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Research Article

Improved synthesis of the antifungal isobutyl *o*-coumarate catalyzed by the *Aspergillus terreus* type B feruloyl esterase ☆

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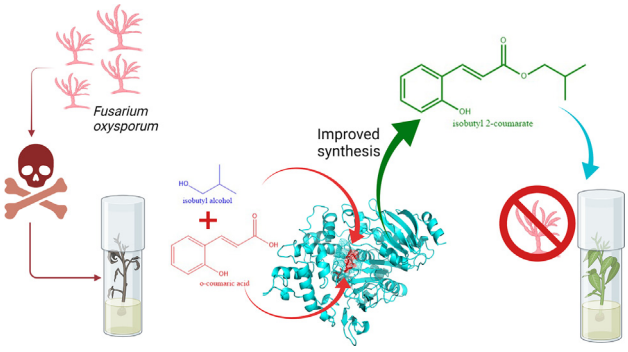
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Hydroxycinnamic acids and some of their derivatives are molecules with interesting biological activities; for instance, hydroxylated hydroxycinnamic esters have proved to have antifungal properties, and thus the generation of these molecules is of industrial importance. In this study, the direct esterification capacity of the pure recombinant type B feruloyl esterase from *Aspergillus terreus* (AtFAE B) was evaluated by its ability to catalyze the synthesis of isobutyl *o*-coumarate, an interesting antifungal molecule. A ternary solvent system (isooctane/isobutanol/water) was employed to improve the synthesis of isobutyl *o*-coumarate, assessing different substrate concentrations, enzyme load, water percentages and pH and temperature values.
Results: AtFAE B showed the highest initial rate at 18% (v/v) isobutanol and 50 mM *o*-coumaric acid, 0.04 mg/ml of enzyme, 4% (v/v) water without buffer and 40°C. AtFAE B half-lives at 30°C, 40°C and 50°C were 16.5 h, 1.75 h and 3.5 min, respectively. Thus, we decided to evaluate the bioconversion yield at 30°C, where the enzyme showed the highest operational stability. At this temperature, we obtained a yield of ~80% after only 8 h of reaction, using a 78:18:4 isooctane:isobutanol:water ternary solvent system, with 50 mM of *o*-coumaric acid.

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Conclusions: Under these improved conditions, the productivity was 1.06 g isobutyl *o*-coumarate/L*h with a biocatalyst yield of 209.6 kg isobutyl *o*-coumarate/kg free AtFAE B, demonstrating the promising potential of AtFAE B to accept the non-canonical *o*-coumaric acid as the substrate and to achieve the synthesis of isobutyl *o*-coumarate.

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1. Introduction

Hydroxycinnamic acids are secondary plant metabolites of phenylalanine; they are found predominantly in cereals, legumes, oilseeds, fruits, and vegetables. The most common and studied are ferulic, sinapic, *p*-coumaric and caffeic acid. Covalently linked to lignin and proteins by ether bonds and with polysaccharides by ester bonds, ferulic acid plays a key role in the cessation of cell growth, anchoring lignin in vegetal cell wall polysaccharides and restricting the accessibility of plant pathogens [1]. Hydroxycinnamic acids and their derivatives possess many health benefits due to their anti-inflammatory, antibacterial, antioxidant and antifungal properties [2,3,4]. Molecules with antifungal properties, such as the hydroxycinnamates, have attracted significant attention in the recent years, especially in the agricultural sector owing to the significant losses in agriculture worldwide caused by phytopathogenic fungi [5,6,7]. A study of the antifungal activity of a series of cinnamic acid esters and their derivatives found that hydroxycinnamic acids with a hydroxy substitution in the *o*-position of the phenyl ring, such as the *o*-coumaric acid and its *t*-butyl ester, show the highest antifungal activity against important phytopathogenic fungi [8]. Another example of the activity of *o*-coumaric acid and its derivatives was demonstrated against *Venturia inaequalis,* the causal fungus of apple scab. In order to improve the effectiveness of *o*-coumaric acid, a series of esters were chemically synthesized, with isobutyl *o*-coumarate being the one that showed the highest antifungal activity [9]. Subsequently, the antifungal activity of a series of chemically synthesized hydroxycinnamates was evaluated, with isobutyl *o*-coumarate showing the highest inhibitory effect over *Fusarium oxysporum* f. sp. *lycopersici, Phytophthora infestans,* and *Phytophthora capsici* [10]. Similarly, isobutyl alcohol has been recognized as a semiochemical produced by certain types of fungi [11]. Thus, the isobutyl ester of *o*-coumaric acid is a promising molecule with great potential as an antifungal for agricultural applications. Given the potential of isobutyl *o*-coumarate in the agricultural sector, it is of great interest to find greener biocatalytic processes that can use *o*-coumaric acid as a substrate to synthesize its corresponding isobutyl ester.

Some enzymes, such as lipases and feruloyl esterases, can perform the direct esterification or transesterification of certain hydroxycinnamic acids, generating molecules with enhanced properties. Lipases (EC. 3.1.1.3) are extensively studied enzymes that naturally hydrolyze triglycerides; however, they are active and stable in nonaqueous organic solvents and can accept a wide range of substrates; therefore, lipases are widely used to synthesize several interesting esters [12,13,14]. Some lipases have been used to synthesize hydroxycinnamic acid esters, especially esters of ferulic acid [15,16,17]. Nonetheless, to achieve the synthesis of coumaric acid esters, a few studies have reported the use of lipases [18,19]; furthermore, the productivities and biocatalyst yields for *o*-coumaric acid derivatives are low: the lipase from *Rhizomucor miehei* was used to synthesize ethyl *o*-coumarate, achieving 8 mg/L*h productivity and with a biocatalyst yield of 0.8 kg product/kg immobilized enzyme [19].

On the other hand, feruloyl esterases (EC. 3.1.1.73) are a subclass of carboxylic acid esterases, which hydrolyze the ester bond between hydroxycinnamic acids and sugars present in plant cell walls. Some plants, such as maize, express FAEs in order to regulate the levels of cell wall ester-linked ferulic acid and this expression is correlated to the exposure of the plant to abiotic stress [20,21]. FAEs are classified into four subclasses (types A, B, C and D) based on their preference to hydrolyze hydroxycinnamic methyl esters and their sequence homology. Type A FAEs preferentially hydrolyze methyl esters of methoxylated hydroxycinnamic acids such as ferulic or sinapic acid; complementary, type B FAEs hydrolyze methyl esters of hydroxylated hydroxycinnamic acids such as caffeic and *p*-coumaric acid; type C and D have no preference for either substrate [22]. Based on the available sequence information on 365 FAEs and different computational tools, a new classification scheme that comprises twelve families was proposed in 2011 [23]. More recently updated phylogenetic analysis of published fungal genomes resulted in the classification of FAEs into 13 subfamilies (SF1–SF13) [24].

FAEs are mostly applied in synergy with xylanases to obtain, after hydrolysis, mono- and polysaccharides from lignocellulose for bioethanol production or for the release of phenolic acids useful in the food, cosmetic and pharmaceutical industry [25,26]. Unlike lipases, hydroxycinnamic acids are the natural substrates of FAEs, a property that may be useful for the synthesis of different hydroxycinnamic acid esters; however, research in this area is still scarce. In recent years, a few FAEs have been reported for the synthesis, mostly by transesterification, of some bioactive hydroxycinnamic esters, achieving yields below 75% and with reaction times above 200 h [27,28,29,30,31]. Synthesis reactions of coumaric derivatives using type B FAEs in a ternary solvent system (hexane:1-butanol: water/47.2:50.8:2/v:v:v) include the FAEs of *Sporotrichum thermophile* and *Fusarium oxysporum* (FoFAE-I), employed for the transesterification of methyl *p*-coumarate into butyl *p*-coumarate, reaching conversions of <60% and <75%, respectively, after 140 h [29,32]. Even though better productivities (>70% yield in $\leq$24 h) have been reported for the transesterification of vinyl ferulate with prenol [33,34], to the best of our knowledge, there are no studies that report the use of FAEs for the esterification of the non-canonical hydroxylated *o*-coumaric acid.

In this study, the reaction conditions were improved by using a ternary solvent system for the enzymatic synthesis of the antifungal isobutyl *o*-coumarate, showing for the first time the outstanding direct esterification activity of AtFAE B, using the non-canonical *o*-coumaric acid as a substrate.

2. Materials and methods

2.1. Reagents and media

o-, *m*- and *p*- coumaric acids, bovine serum albumin (BSA), TLC plates with fluorescent indicator, isooctane, buffers, yeast extract peptone dextrose broth (YPD broth) and potato dextrose broth

(PDB) were purchased from Sigma-Aldrich. Isobutyl alcohol was purchased from Analytyka.

2.2. Antifungal activity of isobutyl o-coumarate over Fusarium oxysporum f. sp capsici

The antifungal activity of isobutyl *o*-coumarate was determined by the bioautography method [35]. 0.1, 0.5, 1, 2, 4 and 5 mM of isobutyl *o*-coumarate in 10 μl of ethyl acetate were injected in a TLC plate with a fluorescent indicator using a Linomat 5 from CAMAG and developed using hexane:ethyl acetate (3:1, v/v) as the mobile phase. The plate was inoculated with a solution of *Fusarium oxysporum* spores (1 exp 6 spores/ml) in PDB and incubated for 3 d at 24°C with 12-h light irradiation. The antifungal activity was analyzed by measuring the inhibition spots at 600 nm with a Scanner 3 from CAMAG and the program WinCATS; the inhibition area was obtained with the program ImageJ. Finally, the IC50 value was calculated using the program NCSS 12.

2.3. Production and purification of feruloyl esterases

The type B FAE (AtFAE B) and the type C FAE (AtFAE C) from *Aspergillus terreus* were produced by submerged fermentation in 1 L baffled Erlenmeyer flasks with 200 mL of YPD broth. The pGAPZ A vector was used for the protein expression of AtFAE B (GenBank accession no. **Q0CI21.2**) and the pGAPZαA vector was used for the protein expression of AtFAE C (GenBank accession no. **Q0CDX2.1**). *Pichia pastoris* SMD1168H (ThermoFischer Scientific) was transformed with each vector and the transformants were selected through zeocin™ (Invitrogen) resistance. After 96 h of fermentation at 30°C, the obtained culture broth was clarified by centrifugation and protein was precipitated with 96% ethanol at 4°C in a culture broth:ethanol ratio of 1:10 (v:v). The mixture was centrifuged (13,000 rpm, 20 min, 4°C) and the precipitate containing FAE activity was dissolved in 100 mL of MOPS 2.5 mM pH 7.2 to obtain a concentrate. Both enzyme concentrates were purified by fast protein liquid chromatography after two purification steps: a) weak anion exchange through a HiPrep™ DEAE FF 16/10 (GE Healthcare) and b) hydrophobic interaction through Phenyl Sepharose® 6 FF High Sub (GE Healthcare). The recombinant *Aspergillus niger* strain producing type B FAE (AnFAE B) from the same fungus was kindly donated by the UMR1163 Biotechnologie des Champignons Filamenteux, Marseille, France. AnFAE B was produced and purified as described by Levasseur et al. [36]. Finally, the type C FAE from *Aspergillus ochraceus* was produced and purified as described by Romero-Borbón [37]. All purified enzymes were aliquoted and stored at −30°C; aliquots were thawed for each synthesis or hydrolysis reaction. FAE hydrolysis activity was measured using methyl *o*-coumarate (MoC), methyl *m*-coumarate (MmC) and methyl *p*-coumarate (MpC) as described by Ramírez [38] using an Eon™ microplate spectrophotometer.

2.4. Synthesis reaction of isobutyl o-coumarate by AtFAE B

The synthesis reaction of the isobutyl *o*-coumarate catalyzed by AtFAE B was performed in a ternary solvent system composed of isooctane as the main solvent and different amounts of isobutyl alcohol, water and *o*-coumaric acid as substrate. The temperature was maintained constant with shaking using a thermomixer (ThermoFisher®) at 1400 rpm and a specific temperature for each experiment. The reaction was carried out in 1.5 mL screw-capped tubes with 1 mL of reaction volume. Isobutyl alcohol, *o*-coumaric acid and water were added to each tube containing isooctane. The reaction was initiated by the addition of AtFAE B containing 5 mg of BSA as a carrier. Samples of the reaction were taken at 0.5, 1, 2, 4, 8 and 24 h in order to determine the initial reaction rates and conversion yields. All the reactions were performed in duplicate and control trials without enzyme were carried out.

2.5. Determination of initial reaction rates and conversion yields by high-performance thin layer chromatography (HPTLC)

The reaction kinetics of isobutyl *o*-coumarate synthesis was monitored by HPTLC. All samples were injected at a convenient dilution, using a Linomat 5 from CAMAG. TLC plates with a fluorescent indicator (SIGMA) and hexane:ethyl acetate (3:1, v/v) as the mobile phase were used to migrate all injected samples. TLC plates were analyzed with a Scanner 3 from CAMAG at 310 nm and the chromatograms were obtained with the winCats computer program. Product concentration was quantified with a standard curve from 0 to 18.5 mM of isobutyl *o*-coumarate (>99%), and the data were used to estimate the initial reaction rates and conversion yields. Isobutyl *o*-coumarate standard was previously synthesized by the reaction of *o*-coumaric acid with isobutyl alcohol, using 4% (v/v) hydrochloric acid as the catalyst. The reaction was maintained at 60°C over 12 h. Purification of the isobutyl *o*-coumarate was performed by flash chromatography with petroleum ether/diethyl ether (70:30) as the mobile phase [10].

2.6. Characterization of recombinant AtFAE B on isobutyl o-coumarate synthesis

2.6.1. Effect of substrate molar ratio

The effects of isobutyl alcohol and *o*-coumaric acid molar ratios on the initial reaction rate of isobutyl *o*-coumarate synthesis were evaluated. To analyze the effect of isobutyl alcohol, 50 mM of *o*-coumaric acid (8.2 mg) was weighted and the mixtures of isooctane/isobutyl alcohol were added in order to achieve 0.5, 1, 2, 4 and 10.7 M concentrations (solvent-free) of isobutyl alcohol. To evaluate the effect of the *o*-coumaric acid, the isobutyl alcohol concentration was fixed at 2 M; a 277 mM *o*-coumaric acid stock solution in isobutyl alcohol was used to achieve 6.25, 12.5, 25, 50, 100, 200 and 400 mM concentrations of *o*-coumaric acid (for 200 and 400 mM *o*-coumaric was weighted). All reactions were carried out at 30°C and initiated after the addition of 20 μL (2%) of distilled water, 670 mU (40 μg) of pure AtFAE B and 5 mg of BSA to each 1.5 mL tube with 1 mL of reaction volume.

2.6.2. Effect of water percentage and enzyme concentration

The effect of water percentage on the initial reaction rate of isobutyl *o*-coumarate synthesis was evaluated at 0, 1, 2, 4 and 8% of water in the ternary solvent reaction system. The reaction mixture contained 2 M of isobutyl alcohol, 50 mM of *o*-coumaric acid, different assay water percentages and a sufficient amount of isooctane to complete the final reaction volume (1 mL). For 0 and 1% of water, the enzyme along with 5 mg of BSA were dried in a speed vacuum evaporator, whereas for 2, 4 and 8%, water and enzyme were added to initiate the reaction.

To evaluate the AtFAE B proportionality in the initial reaction rate of isobutyl *o*-coumarate synthesis, the reaction mixture contained 2 M of isobutyl alcohol, 50 mM of *o*-coumaric acid, 4% of distillate water and 50, 100, 200, 400, 670, 800, 1600 and 3200 of enzymatic mU. All reactions were carried out in screw-capped 1.5 mL tubes at 30°C and initiated after the addition of the corresponding distilled water and enzyme load, containing 5 mg of BSA.

2.6.3. Effect of apparent pH, temperature and thermal stability

The effect of apparent pH and temperature in the initial reaction rate of isobutyl *o*-coumarate synthesis was evaluated as follows: 2 M of isobutyl alcohol and 50 mM of *o*-coumaric acid were mixed in isooctane, containing 4% of different 50 mM buffered solutions (citrate pH 5, MES pH 6, MOPS pH 7, Tris-HCl pH 8, Glycine-

NaOH pH 9 and water pH 6.3 as control) for apparent pH assays, or by measuring the initial synthesis rate at 10, 20, 30, 40, 50 and 60°C, containing 4% of water without buffering, for temperature assays.

The thermal stability of AtFAE B was determined by measuring the remaining activity after incubating the enzyme at 30, 40 and 50°C in isooctane, containing 2 M of isobutyl alcohol and 4% of water. After the incubation time of 15, 30, 60, 120 and 140 min, reactions were initiated by transferring the contents of each tube to a new 1.5 mL screw-capped tube containing 50 mM of *o*-coumaric acid, where the initial synthesis rate was measured. In all cases, 670 mU of pure AtFAE B with 5 mg BSA was employed.

3. Results

3.1. Antifungal activity of isobutyl o-coumarate against Fusarium oxysporum f. sp capsici

The antifungal activity of isobutyl *o*-coumarate was demonstrated by bioautography determination (Fig. 1A, B). The isobutyl *o*-coumarate spot is located at a Rf of 0.7 (Fig. 1A) and after the inoculation and growth of *Fusarium oxyporum* in the same plate an inhibition spot is observed at the same Rf (Fig. 1B). Once the inhibition was proved, a probit analysis (Fig. 1C) was peformed in order to calculate the IC50 value of the isobutyl *o*-coumarate, and a value of 1.28 mM was obtained.

3.2. Feruloyl esterase specific molar activity against methyl coumarates

After purification, the enzymatic preparations of AtFAE B, AtFAE C, AnFAE B (Fig. S1) and AocFAE C [37] with a purity > 98% were used for the hydrolysis determinations and the enzymatic preparation of AtFAE B was used for all isobutyl *o*-coumarate synthesis assays.

In order to compare, mole to mole, the ability of FAEs to catalyze the hydrolysis reaction using different methyl coumarate isomers, the specific molar activity (U/μmol of enzyme) of the four purified FAEs was determined against the methyl esters of the *o*-, *m*- and *p*- coumaric acids (Fig. 2). The type B FAEs evaluated had a substrate preference for the methyl *p*-coumarate: the AnFAE B MpC activity (3026.5 U/μmol of enzyme) was 4.74- and 1000-fold higher than MmC and MoC activities, while AtFAE B MpC activity (7482.9 U/μmol of enzyme) was 1.3- and 4.2-fold higher than MmC and MoC activities. On the other hand, the type C FAEs evaluated had substrate preference for the methyl *m*-coumarate (MmC); both AocFAE C and AtFAE C MmC activities were ~1.2- and ~3.71-fold higher than MpC and MoC activities. Although MoC was the worst substrate for the four evaluated enzymes,

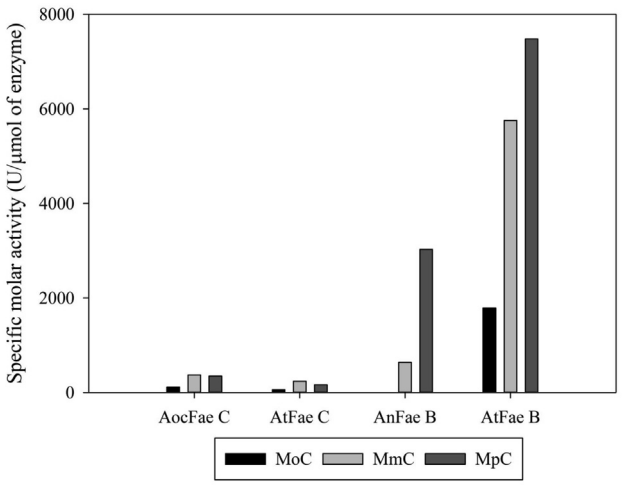

Fig. 2. Specific molar activity against methyl *o*-coumarate (MoC), methyl *m*-coumarate (MmC) and methyl *p*-coumarate (MpC) of the type C FAE of *Aspergillus ochraceus* (AocFAE C), the type C FAE of *Aspergillus terreus* (AtFAE C), the type B FAE of *Aspergillus niger* (AnFAE B) and the type B FAE of *Aspergillus terreus* (AtFAE B).

AtFAE B showed the highest enzymatic activity against MoC (1790.1 U/μmol of enzyme), 15-fold higher than that of AocFAE C (113.8 U/μmol of enzyme), 31-fold higher than that of AtFAE C (57.7 U/μmol of enzyme) and 668-fold higher than that of AnFAE B (2.7 U/μmol of enzyme); hence, AtFAE B was selected for the isobutyl *o*-coumarate synthesis assays.

3.3. Effect of substrate molar ratio on isobutyl o-coumarate synthesis

Given that the substrate molar ratio is determinant for the reaction rate and conversion yield, we decided to evaluate the effect of isobutyl alcohol and *o*-coumaric acid concentration in the reaction system (Fig. 3) by the methodology described in section 2.5.1. Fig. 3A shows the effect of isobutyl alcohol concentration; it can be seen that the reaction rate increases from 29.73 ± 0.499 nmol/min (0.5 M) to 47.78 ± 4.92 nmol/min (2 M). However, for 4 M, the reaction rate decreased, reaching the lowest value under solvent-free conditions, where the reaction rate decreased 23-fold compared with the rate at 2 M and 20-fold compared with the rate at 1 M. Even though the values between 1 M and 2 M are statistically equal, 2 M of isobutyl alcohol was retained in the subsequent experiments in order to achieve a better solubility of the *o*-coumaric acid in the reaction media. Regarding the *o*-coumaric acid concentration (Fig. 3B), the data show an increase in the reaction rate from 9.4 ± 0.319 nmol/min (6.25 mM) to 47.78 ± 4.92 nmol/min (50 mM). Nevertheless, the reaction rate diminished

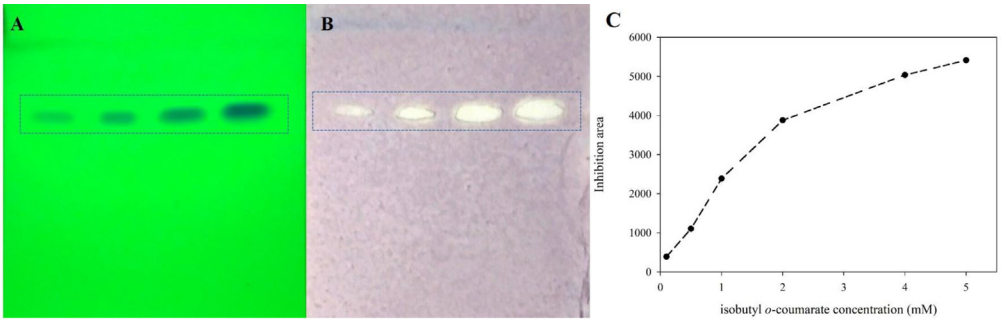

Fig. 1. Antifungal determination of isobutyl *o*-coumarate against *Fusarium oxysporum* f. sp. *capsici*. A. TLC plate with increasing concentrations of isobutyl *o*-coumarate (10–80 *n*moles) under UV light (254 nm). B. TLC plate with increasing concentrations of isobutyl *o*-coumarate (10–80 *n*moles) and growing of *Fusarium oxysporum* f. sp. *capsici* mycelium (after 3 d). C. IC50 determination of isobutyl *o*-coumarate against *Fusarium oxysporum* f. sp. *capsici*.

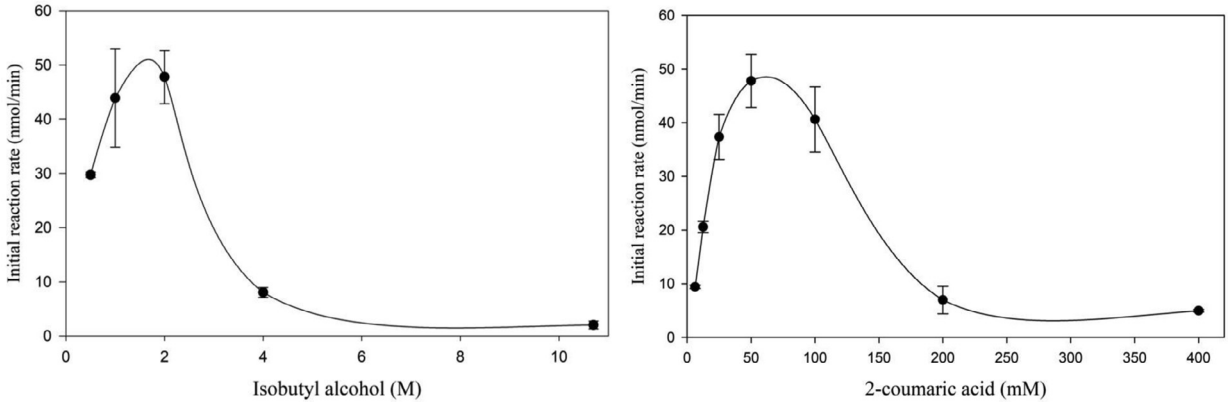

Fig. 3. Effect of the substrate molar ratio on the AtFAE B synthesis rate of isobutyl *o*-coumarate. A. Effect of isobutyl alcohol concentration with 50 mM of *o*-coumaric acid, 2% of water and isooctane as the solvent. B. Effect of *o*-coumaric acid concentration with 2 M of isobutyl alcohol, 2% of water and isooctane as the solvent. The reaction was carried out at 30°C using 0.04 mg of AtFAE B.

15, 86 and 90% with 100 mM, 200 mM and 400 mM concentrations, respectively. Based on these results, we selected 2 M of isobutyl alcohol and 50 mM of *o*-coumaric acid as the initial concentrations for further experiments.

3.4. Effect of water percentage and enzyme concentration on isobutyl o-coumarate synthesis

The results of the water percentage experiment show that AtFAE B requires at least 2% of water in the reaction media in order to catalyze effectively. As seen in Fig. 4, no product was detected with 0 and 1% of water; however, by using 2, 4 and 8% of water, the reaction rate was 34.7 ± 6.26 nmol/min, 48.3 ± 0.636 nmol/m in and 43.5 ± 3.61 nmol/min, respectively. Interestingly, the highest values for the reaction rate and conversion yield were achieved under different conditions; 4% of water was the most effective in terms of the reaction rate, whereas 2% water was the most effective in terms of the bioconversion yield. Since the objective of this study was to improve the reaction rate, 4% of water was used in the subsequent experiments.

The enzyme concentration present in the synthesis vessel is also an important parameter to improve the initial reaction rate and bioconversion yield. In order to find the lowest enzyme load in which the initial synthesis rate is still proportional, we evaluated eight different AtFAE B concentrations. The results of this experiment (Fig. 5) show that between 0.003 and 0.04 mg/ml of AtFAE B, the initial reaction rate exhibits a linear trend (R^2 = 0.987). However, beyond 0.04 mg/ml, the linear tendency is not observed; thus, we selected 0.04 mg/ml of AtFAE B as the enzyme amount for use in further experiments.

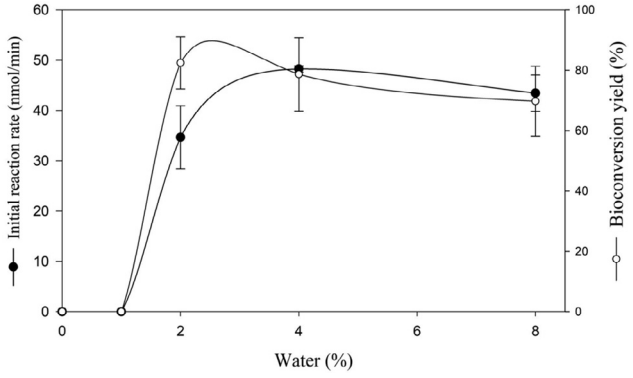

Fig. 4. Effect of water percentage in the reaction media on the initial synthesis rate (●) and conversion yield (○) of the isobutyl *o*-coumarate synthesis. The reaction was carried out at 30°C, using 0.04 mg of AtFAE B, 50 mM of *o*-coumaric acid, 2 M of isobutyl alcohol and isooctane as a solvent.

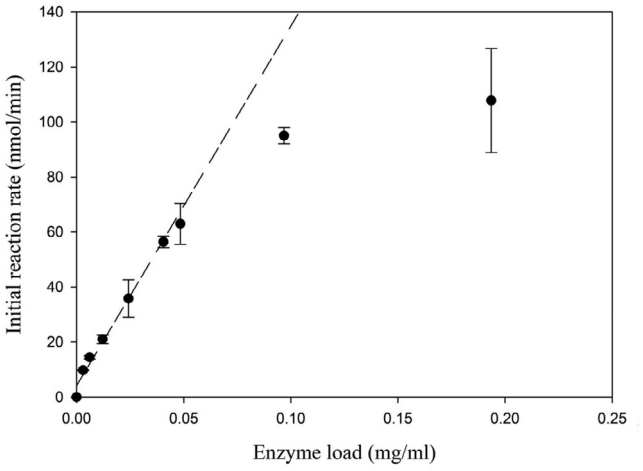

Fig. 5. Effect of the AtFAE B concentration (mg/ml) on the initial synthesis rate of the isobutyl *o*-coumarate. The reaction was carried out at 30°C, using 50 mM of *o*-coumaric acid, 2 M of isobutyl alcohol, 4% of water and isooctane as a solvent.

3.5. Effect of apparent pH, temperature and thermal stability on isobutyl o-coumarate synthesis

In a reaction media composed mostly of organic solvent, we encounter an apparent pH, whose effect was evaluated in the following experiment by the methodology described in section 2.5.3. The results shown in Fig. 6 indicate no statistical difference in the initial reaction rate of AtFAE B measured using distilled water (pH 6.3), or buffered solutions between pH values of 5 and 8; however, at an apparent pH 9, the isobutyl *o*-coumarate synthesis rate decreases almost 50%. Given that there is no difference between a buffered solution and distilled water, we decided to use distilled water in the reaction media in further experiments.

Finally, the effect of temperature and thermostability on AtFAE B was evaluated (Fig. 7). The results in Fig. 7A show that AtFAE B reached its maximum synthesis reaction rate within 40 and 50°C and has an activation energy of 43 kJ/mol. However, the thermostability study showed that the half-life of the enzyme after incubation in the solvent reaction media at 50°C is barely of 3.5 min, while at 40°C, it increases to 1.75 h and at 30°C, the AtFAE

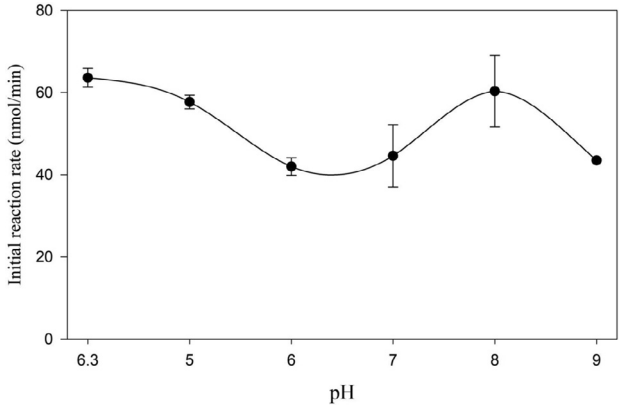

Fig. 6. Effect of apparent pH of the reaction media on the AtFAE B synthesis rate of isobutyl *o*-coumarate. The reaction was carried out at 30°C, using 0.04 mg of AtFAE B, 50 mM of *o*-coumaric acid, 2 M of isobutyl alcohol, 4% of water and isooctane as a solvent.

B has a half-life of 16.5 h (Fig. 7B). Hence, the temperature that we recommend for operational purposes is 30°C.

3.6. Kinetics of isobutyl o-coumarate synthesis

After the analysis and selection of the best substrate molar ratio, water percentage, AtFAE B load, pH and temperature conditions, we achieved after 8 hours of direct esterification a 77% yield of isobutyl *o*-coumarate (Fig. 8; Fig. S2). This equals a productivity of 1.06 g isobutyl *o*-coumarate/L*h. This productivity value was achieved using 0.04 mg/ml of AtFAE B which resulted in a biocatalyst yield of 209.6 kg isobutyl *o*-coumarate/kg free AtFAE B.

4. Discussion

The antifungal activity of the isobutyl *o*-coumarate was evaluated against *Fusarium oxysporum* f. sp. *capsici*, finding an IC50 of 1.28 mM. Previous studies on the antifungal activity of *o*-coumaric acid derivatives report inhibitions of 84.7% using 0.5 mM of the *t*-butyl *o*-coumarate against *Fusarium solani* [8] and 69.7% inhibition at 1.2 mM of isobutyl *o*-coumarate against *Fusarium oxysporum* f. sp *lycopersici* [10]. Given the antifungal activity of the *o*-coumaric acid derivatives, it is of great interest to find enzymes capable of synthesizing *o*-coumaric acid esters.

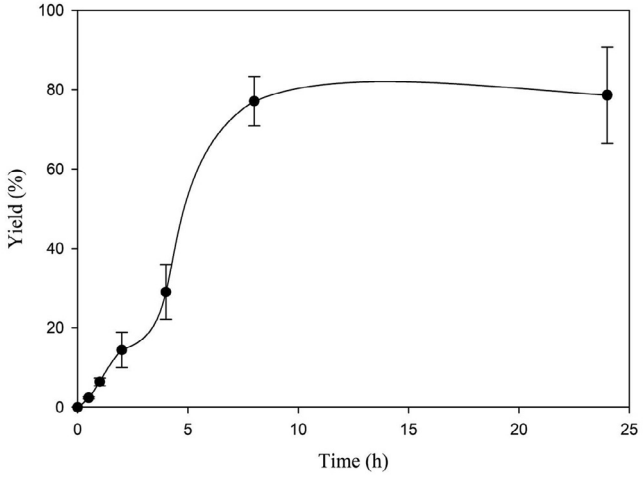

Fig. 8. Kinetics of isobutyl *o*-coumarate synthesis. The reaction was carried out at 30°C, using 0.04 mg of AtFae B, 50 mM of *o*-coumaric acid, 2 M of isobutyl alcohol, 4% of water and isooctane as the solvent.

AocFAE C, AtFAE C, AnFAE B and AtFAE B exhibited a lower FAE specific molar activity against the methyl ester of *o*-coumaric acid (MoC) compared with the activity against the methyl esters of *m*- and *p*-coumaric acids. The evaluated type B FAEs have a substrate preference in the order MpC > MmC > MoC while the evaluated type C FAEs have a substrate preference in the order MmC > MpC > MoC; this result is similar to those of previous reports on type C FAEs [31]. The low preference for MoC could be explained by analyzing the natural occurrence of the three isomers of coumaric acid: *p*-coumaric acid is the most commonly occurring isomer in nature [39], while *o*-coumaric acid is the least abundant; unlike *m*- and *p*-coumaric acid, *o*-coumaric acid is an intermediary in the salicylic acid metabolic route in plants, which few organisms produce [40]; consequently, most enzymes have a low preference for *o*-coumaric acid as the substrate. However, the activity of AtFAE B against MoC is the highest among the four evaluated enzymes, with 15- to 668-fold higher activity. The AtFAE B sequence has not been studied phylogenetically; however, it has significant homology with tannases (up to 85%), specialized enzymes with the ability to accept polyhydroxylated and sterically hindered substrates. This feature could probably be one of the reasons that can explain the interesting activity of AtFAE B towards *o*-coumaric acid derivatives. MoC proved to be a challenging substrate, highlighting

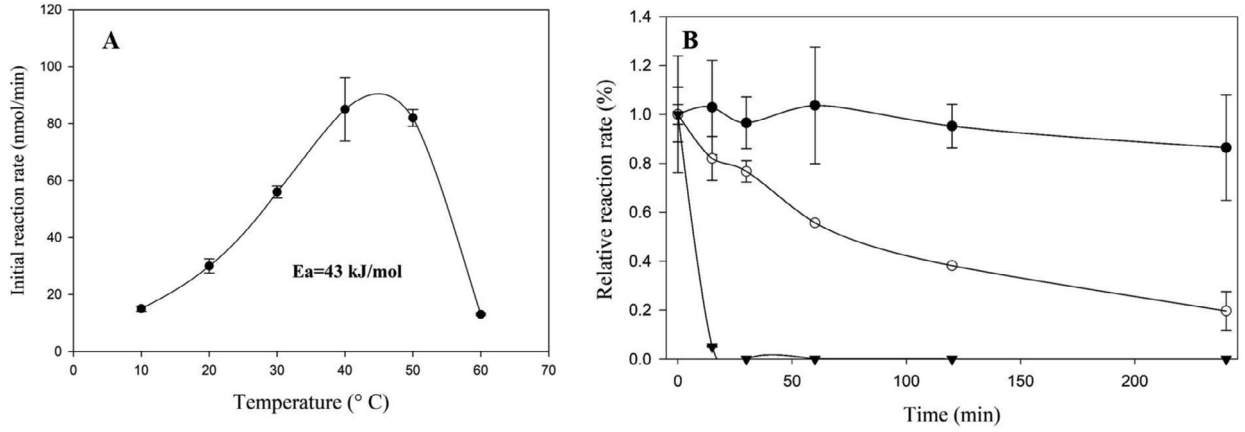

Fig. 7. Effect of temperature A. and thermostability B. on the AtFAE B synthesis rate of isobutyl *o*-coumarate. The reaction was carried out at 30°C for A. and incubations at 30°C (●), 40°C (○) and 50°C (▼) for B., using 0.04 mg of AtFAE B, 50 mM of *o*-coumaric acid, 2 M of isobutyl alcohol, 4% of water and isooctane as the solvent.

the importance of finding the right enzyme and reaction conditions for the production of a valuable derivative of *o*-coumaric acid, a difficult substrate even for the FAEs whose natural substrates are hydroxycinnamic acids.

Several type B FAE synthesis studies have reported ternary systems composed of hexane:alcohol:water in a ratio of 47.2:50.8:2 (v:v:v) [28,29,30]. Our data suggest that 2 M of isobutyl alcohol (18% v/v) is the concentration that produces the highest reaction rate among the evaluated conditions, affording 20% yield in 3 h reaction time. Interestingly, the optimal alcohol concentration observed in this study is much lower than that reported previously (50.8%) for type B FAEs; however, the yields obtained in these studies are below 75% and 15% for transesterification and direct esterification, respectively, both in above 200 h reaction time [28,29]. Besides, beyond 2 M of isobutyl alcohol (18% v/v), we observed a substrate inhibition phenomenon, noticeable from 4 M of isobutyl alcohol (36% v/v); this result is similar to the substrate inhibition observed for AocFAE C, whose optimal alcohol concentration is 25% (v/v) and presents substrate inhibition at 50% (v/v) alcohol [37]. When analyzing the solvent-free conditions (10.7 M isobutyl alcohol), we found a sharp decline in the reaction rate to 2.029 ± 0. 7142 nmol/min and the yield was only 11% after 50 h (data not shown); these results are similar to those observed during the solvent-free system catalysis of butyl ferulate by Depol 740L where the yield was just above the detection limit [27] and catalysis of butyl *p*-coumarate by AnFAE A where a yield of 20% in 72 h was reported [41]; however, our results differ from those observed during the butyl ferulate synthesis catalyzed by AnFAE A, because for this substrate, the yield was above 80% in 48 h in a solvent-free system [41].

Overall, these results suggest that the AtFAE B has a low tolerance towards high alcohol concentrations in the reaction media, and in order to obtain high yields and reaction rates, the alcohol concentration must remain below 18% (v/v) when using this enzyme.

Among the few direct esterification studies using FAEs, the reported hydroxycinnamic acid concentration is 30–50 mM [41,42]; beyond this concentration, a decrease in the enzymatic activity has been reported due to acidification of the reaction media and thus denaturalization of the protein [42]. Similar to previous reports, the data obtained in this study by the effect of the *o*-coumaric acid concentration show that the reaction rate decreases beyond 50 mM of *o*-coumaric acid, which, in this case, could be due to substrate inhibition; however, it is important to mention that even though the mixture of isooctane:isobutanol:water in a ratio of 78:18:4 (v:v:v) is a detergentless microemulsion at low *o*-coumaric acid concentrations ($\leq$5 mM), the solubility of *o*-coumaric acid at 50 mM in the reaction media is poor; thus, further studies must be conducted in homogeneous reaction media to evaluate this hypothesis.

Based on our results, we can conclude that AtFAE B needs at least 2% water in the reaction media in order to catalyze a synthesis reaction. There are few reports on the catalysis of FAEs in reaction media without water such as the synthesis of butyl hydroxycinnamates catalyzed by type A FAE of *Aspergillus niger* in solvent-free no-water reaction media [41]. Nevertheless, most FAE synthesis reports utilize ternary reaction media, which include a water percentage of 2–3% [28,29,32]. In this study, 4% of water in the reaction media resulted in the best reaction rate for the synthesis of isobutyl *o*-coumarate, affording 77% yield in 8 h. Even though the reaction rate at 2% water is lower than that at 4% water, the yield achieved with 2% water is 83%, 5% higher than that obtained with 4% water. This could be explained by the equilibrium in the reaction; at a higher water content, the equilibrium is reached sooner resulting in a lower reaction yield. Nonetheless, both yields are higher than the yields reported in previous reports of direct esterification in ternary systems with FAEs; in these reports, the reaction times are higher than 200 h and yields are lower than 20% [28].

When analyzing the data on the apparent pH in the reaction media, we concluded that apparent pH from 5 to 8 did not have any influence on the initial reaction rate; this may be due to the significantly reduced amount of water in the reaction media. It has been reported that enzymes surrounded by nonpolar solvents, retain a 'pH memory' of the last aqueous solution the enzyme was exposed to [43,44]; furthermore, an analysis of the catalytic activity of AtFAE B showed that the binding pocket does not have many ionic amino acids that can be affected by the apparent pH in the reaction media. However, we observed a moderate decrease in the reaction rate at pH 9; similar to this finding, previous characterized FAEs from *Aspergillus terreus* showed a decrease in activity from pH 9 onwards [45].

The results of the enzyme concentration show that there is a linear correlation between enzyme load and the initial reaction rate from 0.003 to 0.04 mg/ml of AtFAE B; beyond this enzyme load, the catalytic activity was no longer proportional to the load of the enzyme, probably because of a mass transfer limitation [46]. Thus, the recommended AtFAE B load for the esterification of isobutyl *o*-coumarate is 0.04 mg/ml (670 mU against methyl *o*-coumarate). The optimal enzyme concentration of the type C FAE of *Aspergillus ochraceus* for the synthesis by transesterification of methyl ferulate with butanol (a typical model reaction) is 0.01 mg/ml [37]. Similarly, the optimal enzyme load for the *Humicola* spp FAE in Depol 740 L was studied for the synthesis of raffinose ferulate; in this case, 289 to 348 U of enzyme was required for the synthesis of raffinose ferulate [42]. In order to make a true comparison, we ought to perform the enzyme load experiment with the same reaction for every enzyme; however, we could observe that the enzyme load required to efficiently catalyze the esterification of the antifungal isobutyl *o*-coumarate by AtFAE B is only 4 times greater than the amount required for the model reaction of the transesterification of methyl ferulate by the type C FAE of *Aspergillus ochraceus*; nevertheless, the amount of AtFAE B needed to synthesize the functional isobutyl *o*-coumarate is around 500 times lower than the amount required for the esterification of the also functional raffinose ferulate by the *Humicola* spp FAE in Depol 740 L. These results suggest that the ability of AtFAE B to achieve the synthesis of challenging products, as derivatives of *o*-coumaric acid, makes it stand out among common FAEs.

With respect to the effect of temperature on the reaction, we observed that the maximum reduction in the reaction rate occurs between 40 and 50°C. This result agrees with the optimal temperature for the transesterification of methyl ferulate with L-arabinose catalyzed by the type C FAE of *Sporotrichum thermophile* and *Talaromyces stipitatus* [31,47]. Complementary to this study, most of the synthesis reactions catalyzed by FAEs report temperatures between 30 and 40°C [28,29,30]. Also, with the data obtained in this experiment, we determined 43 kJ/mol as the activation energy for this reaction. This activation energy is 1.7-fold greater than that reported previously for the type C FAE of *Aspergillus ochraceus* (24. 9 kJ/mol) for the hydrolysis of methyl ferulate [37]. This difference could be attributed to the increase in the activation energy in a system with an organic solvent in which the enzyme is less flexible than in water reaction media such as the ones used for hydrolysis reactions.

When analyzing the initial synthesis reaction rate for the different temperatures, we observed that at 50 and 60°C, the isobutyl *o*-coumarate production presented an asymptotic tendency beyond 15 min (data not shown), which indicates enzyme denaturalization at these temperatures. Thus, a thermostability study mimicking the synthesis conditions was conducted; the half-lives of the enzyme were 16.5 h, 1.75 h and 3.5 min determined at 30, 40

and 50°C, respectively. The data showed a dramatic decrease in the half-lives of the enzymes beyond 30°C, suggesting that AtFAE B is not operationally stable at a temperature higher than 30°C in the ternary reaction system used in this study. There are reports of FAEs with higher thermostability than that reported for AtFAE B, for example, the half-life of the type A FAE of *Aspergillus niger* (AnFAE A) was 78 min in a solvent-free system at 60°C [41]. This difference could be due to the presence and absence of water in the reaction media for AtFAE B and AnFAE A, respectively, given that the half-life of an enzyme in an organic solvent decreases when the water content is increased, as reported in prior studies [48,49]. Another example of higher thermostability is the type D FAE of *Aspergillus terreus*, which retained 15% of residual activity against methyl ferulate after 30 min of incubation at 60°C [50]. Thus, the half-life observed for AtFAE B in this study indicates the necessity of developing strategies to increase the stability of this enzyme.

Finally, after the experiments carried through this study, we found reaction conditions that allow, for the first time, to obtain yields above 75% in 8 h for a direct esterification, resulting in a productivity of 1.06 g isobutyl *o*-coumarate/L*h. Such productivity values have never been reported before in a direct esterification reaction using FAEs or lipases. Moreover, through hydrolysis studies, it has been demonstrated that AtFAE B presents 4-fold less specific activity against methyl *o*-coumarate than methyl *p*-coumarate, which suggests that isobutyl *o*-coumarate synthesis may not be the easiest reaction for this enzyme and definitely not a model reaction such as previous reports of esterification with FAEs. Recently, a type D FAE from *Aspergillus terreus* has been used for the transesterification of hydroxycinnamates, achieving high yields (~90% after 24 h) for methoxylated hydroxycinnamic acid derivatives (glyceryl ferulate), but low yields (>5% after 24 h) for hydroxylated hydroxycinnamic acid derivatives (prenyl caffeate) [51]. This example highlights the importance of studying FAEs with a preference for hydroxylated hydroxycinnamic acid as substrates. In this regard, the highest esterification yields reported for type B FAEs are less than 15% in more than 200 h reaction time for model reactions such as propyl *p*-coumarate [29] or 1.4% yield in 96 h for the esterification of prenyl ferulate [34] and 8 mg product/L*h productivity using lipases for the esterification of ethyl *o*-coumarate [19]. Another important parameter when evaluating a biocatalyst is the biocatalyst yield (BY) [46]; the BY for the esterification of the isobutyl *o*-coumarate by AtFAE B is 209.6 kg isobutyl *o*-coumarate/kg free AtFAE B; even though there are BYs of more than 300 kg product/kg free enzyme reported for transesterification reactions with FAEs [33], the yield achieved in this study is within the acceptable BY for pharmaceutical products (100–250 kg product/kg free enzyme) [52] and the direct esterification strategy confers important process advantages over transesterification. Moreover, when the enzyme is recycled, the BY increases substantially and this can be achieved through immobilization.

5. Conclusions

The productivity values obtained in this study have never been reported before for a direct esterification reaction using type B FAEs. Furthermore, we introduced a greener alternative for the synthesis of a potentially valuable industrial molecule: the antifungal isobutyl *o*-coumarate. The outstanding synthetic capacity of AtFAE B and the reaction media conditions observed in this study enabled us to achieve a productivity suitable for greener industrial applications: 1.06 g isobutyl *o*-coumarate/L*h and a biocatalytic yield of 209.6 kg isobutyl *o*-coumarate/kg free AtFAE B.

Given these results, it is of great interest to conduct immobilization studies of AtFAE B in order to enable the reuse of the biocatalyst at temperatures above 40°C and increase its biocatalytic yield. Immobilization studies on AtFAE B are currently underway.

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Conflict of interest

This manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with the submission to the Electronic Journal of Biotechnology.

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Supplementary material

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