ml^{-1} in 60 h using *Rhizopus oryzae* in a semi-solid fermentation system, whereas Sabu et al. [34] reported 13.03 U g^{-1} DM and 6.44 U g^{-1} DM using *A. niger* ATCC 16620 and palm kernel cake and tamarind seed powder as solid substrate, respectively. Kumar et al. reached a maximum yield of 69 U g^{-1} DM (jamun leaves) after 96 h of incubation of *Aspergillus ruber* under solid state at 30 °C [35].

Finally, chlorogenate esterase activity was only present in two strains, AS III and Café I (Table 2.). Both were classified into the *Aspergillus* genera. The maximal enzymatic activity achieved was three times higher for AS III (133 mU g^{-1} DM after 6 days) than for Café I (39.27 mU g^{-1} DM after 3 days; see Fig. 2). In the work of Asther et al. [8], a chlorogenate esterase from *A. niger* in submerged fermentation (SF) was produced using sugar beet pulp as inducer; these authors found a maximum enzymatic activity of 0.46 nkat ml^{-1} after 11 days of fermentation which corresponds to a calculated productivity of 2.5 mU ml^{-1} day^{-1}. In our study, the chlorogenate esterase productivities calculated for 3 days of fermentation were 37 and 13 mU g^{-1} day^{-1} for ASIII and Café I, respectively. Thus, the productivity for ASIII and Café I were, respectively, 15- and fivefold higher than the productivity observed in Asther's work [8]. The production of chlorogenate esterase in SSF was shown to be better and faster than that of SF experiments such as of Asther et al. [8]. It should be emphasized that, as the ASIII strain seemed to be a new chlorogenate esterase producer, since morphological observations do not classify it into the fungal species reported in the literature (i.e., *A. niger* [8, 17] and *Aspergillus japonicus* [12]) as able to produce this kind of enzymes, we decided to further characterize this strain. ASIII strain identified by molecular methods as *A. ochraceus* is a new source of esterases; therefore, our screening method is able to perform rapid and simultaneous detection of new esterases of biotechnological interest, thus showing the potential of the method for high-throughput screening of new enzymes. Presently, this screening method is applied in a study for the selective production of the *A. ochraceus* strain AS III chlorogenate esterase by medium tuning in SSF on coffee wastes equipped with simultaneous monitoring of other esterase activities.

Work is ongoing to implement our screening for the selective production of chlorogenate esterase from *A. ochraceus* by medium tuning in solid state fermentation on coffee wastes. Furthermore, purification and characterization of *A. ochraceus* chlorogenate estrerase will be presented in a separate publication.

Conclusions

The assays used to screen the biocatalyst diversity are currently considered as a bottleneck, specifically for identifying a desired target. In this study, a new microplate screening method for the simultaneous screening of feruloyl esterases, tannases, and chlorogenate esterases as potential tools for phenolic modifications was successfully implemented. Kinetics of the esterase production patterns were obtained for seven fungi strains. Among the isolates screened, a new chlorogenate esterase from *A. ochraceus* strain AS III was found to have a 15-fold higher productivity in SSF than previously observed for *A. niger* by Asther et al. Thus, the screening method here implemented is an efficient way to find improved or new esterase activities simultaneously among large amounts of samples.

Acknowledgements This research was supported by the FOMIX-CONACYT-Gobierno del Estado de Chiapas, project CHIS-2005-C04-15002, and Bruno Giesemman Eversbusch from the coffee company Diversificados

Argovia S.A de C.V. We would like to thank Alejandra Rodríguez Camacho from ECOSUR, Karime Rivera form the University of Guadalajara, Adriana Espino del Castillo and Concepción León Cano from the UNAM for technical support and peace corps volunteers Winston Smith and Helen Ashraf for English revisions.

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