Research article

# Type C feruloyl esterase from *Aspergillus ochraceus*: A butanol specific biocatalyst for the synthesis of hydroxycinnamates in a ternary solvent system

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## ARTICLE INFO

## ABSTRACT

*Background:* Aspergillus ochraceus was isolated from coffee pulp and selected as an interesting hydroxycinnamoyl esterase strain producer, using an activity microplate high-throughput screening method. In this work, we purified and characterized a new type C *A. ochraceus* feruloyl esterase (AocFaeC), which synthesized specifically butyl hydroxycinnamates in a ternary solvent system.
*Results:* AocFaeC was produced by solid state fermentation, reaching its maximal activity (1.1 U/g) after 48 h of culture. After purification, the monomeric protein (34 kDa) showed a specific activity of 57.9 U/mg towards methyl ferulate. AocFaeC biochemical characterization confirmed its identity as a type C feruloyl esterase and suggested the presence of a catalytic serine in the active site. Its maximum hydrolytic activity was achieved at 40°C and pH 6.5 and increased by 109 and 77% with $Ca^{2+}$ and $Mg^{2+}$, but decreased by 90 and 45% with $Hg^{2+}$ and $Cu^{2+}$, respectively. The initial butyl ferulate synthesis rate increased from 0.8 to 23.7 nmol/min after transesterification condition improvement, using an isooctane:butanol:water ternary solvent system, surprisingly the synthesis activity using other alcohols was negligible. At these conditions, the synthesis specific activities for butyl *p*-coumarate, sinapinate, ferulate, and caffeate were 87.3, 97.6, 168.2, and 234 U/μmol, respectively. Remarkably, AocFaeC showed 5 folds higher butyl caffeate synthesis rate compared to type B *Aspergillus niger* feruloyl esterase, a well-known enzyme for its elevated activity towards caffeic acid esters.
*Conclusions:* Type C feruloyl esterase from *A. ochraceus* is a butanol specific biocatalyst for the synthesis of hydroxycinnamates in a ternary solvent system.
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## 1. Introduction

Feruloyl esterases (Faes; E.C. 3.1.1.73) are a group of hydrolases broadly distributed in plants and microorganisms that catalyze the hydrolysis or condensation between hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, and sinapinic acid) and an alcohol moiety. Their substrate specificity against four methyl hydroxycinnamates and their primary sequence identity allows the Faes to be classified into four types (A-D) [1]. Type A Faes show a preference for methyl ferulate and sinapinate, type B Faes for methyl *p*-coumarate and caffeate, while type C and D Faes have a broad specificity, hydrolyzing all methyl hydroxycinnamates.

Nowadays, Faes from several fungi of the *Aspergillus* genus are produced by solid state fermentation (SSF) with several advantages, such as a high volumetric productivity. Furthermore, many agro-industrial by-products containing hydroxycinnamic acids, may be used as supports/substrates for the induction of Fae activity [2,3]. For example, wheat bran and sugar beet pulp are inducers for type A (AnFaeA) and type B (AnFaeB) feruloyl esterases from *Aspergillus niger*, respectively [4,5], while maize bran induces a type C feruloyl esterase from *Aspergillus terreus* [6].

Faes have considerable roles in biotechnological processes in a wide variety of applications such as in biofuel, food, feed, pulp, paper, cosmetics, and pharmaceutical industries [7,8]. They have the ability to release ferulic acid and other hydroxycinnamic acids from plant biomass, which have been demonstrated to possess antioxidant, anti-inflammatory, antitumoral, antimicrobial and photoprotective activities [9] and are precursors of high economic value molecules [10,11,12]. In addition, when the acidic moiety of a hydroxycinnamic acid is esterified with an alcohol (aliphatic or sugar), its biological activities might be enhanced or modified. Furthermore, the hydrophilic–lipophilic balance modification of hydroxycinnamic acids after esterification may also allow its incorporation into water or oil based formulations, which could be of interest in the cosmetic, food, agro-, and pharmaceutical industries [9].

During the last 15 years, several research works demonstrated that alkyl hydroxycinnamates biological activities show a cut-off effect between $C_4$ and $C_8$ alkyl chain length [13,14,15]. In this respect, butyl and octyl esters of p-coumaric and caffeic acid, showed the highest in vitro and in vivo anti-inflammatory activity through NF-κβ pathway inhibition, suppressing the secretion of PGE2 [16]. Moreover, short and medium alkyl caffeate esters (<C5) showed enhanced antitumoral activities against cervical and gastric cancer cells in comparison with the non-esterified acids [17], while the butyl ester of sinapinic acid is reported as a potent antioxidant inhibiting lipid oxidation [18]. Additionally, butyl esters of coumaric acid showed the highest antifungal activity against Pyricularia grisea, Valsa mali or Botryosphaeria dothidea [19]. Finally, it has been demonstrated that butyl ferulate is an important precursor of a novel prodrug, used as a lipophilic antioxidant [20]. All these previous works prove the biological importance of butyl hydroxycinnamates and the need of alkyl specific enzymes for their synthesis.

The derivatization of hydroxycinnamic acids with an alkyl alcohol has been performed by esterification or transesterification using Faes in a ternary solvent system [21,22,23,24]. This system contains a non-polar solvent, an alcohol (used as the substrate), and an aqueous phase containing the enzyme. It has been shown that the concentrations of the solvent, alcohol, and water in this reaction system strongly influence the Fae conversion yield [21,23,25]. The synthesis of hydroxycinnamic esters using Faes instead of chemical catalysts reduces the formation of secondary by-products, using environmentally friendly processes. Type C Faes possess a broad specificity during the synthesis of hydroxycinnamic acid esters. Hence, type C Faes from Fusarium oxysporum [26], Myceliophthora thermophila (formerly Sporotrichum thermophile) [25], and Talaromyces stipitatus [27] were successfully used in the synthesis of propyl, butyl and arabinosyl hydroxycinnamic acid esters reaching maximum yields of approximately 70% at mild temperatures (30–37°C). However, at our knowledge Faes alkyl specificity during butyl hydroxycinnamates synthesis has been poorly studied. Therefore, bioprospecting for alkyl specific Faes capable of synthesizing hydroxycinnamic butyl esters is still required.

In a previous work, A. ochraceus was selected among a fungal strain collection isolated from coffee waste and used to produce an enzymatic extract containing Fae activity, which released caffeic acid from coffee pulp [28,29]. In this work, a new type C feruloyl esterase from A. ochraceus (AocFaeC) was produced by SSF using maize bran as an inducer, purified, biochemically characterized and its biocatalytic potential was demonstrated for the alkyl specific synthesis of butyl esters of hydroxycinnamic acids, which own interesting biotechnological applications.

## 2. Materials and methods

### 2.1. Materials and strains

Bradford reagent, phenylmethanesulfonyl fluoride (PMSF), $MgSO_4$, $K_2HPO_4$, HCl, formic acid, urea, ammonium sulfate, hydroxycinnamic acids, methanol, ethanol, propanol, butanol, tert-butanol, octanol,

dodecanol, β- mercaptoethanol, bacteriological agar, bovine serum albumin (BSA), Tween 80, n-hexane, ethyl acetate, petroleum ether, diethyl ether, acetonitrile, buffer salts, and metal ion salts were purchased from Sigma (Toluca, Mexico). The broad range molecular weight standard was purchased from Bio-Rad (Ciudad de Mexico, Mexico). Maize bran was kindly provided by Minsa® (Los Mochis, Sinaloa, Mexico). A. niger strains for type A and type B feruloyl esterase production were kindly donated by Dr. Record and Dr. Levasseur from INRA, France.

### 2.2. Fungal strain conservation and inoculum preparation

A. niger and A. ochraceus spores were maintained in 50% v/v glycerol at −80°C. The spore suspension used as inoculum was prepared in 250 ml Erlenmeyer flasks each containing 50 ml of the media reported by Record et al. [30] and Rodríguez et al. [31] for A. niger and A. ochraceus, respectively. For A. ochraceus, washed maize bran with a particle size of approximately 0.15 mm (40 g/l) was used as an inducer. Flasks were incubated for 4 d at 30°C, and spores were collected by stirring 50 ml of 0.01% w/v Tween 80 sterile solution.

### 2.3. Feruloyl esterase production in SSF

SSF was performed essentially as described by Rodríguez et al. [31]. Prior to fermentation, maize bran (substrate) was washed thoroughly with tap water to eliminate the soluble sugars. Then, it was dried at 30°C for 3 d and finally milled and sieved to achieve a particle size of approximately 0.42 to 0.59 mm. Polyurethane foam was cut into 0.5 cm cubes and employed as a support for the SSF. The impregnation media composition (g/l) was as follows: $MgSO_4$ (1); $K_2HPO_4$ (5); and urea (4). The support and substrate were mixed in a 1/3 (w/w) proportion, and then, each gram of mixture was added with 1.5-fold concentrated impregnation media at a pH of 6.5 and sterilized at 121°C for 15 min. The spore suspension was added to achieve $3 \times 10^7$ spores per gram of dry matter (gdm). The humidity and temperature were adjusted to 75% w/w and 30°C, respectively. After culture, the solid ferment of each column was placed in a plastic bag. The enzymatic extract was obtained after mixing the solid ferment with 2.5 mM MOPS at a pH of 7.2 in a 1/2 (w/v) proportion and applying slight manual pressure to each bag. The enzymatic extract was centrifuged at 14,000 ×g for 5 min to eliminate fungal residues and suspended matter. Two fermentation columns were removed every 24 h to measure the Fae hydrolytic activity in the enzymatic extracts using methyl ferulate (MF) as the substrate and the spectrophotometric assay described below. Average values and standard deviation were reported.

### 2.4. Synthesis of methyl and butyl hydroxycinnamates

Each hydroxycinnamic acid was dissolved in methanol or butanol to obtain a 0.5 M solution, and then, 4% (v/v) HCl was added. The reaction mixture was heated under reflux for 18 h. The reaction progress was followed by thin layer chromatography (n-hexane/ethyl acetate; 2/1) and after complete reaction, the solvent was evaporated under reduced pressure using a rotary evaporator. The obtained powder was purified by silica gel column chromatography using different petroleum ether/diethyl ether mixtures. Fractions with the corresponding ester were pooled and dried to obtain white crystals with a purity of >98%.

### 2.5. Feruloyl esterase hydrolytic activity assays

#### 2.5.1. Spectrophotometric assay

The Fae activity was determined by measuring the hydrolysis of different methyl hydroxycinnamates (5 mM) in the presence of the pH indicator 4-nitrophenol (0.5 mM), as described by Ramírez et al. [28]. The substrate solution was prepared using tert-butanol and 2.5 mM MOPS at a pH of 7.2 in a 1/9 (v/v) proportion. The decrease in

absorbance at 410 nm was continuously monitored in a microplate reader X-Mark from BioRad (Ciudad de Mexico, Mexico) at 30°C and a pH of 7.2 over 15 min. The reaction rate was calculated using the extinction coefficient obtained from standard curves (0 to 1 mM) for each hydroxycinnamic acid. Blank experiments were carried out using 2.5 mM MOPS buffer at a pH of 7.2. One unit corresponds to 1 μmol of hydroxycinnamic acid released per minute.

### 2.5.2. Chromatographic assay

The Fae activity was also measured by quantifying the hydroxycinnamic acid released after 30 min of enzymatic hydrolysis of the corresponding ester in 50 mM potassium phosphate buffer at a pH of 7 and 30°C. Reaction products were monitored at 310 nm in a Varian HPLC System (California, USA) with a ZORBAX Eclipse XDB-C18 column (4.6 mm, 3 × 250 mm, Agilent) at 30°C using water with 1% (v/v) formic acid and acetonitrile (60/40, v/v) as the mobile phase. The reaction rate was calculated using the extinction coefficient of each hydroxycinnamic acid, which was obtained from standard curves (0 to 5 mM). Blank experiments were carried out using 50 mM potassium phosphate buffer at a pH of 7. HPLC grade solvents were used during analysis. One unit corresponds to 1 μmol of hydroxycinnamic acid released per minute.

### 2.6. Feruloyl esterase purification

Ammonium sulfate (2 M) was added to the enzymatic extract, which was filtered through a 0.45 μm cellulose membrane (Millipore) and then loaded into a HiPrep Phenyl Sepharose FF 16/10 (Phenyl S) column from GE (Little Chalfont, United Kingdom) pre-equilibrated with 2.5 mM MOPS at a pH of 7.2 (buffer A) containing 2 M ammonium sulfate. The column was washed with 10 column volumes (CVs) of the same buffer to remove any unbound proteins. The elution was performed in 20 CVs using a linear gradient of 2–0 M ammonium sulfate in buffer A at a flow rate of 1 ml/min. Active fractions were pooled and dialyzed overnight against buffer A. Afterwards, the pooled fractions were loaded into a DEAE 16/10 from GE (Little Chalfont, United Kingdom) pre-equilibrated with buffer A, and then, the column was washed with 10 CVs using the same buffer. The protein elution was performed in 10 CVs with a linear gradient of 0–0.5 M NaCl in buffer A at a flow rate of 1 ml/min. Active fractions were pooled and concentrated to 200 μl using an Amicon cellulose membrane from Millipore (10 kDa molecular mass cut-off) and loaded into a Superdex G200 16/60 from GE (Little Chalfont, United Kingdom) equilibrated with buffer A containing 150 mM NaCl. The elution was performed in 1.5 CVs, and active fractions were pooled and loaded onto a Mono Q 5/50 GL column from GE (Little Chalfont, United Kingdom) pre-equilibrated with buffer A. The purified protein was eluted in 10 CVs with a linear gradient of 0–0.5 M NaCl in buffer A at a flow rate of 1 ml/min. Recombinant type A and type B Faes from *A. niger* were produced and purified according to Record et al. [30] and Levasseur et al. [32], respectively.

### 2.7. Protein and electrophoresis assays

The protein concentration was determined using the Bradford microassay [33] and BSA as the reference protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 12% was performed to verify protein purity and to estimate the Fae molecular weight [34]. Zymography was carried out in 12% PAGE under non-denaturing conditions according to Camacho et al. [35] using MF as the substrate for the detection of the activity bands.

### 2.8. N-terminal amino acid sequence determination

The N-terminal sequence was determined by Edman degradation of an electroblotted pure deglycosylated protein sample (40 μg) obtained from a polyvinylidene difluoride membrane using a Trans-Blot Turbo Transfer Starter System from Bio-Rad (Ciudad de Mexico, Mexico). Analysis was carried out on a Microflex II instrument from Bruker (Massachusetts, USA).

### 2.9. Biochemical characterization of type C feruloyl esterase from A. ochraceus

#### 2.9.1. Effects of the temperature and pH

The hydrolytic activity of the purified protein was evaluated at different temperatures (30 to 70°C) and pH values (4 to 10) using the chromatographic assay and MF as the substrate. For the pH assays, different solutions at 50 mM were used including the following: citrate buffer (pH = 4 to 5.5), sodium phosphate (pH = 6 to 7), Tris–HCl (pH = 7.5 to 8.5), and glycine-NaOH (pH = 9 to 10). Assay was performed by triplicate. Average values and standard deviation were reported.

#### 2.9.2. Effects of metal ions and inhibitors

The effects of various metal ions ($Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Cu^{2+}$, and $Hg^{2+}$) and inhibitors (PMSF and β-mercaptoethanol) were evaluated by measuring the hydrolytic activity after 1 h of incubation using the chromatographic assay and MF as the substrate. The final concentration of all effectors was 1 mM. Assay was performed by triplicate. Average values and standard deviation were reported.

#### 2.9.3. Kinetic parameters

The initial hydrolysis rates at different (0.01 to 4 mM) methyl caffeate (MC), methyl *p*-coumarate (M*p*C), MF, and methyl sinapinate (MS) concentrations were evaluated using the chromatographic assay. Afterwards, $K_m$ and $V_{max}$ were calculated using the Michaelis–Menten plot, Lineweaver–Burk regression plot and SigmaPlot 12.0 (Systat Software Inc.). For calculation of the $k_{cat}$, the molecular weight was estimated by SDS-PAGE. Assay was performed by triplicate.

### 2.10. Feruloyl esterase synthetic activity assays

#### 2.10.1. Selection of the ternary solvent system for butyl ferulate synthesis

The synthesis of butyl ferulate was carried out by the transesterification of 50 mM MF in a ternary solvent system (isooctane:butanol:water) at 30°C, a pH of 7, and 1500 rpm in an Eppendorf Thermomixer®. The ternary solvent system was prepared according to Topakas et al. [21], with 1 ml being used in 1.5 ml sealed microtubes. To select butanol and water content in the ternary reaction system, seven isooctane: butanol: water relations (97.5:0.5:2; 92:6:2; 85.5:12.5:2; 73:25:2; 48:50:2; 74:25:1 and 71:25:4) were evaluated on the initial synthetic rate. The effect of enzyme concentration (1.3 to 20.7 μg/ml), BSA concentration (0 and 1 mg/ml), and initial aqueous pH (3 to 9) was evaluated after 30 min of reaction on the initial synthetic rate in the ternary solvent system previously selected. For the pH assays, different solutions at 50 mM were used including the following: citrate buffer (pH = 4 to 5), MES (pH = 6 to 6.5), MOPS (pH = 7), Tris–HCl (pH = 8), and glycine-NaOH (pH = 9). Reaction samples (20 μl) were taken at 0 as well as 30 min and stored at −20°C until HPLC analysis. Blank experiments were carried out without enzyme using the corresponding buffer. Assay was performed by triplicate. Average values and standard deviation were reported.

#### 2.10.2. Synthesis of alkyl hydroxycinnamates

The transesterification of different methyl hydroxycinnamates (50 mM) with ethanol, propanol, butanol, octanol, and dodecanol was carried out in a ternary solvent system (isooctane:alcohol:water, 73:25:2, v:v:v) with 1 mg/ml BSA using 20 μl of the purified enzyme (0.52 mg/ml) dissolved in 50 mM Tris–HCl buffer at pH 8. Reaction were performed in a 1 ml volume at 30°C and 1500 rpm for 4 h. Reaction samples (20 μl) were removed at 0, 15, 30, 60, and 240 min, followed by storage at −20°C until HPLC analysis. Blank experiments were carried out using 50 mM Tris–HCl buffer at a pH of 8. Assay was
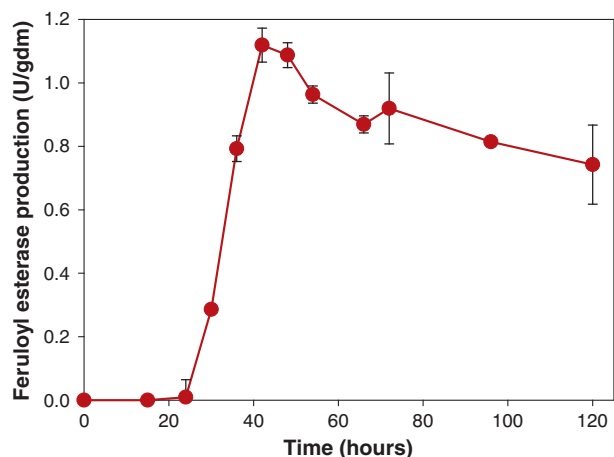
**Fig. 1.** Kinetics of *Aspergillus ochraceus* feruloyl esterase production by SSF. The production of feruloyl esterase is expressed in units (U) per gram of dry matter (gdm) using MF as a substrate.

performed by triplicate. Average values and standard deviation were reported.

### 2.10.3. Determination of feruloyl esterase synthetic activity by HPLC

Synthesis reaction samples (20 μl) were diluted 10-fold with ethyl acetate and filtered through a 0.45 μm filter before HPLC analysis. The reaction products were monitored at 310 nm in a Varian HPLC System (California, USA) using a Luna® 5 μm silica (2) Phenomenex® column (100 Å, 150 × 4.6 mm, Agilent) at 30°C using *n*-hexane/ethyl acetate (80/20) as the mobile phase. The reaction rate was calculated using the extinction coefficient of each butyl hydroxycinnamate, which was obtained from standard curve (0 to 1 mM). One unit corresponds to 1 μmol of butyl hydroxycinnamate produced per minute. HPLC grade solvents were used during analysis. Assay was performed by triplicate. Average values and standard deviation were reported.

## 3. Results

### 3.1. Production and purification of type C feruloyl esterase from A. ochraceus (AocFaeC)

AocFaeC was produced by SSF using maize bran as an inducer (Fig. 1). Fae activity against MF was observed after 24 h, and the maximum activity of 1.1 U/gdm was reached around at 42 to 48 h of culture. Afterwards, the Fae activity decreased to 0.7 U/gdm after 120 h of fermentation. Thus, 200 g of solid ferment production was performed to accomplish the purification and characterization assays. The culture was stopped after 48 h to avoid possible protein proteolysis.

AocFaeC was purified using a four-step chromatographic strategy from 1.3 l of enzymatic extract (Table 1). For the first chromatography step, a hydrophobic Phenyl S column was used. This step allows 58-fold enrichment of the protein and the recovery of 109% of the total initial activity. In the second chromatography step, a weak anion exchange DEAE column was employed and allowed the protein to be enriched 80-fold with a 71.4% yield. Then, a molecular exclusion step

using a Superdex G200 column increased the purification factor by 295 with a 56.8% yield. Finally, a strong anion exchange Mono Q column led to the recovery of 0.23 mg of the pure protein (11.3% yield) with a purification factor of 483. The eluted protein was dialyzed overnight against 2.5 mM MOPS buffer at a pH of 7.2 to remove the residual NaCl. The homogeneity of the purified protein was verified by SDS-PAGE, Fig. 2a, showing a single band after purification. The specific activity of the pure AocFaeC was 57.9 U/mg using MF as a substrate (Table 1). The zymographic analysis through each purification step showed only one activity band when using MF as a substrate, Fig. 2b.

### 3.2. Biochemical characterization of AocFaeC

The molecular mass of AocFaeC was estimated to be approximately 34.6 kDa by SDS-PAGE, Fig. 2a, and 26.5 kDa (data no shown) by exclusion chromatography using a Superdex G200 column. The kinetic constants ($K_m$, $V_{max}$, and $k_{cat}$) were determined by measuring the initial hydrolysis rates of methyl hydroxycinnamates at different substrate concentrations (Table 2). The purified protein obeys Michaelis–Menten kinetics in the substrate concentration range of 0.01 to 4 mM. The $K_m$ constants for all methyl hydroxycinnamates tested are shown in Table 2; for MS (1.11), the constant was approximately 2-fold higher than for MC (0.53), M*p*C (0.58), and MF (0.64). The $V_{max}$ values against MC (40), M*p*C (47), MF (58) and MS (40) showed a broad hydrolytic capacity for *A. ochraceus* Fae. The substrate specificity was estimated by the specificity constant ($k_{cat}/K_m$). AocFaeC showed a slight preference for MF (31 356), M*p*C (28 037), and MC (26 113) with regards to MS (12 468). The AocFaeC N-terminal amino acid sequence is shown in Table 3. A BLAST analysis using the AocFaeC N-terminal sequence revealed more than 60% homology with other known N-terminal sequences of type C Faes from *Aspergillus* species and 100% homology with a putative type C Fae from *A. niger* (Table 3).

On the other hand, the activity of AocFaeC was measured as function of the temperature using MF as the substrate, Fig. 3a. AocFaeC showed a maximum activity at 40°C, and then it decreased sharply to 7% of the maximum activity at 60°C. The AocFaeC activation energy (Ea) was 24.9 kJ/mol, Fig. 3a. In the same way, the influence of the pH on the AocFaeC activity was measured in a pH range of 4 to 10, Fig. 3b. The maximum AocFaeC activity was observed at a pH of 6.5 using MF as a substrate, and surprisingly, the enzyme retained 40% of its maximum activity at a pH of 10.

The effects of various divalent metal ions and inhibitors on the AocFaeC hydrolytic activity using MF as the substrate are shown in Table 4. The AocFaeC activity increased with the addition of $Ca^{2+}$ (209%), $Mg^{2+}$ (117%), $Mn^{2+}$ (143%), and $Zn^{2+}$ (112%); was partially inhibited by β-mercaptoethanol (83%) and $Cu^{2+}$ (55%); and a significant decrease in Fae activity was detected in the presence of $Hg^{2+}$ (10%). Besides, the addition of PMSF resulted in a total loss of AocFaeC activity.

### 3.3. Effect of the ternary solvent system composition on the AocFaeC initial synthetic rate of butyl ferulate

The synthesis of butyl ferulate by the transesterification of MF with butanol was performed in an isooctane:butanol:water ternary system using AocFaeC at 30°C (Fig. 4). The effects of the butanol, Fig. 4a, and

**Table 1**

Purification summary of *Aspergillus ochraceus* type C feruloyl esterase.

| Step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification factor[a] |
|---|---|---|---|---|---|
| Crude extract | 118.4 | 1029.3 | 0.12 | 100 | 1 |
| Phenyl Sepharose | 129.1 | 18.5 | 6.9 | 109 | 58 |
| DEAE | 84.6 | 8.7 | 9.6 | 71.4 | 80 |
| Superdex G200 | 67.2 | 1.9 | 35.4 | 56.8 | 295 |
| Mono Q | 13.4 | 0.23 | 57.9 | 11.3 | 483 |

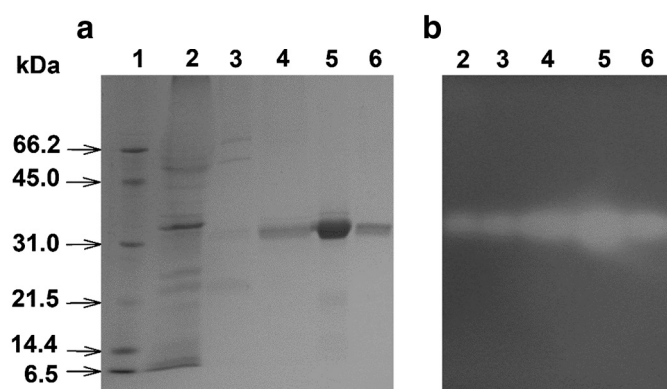[a] Purification factor = (Specific activity of each step) / (Specific activity of the crude extract).

**Fig. 2.** Electrophoretic analysis of the purified type C feruloyl esterase from *Aspergillus ochraceus*. (a) SDS-PAGE: lane 1, broad range molecular weight standards; lane 2, enzymatic crude extract; lane 3, pooled fractions eluted from the phenyl sepharose purification step; line 4, pooled fractions eluted from the DEAE purification step; line 5, pooled fractions eluted from the superdex G-200 purification step; and line 6, pooled fractions eluted from the mono Q purification step. The gel was stained with coomassie brilliant blue. (b) The Fae activity zymogram was determined using MF as the substrate.

water concentrations, Fig. 4b, as well as the pH in the aqueous phase, Fig. 4c, and enzyme concentration, Fig. 4d, were evaluated. The maximum initial synthetic rate of 2.8 nmol/min was reached at 25% ($v$/v) butanol, using an isooctane:butanol:water relation of 73:25:2 as seen in Fig. 4a. A higher butanol concentration using an isooctane:butanol:water relation of 48:50:2 sharply decreased the initial synthetic rate to 1.2 nmol/min (Fig. 4a). A lower (isooctane:butanol:water, 74:25:1 relation) or higher (isooctane:butanol:water, 71:25:4 relation) water concentration, decreased the initial synthetic rates to 0.9 nmol/min and 1.3 nmol/min, respectively (Fig. 4b). Thus, further experiments were carried out at an isooctane:butanol:water relation of 73:25:2. Afterwards, the addition of 1 mg/ml BSA into the aqueous phase of the ternary system reaction was evaluated. The addition of BSA increased the initial synthetic rate by 2.8-fold to 7.8 nmol/min. Regarding the effect of the initial aqueous phase pH on the initial butyl ferulate synthetic rate, AocFaeC was not active at an aqueous phase pH of 4, although the initial synthetic rate increased to 9.3 nmol/min at an aqueous phase pH of 9. It is important to mention that butyl ferulate was not detected using the corresponding buffer without enzyme, showing that alkaline catalysis is not occurring under the reaction conditions. Further studies were assessed at an aqueous phase pH of 8 to avoid possible methyl hydroxycinnamate degradation. Finally, the effect of the AocFaeC concentration on the initial synthetic rate was evaluated, Fig. 4d. The initial synthetic rate increased linearly to reach its maximum of 23.7 nmol/min using 10 μg of AocFaeC. A higher enzyme concentration (20 μg) did not increase the initial synthetic rate (23 nmol/min). Thus, further studies were performed using a ternary solvent composition of isooctane:butanol:water 73:25:2, an AocFaeC concentration of 10 μg, 1 mg/ml BSA and an initial aqueous phase pH of 8, which increased the initial transesterification synthetic rate for obtaining butyl ferulate up to 30-fold (from 0.8 nmol/min to 23.7 nmol/min).

**Table 2**
Kinetic parameters of AocFaeC for different methyl hydroxycinnamates.

| Substrate | $K_m$ (mM)[a] | $V_{max}$ (μmol/min)/mg[a] | $k_{cat}$ (1/min) | $k_{cat}/K_m$ (1/mM min) |
|---|---|---|---|---|
| MC | 0.53 | 40 | 13,840 | 26,113 |
| M*p*C | 0.58 | 47 | 16,262 | 28,037 |
| MF | 0.64 | 58 | 20,068 | 31,356 |
| MS | 1.11 | 40 | 13,840 | 12,468 |

[a] Kinetic parameters were determined using methyl caffeate (MC), methyl *p*-coumarate (M*p*C), methyl ferulate (MF), and methyl sinapinate (MS) as substrates and determined from Lineweaver–Burk plots.

**Table 3**
N-terminal amino acid sequences of type C Faes from the *Aspergillus* genus.

| Type C Fae source | N-terminal amino acid sequences | NCBI reference sequence |
|---|---|---|
| *A. ochraceus* | ANSPGCGKNP | |
| *A. niger* | ANSPGCGKNP | XP_001395336.1 |
| *A. oryzae* | ANSSGCGKQP | XP_001820736.2 |
| *A. udagawae* | TRSSGCGKQP | GAO87330.1 |
| *A. lentulus* | TRSSGCGKQP | GAQ06322.1 |

### 3.4. Potential of AocFaeC for the synthesis of alkyl hydroxycinnamates

The transesterification of methyl to alkyl ferulates in the ternary solvent system conditions previously selected were performed using pure preparations of AnFaeA, AnFaeB, and AocFaeC as biocatalysts, in order to evaluate its synthetic potential. The specific synthetic activity per μmol of pure enzyme for each Fae is shown in Fig. 5. The specific synthetic activities of AnFaeA for ethyl, propyl, butyl, octyl, and dodecyl ferulate were 19.0, 33.4, 20.5, 4.5, and 3.2 U/μmol of enzyme, respectively, showing a preference for the propyl chain. Aversely, AnFaeB shows low specific synthetic activity for all alkyl ferulate (around 2.9–5.4 U/μmol), without showing any alkyl synthesis preference. Interestingly, AocFaeC was able to synthesize only butyl and octyl ferulate with a specific synthetic activity of 168.2 and
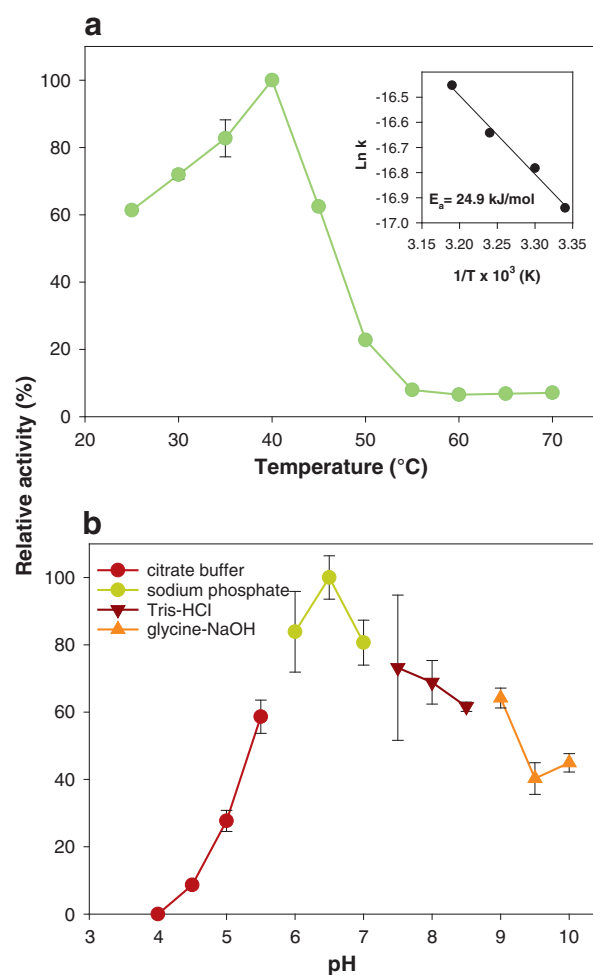


**Fig. 3.** Effects of temperature and pH on the hydrolytic activity of AocFaeC. The relative activity was measured at different temperatures (a) and pH values using citrate buffer, sodium phosphate, Tris–HCl, and glycine-NaOH (b). The activation energy ($E_a$) was calculated using the Arrhenius equation. The activity was measured using MF as the substrate.

**Table 4**
Effects of metal ions and inhibitors on the AocFaeC hydrolytic activity.

| Substance | Relative activity (%) |
|---|---|
| Control | 100 |
| $CaCl_2$ | 209 ± 20 |
| $MgCl_2$ | 177 ± 4 |
| $MnCl_2$ | 143 ± 23 |
| $ZnCl_2$ | 112 ± 8 |
| $CuCl_2$ | 55 ± 4 |
| $HgCl_2$ | 10 ± 1 |
| β-mercaptoethanol | 83 ± 3 |
| PMSF | 0 |

5.3 U/μmol, respectively. As the specific synthetic activity of AocFaeC using other alcohols during transesterification reaction was negligible, it might be considered as a butyl chain specific Fae.

Finally, the transesterification of methyl to butyl hydroxycinnamates using AnFaeA, AnFaeB, and AocFaeC were performed to evaluate the acyl substituent preference (Fig. 6). The specific synthetic activities of AnFaeA for butyl p-coumarate, ferulate and sinapinate transesterification were 4.1, 20.5, and 39.3 U/μmol, respectively, showing a preference for butyl sinapinate synthesis. The specific synthetic activities of AnFaeB for butyl ferulate, caffeate, and p-coumarate transesterification were 5.4, 47.7, and 80 U/μmol, respectively, showing a preference for the synthesis of hydroxylated hydroxycinnamates. Interestingly, the specific synthetic activities of AocFaeC for the transesterification of butyl p-coumarate, sinapinate, ferulate, and caffeate were 87.3, 97.6, 168.2, and 234 U/μmol, respectively, showing the highest activity for the synthesis of butyl caffeate. AocFaeC shows 2.5- and 5-fold higher specific synthetic activities than AnFaeA and AnFaeB for butyl sinapinate and caffeate, respectively. These results demonstrate that at the conditions described in this work, AocFaeC was able to specific synthesize all butyl hydroxycinnamates tested, at higher rates than AnFaeA and AnFaeB.

## 4. Discussion

After the first purification step, 109% of the total initial activity was recovered probably due to the presence of an inhibitor in the crude extract. AocFaeC is a monomeric protein and a type C feruloyl esterase like Faes from *Aspergillus oryzae* (AoFaeC) [36], *Talaromyces stipitatus* (TsFaeC) [37], *Myceliophthora thermophila* (StFaeC) [38], and *Fusarium oxysporum* (FoFaeC) [39]. The molecular weight for AocFaeC estimated by SDS-PAGE (34.6 kDa) was between those of AtFae-2 (23 kDa) and AtFae-3 (36 kDa) from *A. terreus* [8] and between those of StFaeC (23 kDa) [38], TsFaeC (55.3 kDa) [37], FoFaeC (62 kDa) [39], and AoFaeC (75 kDa) [40]. Thus, the type C feruloyl esterase group is composed of a wide range of molecular weight proteins and AocFaeC belongs to the medium molecular weight ones. The AocFaeC specific hydrolysis activity against MF (57.9 U/mg) was similar to the activity of TsFaeC (68.5 U/mg) [37] and significantly greater than that of StFaeC (0.7 U/mg) [25].

The comparison of $k_{cat}/K_m$ values showed the broad specificity of AocFaeC against methyl hydroxycinnamates. Presently, the other reported fungi Faes that exhibit such a broad activity spectrum towards hydroxycinnamic esters are AtFae-2 [8], AtFae-3 [8], FoFae-I [21], FoFaeC [39], TsFaeC [37], AoFaeC [40], and StFaeC [25]. Comparing different type C Faes, the AocFaeC specificity constant using MF as a substrate is 3- and 330-fold higher than those of AoFaeC [40] and StFaeC [25], respectively, but is half of that reported for FoFaeC [39]. Nonetheless when comparing AocFaeC with type A Faes, the specificity constant using MF as a substrate is 6- and 18-fold greater than those values reported by Faulds et al. [41] and Schär et al. [42] for AnFaeA, which is an enzyme evolutionarily adapted to hydrolyze hydroxycinnamic acid esters containing methoxy substituents in the phenolic ring [43]. Thus, the broad substrate specificity and the N-terminal amino acid sequence reveals that the Fae from *A. ochraceus* corresponds to a type C feruloyl esterase [3].

The maximum activity of AocFaeC was reached at 40°C, similar to AtFae-2 and AtFae-3 from *A. terreus* [8]. However, this temperature
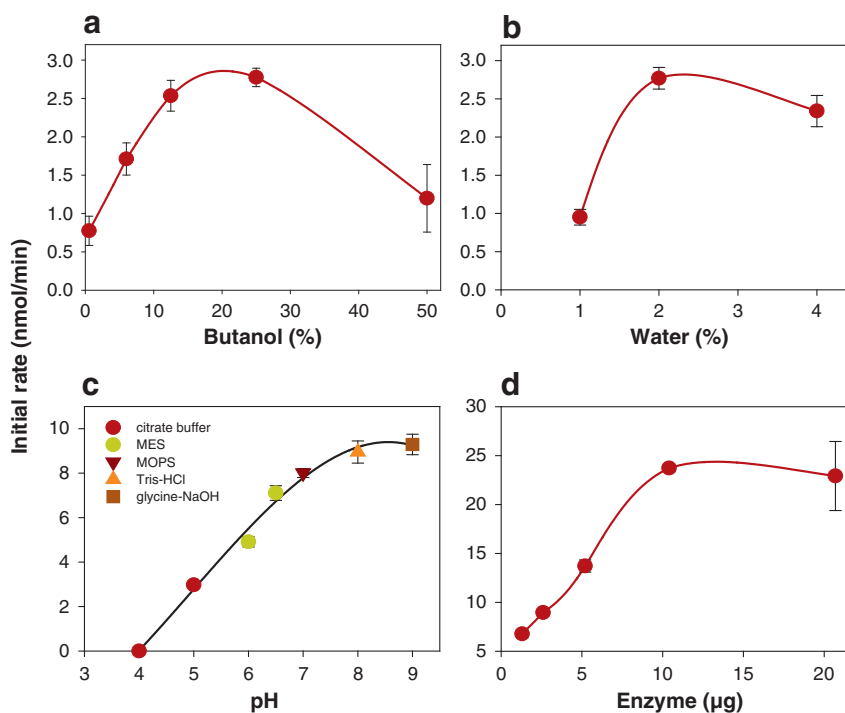


**Fig. 4.** Influence of the ternary solvent system composition on the AocFaeC initial transesterification synthetic rate of MF to BF. (a) Effect of different butanol concentrations. (b) Effect of different water concentrations. (c) Effect of the initial aqueous phase pH: citrate buffer, MES, MOPS, Tris–HCl, and glycine-NaOH. (d) Effect of different enzyme concentrations. All reactions were performed at 30°C.
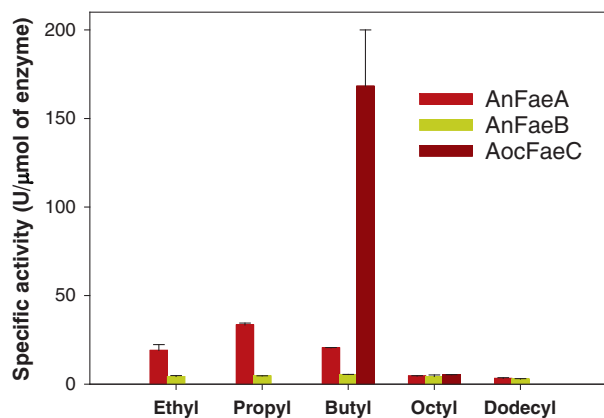
Fig. 5. Specific activities of different feruloyl esterases in the synthesis of alkyl ferulates by transesterification. Comparison of the specific synthetic activity per μmol of pure preparations of feruloyl esterase type A from *Aspergillus niger* (AnFaeA), type B from *Aspergillus niger* (AnFaeB) and type C from *Aspergillus ochraceus* (AocFaeC). The transesterification reactions were performed using MF as substrates and a ternary solvent system isooctane:alcohol:water, 73:25:2 containing 1 mg/ml of BSA at a pH of 8 and 30°C.

differs from those reported for other mesophilic fungi type C Faes such as *Fusarium oxysporum* (65°C) [39] and *Talaromyces stipitatus* (60°C) [37]. On the other hand, the activation energy ($E_a$) of AocFaeC (24.9 kJ/mol) is similar to that of the type A Fae from *Aspergillus awamori* (21.4 kJ/mol) [44]. The effect of pH on AocFaeC hydrolytic activity towards MF reveals that the maximum activity was reached at a pH of 6.5, a value slightly higher than those reported for mesophilic fungi type C Faes, which reported pH values of 5 or 6 [8,37,39,40]. As shown in Fig. 3b, AocFaeC retains more than 60% of its maximum activity at pH values from 5.5 to 9.0, a property that might be exploited in alkaline processes.

In the effectors study, most metal ions tested increased the AocFaeC hydrolytic activity towards MF. The activation of AocFaeC hydrolytic activity by $Ca^{2+}$ using MF as a substrate (209%), was higher than the activation of *Penicillium pinophilum* Fae using the same substrate (173%) [45] and that found for AwFaeA using sugarcane bagasse as a substrate (170%) [44]. Similar behaviors by $Mg^{2+}$ and $Mn^{2+}$ have been reported for the *Penicillium pinophilum* Fae [45] and a soil metagenomic library Tan410 Fae [46], respectively. The AocFaeC
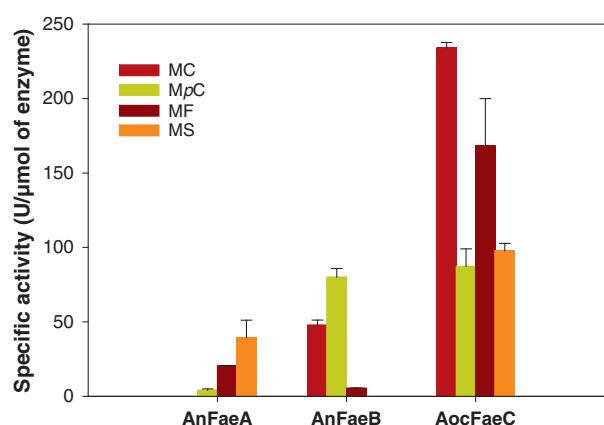


Fig. 6. Specific activities of different feruloyl esterases in the synthesis of butyl hydroxycinnamates by transesterification. Comparison of the specific synthetic activity per μmol of pure preparations of feruloyl esterase type A from *Aspergillus niger* (AnFaeA), type B from *Aspergillus niger* (AnFaeB) and type C from *Aspergillus ochraceus* (AocFaeC). The transesterification reactions were performed using MC, MpC, MF, and MS as substrates and a ternary solvent system with the following composition: isooctane:butanol:water, 73:25:2 containing 1 mg/ml of BSA at a pH of 8 and 30°C.

hydrolytic activity increased to 112% after incubation with $Zn^{2+}$, unlike *Russula virescens* Fae, whose hydrolytic activity measured with MF was reduced to 98% [47]. Heavy metals such as $Hg^{2+}$ and $Cu^{2+}$ are thiol group and disulfide bond reducing agents [48], which inhibited AocFaeC activity by 90 and 45%, respectively. As AocFaeC maintains 83% of its activity after incubation with β-mercaptoethanol, a disulfide bond reducing agent [49], it can be suggested that thiol groups may play an important role in Fae activity. The complete loss of AocFaeC activity by PMSF reveals the presence of a serine in the active site [50].

In the transesterification of MF to butyl ferulate using the best ternary solvent system conditions found in this work (isooctane: butanol:water, 73:25:2, v:v:v), the AocFaeC initial synthetic rate (23.7 nmol/min or 2.8% conversion/h) was 16- and 35-fold higher than those of StFaeC (0.18% conversion/h) [25] and FoFae-I (0.08% conversion/h) [21] using hexane as a co-solvent, 43.4 and 50.8% of butanol, and 3.2 and 2% of water, respectively. In our case, reducing the butanol concentration in the ternary solvent system causes a loss of the microemulsion stability. Interestingly, AocFaeC initial synthesis rate increases with pH, as reported for FaeA1 and MtFae1a expressed in *Myceliophtora thermophile*, whose maximal hydrolytic activity was reached at pH 7 [51,52], while optimal synthesis activity was found at pH 8 [53]. This could probably be explained by the different ionization state of AocFaeC at the evaluated conditions, as stated by Antonopoulou et al. [53]. As in hydrolysis, AocFaeC shows a broad synthetic activity spectrum towards hydroxycinnamic methyl esters. However, differences between the hydrolytic and synthetic activity patterns were observed. AocFaeC preferentially hydrolyzes MF, while in transesterification reactions, it preferentially uses MC as a substrate, similar to as found for FoFae-I [21] and StFaeC [25]. Hence, this preference switch was probably caused by the ternary solvent system composition [54]. Nowadays, Faes alkyl chain specificity during transesterification of methyl hydroxycinamates has been poorly studied. Schär and Nyström [55] evaluated the alkyl chain specificity of *Rhizomucor miehei* lipase in the synthesis of alkyl ferulates by direct esterification, proving its short alkyl chain preference ($C_2$-$C_4$). To the best of our knowledge, AocFaeC is the only esterase that may perform the specific synthesis of butyl ferulate by transesterification of methyl ferulate. Finally, the AocFaeC synthetic specific activity per μmol of biocatalyst in the transesterifications of MF and MC to butyl ferulate and butyl caffeate was higher than that of AnFaeA and AnFaeB, which are naturally adapted for catalyzing methyl hydroxycinnamates with methoxylated or hydroxylated substituents in the aromatic ring, respectively. In this study, it was demonstrated that *Aspergillus ochraceus* type C Fae possesses a broad specificity for hydrolyzing methyl hydroxycinnamates and has the potential of carrying out the specific synthesis of butyl hydroxycinnamates, particularly by butyl caffeate, which possess all interesting antioxidant and anticarcinogenic properties [56].

## Declarations of interest

None.

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## References

[1] Crepin VF, Faulds CB, Connerton IF. Functional classification of the microbial feruloyl esterases. Appl Microbiol Biotechnol 2004;63(6):647–52. https://doi.org/10.1007/s00253-003-1476-3.

[2] Asther M, Haon M, Roussos S, et al. Feruloyl esterase from Aspergillus niger: A comparison of the production in solid state and submerged fermentation. Process Biochem 2002;38(5):685–91. https://doi.org/10.1016/S0032-9592(02)00196-6.

[3] Kumar CG, Kamle A, Mongolla P, et al. Parametric optimization of feruloyl esterase production from Aspergillus terreus strain GA2 isolated from tropical agro-ecosystems cultivating sweet sorghum. J Microbiol Biotechnol 2011;21(9):947–53. https://doi.org/10.4014/jmb.1104.04014.

[4] Faulds CB, Devries RP, Kroon PA, et al. Influence of ferulic acid on the production of feruloyl esterases by Aspergillus niger. FEMS Microbiol Lett 1997;157(2):239–44. https://doi.org/10.1016/S0378-1097(97)00481-3.

[5] Brézillon C, Kroon AP, Faulds BC, et al. Novel ferulic acid esterases are induced by growth of Aspergillus niger on sugar-beet pulp. Appl Microbiol Biotechnol 1996;45(3):371–6. https://doi.org/10.1007/s002530050698.

[6] Kumar CG, Kamle A, Kamal A. Purification and biochemical characterization of feruloyl esterases from Aspergillus terreus MTCC 11096. Biotechnol Prog 2013;29(4):924–32. https://doi.org/10.1002/btpr.1729.

[7] Fazary AE, Ju YH. The large-scale use of feruloyl esterases in industry. Biotechnol Mol Biol Rev 2013;3(5):95–110.

[8] Dilokpimol A, Mäkelä MR, Aguilar-Pontes MV, et al. Diversity of fungal feruloyl esterases: Updated phylogenetic classification, properties, and industrial applications. Biotechnol Biofuels 2016;9(1):231–49. https://doi.org/10.1186/s13068-016-0651-6.

[9] Figueroa-Espinoza MC, Villeneuve P. Phenolic acids enzymatic lipophilization. J Agric Food Chem 2005;53(8):2779–87. https://doi.org/10.1021/jf0484273.

[10] Benoit I, Danchin EGJ, Bleichrodt RJ, et al. Biotechnological applications and potential of fungal feruloyl esterases based on prevalence, classification and biochemical diversity. Biotechnol Lett 2008;30(3):387–96. https://doi.org/10.1007/s10529-007-9564-6.

[11] Estrada Alvarado I, Lomascolo A, Navarro D, et al. Evidence of a new biotransformation pathway of p-coumaric acid into p-hydroxybenzaldehyde in Pycnoporus cinnabarinus. Appl Microbiol Biotechnol 2001;57(5–6):725–30. https://doi.org/10.1007/s002530100761.

[12] Bel-Rhlid R, Thapa D, Kraehenbuehl K, et al. Biotransformation of caffeoyl quinic acids from green coffee extracts by Lactobacillus johnsonii NCC 533. AMB Express 2013;3(28):1–7. https://doi.org/10.1186/2191-0855-3-28.

[13] Sørensen A-DM, Durand E, Laguerre M, et al. Antioxidant properties and efficacies of synthesized alkyl caffeates, ferulates, and coumarates. J Agric Food Chem 2014;62(52):12553–62. https://doi.org/10.1021/jf500588s.

[14] Suárez-Quiroz ML, Alonso Campos A, Valerio Alfaro G, et al. Anti-Aspergillus activity of green coffee 5-O-caffeoyl quinic acid and its alkyl esters. Microb Pathog 2013;61-62:51–6. https://doi.org/10.1016/j.micpath.2013.05.005.

[15] da Cunha FM, Duma D, Assreuy J, et al. Caffeic acid derivatives: In vitro and in vivo anti-inflammatory properties. Free Radic Res 2004;38(11):1241–53. https://doi.org/10.1080/10715764000016139.

[16] Alam MA, Subhan N, Hossain H, et al. Hydroxycinnamic acid derivatives: A potential class of natural compounds for the management of lipid metabolism and obesity. Nutr Metab (Lond) 2016;13(27):1–13. https://doi.org/10.1186/s12986-016-0080-3.

[17] Wang J, Gu SS, Pang N, et al. Alkyl caffeates improve the antioxidant activity, antitumor property and oxidation stability of edible oil. PLoS One 2014;9(4):e95909. https://doi.org/10.1371/journal.pone.0095909.

[18] Gaspar A, Martins M, Silva P, et al. Dietary phenolic acids and derivatives. Evaluation of the antioxidant activity of sinapic acid and its alkyl esters. J Agric Food Chem 2010;58(21):11273–80. https://doi.org/10.1021/jf103075r.

[19] Zhou K, Chen D, Li B, et al. Bioactivity and structure-activity relationship of cinnamic acid esters and their derivatives as potential antifungal agents for plant protection. PLoS One 2017;12(4):e0176189. https://doi.org/10.1371/journal.pone.0176189.

[20] Kaki SS, Kunduru KR, Kanjilal S, et al. Synthesis and characterization of a novel phenolic lipid for use as potential lipophilic antioxidant and as a prodrug of butyric acid. J Oleo Sci 2015;64(8):845–52. https://doi.org/10.5650/jos.ess15035.

[21] Topakas E, Stamatis H, Mastihobova M, et al. Purification and characterization of a Fusarium oxysporum feruloyl esterase (FoFAE-I) catalysing transesterification of phenolic acid esters. Enzyme Microb Technol 2003;33(5):729–37. https://doi.org/10.1016/S0141-0229(03)00213-8.

[22] Tsuchiyama M, Sakamoto T, Tanimori S, et al. Enzymatic synthesis of hydroxycinnamic acid glycerol esters using type A feruloyl esterase from Aspergillus niger. Biosci Biotechnol Biochem 2007;71(10):2606–9. https://doi.org/10.1271/bbb.70382.

[23] Vafiadi C, Topakas E, Alissandratos A, et al. Enzymatic synthesis of butyl hydroxycinnamates and their inhibitory effects on LDL-oxidation. J Biotechnol 2008;133(4):497–504. https://doi.org/10.1016/j.jbiotec.2007.11.004.

[24] Vafiadi C, Topakas E, Christakopoulos P. Regioselective esterase-catalyzed feruloylation of L-arabinobiose. Carbohydr Res 2006;341(12):1992–7. https://doi.org/10.1016/j.carres.2006.05.022.

[25] Topakas E, Vafiadi C, Stamatis H, et al. Sporotrichum thermophile type C feruloyl esterase (StFaeC): Purification, characterization, and its use for phenolic acid (sugar) ester synthesis. Enzyme Microb Technol 2005;36(5–6):729–36. https://doi.org/10.1016/j.enzmictec.2004.12.020.

[26] Topakas E, Stamatis H, Biely P, et al. Purification and characterization of a feruloyl esterase from Fusarium oxysporum catalyzing esterification of phenolic acids in ternary water-organic solvent mixtures. J Biotechnol 2003;102(1):33–44. https://doi.org/10.1016/S0168-1656(02)00363-2.

[27] Vafiadi C, Topakas E, Christakopoulos P, et al. The feruloyl esterase system of Talaromyces stipitatus: Determining the hydrolytic and synthetic specificity of TsFaeC. J Biotechnol 2006;125(2):210–21. https://doi.org/doi.org/10.1016/j.jbiotec.2006.02.009.

[28] Ramírez L, Arrizon J, Sandoval G, et al. A new microplate screening method for the simultaneous activity quantification of feruloyl esterases, tannases, and chlorogenate esterases. Appl Biochem Biotechnol 2008;151(2–3):711–23. https://doi.org/10.1007/s12010-008-8319-8.

[29] Ramírez-Velasco L, Armendáriz-Ruiz M, Arrizon J, et al. Liberation of caffeic acid from coffee pulp using an extract with chlorogenate esterase activity of Aspergillus ochraceus produced by solid state fermentation. Rev Mex Ing Quim 2016;15(2):503–12.

[30] Record E, Asther M, Sigoillot C, et al. Overproduction of the Aspergillus niger feruloyl esterase for pulp bleaching application. Appl Microbiol Biotechnol 2003;62(4):349–55. https://doi.org/10.1007/s00253-003-1325-4.

[31] Rodríguez JA, Mateos JC, Nungaray J, et al. Improving lipase production by nutrient source modification using Rhizopus homothallicus cultured in solid state fermentation. Process Biochem 2006;41(11):2264–9. https://doi.org/10.1016/j.procbio.2006.05.017.

[32] Levasseur A, Benoit I, Asther M, et al. Homologous expression of the feruloyl esterase B gene from Aspergillus niger and characterization of the recombinant enzyme. Protein Expr Purif 2004;37(1):126–33. https://doi.org/10.1016/j.pep.2004.05.019.

[33] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72(1–2):248–54. https://doi.org/10.1016/0003-2697(76)90527-3.

[34] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227(5259):680–5. https://doi.org/10.1038/227680a0.

[35] Camacho-Ruíz MA, Camacho-Ruíz RM, Armendáriz-Ruiz M, et al. Cornbran as potential substrate high production of feruloyl and acetylxylan esterases by solid state fermentation. Rev Mex Ing Quim 2016;15(1):11–21.

[36] Tenkanen M, Schuseil J, Puls J, et al. Production, purification and characterization of an esterase liberating phenolic acids from lignocellulosics. J Biotechnol 1991;18(1–2):69–83. https://doi.org/10.1016/0168-1656(91)90236-O.

[37] Garcia-Conesa MT, Crepin VF, Goldson AJ, et al. The feruloyl esterase system of Talaromyces stipitatus: Production of three discrete feruloyl esterases, including a novel enzyme, TsFaeC, with a broad substrate specificity. J Biotechnol 2004;108(3):227–41. https://doi.org/10.1016/j.jbiotec.2003.12.003.

[38] Topakas E, Kalogeris E, Kekos D, et al. Production and partial characterisation of feruloyl esterase by Sporotrichum thermophile in solid-state fermentation. Process Biochem 2003;38(11):1539–43. https://doi.org/10.1016/S0032-9592(03)00044-X.

[39] Moukouli M, Topakas E, Christakopoulos P. Cloning, characterization and functional expression of an alkalitolerant type C feruloyl esterase from Fusarium oxysporum. Appl Microbiol Biotechnol 2008;79(2):245–54. https://doi.org/10.1007/s00253-008-1432-3.

[40] Koseki T, Hori A, Seki S, et al. Characterization of two distinct feruloyl esterases, AoFaeB and AoFaeC, from Aspergillus oryzae. Appl Microbiol Biotechnol 2009;83(4):689–96. https://doi.org/10.1007/s00253-009-1913-z.

[41] Faulds C, Molina R, Gonzalez R, et al. Probing the determinants of substrate specificity of a feruloyl esterase, AnFaeA, from Aspergillus niger. FEBS J 2005;272(17):4362–71. https://doi.org/10.1111/j.1742-4658.2005.04849.x.

[42] Schär A, Sprecher I, Topakas E, et al. Hydrolysis of nonpolar n-alkyl ferulates by feruloyl esterases. J Agric Food Chem 2016;64(45):8549–54. https://doi.org/10.1021/acs.jafc.6b02694.

[43] Levasseur A, Gouret P, Lesage-Meessen L, et al. Tracking the connection between evolutionary and functional shifts using the fungal lipase/feruloyl esterase A family. BMC Evol Biol 2006;6(92):1–11. https://doi.org/10.1186/1471-2148-6-92.

[44] Fazary AE, Ismadji S, Ju YH. Stability and solubility studies of native and activated Aspergillus awamori feruloyl esterase. J Mol Catal B: Enzym 2009;59(1–3):190–6. https://doi.org/10.1016/j.molcatb.2009.02.011.

[45] Castanares A, McCrae SI, Wood TM. Purification and properties of a feruloyl/p-coumaroyl esterase from the fungus Penicillium pinophilum. Enzyme Microb Technol 1992;14(11):875–84. https://doi.org/10.1016/0141-0229(92)90050-X.

[46] Yao J, Chen QL, Shen AX, et al. A novel feruloyl esterase from a soil metagenomic library with tannase activity. J Mol Catal B: Enzym 2013;95:55–61. https://doi.org/10.1016/j.molcatb.2013.05.026.

[47] Wang L, Zhang R, Ma Z, et al. A feruloyl esterase (FAE) characterized by relatively high thermostability from the edible mushroom Russula virescens. Appl Biochem Biotechnol 2014;172(2):993–1003. https://doi.org/10.1007/s12010-013-0536-0.

[48] Krawczyński vel Krawczyk T, Moszczyńska M, Trojanowicz M. Inhibitive determination of mercury and other metal ions by potentiometric urea biosensor. Biosens Bioelectron 2000;15(11–12):681–91. https://doi.org/10.1016/S0956-5663(00)00085-3.

[49] Colak A, Camedan Y, Faiz Ö, et al. An esterolytic activity from a wild edible mushroom, Lycoperdon perlatum. J Food Biochem 2009;33(4):482–99. https://doi.org/10.1111/j.1745-4514.2009.00232.x.

[50] Hegde S, Muralikrishna G. Isolation and partial characterization of alkaline feruloyl esterases from Aspergillus niger CFR 1105 grown on wheat bran. World J Microbiol Biotechnol 2009;25(11):1963–9. https://doi.org/10.1007/s11274-009-0095-2.

[51] Kühnel S, Pouvreau L, Appeldoorn MM, et al. The ferulic acid esterases of Chrysosporium lucknowense C1: Purification, characterization and their potential application in biorefinery. Enzyme Microb Technol 2012;50(1):77–85. https://doi.org/10.1016/j.enzmictec.2011.09.008.

[52] Topakas E, Moukouli M, Dimarogona M, et al. Expression, characterization and structural modelling of a feruloyl esterase from the thermophilic fungus Myceliophthora thermophila. Appl Microbiol Biotechnol 2012;94(2):399–411. https://doi.org/10.1007/s00253-011-3612-9.

[53] Antonopoulou I, Leonov L, Jütten P, et al. Optimized synthesis of novel prenyl ferulate performed by feruloyl esterases from *Myceliophthora thermophila* in microemulsions. Appl Microbiol Biotechnol 2017;101(8):3213–26. https://doi.org/10.1007/s00253-017-8631-8.

[54] Klibanov MA. Enzymatic catalysis in anhydrous organic solvents. Trends Biochem Sci 1989;14(4):141–4. https://doi.org/10.1016/0968-0004(89)90146-1.

[55] Schär A, Nyström L. High yielding and direct enzymatic lipophilization of ferulic acid using lipase from *Rhizomucor miehei*. J Mol Catal B: Enzym 2015;118:29–35. https://doi.org/10.1016/j.molcatb.2015.04.011.

[56] Serafim TL, Carvalho FS, Marques MPM, et al. Lipophilic caffeic and ferulic acid derivatives presenting cytotoxicity against human breast cancer cells. Chem Res Toxicol 2011;24(5):763–74. https://doi.org/10.1021/tx200126r.