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PII: S1871-6784(17)30679-9
DOI: https://doi.org/10.1016/j.nbt.2019.01.008
Reference: NBT 1159

To appear in:

Received date: 6 December 2017
Revised date: 9 January 2019
Accepted date: 23 January 2019

Please cite this article as: Cerullo G, Varriale S, Bozonnet S, Antonopoulou I, Christakopoulos P, Rova U, Gherbovet O, Fauré R, Piechot A, J*ü*tten P, Brás JLA, Fontes CMGA, Faraco V, Directed evolution of the type C feruloyl esterase from *Fusarium oxysporum* FoFaeC and molecular docking analysis of its improved variants, *New BIOTECHNOLOGY* (2019), https://doi.org/10.1016/j.nbt.2019.01.008

Directed evolution of the type C feruloyl esterase from *Fusarium oxysporum* FoFaeC and molecular docking analysis of its improved variants

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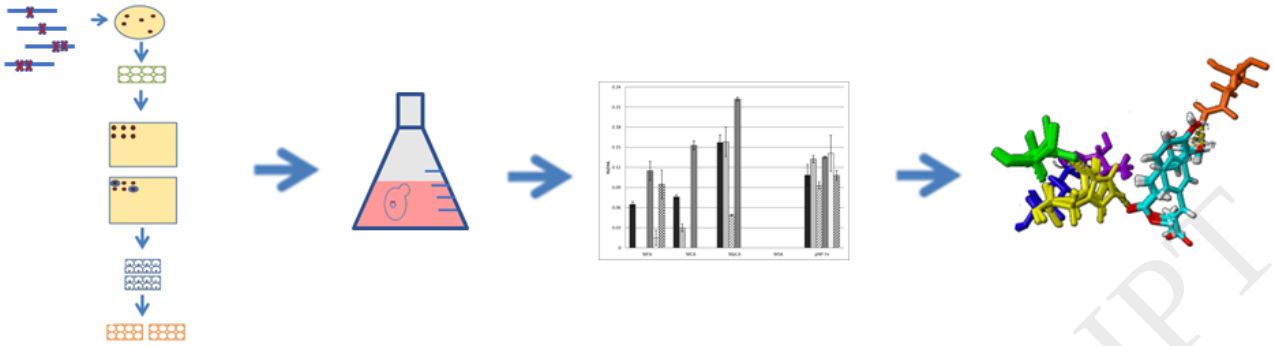
Graphical abstract



Highlights

- A library of around 30,000 random mutants of FoFaeC was generated
- Screening for enzymatic activity led to the selection of 96 improved variants
- The best five mutants were characterized for their substrate specificity
- Small molecule docking simulations were applied to the selected variants

Abstract

The need to develop competitive and eco-friendly processes in the cosmetic industry leads to the search for new enzymes with improved properties for industrial bioconversions in this sector. In the present study, a complete methodology to generate, express and screen diversity for the type C feruloyl esterase from *Fusarium oxysporium* FoFaeC was set up in a high-throughput fashion. A library of around 30,000 random mutants of FoFaeC was generated by error prone PCR of *fofaec* cDNA and expressed in *Yarrowia lipolytica.* Screening for enzymatic activity towards the substrates 5-bromo-4-chloroindol-3-yl and 4-nitrocatechol-1-yl ferulates allowed the selection of 96 enzyme variants endowed with improved enzymatic activity that were then characterized for thermo- and solvent- tolerance. The five best mutants in terms of higher activity, thermo- and

solvent- tolerance were selected for analysis of substrate specificity. Variant L432I was shown to be able to hydrolyze all the tested substrates, except methyl sinapate, with higher activity than wild type FoFaeC towards methyl *p*-coumarate, methyl ferulate and methyl caffeate. Moreover, the E455D variant was found to maintain completely its hydrolytic activity after two hour incubation at 55°C, whereas the L284Q/V405I variant showed both higher thermo- and solvent- tolerance than wild type FoFaeC. Small molecule docking simulations were applied to the five novel selected variants in order to examine the binding pattern of substrates used for enzyme characterization of wild type FoFaeC and the evolved variants.

Abbreviations: 4NTC-Fe 4-nitrocatechol-1-yl ferulate; ep-PCR error prone polymerase chain reaction; FAEs ferulic acid esterases; MCA methyl caffeate; MD molecular dynamics; MFA methyl ferulate; M*p*CA methyl *p*-coumarate; MSA methyl sinapate; *p*NP-Fe 4-nitrophenyl ferulate; RMSD root-mean-square deviation; SMD small molecule docking; X-Fe 5-bromo-4-chloroindol-3-yl ferulate.

Keywords: directed evolution, high-throughput screening, *Fusarium oxysporum*, library, feruloyl esterase

Introduction

The interest in feruloyl esterases (ferulic acid esterases, FAEs), also known as cinnamoyl esterases (E.C. 3.1.1.73), is growing due to the large number of potential biotechnological applications in several industrial sectors based on their ability to hydrolyze the ester bond between hydroxycinnamoyl motifs and sugars present in plant cell walls [1]. The enzymes release phenolic components, such as ferulic, *p*-coumaric, caffeic and sinapic acids [2], with widespread industrial potential due to their antioxidant and antimicrobial properties [3,4]. The modification of these compounds via esterification with aliphatic molecules (such as alcohols) can be used as a tool to alter solubility in oil-based formulas and emulsions, making them ideal candidates for application in

oil-based industries, maintaining the antioxidant activity of the starting acid. The reaction catalyzed by FAEs in the absence/low content of water represents a further route of application of these enzymes that is receiving increasing interest for its industrial potential [5]. As a consequence, the need to develop competitive and eco-friendlier bioconversions based on esterification reactions catalyzed by FAEs leads to a search for new enzymes with improved properties in industrial applications. Directed evolution, which mimics natural evolution, has proved to be a strategy suitable for improving or altering enzyme properties such as specificities, activity, stability and solubility by methods of genetic diversity integration [6]. However, it is noteworthy that this approach, of a time-consuming and cost-intensive nature, is based on procedures that use model substrates to detect target activities in order to provide more detailed qualitative data on enzyme properties. So far, the analysis of FAE activity has not been straightforward, mainly due to a lack of suitable compounds for practical high-throughput assays [7,8].

In a previous study, it was reported that the fungus *Fusarium oxysporum* showed multiple FAE enzymes enabling its ability to grow on varied materials such as wheat straw and corn cobs [9,10]. In particular, heterologous recombinant expression of type C FAE, belonging to the SF2 subfamily, was carried out in *Pichia pastoris* and the recombinant enzyme was purified and characterized using different substrates including methyl esters of hydroxycinnamates [11].

This study was aimed at developing evolved variants of FoFaeC with higher activity than the wild type enzyme and improved resistance to temperature and solvent exposure. The objectives were therefore to generate a library of mutants by error-prone polymerase chain reaction (ep-PCR) in *Yarrowia lipolytica* and apply this platform in conjunction with a high-throughput method to select the best variants. In addition, docking studies were employed to examine the affinity of the different substrates with the wild type and the selected evolved variants of FoFaeC.

Materials and methods

Chemicals

Yeast extract, bacto tryptone, bacto peptone and yeast nitrogen base (without amino acids and without ammonium sulphate) were purchased from Difco (Difco, Paris, France). QIAprep kit from Qiagen (Hilden, Germany) was used for plasmid extraction and PCR fragment purifications. Enzymes were purchased from Promega, Wisconsin, USA and methyl cinnamate substrates were provided by Apin Chemicals ltd, Oxford, UK. 5-Bromo-4-chloroindol-3-yl ferulate (X-Fe) was provided by LISBP (Université de Toulouse, CNRS, INRA, INSA, Toulouse, France). 4-nitrophenyl ferulate (*p*NP-Fe) and 4-nitrocatechol-1-yl ferulate (4NTC-Fe) [7] were provided by Taros Chemicals (Dortmund, Germany). Other chemicals were purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO).

Vectors, strains and culture media

The *Escherichia coli* strain Top 10 was used for transformations and manipulations of recombinant plasmids and its growth was performed at 37°C in Luria–Bertani (LB) medium (10 g/L bacto tryptone, 10 g/L NaCl, and 5 g/L yeast extract) supplemented with 100 µg/ mL of ampicillin or 40 µg/mL of kanamycin to select transformed clones.

The JMP62-TEF-ppLIP2-LIP2 expression vector [12] was used for the cloning of the *faeC* cDNA from *F. oxysporum* and its mutants in *Y. lipolytica* strain JMP1212. The Ura3 transformants obtained by yeast transformation, were selected on YNBG medium (1.7 g/L yeast nitrogen base, sterilized by filtration; 10 g/L glucose; 5 g/L NH_4Cl; 50 mM phosphates buffer pH 6.8 phosphates buffer Na/K (10%, v/v); 2 g/L casamino acids, sterilized by filtration) and grown in rich medium YPD (10 g/L bactopeptone; 10 g/L yeast extract; 10 g/L glucose) and YT2DH5 (10 g/L yeast extract; 20 g/L tryptone; 50 mM phosphates buffer pH 6.8 Na/K (20%, v/v) supplemented with 50 g/L glucose). For solid media, 20 g/L agar was added.

Recombinant vectors construction

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Cerullo, G., Varriale, S., Bozonnet, S., Antonopoulou, I., Christakopoulos, P., Rova, U., Gherbovet, O., Fauré, R., Piechot, A., Jütten, P., Brás, J. L., Fontes, C. M., Faraco, V. (2019). Directed evolution of the type C feruloyl esterase from Fusarium oxysporum FoFaeC and molecular docking analysis of its improved variants. New Biotechnology, 51, 14-20. , DOI : 10.1016/j.nbt.2019.01.008

Two types of cloning were performed using the cDNA sequence of *fofaec* cDNA, synthesized and optimized following *Y. lipolytica* codon usage (NZYTech, Lisbon, Portugal), in which three different restriction sites, BamHI, BsrGI and AvrII, were inserted. The cDNA was cloned either with its own optimized native signal sequence (indicated as FoFaeC + SP) or by fusing the sequence of the mature protein with preproLIP2 (indicated as FoFaeC – SP), a pro-peptide that has been shown in some cases to increase the level of extracellular recombinant protein [13].

The plasmid JMP62-TEF-ppLIP2-LIP2 and *fofaec* cDNA were digested by restriction enzymes BamHI/AvrII and BsrgI/AvrII (Promega, Wisconsin, USA). In order to prevent circularization and re-ligation of linearized DNA, dephosphorylation of the linearized vector ends was performed using Calf intestinal alkaline phosphatase (CIAP) (Promega, Wisconsin, USA).

The ligation of DNA fragments with cohesive ends was carried out overnight at 4°C in presence of T4 DNA ligase (Promega, Wisconsin, USA) and, after plasmid amplification in *E. coli*, linearization by NotI (Promega, Wisconsin, USA) was performed according to manufacturer's instruction.

Error-prone PCR strategy

The expression cassette dedicated to the library construction of mutants was obtained by overlapping PCR amplification as described by Bordes et al. [14]. PCR reactions were carried out in order to amplify DNA sequence of interest using MyCyclerTIM thermal cycler (Bio-Rad, North America). Primers used in PCR reactions are listed in **Table 1**.

Y. lipolytica preparation and transformation

Competent cells preparation and transformation of *Y. lipolytica* wild type was performed as previously described in [13].

Wild type FoFaeC purification

The crude cell-free extract was concentrated by ultrafiltration (Amicon chamber 8200, cut off 10kDa membrane Millipore, Billerica, MA) and purified by immobilized metal ion affinity chromatography (IMAC). The concentrated sample was loaded onto a HisTrap 1 mL column (GE Healthcare). The column was first washed with 20 mM sodium phosphate buffer (pH 7) containing 100 mM sodium chloride, 10 mM imidazole and then a linear salt gradient was applied, at a flow rate of 1mL/min, from 0 to 100% of elution buffer (20 mM sodium phosphate buffer pH 7 containing 100 mM NaCl and 500 mM imidazole). The active fractions were pooled and analyzed by means of SDS-PAGE.

Library screening

The screening strategy was set up in high-throughput version using an automated workstation including the robot colony picker QPIX 450 (Molecular Devices, LLC, CA, USA) and the robot BioMek NXP (Beckman Coulter, CA, USA). Using the screening strategy adapted from Bordes et al. [15], clones of *Y. lipolytica* obtained by transformation with ep-PCR product were grown on selective medium on Petri dishes at 28°C. Colonies obtained from different sets of transformation were transferred to 200 µL of liquid medium in 96-well plates using the robot colony picker QPIX 450. After growth at 28°C for 16 h, mutants were transferred on Q-Tray containing selective medium supplemented with chromogenic substrate. A first layer of solid medium without substrate was poured and after complete solidification, a second layer supplemented with 60 µg/mL of X-Fe from a stock solution of 60 mg/mL X-Fe in DMSO was deposited [16]. Plates were incubated for 4-12 h at 28°C and active clones were detected by the hydrolysis of the substrate into an insoluble blue precipitate forming a halo around colonies. In order to analyze the activity towards 4NTC-Fe as described in [7], the positive clones were selected and transferred from 96-well plates to 96 deep-well microplates for growth at 28°C for 16 h. Thereafter, 100 µL of preculture were transferred in 900 µL of liquid medium in 96 deep-well microplates using the robot BioMek NXP and the cultures

were incubated at 28°C for 48 h. After biomass removal by centrifugation, samples of culture supernatant were analysed for FAE activity production by 4NTC-Fe assay in microscale.

Characterization toward methyl cinnamates

Activity of wild type and mutated FoFaeC crude supernatants was assayed against the substrates methyl ferulate (MFA), methyl sinapate (MSA), methyl caffeate (MCA), and methyl *p*-coumarate (M*p*CA). 1.18 mM substrate stocks were prepared in 100 mM MOPS buffer pH 6.0. Esterase activity measurement was performed in 1 mL of reaction mixture (30 µL substrate stocks in 100 mM MOPS buffer pH 6 and complemented with 100 µL culture supernatant) at 37 °C, monitoring the consumption of substrate over 15 min by spectrophotometer (Beckman DU7500).

Analysis of variance (ANOVA) was performed on the results from the screening of selected optimized mutants and wild type FoFaeC to estimate the statistical parameters.

Thermo- and solvent tolerance conditions

The cultures were incubated at 28°C in 20 mL YT2DH5 for 48 h and, after biomass removal, the crude supernatant was analyzed for FAE activity against 4NTC-Fe. The crude broths were analyzed for enzyme thermo-tolerance at 55°C for 2 h and tolerance to 25% acetone (v/v) at 37°C for 30 min, measuring the residual activity towards 4NTC-Fe.

DNA extraction and sequencing

The genomic DNA was extracted following the protocol reported in [17]. Mutated *fofaec* genes were amplified by high-fidelity PCR and sequenced (Eurofins Genomics s.r.l.) using primers PCR2_d and PCR3_RT.

Molecular docking and molecular dynamics simulations

A docking study was designed to examine the affinity of different substrates for the wild type and selected evolved variants of FoFaeC. A hybrid structure of FoFaeC wild type was built by

homology modeling using YASARA Structure and based on the known structure of a feruloyl esterase from *Aspergillus oryzae* (AoFaeB) (3WMT_A [18]; query cover 94%, identities 49%, positives 66%, gaps 2%) as described in a previous study [28]. The acquired model passed quality tests. Small molecule docking (SMD), molecular dynamics (MD) simulations and protein visualization were performed using YASARA Structure. Mutations were added to the wild type hybrid model by swapping residues resulting in five mutant models: M278L/V313I, R308H, L432I, E455D and L284Q/V405I. Amino acids of the FoFaeC hybrid structure were numbered according to the sequence adopted for the generation of the model, with a difference of -53 amino acids compared to the sequences of the proteins secreted by *Y. lipolytica*. Heteroatoms were removed, the protein molecules 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. Following this, structures were energy minimized using AMBER14 force field. Prior to SMD, 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 structures were cleaned and their geometries optimized. SMD was carried out on wild type and mutants by Autodock VINA [19] performing 25 docking runs per simulation. Results were evaluated based on the resulting binding energy, the dissociation constant, the number of clusters, the number of genetic runs per cluster, the orientation of ligand per cluster and the distance of carbonyl carbon from the catalytic serine. More positive energies indicate stronger binding and negative energies mean no binding. 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% water, 0.9% NaCl water at defined temperature for specific simulation time. The system was energy minimized prior to simulation while receptor structures were used after the simulation for docking against 4NTC-Fe.

Results and Discussion

Development of recombinant expression system of FoFaeC

In order to develop improved variants of the feruloyl esterase from *F. oxysporium* FoFaeC by directed evolution, a recombinant expression system for this enzyme was developed using *Y. lipolytica*. Among the higher eukaryotic systems currently used for heterologous gene expression, *Y. lipolytica* generally provides higher yields of industrially relevant enzymes than *P. pastoris* and *Saccharomyces cerevisiae*, together with a high secretion ability, efficient tools for post-translational modification and appropriate glycosylation [12,20]. Moreover, *Y. lipolytica* represents a more appropriate platform for directed evolution than *P. pastoris* [11,15] which is characterized by low transformation efficiency and random gene integration in its genome [13]. In this study, *Y. lipolytica* was used in conjunction with the JMP62-TEF-ppLIP2-LIP2 expression vector that has been previously shown to be a correct platform for expressing and optimizing a lipase gene [12,14].

With the aim of optimizing the level of extracellular recombinant protein, the cDNA coding for the feruloyl esterase FoFaeC including the signal peptide was synthesized with the sequence optimized for the codon usage of the host and two different cloning strategies were performed to express the enzyme either with its own native secretion signal (JMP62/FoFaeC +PS) or as a fusion with LIP2 prepropeptide (ppLIP2), to direct the secretion of recombinant proteins (JMP62/FoFaeC -PS). Transformation efficiency of *Y. lipolytica* ($4x10^3$ CFU/µg) was compatible with the construction of large mutant libraries.

Validation of the recombinant platform

In order to check if clonal variability took place among the different *Y. lipolytica* transformed clones, ten mutants for each transforming vector were cultured in liquid medium and the time-course of FAE activity production was evaluated until the day 8 of growth. Since no clonal variability among different clones was revealed, transformants for each recombinant expression

system were chosen at random and subjected to further analysis of activity production. The two recombinant systems were compared analyzing FAE activity on solid growth medium containing the chromogenic substrate X-Fe. Based on these analyses, the best results were obtained for the construct containing the native secretion signal (FoFaeC + SP), since only the yeast transformed with FoFaeC + SP construct showed blue halo activity on solid medium. Therefore, the FoFaeC + SP was selected for further experiments of mutant library generation.

Construction and screening of FoFAEc mutant library

The system *Y. lipolytica*/JMP62/FoFaeC + SP was shown to be a suitable platform for expressing the *fofaec* cDNA and was therefore adopted for directed evolution. The variants of FoFaeC esterase were created using the strategy previously reported [14] applying a medium mutation frequency for ep-PCR (4.5 – 9 mutations/kb). At the end of the overlapping step of PCR providing for the construction of the expression cassette, the resulting mutated fragments were introduced into the genome of strain JMP1212 through the yeast homologous recombination system.

Around 30,000 mutants were prepared and analyzed by high-throughput screening (HTS) using an automated workstation. To identify active mutants, a primary screening on YNBG agar plates containing X-Fe was performed. Around 10% of the complete library, corresponding to 3,313 clones, proved to be positive on the solid culture medium with the chromogenic substrate and were selected for a secondary screening focused on the detection of FoFaeC variants with higher activity than the wild type enzyme in liquid culture medium. The use of *Y. lipolytica* as expression host allowed the screening of the evolved variants library for higher hydrolytic activity than the wild type enzyme. Previous reports have focused on the directed evolution of FAEs to improve their thermostability by expressing the enzymes in *P. pastoris* with the limitation of evaluating only the residual activity after heat exposure [21,22]. Moreover, the application of a primary screen on solid media using a chromogenic substrate allowed a reduced number of variants to be screened in liquid medium by selecting only the evolved variants which showed blue halos of hydrolytic activity.

Positive clones from the primary screen were grown in microscale for 48 h and culture supernatants were subjected to analysis of FAE activity production using of 4NTC-Fe as substrate in microscale.

The 96 most active evolved variants having at least two-fold higher activity than the wild type enzyme towards 4NTC-Fe were chosen for scale-up of growth in 20 mL of YT2DH5 medium. They were analyzed for FAE activity production towards 4NTC-Fe, their thermo-tolerance at 55°C for 2 h and solvent tolerance in 25% acetone (v/v). The five best mutants in terms of higher activity, thermo- and solvent tolerance were sequenced and the corresponding mutations and the activity values of crude supernatants of the selected clones and percentages of residual activity after solvent and heat exposure are reported in **Table 2**.

The choice of conditions for the evaluation of improved thermo and solvent stability was established for the possible application of these enzymes as biocatalysts in chemo-enzymatic synthesis of bioactive compounds for the cosmetics industry. These transesterification reactions have been performed using a ternary system in which a percentage of organic solvents is applied, hence the testing of the tolerance of the enzymes to acetone. Some examples of transesterification reactions have been reported by Antonopoulou et al. [23,24] in which a temperature range of between 40 and 60°C for several hours has been tested for the synthesis of bioactive compounds for the cosmetics industry.

Scale up of the growth of the evolved variants to 20 mL in flask resulted in lower FAE activity production. This is due to changes in growth conditions compared with microscale [21]. Table 2 reports the results in flask for the five best evolved variants showing at least a two-fold higher activity than wild type FoFaeC and improved thermo- and solvent tolerance. In particular, the variants R308H, L432I and M278L/V313I showed 2-fold, 3-fold and 4-fold increased activities compared to wild type FoFaeC. Moreover, variant E455D showed a 1.3–fold higher solvent tolerance and variant L284Q/V405I showed both a 1.3-fold higher thermo-tolerance and a 1.6–fold higher solvent tolerance than wild type FoFaeC.

Test of hydrolytic capabilities of selected optimized mutants and wild type FoFaeC

To investigate substrate specificities of the variants, the hydrolytic ability of the crude supernatants of selected mutants was analyzed by assaying their activities towards a synthetic substrate, *p*NP-Fe and methyl cinnamates, MFA, MCA, M*p*Ca and MSA, in comparison to the wild type (**Table 3**). Since the purpose of our study was to develop biocatalysts whose industrial application would be more competitive, avoiding purification steps, the comparison between evolved variants and wild type enzyme activity was performed using unpurified enzymes.

FoFaeC wild type followed a M*p*CA > MCA> MFA activity pattern but showed no activity for MSA. This result is consistent with findings by Moukouli et al. [11] for FoFaeC recombinantly expressed in *P. pastoris,* which was found not to have broad substrate specificity despite being a type C FAE according to the Crepin et al. [27] classification. According to the new phylogenetic classification of FAEs [28], FoFaeC falls into SF2, together with AwFaeG (*A. wentii*) and GlFae1 (*Gymnopus luxurians*). AwFaeG showed low activity towards MSA as did FoFaeC, but did not hydrolyse M*p*CA. On the other hand, GlFae1 showed the highest activity towards MSA and lower activity towards MFA, but no activitytowards M*p*CA and MCA. Similarly to the wild type FoFaeC, the crude supernatants [29] of the evolved variants showed activity with MFA, MCA and M*p*CA, but none of with MSA. The crude supernatant of variant L432I hydrolyzed all the tested substrates, except MSA, with higher activity than the supernatant of wild type FoFaeC towards M*p*CA, MFA and MCA (1.4-fold, 1.8-fold and 2-fold, respectively). The supernatant of variant L432I showed an improved activity of 1.5-fold towards M*p*CA compared to FoFaeC wild type. Moreover, the supernatant of variant E455D completely maintained its hydrolytic activity after 2 h incubation at 55°C, while that of variant L284Q/V405I showed both higher thermo- and solvent tolerance (1.3-fold and 1.6-fold, respectively) than wild type FoFaeC.

Interestingly, L432I is the only variant bearing a single mutation in the region of the substrate binding pocket, reflected in an improvement of activity towards *p*NP-Fe as well as all the

methylated cinnamic acids (except for MSA). Rational design experiments have been applied to FoFaeC in a previous work, in order to broaden its substrate specificity [28].

All the other substitutions of the FoFaeC evolved variants occurred on the surface of the enzyme, particularly in the area forming a lid (residues 191-350 as determined by Suzuki *et al.* [18]). It is also noteworthy that the R308H variant, having a single amino acid substitution, lost wild type FoFaeC ability to hydrolyze MCA and M*p*CA. The same applies to the E455D variant, which lost the original activity towards MFA and MCA. Variant L284Q/V405I with two substitutions maintained activity exclusively towards MFA.

Analysis of variance (ANOVA) was performed on the results from the screening of selected optimized mutants and wild type FoFaeC and their hydrolytic abilities. Resulting *p*-values were less than the α level set at 0.05, confirming the effect of the mutations on the performance of the FoFaeC mutants and wild type. ANOVA analysis results are summarized in Table S1 (supplementary material) reporting the parameters for levels of variability.

Docking of hydroxycinnamic acid esters on the hybrid structure of FoFaeC and mutants

The feruloyl esterase from *Aspergillus oryzae* AoFaeB [18] was chosen as template (3WMT_A from PDB) to identify the position of the putative catalytic triad and disulfide bonds of FoFaeC using YASARA software, since it displays a 49% identity with the FoFaeC sequence (assessed using BLASTp). The docking process was employed to understand the binding pattern of substrates used for enzyme characterization to wild type FoFaeC and its selected evolved variants. It was found that ligands dock onto the enzyme active sites (wild type and mutants) in the following order of preference in terms of mean/highest binding energy (determined with the correct orientation, where the hydroxycinnamoyl moiety is placed inside the binding cavity while the substitution extends outside the cavity): *p*NP-Fe, 4NTC-Fe (>7 kcal/mol) > methyl esters (5-5.8 kcal/mol) (Table S2 of supplementary material). Furthermore, it was determined that for all methyl substituted

ligands, three main clusters were formed in a descending order of binding energy: docking in the correct orientation (with substitution extending outside the binding cavity), in the reversed orientation (with substitution accommodated inside the binding cavity) and in a "displaced" reversed orientation (ligand stabilized outside the cavity with the ester substitution extending towards the cavity). The latter, allowed stabilization of ligand in the highest proximity to the catalytic serine (<2.8 Å) compared to other clusters, included the majority of genetic runs per simulation (6-8 runs out of 20) but resulted in lower binding energies (4-4.8 kcal/mol). In regard to the size of substitutions, MSA (containing two methoxy groups) was docked most often in the reversed orientation (with higher mean binding energy), while the two most favorable acquired orientations (reversed and correct) resulted in a non-catalytic distance between the catalytic serine (S219) and the ester carbonyl carbon (>5 Å) for MSA. This phenomenon could explain the absence of activity of FoFaeC towards MSA and aligns with previous findings [30]. On the other hand, bulky substituted esters such as *p*NP-Fe and 4NTC-Fe docked preferably in the reversed orientation, but all main clusters (with correct, reversed and displaced orientations) resulted in high proximity to the catalytic serine, potentially explaining the increased activity towards these substrates. An additional indication for increased activity towards *p*NP-Fe and 4NTC-Fe is the stabilization of both the methoxy moiety by N483 and nitryl moiety by Q252. Although no systematic differences could be detected for binding of ligands to the selected mutants and wild type, it was shown that ligands M*p*CA, *p*NP-Fe and 4NTC-Fe had the smallest deviation in docking orientation (**Figure 1**) among wild type and mutants, supporting the increased activity observed against these substrates. The contact residues during docking of ligands on the predicted structures of wild type and mutants are presented in Table S3 (supplementary material). The methoxy groups of the benzoyl ring of hydroxycinnamates are stabilized by L251 and F248, respectively, when substrates are docked in the correct orientation.

MD simulations in 25% acetone (v/v) and at 55°C resulted in distorted structures with RMSDs up to 1.8 Å for acetone exposure and up to 2.3 Å for thermal exposure (Table S4, supplementary

material). This aligns with experimental findings where thermal exposure has a greater impact than acetone in residual activity. Furthermore, MD simulations imposed an increase in volume of the binding cavity that was higher after thermal exposure than solvent exposure (Table S5, supplementary material), allowing stabilization of 4NTC-Fe in the "correct" orientation (clusters with higher mean binding energy and/or genetic runs) for M278L/V313I, L432I and R308H. Binding of 4NTC-Fe remained in the preferred initial "reversed" orientation (allowing proximity with catalytic serine) for wild type, E455D and L284Q/V405I (Table S6, supplementary material). This could explain the increased stability of these enzymes to both heat (80 and 102% residual activity, respectively) and acetone (102 and 114% residual activity, respectively) (Figure 2). The contact residues during docking of 4NTC-Fe on the predicted structures of wild type and mutants after MD simulations are presented in Table S7 (supplementary material).

Conclusions

A library of around 30,000 evolved variants of FoFaeC was generated by epPCR of *fofaec* cDNA and recombinantly expressed in *Y. lipolytica.* Screening of the collection for extracellular enzymatic activity towards X-Fe and 4NTC-Fe led to the selection of five variants producing higher activity than wild type enzyme, of which two were subsequently found to exhibit improved thermo- and solvent tolerance. Crude supernatant of the L432I variant was shown to hydrolyze all the tested substrates, except MSA, with higher activity than wild type FoFaeC towards M*p*CA, MFA and MCA. SMD simulations on the five selected evolved variants revealed that M*p*CA and *p*NP-Fe had the smallest deviation in docking orientation among wild type and mutants, explaining the increased activity determined on these substrates. Moreover, the E455D variant crude supernatant was found to maintain completely its hydrolytic activity after two hours incubation at 55°C, while that of the L284Q/V405I variant showed both higher thermo- and solvent tolerance than wild type FoFaeC. These findings were confirmed by MD simulations in 25% acetone and at 55ºC, which showed that

binding of 4NTC-Fe remained in the preferred initial reversed orientation allowing proximity to the catalytic serine. In conclusion, generation and screening of 30,000 evolved variants library of FoFaeC allowed the selection of variants endowed with improved characteristics compared to the wild type enzyme. Properties of these enzymes could be exploited for industrial bioconversions.

Acknowledgements

This work was supported by the European Union, Grant agreement no: 613868 (OPTIBIOCAT).

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Figure and Table Legends

Figure 1. Docking of hydroxycinnamates onto the binding cavity of FoFaeC and evolved mutants. Numbering of residues does not include the signal peptide. (A) MFA, (B) MCA, (C) MpCA, (D) MSA, (E) pNP-Fe, (F) 4NTC-Fe. The selected cluster reflects highest binding energy. Purple: catalytic serine (S219), green: catalytic aspartate (Asp430), yellow: catalytic histidine (H470), Blue: cysteine (C218, C471, participating in formation of disulfide bridge), orange: glutamine, asparagine (aminoacids participating in the stabilization of substrates).

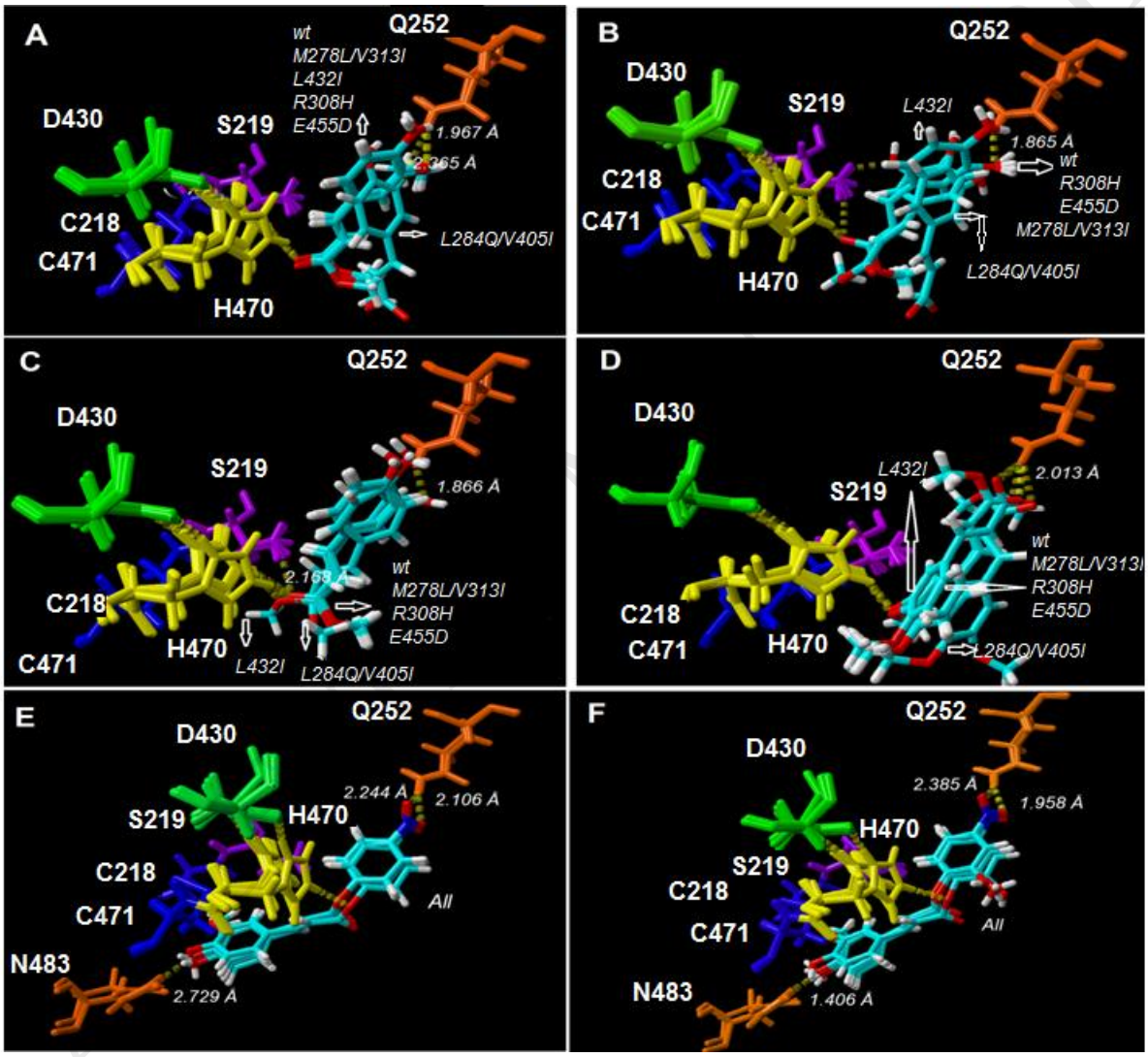

Figure 2. Position of mutations on the FoFaeC mutants. (A) wild type (no mutation); (B) M278L/V313I; (C) L432I; (D) R308H; (E) E455D; (F) L284Q/V405I with hydrophilic aminoacids removed in order to locate the mutations in the protein core. Blue: hydrophilic

aminoacids, red: hydrophobic aminoacids; aqua: hydrophilic mutation; pink: hydrophobic mutation; purple: catalytic serine (S219); green: catalytic aspartate (Asp430); yellow: catalytic histidine (H470).

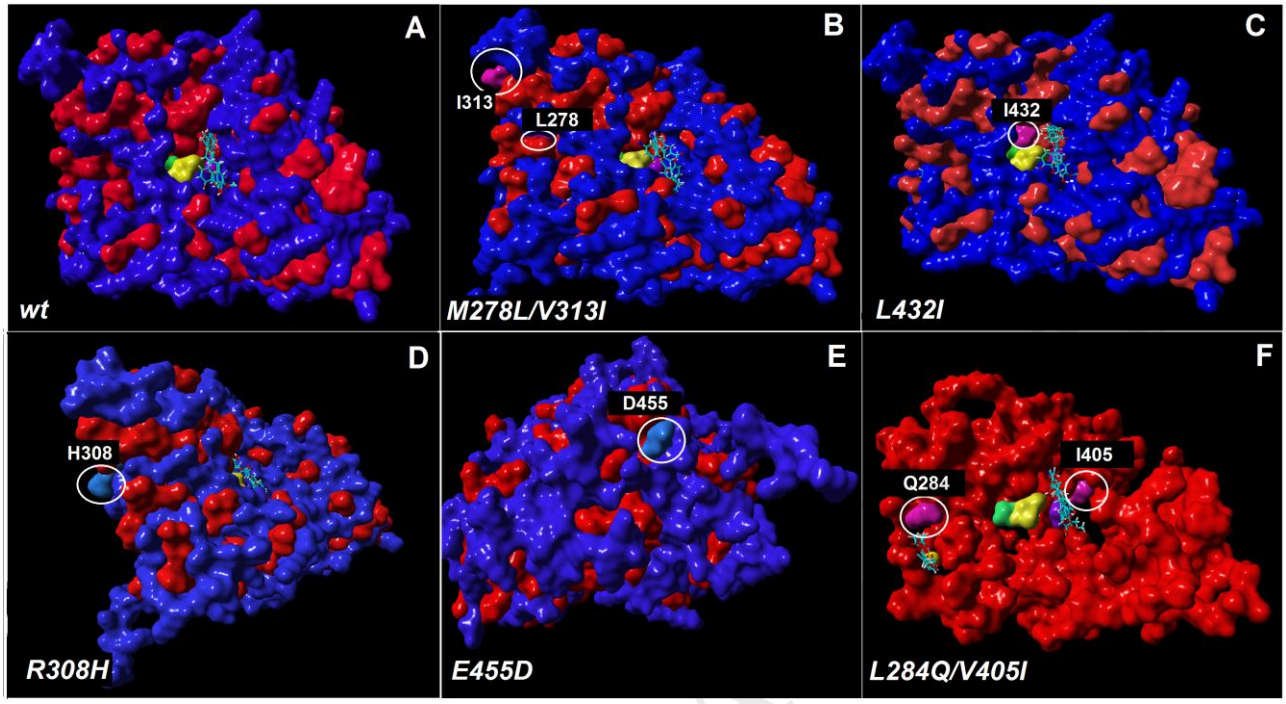

Table 1: Primers used into the PCR strategy.

Primer name	PCR reaction	Sequence
PCR1_d	upstream region/faithfull	GATCCCCACCGGAATTGC
PCR1_RT		GCACCTGGGGAATGAAGCCGAGC ACGAGCACG
PCR2_d	cDNA of interest + downstream Zeta/epPCR	CGTGCTCGTGCTCGGCTTCATTCC CCAGGTGC
PCR2_RT		GGAGTTCTTCGCCACCCC
PCR3_d	Fusion of PCR fragments PCR 1and 2	CCGCCTGTCGGGAACCGCGTTCAG GTGGAACAGGACCACC
PCR3_RT		CCGCACTGAAGGGCTTTGTGAGAG AGGTAACGCCG

Table 2: Activity values of crude supernatants of FoFaeC selected clones and percentages of residual activity after solvent and heat exposure.

	Activity (mIU/OD)(100mL-Flask)	Thermo-tolerance (%) (2 h at 55°C)	Solvent resistance (%)(30min in 25% acetone, v/v)
FoFaeC wt	2.28 ± 0.35	80±8	70±18
M278L/V313I	9.02 ± 1.20	42±11	55±12
R308H	4.67 ± 0.32	nd[a]	63±17
L432I	6.63 ± 0.59	45±12	54±9
E455D	1.66 ± 0.24	69±25	94±13
L284Q/V405I	2.74 ± 0.10	102±15	114±13

[a]nd, not detected. (The reported data are the average of three experiments, each one carried out in duplicate).

Table 3: Activity values of crude supernatants of selected FoFaeC evolved variants towards methyl cinnamates and *p*NP-Fe.

	Hydrolytic activity (mIU/mL)				
	MFA	**MCA**	**MpCA**	**MSA**	**pNP-Fe**
FoFaeC wt	65 ± 4.3	76 ± 3.1	157.1 ± 11.5	na	105.7 ± 9.4
M278L/V313I	na	31.8 ± 2.9	153.2 ± 13.8	na	132.5 ± 5.4
R308H	18.5 ± 1.9	na	na	na	142.8 ± 14.8
L432I	113.5 ± 11.8	153 ± 6.5	222 ± 1.5	na	135.3 ± 1.4
E455D	na	na	49.02 ± 0.7	na	93.2 ± 4.9
L284Q/V405I	97.3 ± 8.9	na	na	na	108.2 ± 7.3

na, no activity detected