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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Evolution of the feruloyl esterase MtFae1a from *Myceliophthora thermophila* towards improved catalysts for antioxidants synthesis

Simona Varriale[1] · Gabriella Cerullo[1] · Io Antonopoulou[2] · Paul Christakopoulos[2] · Ulrika Rova[2] · Thierry Tron[3] · Régis Fauré[4] · Peter Jütten[5] · Alexander Piechot[5] · Joana L. A. Brás[6] · Carlos M. G. A. Fontes[6] · Vincenza Faraco[1]

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Abstract

The chemical syntheses currently employed for industrial purposes, including in the manufacture of cosmetics, present limitations such as unwanted side reactions and the need for harsh chemical reaction conditions. In order to overcome these drawbacks, novel enzymes are developed to catalyze the targeted bioconversions. In the present study, a methodology for the construction and the automated screening of evolved variants library of a Type B feruloyl esterase from *Myceliophthora thermophila* (MtFae1a) was developed and applied to generation of 30,000 mutants and their screening for selecting the variants with higher activity than the wild-type enzyme. The library was generated by error-prone PCR of *mtfae1a* cDNA and expressed in *Saccharomyces cerevisiae*. Screening for extracellular enzymatic activity towards 4-nitrocatechol-1-yl ferulate, a new substrate developed ad hoc for high-throughput assays of feruloyl esterases, led to the selection of 30 improved enzyme variants. The best four variants and the wild-type MtFae1a were investigated in docking experiments with hydroxycinnamic acid esters using a model of 3D structure of MtFae1a. These variants were also used as biocatalysts in transesterification reactions leading to different target products in detergentless microemulsions and showed enhanced synthetic activities, although the screening strategy had been based on improved hydrolytic activity.

Keywords Directed evolution · High-throughput screening · *Myceliophthora thermophila* · Library · Feruloyl esterase

Introduction

Feruloyl esterases (FAEs, also known as ferulic or cinnamic acid esterases, EC 3.1.1.73) are enzymes whose function in nature is to remove L-arabonofuranosyl-linked monomeric or dimeric ferulate moieties from the polysaccharide main chain of xylan in plant cell wall. They act as accessory (or auxiliary) enzymes that facilitate other enzymes, such as xylanases, xylosidases, arabinofuranosidases, etc., in accessing to their site of action during biomass conversion (Wong 2006; Faulds 2010; Dumon et al. 2012). FAE classification is based on their specificity towards the hydrolysis of methyl esters of hydroxycinnamic acids: methyl caffeate (MCA), methyl ferulate (MFA), methyl sinapate (MSA), and methyl *p*-coumarate (M*p*CA) (Crepin et al. 2004; Benoit et al. 2008; Udatha et al. 2011). However, the discovery of new FAEs using genome mining and phylogenetic analysis of current publicly accessible fungal genomes led to the development of a new subfamily classification of fungal FAEs considering both phylogeny and substrate specificity (Dilokpimol et al. 2016; Dilokpimol et al. 2018).

Simona Varriale and Gabriella Cerullo contributed equally to this work.

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✉ Vincenza Faraco
vfaraco@unina.it

[1] Department of Chemical Sciences, Complesso Universitario Monte S. Angelo, University of Naples Federico II, via Cintia 4, 80126 Naples, Italy

[2] Biochemical Process Engineering, Division of Chemical Engineering, Department of Civil, Environmental and Natural Resources Engineering, Luleå University of Technology, Luleå, Sweden

[3] Centrale Marseille, CNRS, Aix Marseille Université, iSm2 UMR, 7313 Marseille, France

[4] LISBP, CNRS, INRA, INSA, Université de Toulouse, Toulouse, France

[5] Taros Chemicals GmbH & Co. KG, Dortmund, Germany

[6] NzyTech LDA, Estrada Do Paco Do Lumiar Campus Do Lumiar Ed. E - R/C, Lisbon, Portugal

In addition to their hydrolytic ability, in appropriate tailor-made operative conditions, some FAEs are able to perform (trans)esterification reactions, conjugating hydroxycinnamic motif from its acid or related ester forms and leading to the synthesis of compounds with attractive properties for the cosmeceutical industry (Antonopoulou et al. 2016). Enzymatic (trans)esterification meets the requirement for greener processes and the consumers' preference for natural products, encouraging the development of sustainable and competitive biotechnological processes which can give several advantages in alternative to the entirely chemo-catalyzed processes such as milder reaction conditions, high selectivities, and shorter synthetic pathways (Kiran and Divakar 2001).

Type B FAE from the thermophilic fungus *Myceliophthora thermophila* ATCC 42464 (synonym *Sporotrichum thermophile*) (MtFae1a) belonging to CE1 family of the CAZy database (Lombard et al. 2014) (SF 6 according to Dilokpimol et al. 2016) has been previously heterologously expressed in *Pichia pastoris* and characterized (Topakas et al. 2012a). Besides its hydrolytic capabilities (Topakas and Christakopoulos 2004; Karnaouri et al. 2014), MtFae1a has also been applied to the synthesis of a variety of esters and in particular of prenyl ferulate (PFA) and 5-*O*-feruloyl-L-arabinose (AFA) in detergentless microemulsions (Antonopoulou et al. 2017a; Antonopoulou et al. 2017b).

This study was aimed at developing a methodology for the construction and the automated screening of evolved variants library of MtFae1a, allowing the selection of variants with higher activity than the wild-type enzyme. Therefore, the objectives were to generate a library of mutants by error-prone polymerase chain reaction (ep-PCR) in *Saccharomyces cerevisiae*, using an expression platform previously adopted in our laboratory (Giacobbe et al. 2014) and apply this strategy in conjunction with a high-throughput method to select the best variants. Crude supernatants of these new variants were characterized for their thermo- and solvent tolerance, and the best four variants were investigated in docking experiments on hydroxycinnamic esters using a hybrid structure of MtFae1a. Moreover, the best four variants were tested in transesterification reactions in detergentless microemulsions for the production of target compounds selected for their potential antioxidant activity.

Materials and methods

Chemicals

Casaminoacids, yeast nitrogen base (without amino acids and without ammonium sulfate) and agar were purchased from Difco (Paris, France). QIAprep kit from Qiagen (Hilden, Germany) was used for plasmid extraction and PCR fragment purifications. Restriction enzymes were purchased from Promega, (Wisconsin, USA) and methyl esters of cinnamic acids substrates were provided by Apin Chemicals ltd, Oxford, UK. 4-Nitrophenyl ferulate (*p*NP-Fe) (Mastihuba et al. 2002; Hegde et al. 2009) and 4-nitrocatechol-1-yl ferulate (4NTC-Fe) (Gherbovet et al. 2016), which was necessary for the screening of the complete library, were provided by Taros Chemicals (Dortmund, Germany). Other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

Vectors, strains, and culture media

The *Escherichia coli* strain Top 10 (*F-mcrA D* (*mrr-hsdRMS-mcrBC*) *f80lacZDM15 DlacX74 deoR recA1 araD139 D* (*ara-leu*) *7697 galU galK rpsL* (*StrR*) *endA1 nupG*) was used in all DNA manipulations, and its growth was performed in Luria–Bertani (LB) medium (in g l^{-1}: 10 bactotryptone, 10 NaCl, 5 yeast extract), supplemented with 100 μg ml^{-1} of ampicillin for the selection of transformed clones.

The *S. cerevisiae* strain used for heterologous expression was W303-1A (*MATa*, *ade2-1*, *his3-11*, *15*, *leu2-3*, *112*, *trp1-1*, *ura3-1*, *can1-100*). The plasmids used for *S. cerevisiae* expression were pSAL4 (copper-inducible CUP1 promoter) carrying URA3 gene for auxotrophic selection (Mascorro-Gallardo et al. 1996). Both *S. cerevisiae* strain and pSAL4 plasmid were kindly provided by Dr. T. Tron (Institut des Sciences Moléculaires de Marseille CNRS, Aix Marseille Université Service 342, Faculté des Sciences de Saint-Jérôme). The cDNA coding for MtFae1a was codon optimized for expression in *S. cerevisiae* and synthesized by NZYTech (Lisbon, Portugal). The vector pSAL4-*mtfae1a* used for recombinant expression of MtFae1a in *S. cerevisiae* was prepared by cloning *mtfae1a* cDNA in pSAL4 (in *Eco*RI/*Hin*dIII) under control of copper-inducible CUP1 promoter (Piscitelli et al. 2005). *S. cerevisiae* was grown on a selective medium (SG)(6.7 g l^{-1} yeast nitrogen base without amino acids and ammonium sulfate, 5 g l^{-1} casaminoacids) supplemented with 30 mg l^{-1} adenine, 40 mg l^{-1} tryptophan, 50 mM succinate buffer (pH 5.3), 20 g l^{-1} galactose, and 600 μM copper sulfate. For solid media, 15 g l^{-1} agar was added.

Random mutagenesis

The random mutagenesis was performed by ep-PCR with the GeneMorph II random mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacture's protocol using the recombinant vector pSAL4-*mtfae1a* as template. Primers, pSal4Fw (CCAACGCAATATGG ATTGTCAG) and pSal4Rev (CAAGTGTAGCGGTC ACGCTGCG) used in amplification experiments, are complementary to the two ends of pSAL4 polylinker sequence. Cycling parameters were 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and final elongation step of 72 °C for 10 min. The sequence optimized

for expression of MTFae1a in *S. cerevisiae* was deposited in GenBank database with the accession number MH142911.

Construction of a mutant library

DNA fragments derived from error-prone PCR on *mtfae1a* cDNA were ligated in pSAL4 vector digested with *Eco*RI/*Hin*dIII restriction enzymes, exploiting homologous recombination expression system of *S. cerevisiae*. Transformation was done by using a lithium acetate protocol (Gietz and Woods 2006) using 1 μg *Eco*RI/*Hin*dIII-restricted pSAL4 and 300 ng ep-PCR *mtfae1a* product. The cells were spread on the selective medium and the plates incubated upside down for 4 days at 28 °C.

Library screening

Screening on solid medium was performed on 22 × 22 cm Q-Tray bioassay plates containing 50 mM potassium phosphate buffer (KP) pH 6.5, 1% agarose, and 0.5 mM ammonium iron (III) citrate supplemented with 75 μg/mL of 4NTC-Fe chromogenic substrate, as reported in Gherbovet et al. (2016). The strategy for screening was set up in high-throughput version using the automated workstation including the robot colony picker QPIX 450 (Molecular Devices, LLC, CA, USA) and the robot BioMek NXP (Beckman Coulter, CA, USA). Readings of the absorbance for screening in liquid medium were performed in a plate reader Multi Detection SystemGloMax® Discover System (Promega, Wisconsin, USA).

DNA extraction and sequencing

The recombinant plasmid DNA was extracted following the protocol reported by Robzyk and Kassir (1992) and amplified in *E. coli* TOP10. Plasmid DNA bearing the mutated *mtfae1a* genes were purified and sequenced (Eurofins Genomics s.r.l.) using primers pSal4Fw and pSal4Rev.

Hydrolytic esterase activity measurements

Activity of wild-type and mutated MtFae1a was assayed against the substrates MFA, MSA, MCA, and M*p*CA. Substrate stocks (1.18 mM) were prepared in 100 mM MOPS buffer, pH 6.0. The esterase activity measurement was performed by Beckman DU7500 spectrophotometer in 1 mL of reaction mixture (100 mM MOPS buffer, pH 6; 100 μl culture supernatant; 30 μl substrate stock) adapted from Dilokpimol et al. (2017). The hydrolysis of the substrate was monitored at 320 nm for methyl caffeate, methyl ferulate, and methyl sinapate, and at 308 nm for methyl *p*-coumarate for 15 min at 37 °C.

Activity of wild-type and mutated MtFae1a was assayed towards *p*NP-Fe in 1.1 mL reaction mixtures adapted from Mastihuba et al. (2002). The *p*NP-Fe substrate solution was prepared by mixing 10.5 mM *p*NP-Fe (in dimethyl sulfoxide) and 100 mM potassium phosphate buffer, pH 6.5 containing 2.5% Triton-X (1:9, *v*/*v*). The reactions were performed in the presence of 1 mL *p*NP-Fe substrate solution incubated with 100 μL of culture supernatant at 37 °C for 30 min. The release of *p*-nitrophenol was spectrophotometrically quantified by measuring absorbance at 410 nm.

One unit of feruloyl esterase activity was defined as the amount of enzyme hydrolysing 1 μmol of substrate per minute in the reaction mixture under assay conditions.

Thermo- and solvent tolerance conditions

The cultures were incubated at 28 °C in 20 mL SG for 3 days and, after biomass removal, the supernatant was analyzed for FAE activities against the substrate 4NTC-Fe. The crude broths were analyzed for thermotolerance at 55 °C for 1 h and tolerance to 25% of acetone at 37 °C for 30 min towards 4NTC-Fe. The amount of protein production was detected by the Bradford method (Sigma, Saint-Louis, USA), and the homogeneity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Blue. The FAE content (%, *w*/*w*) was estimated by SDS-PAGE, and subsequent quantification was done by a densitometric method using JustTLC software (Sweday, Sweden).

Simulations

An MtFae1a wild-type model was built by homology modeling based on the recently determined structure of an acetyl xylan esterase from *Aspergillus awamori* (5X6S_A) (Komiya et al. 2017) using SWISS-MODEL (Biasini et al. 2014). The model passed both evaluation tests (GMQE 0.77, QMEAN − 1.69) having high similarity with the template protein (93% query cover, 42% identities, 61% similarities, 2% gaps). Small molecule docking simulations (SMD), molecular dynamic simulations (MD), and visualization were performed in YASARA Structure in one monomer of the dimeric structure. Mutations were added to the MtFae1a model by swapping residues forming mutants, H105Y, F251L/H105Y, G49D, and G49A. Receptors were cleaned, and their hydrogen bonding system was optimized while the pK_a values of the ionisable groups were predicted and assigned protonation states based on pH 6.0, 0.9% NaCl prior to energy minimization that was performed using AMBER14. Prior to SMD simulations, a simulation cell was created around the catalytic serine (cube of approximately 6-Å extensions, forming a cube of 16.95 Å per side), large enough to include ligands but small enough to exclude non-catalytically relevant binding. Ligands

(MFA, MCA, M*p*CA, MSA, *p*NP-Fe, and 4NTC-Fe) were designed, their structure was cleaned, and their geometry was optimized. SMD was done on wild type and mutants by Autodock VINA (Trott and Olson 2009) performing 25 docking runs per simulation. Results were evaluated based on the resulting binding energy (more positive energies indicate stronger binding and negative energies mean no binding), the dissociation constant, the number of clusters (runs are clustered into distinct complex conformations, differing by at least 5.0 Å heavy atom RMSD after superposing on the receptor), the number of genetic runs per cluster, the orientation of ligand per cluster, and the distance of carbonyl carbon from the catalytic serine. MD simulations were performed at desired temperature creating a simulation cell of 10.0 Å around all atoms of wild-type and mutant structures filled with 25% acetone, 75% water, 0.9% NaCl or 100%, 0.9% NaCl, water at defined temperature. The system was energy minimized prior to simulation while structures were used after the simulation for docking against 4NTC-Fe.

Transesterification reactions and quantitative analysis

Transesterification reactions in a ternary system of *n*-hexane:*t*-butanol:buffer forming detergentless microemulsions were performed at adapted conditions based on previous work (Antonopoulou et al. 2017a; Antonopoulou et al. 2017b). Target compounds were prenyl ferulate, prenyl caffeate (PCA), glyceryl ferulate (GFA), *n*-butyl ferulate (BFA), and L-arabinose ferulate. Quantitative analysis of hydroxycinnamates was done on a 100-5 C18 Nucleosil column (Macherey Nagel, Düren, Germany) using an acetonitrile: water linear gradient. Detection was done at 300 nm by a PerkinElmer Flexar UV/Vis detector (Waltham, USA).

Transesterification yield was calculated as the molar amounts of generated transesterification product compared to the initial amount of donor, expressed as a percentage. Product selectivity was defined by the molar concentration of transesterification product divided by the molar concentration of hydrolysis product. Transesterification rate was calculated as the molar concentration of product formed by 1 g of expressed FAE in 1 h (mol/g FAE L h).

Results

Construction and screening of MtFae1a mutant library

To develop improved variants of *M. thermophila* feruloyl esterase MtFae1a, the enzyme was subjected to directed evolution experiments. Random mutations were introduced into *mtfae1a* cDNA sequence by ep-PCR. *S. cerevisiae* system with pSAL4 vector was adopted for the recombinant expression of MtFae1a enzyme. Random mutated *mtfae1a* sequences and pSAL4 vector were used to transform *S. cereviasiae* and create a 30,000 evolved variants collection, applying the system previously adopted for directed evolution of an α-L-arabinofuranosidase from *Pleurotus ostreatus* (Giacobbe et al. 2014).

The MtFae1a mutant library was analyzed by an automated HTS strategy designed and developed to select variants with higher activity than the wild-type enzyme towards 4NTC-Fe, a new substrate developed ad hoc due to the lack of suitable compounds for high-throughput assays of FAEs. An automated workstation including the robot colony picker QPIX 450 (Molecular Devices, LLC, CA, USA) and the robot BioMek NXP (Beckman Coulter, CA, USA) was adopted. The robot colony picker QPIX 450 was used to transfer the obtained clones of *S. cerevisiae* from selective solid medium to liquid medium in 96-well plates (Fig. 1(A), picking). Following growth by incubation at 28 °C for 2 days, the mutants were transferred Q-Tray bioassay plates containing agarose and ammonium iron(III) citrate in the presence of 4NTC-Fe (Fig. 1(B), gridding) and activity was detected after 1 h at 37 °C. A total of around 30,000 mutants were prepared and analyzed by the developed automated HTS. The primary screening performed on agarose growth medium containing 4NTC-Fe and ammonium iron (III) citrate to identify the active mutants allowed us to identify the 2584 positive in the assay with the chromogenic substrate corresponding to around 10% of the library.

The clones showing activity halos were selected and transferred from 96-well plates to 96 deep-well microplates (Fig. 1(C), re-arraying) for the secondary screening, focused on the detection of MtFae1a variants with higher activity than the wild-type enzyme in solution. After growth at 28 °C for 24 h, 100 μL of culture broth were transferred in 900 μL of liquid medium in 96 deep-well microplates by using the robot BioMek NXP (Fig. 1(D)) and the cultures were incubated at 28 °C for 72 h. After biomass removal by centrifugation (Fig. 1(E)), samples of culture supernatant were subjected to analysis of FAE activity production towards 4NTC-Fe (Fig. 1(F)).

Characterization of mutated and wild-type MtFae1a

The 30 most active evolved variants having at least twofold higher activity than the wild-type enzyme towards 4NTC-Fe in microscale were chosen to scale-up the growth in 20 mL of SG medium. Crude supernatants of improved variants were analyzed for FAE activity production towards 4NTC-Fe, their thermotolerance at 55 °C for 1 h and solvent tolerance in 25% acetone evaluating residual activity towards 4NTC-Fe. These analyses allowed the selection of the best four evolved variants, whose crude supernatants showed at least twofold higher activity, around 1.3-fold higher solvent resistance and at least twofold

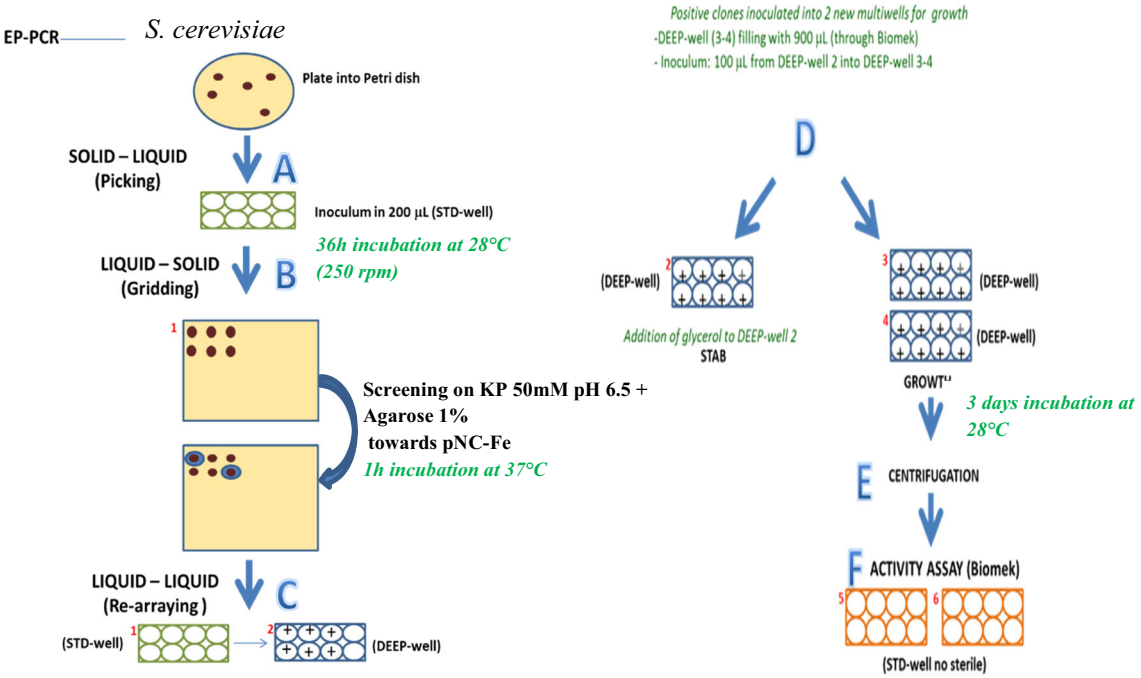

Fig. 1 Schematic representation of the high-throughput screening strategy developed to analyze *S. cerevisiae* mutants expressing feruloyl esterase activity

higher thermotolerance than wild-type MtFae1a (Table 1). These best four mutants were sequenced, and the corresponding mutations of the selected clones are reported in Table 1.

Substrate specificity of the four selected evolved variant crude supernatants was analyzed in comparison to that of MtFAE1a wild type evaluating their activity towards MFA, MCA, MpCA, MSA, and *p*NP-Fe (Fig. 2).

Docking of hydroxycinnamates on the structure of MtFae1a and mutants

Docking of hydroxycinnamates on a structure of MtFae1a and on the selected evolved mutants was performed to study substrate interaction. MtFae1a belongs to the SF6 subfamily of phylogenetic classification evolved from acetyl xylan esterases (Dilokpimol et al. 2016). Homology modeling resulted in a structure with a located putative catalytic triad (Ser118-His257-Asp201) and formation of disulfidic bond between Cys29 and Cys64. The mutants had a low RMSD (< 1 Å) compared to the wild type. Mutations on Gly49 of G49A and G49D and mutation on Phe251 at F251L/H105Y were located in the second shell around the catalytic serine (within 12–13 Å) extending to different directions. Mutation on His105 on F251L/H105Y and H105Y is located further from Ser108 and within a distance up to 30 Å (Fig. 3(B, C)). MD in 25% acetone at 37 °C resulted in low RSMD (0.1955 Å) for H105Y and higher RSMD (2.0303, 1.7128, and 1.8507 Å) for F251L/H105Y, G49D, and G49A, respectively, compared to structures before simulation (Table S1). Binding of 4NTC-Fe on structures before and after solvent exposure revealed that there was a 0.8-kcal/mol reduction (from 6.7660 to 5.9250 kcal/mol) in the highest binding energy for the wild type and a reduction in the genetic runs resulting to a "correct orientation" of ligand (accommodating the hydroxycinnamic moiety inside the cavity according to Suzuki et al. (2014) (Table S2)). Thermal exposure seemed that it had a greater impact on the highest binding energy causing a 1.3775-kcal/mol reduction (from 6.7660 to 5.3910 kcal/mol) for the wild type. Furthermore, the orientation of 4NTC-Fe binding onto MtFae1a wild type was severely modified after MD simulations while it was quite similar after

Table 1 Activity values of crude supernatants MtFae1a selected clones towards 4NTC-Fe and percentages of residual activity after solvent and heat exposure

	Activity (mIU/OD) (100-mL flask)	Thermotolerance (%) (1 h at 55 °C)	Solvent resistance (%) (1 h in 25% acetone, *v*/*v*)
MtFae1a wt	25.7 ± 6.4	10.8	67.2
H105Y	79.3 ± 21.0	34.3	85.3
F251L/H105Y	50.0 ± 14.6	21.4	94.5
G49D	54.5 ± 21.5	46.2	95.9
G49A	49.6 ± 12.8	37.4	95.5

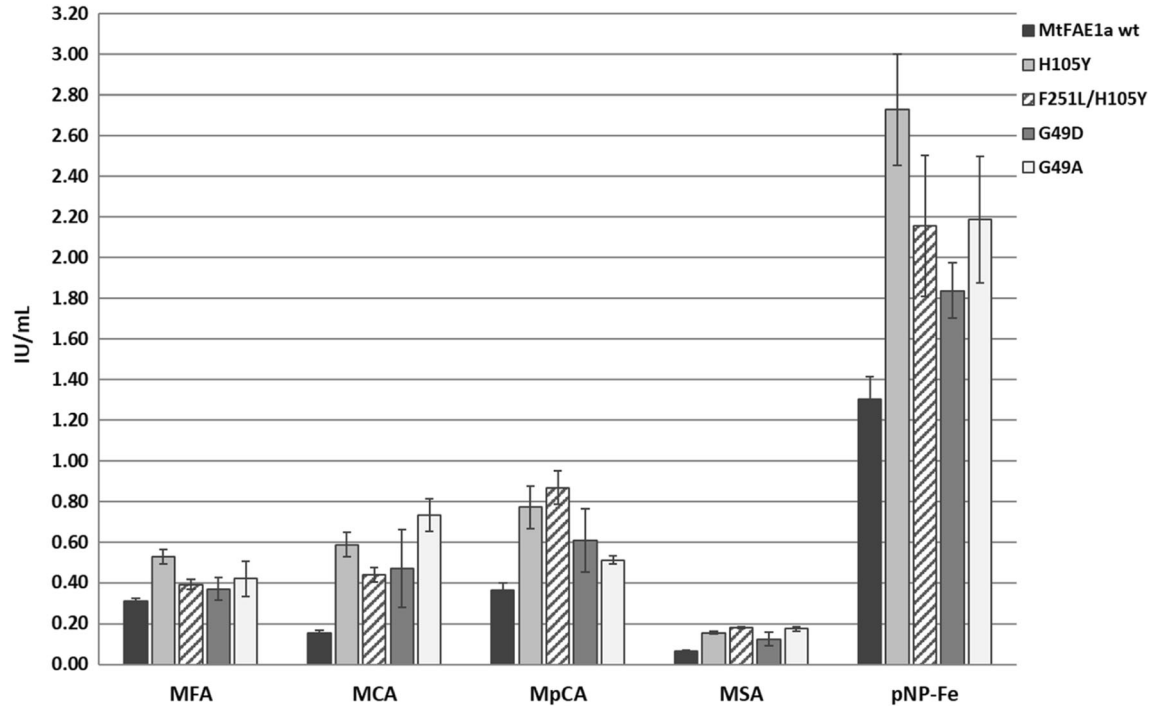

Fig. 2 Activity production of selected MtFae1a evolved variants towards methyl cinnamates and *p*NP-Fe. *Black* MtFae1a wild type, *light gray* H105Y, *striped* F251L/H105Y, *dark gray* G49D, and *white* G49A

solvent and thermal exposure (Fig. 3(D)) possibly contributing to the detrimental impact on residual activities. On the other hand, docking of 4NTC-Fe onto evolved mutants resulted in modification in the ligand orientation but did not affect significantly measures as the binding energy after acetone exposure aligning with the increased determined solvent resistance of evolved mutants. Regarding thermal exposure, highest binding energies were reduced while the distance of carbonyl carbon from catalytic serine was increased.

Binding of methyl hydroxycinnamates and bulkier esters such as *p*NP-Fe and 4NTC-Fe on structures with ionized state at pH 6.0 revealed similar binding energies for all methyl substituted ligands (around 5 kcal/mol) and increased binding energy for *p*NP-Fe and 4NTC-Fe (around 6.5 kcal/mol), perhaps due to better stabilization of bulkier moieties around the active site (Table S3). Generally, results validated the broad specificity of MtFae1a towards hydroxycinnamates. Orientation of binding onto wild type and mutants revealed that there was higher deviation during binding of methoxy substituted methyl esters than hydroxy substituted methyl esters (Fig. 4). The contacting residues during docking of ligands before and after MD simulations are presented in Tables S4 and S5.

Evaluation of synthetic abilities of MtFae1a evolved mutants

Wild-type MtFae1a and the selected four best evolved variants were evaluated for their ability to catalyze the transesterification of vinyl ferulate and caffeate with various fatty alcohols and carbohydrates in a ternary system of *n*-hexane:*t*-butanol:buffer forming detergentless microemulsions. As reported in Fig. 5, all the tested MtFae1a evolved variants and the wild-type enzyme were able to synthesize four target compounds, namely prenyl ferulate, prenyl caffeate, glyceryl ferulate, *n*-butyl ferulate, and 5-*O*-feruloyl-L-arabinose.

Discussion

Environmental concerns and drawbacks related to chemical syntheses currently employed for industrial purposes, including in the manufacture of cosmetics, make necessary the development of sustainable and competitive biotechnological processes. FAE catalyzed (trans)esterification satisfy these requirements by bringing numerous advantages compared to chemo-catalyzed processes such as milder reaction conditions, high selectivities, and shorter synthetic pathways (Kiran and Divakar 2001). Therefore, our research was aimed at replacing chemical processes currently adopted for the production of ingredients for the cosmetic industry with bioconversions characterized by reduced environmental impact (European Union funded OPTIBIOCAT project "Optimized esterase biocatalysts for cost-effective industrial production" (www.optibiocat.eu)). In this context, with the objective to develop competitive and eco-friendlier bioconversions catalyzed by FAEs, *M. thermophila* feruloyl esterase MtFae1a was subjected to directed evolution experiments obtaining biocatalysts with enhanced enzymatic properties in this work.

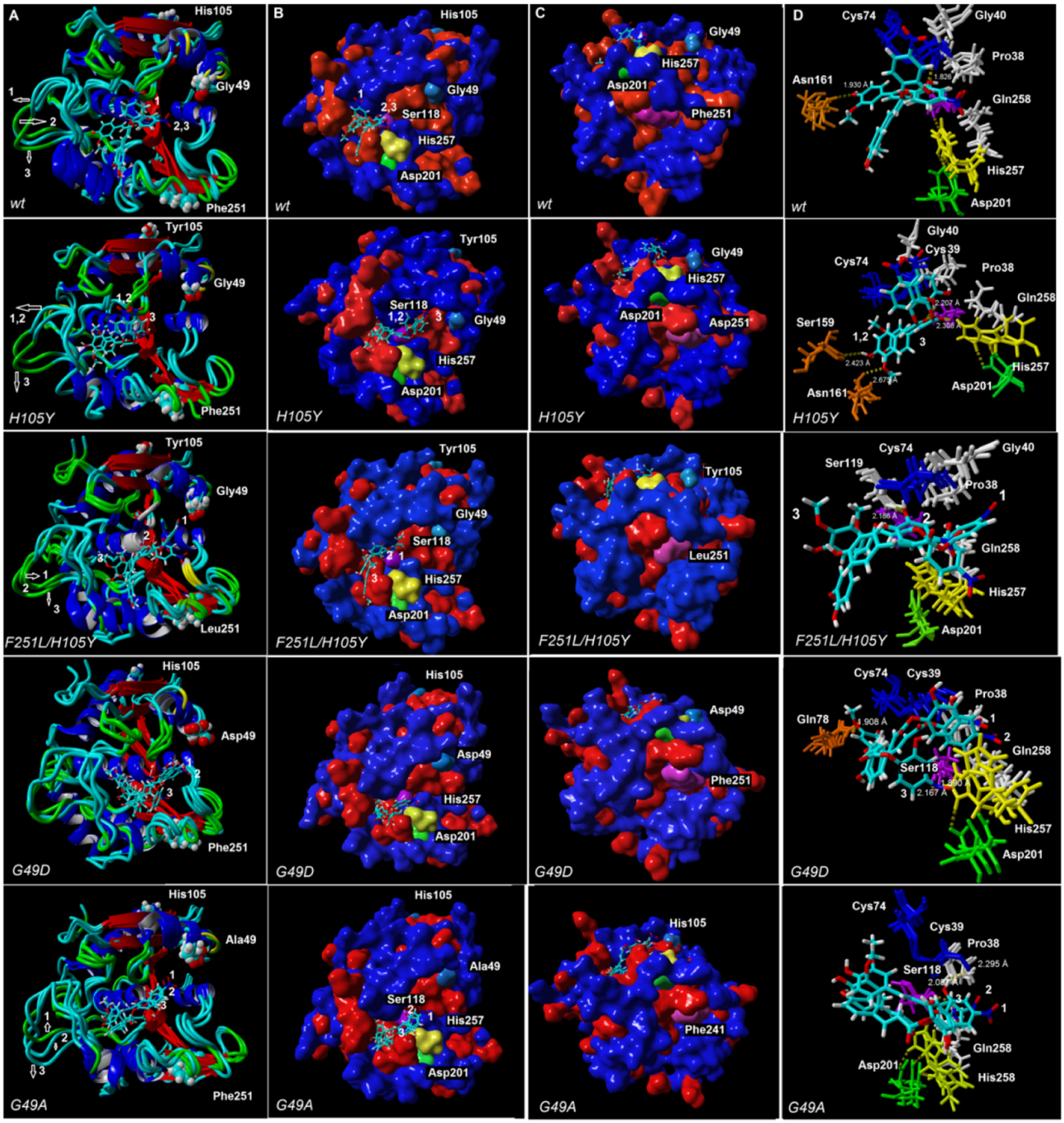

Fig. 3 (A) Superposition of secondary hybrid structures including docking of 4NTC-Fe. (B) and (C) Position of mutations on the surface of receptors. (D) Stabilization of esters in binding cavity: (1) initial condition, (2) after acetone, (3) after thermal exposure. Numbering of residues does not include the signal peptide

The ability of MtFae1a to act in plant biomass biodegradation together with its (trans)esterification capability makes of considerable interest the discovery of new FAEs with novel properties and applications (Topakas et al. 2007; Gopalan et al. 2015). Moreover, enlarging the spectrum of biologically active compounds obtainable by FAE bioconversions and improving the (trans)esterification yields could lead to the synthesis of new hits for the cosmetic sector. In this frame, directed evolution, that mimics the natural evolution, has proven to be a suitable strategy to improve or alter enzyme features such as selectivities, activity, stability, and solubility by methods of genetic diversity integration (Packer and Liu 2015). In the present study, a library of 30,000 directed evolved variants of MtFae1a was generated introducing

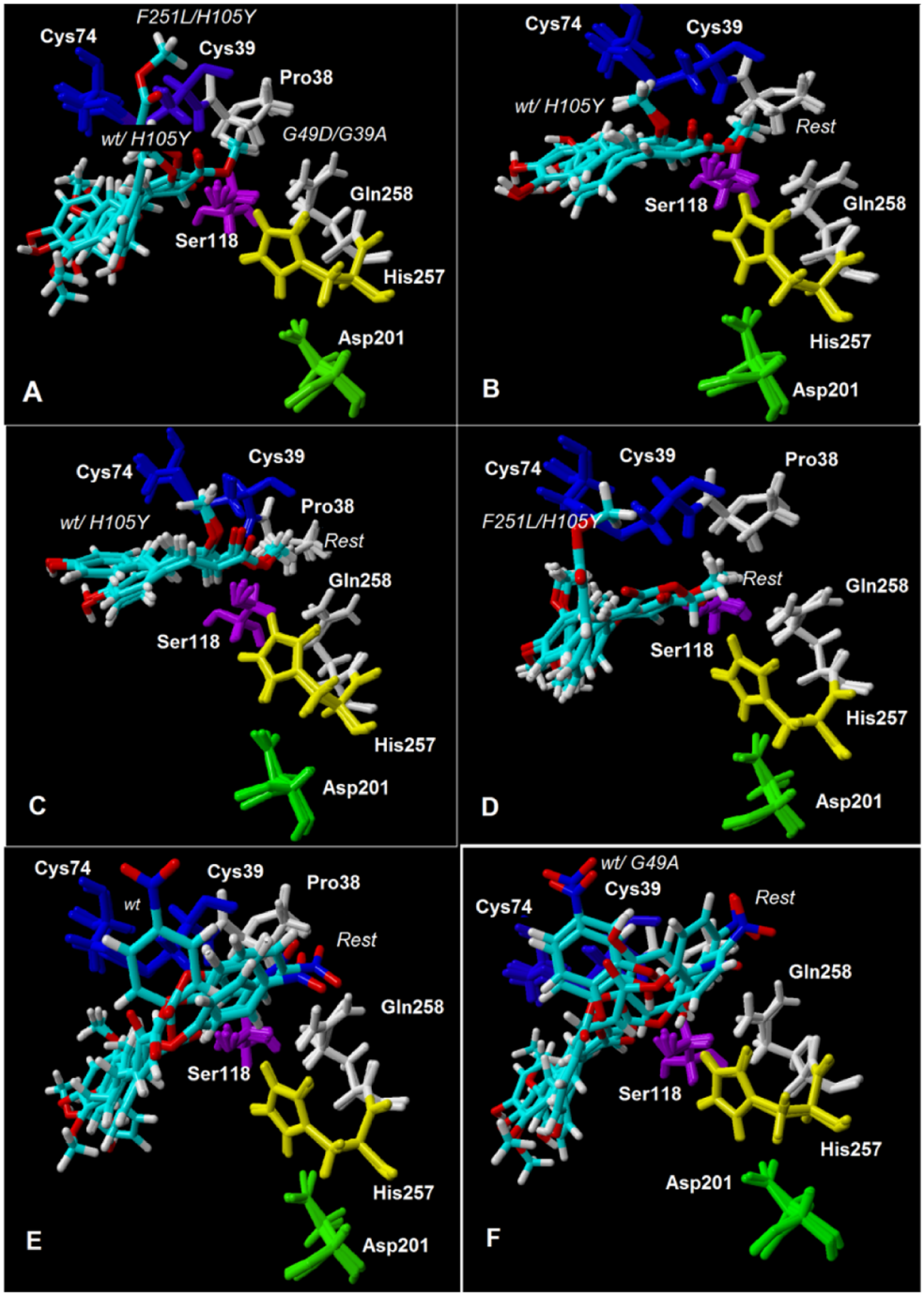

Fig. 4 Docking of hydroxycinnamates onto the binding cavity of MtFae1a and evolved mutants. **a** MFA. **b** MCA. **c** MpCA. **d** MSA. **e** pNP-Fe. **f** 4NTC-Fe. Numbering of residues does not include the signal peptide

random mutations in the corresponding cDNA by ep-PCR and using *S. cerevisiae* system with pSAL4 vector for the recombinant expression of the randomly mutated cDNAs, applying the system previously adopted for directed evolution of an α-L-arabinofuranosidase from *P. ostreatus* (Giacobbe et al. 2014). *S. cerevisiae* was chosen as recombinant expression platform for directed evolution, thanks to its several advantages compared to other yeasts, such as *P. pastoris*, which is able to secrete large amounts of proteins but suffers from low transformation efficiency and random gene integration in the genome limiting its use for high-throughput screening (HTS). *S. cerevisiae* exhibits the possibility to exploit its DNA recombination machinery avoiding ligation steps, together with high transformation efficiency and post-translational modifications (Gonzalez-Perez et al. 2012).

Although directed evolution has been shown to be a potent technology for the improvement of enzymes that are potentially important for industrial applications, there are several limitations that need to be solved, mainly related to the lack of rapid and generalized high-throughput screening systems. In the present

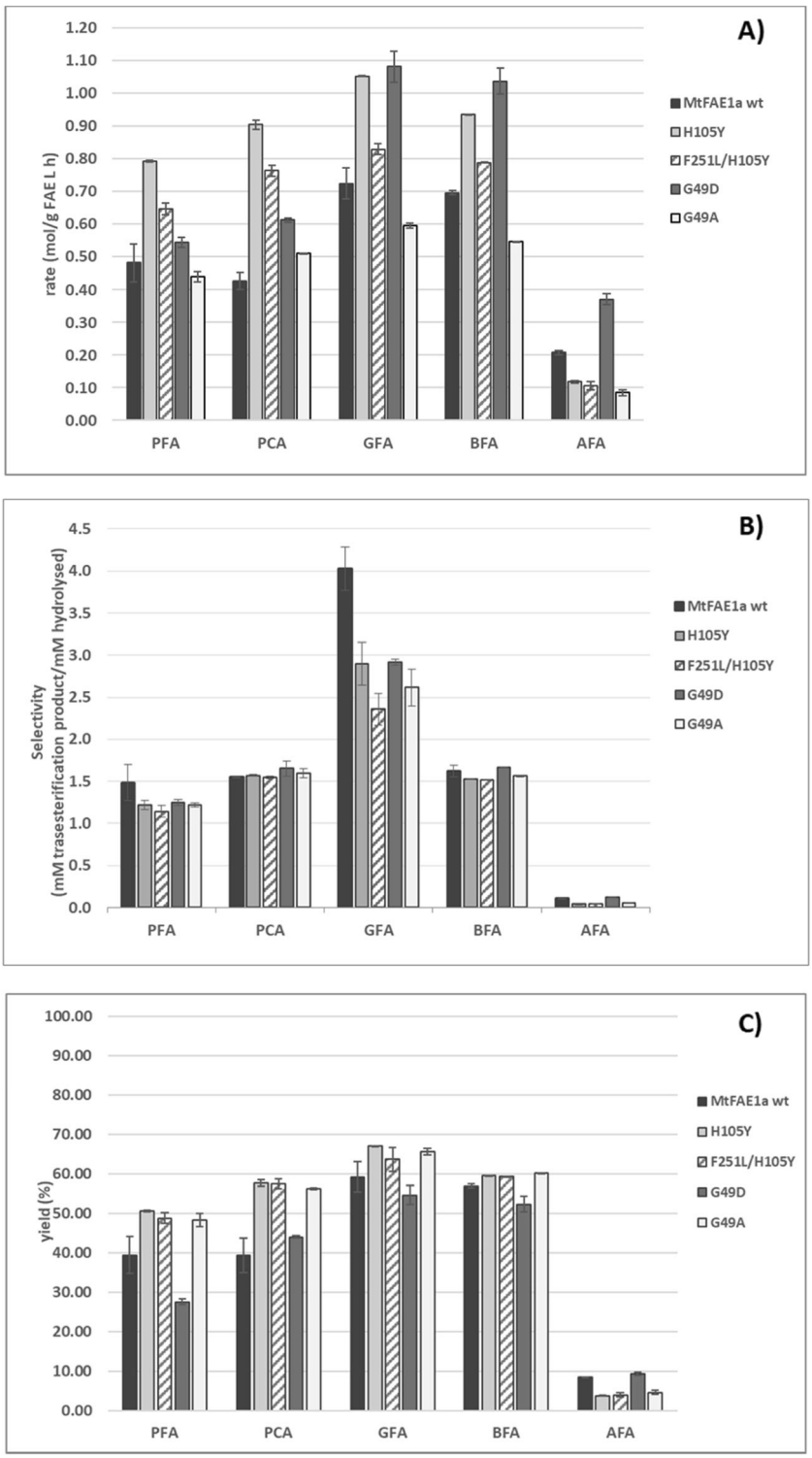

Fig. 5 Synthetic abilities of the wild-type MtFae1a and its evolved variants. **a** Transesterification rate. **b** Enzyme selectivity. **c** Transesterification yield. *Black* wild-type MtFae1a, *light gray* H105Y, *striped* F251L/H105Y, *dark gray* G49D and *white* G49A

study, an ad hoc strategy of automated HTS was designed and developed to select MtFae1a variants with higher activity than the wild-type enzyme towards 4NTC-Fe, a new substrate developed ad hoc due to the lack of suitable compounds for high-throughput assays of FAEs. The absence of a HTS method based on the evaluation of the synthetic abilities of FAEs implied the use of a screening strategy based on the hydrolysis of the ester bond of 4NTC-Fe that leads to 4-nitrocatechol release correlated with a color change, which can be readily monitored. This compound enables the evaluation of FAE activity in both qualitative way in solid culture medium based and quantitative measurement in liquid medium (Gherbovet et al. 2016).

Applying the developed screening strategy to the library of 30,000 directed evolved variants of MtFae1a, the 30 most active evolved variants having at least twofold higher activity than the wild-type enzyme towards 4NTC-Fe in microscale were selected for further experiments of scale-up of the growth and analyses of FAE activity production towards 4NTC-Fe, thermotolerance at 55 °C for 1 h, and solvent tolerance in 25% acetone evaluating residual activity towards 4NTC-Fe, on crude supernatants. These analyses allowed the selection of the best four evolved variants, whose crude supernatants showed at least twofold higher activity, around 1.3-fold higher solvent resistance and at least twofold higher thermotolerance than wild-type MtFae1a.

Sequence analysis of the corresponding mutated *mtfae1a* cDNAs (Tables 1) showed that two out of the selected clones have different amino acid substitutions in the same position (G49D and G49A) while only one variant has a double mutation (F251L/H 105Y), one of which was shared with another variant (H105Y). In particular, G49D crude supernatant showed both the highest residual activity after heat and solvent exposure, fourfold and 1.5-fold increase, respectively, compared to wild-type MtFae1a.

The four selected evolved variants were further investigated for their substrate specificity towards MFA, MCA, MpCA, MSA, and *p*NP-Fe (Fig. 2) in comparison to that of MtFAE1a wild type. MtFae1a and its mutated variants showed activity towards all the tested substrates. Wild-type MtFae1a expressed in *S. cerevisiae* in this work followed an MpCA > MFA > MCA > MSA pattern, showing a slightly different behavior from MtFae1a expressed in *P. pastoris*, as reported by Topakas et al. (2012b). However, MtFae1a expressed in *S. cerevisiae* behavior is consistent with the classification of type B FAEs, which show a preference for the phenolic moiety of the substrate containing one or two hydroxyl substitutions (as found in *p*-coumaric and caffeic acids, respectively) and no activity towards MSA (Crepin et al. 2004). In particular, H105Y crude supernatant exhibited twofold increased activity towards MFA, M*p*CA, and *p*NP-Fe and a threefold increase towards MCA, together with F251L/H105Y. Moreover, G49A crude supernatant showed fourfold higher activity that wild-type MtFae1a crude supernatant towards MCA. SMD simulations using a collection of ligands on the predicted structure of wild-type MtFae1a and respective mutants confirmed that this enzyme has a broad specificity towards hydroxycinnamate esters. MD simulations for prediction of solvent resistance and thermal exposure supported the experimental findings indicating that thermal exposure had a greater impact on substrate binding and increasing catalytic distances.

According to the new phylogenetic classification of FAEs (Dilokpimol et al. 2016), MtFae1a belongs to subfamily SF6 together with NcFae1 and ClFaeB2, which showed activity towards MCA and MpCA but no activity towards MSA (Crepin et al. 2003; Kühnel et al. 2012). The same affinity pattern applies also to FoFae2 and CcFae2, while AsFaeD and MtFae1a showed activity towards all the methyl hydroxycinnamates (Dilokpimol et al. 2018). ChFae and FaeB (*Talaromyces funiculosus*) are also classified in SF6, but the latter exhibits strict substrate specificity with activity only towards MFA (Kroon et al. 2000; Yang et al. 2013).

Wild-type MtFae1a and the selected four best evolved variants showed also the ability to synthesize four target compounds (Fig. 5), namely prenyl ferulate (PFA), prenyl caffeate (PCA), glyceryl ferulate (GFA), *n*-butyl ferulate (BFA), and 5-*O*-feruloyl-L-arabinose (AFA) in a ternary system of *n*-hexane:*t*-butanol:buffer forming detergentless microemulsions. Generally, increased transesterification yields and selectivity were observed when alcohols were used as acceptors while L-arabinose resulted in lower yields (< 10%). However, hydrolytic activity as a side reaction was still detected.

The highest transesterification yield was achieved with H105Y crude supernatant (67%) in GFA synthesis, followed by G49A variant. Moreover, H105Y crude supernatant, together with G49D, showed the highest conversion rates in GFA synthesis (Fig. 5a, c). However, wild-type MtFae1a crude supernatant showed the highest selectivity in GFA synthesis, giving a GFA/FA ratio of 4.0 (Fig. 5b). Despite being the highest transesterification yield for GFA synthesis achieved by our evolved variants, these values are lower than those reported for FAE-PL from *Aspergillus niger* purified from the commercial preparation pectinase PL "Amano" (81%) when using a reaction mixture of glycerol:DMSO:buffer and ferulic acid as a donor (Tsuchiyama et al. 2006). The same enzyme was also applied to a mixture of glycerol and acetate buffer achieving the 77% of conversion yield of ferulic acid as free enzyme and 48% as immobilized onto Chitopearl resin (Matsuo et al. 2008). Nevertheless, optimized reaction conditions for PFA synthesis were developed using MtFae1a expressed in *P. pastoris* (Antonopoulou et al. 2017a), with a transesterification yield similar to that achieved by H105Y, F251L/H105Y, and G49A evolved variants (50.6, 48.8, and 48.3%, respectively) using fixed reaction conditions.

Conversion yields around 60% were achieved in BFA synthesis with G49A, H105Y, and F251L/H105Y crude supernatants. These variants achieved comparable yields in PCA synthesis with similar BFA/FA and PCA/CA ratios. Conversion yields achieved by MtFae1a evolved variants are higher than those reported for StFaeC when using MFA (20%) or ferulic acid (3.4%) as donors (Topakas et al. 2005; Vafiadi et al. 2005). The best transesterification rates in BFA synthesis were obtained using G49D and H105Y crude supernatants, while the best in PCA synthesis were achieved with H105Y and F251L/H105Y variants.

The lowest transesterification yields were achieved in the synthesis of AFA by all the tested enzymes, indicating a less preference of MtFae1a and its evolved variants for sugar acceptors in the reaction conditions applied. Moreover, the AFA/FA ratio was less than 1, showing that the hydrolytic activity

was much more prevalent that the synthetic one. Although showing higher yield than MtFae1a in this work, the same low selectivity for AFA synthesis was observed for wild-type MtFae1a expressed in *P. pastoris* also when optimized reaction conditions were applied (Antonopoulou et al. 2017b).

Overall, MtFae1a and its evolved mutants showed to be superior biocatalytic tools for synthesis of aliphatic hydroxycinnamates.

In conclusion, although the developed screening strategy was based on the selection of evolved variants with improved hydrolytic activity, it was possible to obtain variants with enhanced synthetic activities. In particular, H105Y crude supernatant exhibited twofold increased hydrolytic activity towards MFA, M*p*CA, and *p*NP-Fe and a threefold increased activity towards MCA. Moreover, this variant showed enhanced abilities in GFA, PCA, PFA, and BFA syntheses. Docking of hydroxycinnamates and bulkier esters such as *p*NP-Fe and 4NTC-Fe on a model of 3D structure of MtFae1a and mutants confirmed the preference of the enzymes towards bulkier substitutions and the broad substrate specificity of MtFae1a. Hence, the developed methodology allowed the selection of evolved variants with broader substrate specificity than the wild-type enzyme together with the potential to expand the range of antioxidant compounds through (trans)esterification reactions. The evolved variant novel biocatalysts could serve as the starting point for further optimization and improve cost-efficiency and environmental impact of bioconversions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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