Use of Feruloyl Esterases for Chemoenzymatic Synthesis of Bioactive Compounds

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Biochemical Process Engineering
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Feruloyl esterases (FAEs, EC 3.1.1.73) represent a subclass of carboxylic acid esterases that under normal conditions catalyze the hydrolysis of the ester bond between hydroxycinnamic acids (ferulic acid, sinapic acid, caffeic acid, p-coumaric acid) and arabinose residues in plant cell walls. Based on their specificity towards monoferulates and diferulates, substitutions on the phenolic ring and on their amino acid sequence identity, they have been classified into four types (A-D). The use of FAEs as accessory enzymes for the degradation of lignocellulosic biomass and their synergism with other hemicellulases has been studied for application in many industries, such as the food, the biofuel and the paper pulp industry. In the recent years, the use of FAEs as biosynthetic tools has been underlined. Under low water content, these enzymes are able to catalyze the esterification of hydroxycinnamic acids or the transesterification of their esters resulting in compounds with modified lipophilicity, revealing a great potential for tailor-made modification of natural antioxidants for use in cosmetic, cosmeceutical and pharmaceutical industries.

The first part of the thesis is focused on the use of substrate engineering techniques for the investigation of the basis of the type A classification of a well-studied FAE from Aspergillus niger (AnFaeA) by comparing its activity towards methyl and arabinose hydroxycinnamate esters. For this purpose, L-arabinose ferulate and caffeate were synthesized enzymatically. $k_{cat}/K_m$ ratios revealed that AnFaeA hydrolyzed arabinose ferulate 1600 times and arabinose caffeate 6.5 times more efficiently than methyl esters. This study demonstrated that short alkyl chain hydroxycinnamate esters which are used nowadays for FAE classification can lead to activity misclassification, while L-arabinose esters could potentially substitute synthetic esters in classification.

The second part of the thesis is focused on the use of medium engineering techniques for the optimization of the synthesis of two bioactive esters: prenyl ferulate and L-arabinose ferulate using 5 FAEs (FaeA1, FaeA2, FaeB1, FaeB2 and MtFae1a) from Myceliophthora thermophila in detergentless microemulsions. Reaction conditions were optimized investigating parameters such as the medium composition, the substrate concentration, the enzyme load, the pH, the temperature and the agitation. Regarding the synthesis of prenyl ferulate, FaeB2 offered the highest transesterification yield ($71.5\pm0.2\%$) after 24 h of incubation at 30°C using 60 mM vinyl ferulate, 1 M prenol and 0.02 mg FAE mL$^{-1}$ in a mixture comprising of 53.4: 43.4: 3.2 v/v/v n-hexane: t-butanol: 100 mM MOPS-NaOH pH 6.0. At these conditions, the competitive hydrolysis was 4.7-fold minimized. Regarding the synthesis of L-arabinose ferulate, FaeA1 offered highest transesterification yield ($35.9\pm2.9\%$) after 8 h of incubation at
50°C using 80 mM vinyl ferulate, 55 mM L-arabinose and 0.02 mg FAE mL⁻¹ in a mixture of 19.8: 74.7: 5.5 v/v/v n-hexane: t-butanol: 100 mM MOPS-NaOH pH 8.0. It was revealed that the type B FAEs from *M. thermophila* show higher preference to more lipophilic and smaller acceptors like prenol, while the type A FaeA1 was more efficient in the synthesis of the more hydrophilic and bulkier L-arabinose ferulate.
LIST OF ARTICLES

The thesis is based on the following articles:

**Paper I.** Cameron H, Antonopoulou I, Tanksale A, Rova U, Christakopoulos P, Haritos V Insights into substrate binding of ferulic acid esterases by arabinose and methyl hydroxycinnamate esters and molecular docking. Submitted to Biotechnology and Bioengineering


**Paper III.** Antonopoulou I, Papadopoulou A, Kletsas D, Ralli M, Rova U, Christakopoulos P Optimization of chemoenzymatic synthesis of L-arabinose ferulate catalyzed by feruloyl esterases from *Myceliophthora thermophila* in detergentless microemulsions. To be submitted to Carbohydrate Research
LIST OF ADDITIONAL ARTICLES

Articles:


Book chapter:

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1 INTRODUCTION

1.1 Ferulic acid: role in plant biomass and applications

Ferulic acid (FA) and other hydroxycinnamic acids (p-coumaric acid, pCA; sinapic acid, SA; caffeic acid; CA) have a widespread industrial potential due to their strong antioxidant activity. A variety of hydroxycinnamic acids are present in nature as part of plant cell walls with FA being the most ubiquitous in plant biomass (mainly in trans form) and to a lesser extent pCA. In graminaceous monocots such as maize, wheat and barley, FA is contained at a percentage up to 3% w/w being esterified to the O-5 hydroxyl group of α-L-arabinofuranose of glucuronoarabinoxylan while it is also esterified to the O-4 group of α-D-xylopyranose in xylloglucans in bamboo (Mueller-Harvey et al. 1986; Ishii et al. 1990; Kroon et al. 1999). Few dicots, such as sugar beet and spinach, contain FA up to 1% w/w esterified to pectin at the O-2 or O-5 hydroxyl group of α-L-arabinofuranose in arabinan and at the O-6 hydroxyl group of β-D-galactopyranose in (arabino-)galactan, both of which are neutral side chains of rhamnogalacturonan I (Colquhoun et al. 1994). Linkages between FA and arabinoxylan or lignin in monocots are presented in Figure 1.

FA can be oxidatively cross-linked forming intermolecular ester bonds to another arabinoxylan and ester or ether bonds with lignin (arabinoxylan-ferulate-lignin). Diferulates (diFA) have been mainly detected in the high-arabinose substitution region of arabinoxylan. There are six different detected structures of ferulate dehydrodimers isolated from plant cell walls (mainly 8,5′-, 5,5′-, 8,4′-, 8,8′- and less commonly 8,5′-(benzofuran)- and 8,8′-(aryl)- diFA)(Waldron et al. 1996)(Figure 2). Cross-linking of cell wall polysaccharides with lignin via hydroxycinnamic acids leads to a dramatic increase in mechanical strength of the plant cell wall, decelerates wall extension and acts as a barrier to block hydrolytic enzymes secreted by microorganisms. Fry et al. (2000) suggested that trimers or larger polymers contribute highly to cross-linking between polysaccharides in culture maize cells. The first FA dehydrotrimer was isolated from maize bran insoluble fibers (Bunzel et al. 2003) while more trimers and tetrarners have been identified (Rouaou et al. 2013; Bunzel et al. 2006; Funk et al. 2005; Hemery et al. 2009).

FA along with other hydroxycinnamic acids show a variety of bioactive properties owed to the presence of benzoic ring. Additionally to the antioxidant activity, FA has antibacterial, antitumor, anti-inflammatory, skin-whitening, UV-absorptive, anti-diabetic, anti-thrombosis properties and beneficial effects against Alzheimer’s disease.
constituting it attractive for many applications in the pharmaceutical, cosmetic and cosmeceutical industries (Huang et al. 1998; Graf 1992; Kanski et al. 2002; Ou and Kwok, 2004; Sultana et al. 2005; Vafiadi et al. 2008a; Barone et al. 2009). A limitation for the use of FA in such industries is the poor solubility to both aqueous and oil media. Therefore, a strategy should be implemented for modifying the hydrophilicity or lipophilicity of this compound with potential simultaneous enhancement of the aforementioned properties. For instance, the use of FA in solutions of other antioxidants and photo-protective agents that are readily destabilized by oxygen, such as vitamin E, stabilizes the preparations and doubles its skin photo-protection as the lipophilic vitamin E allows better penetration of FA into the stratum corneum (Lin et al. 2005). Vitamin E ferulate inhibited melanogenesis in human melanoma cells, being an attractive candidate as skin whitening agent (Xin et al. 2011).

Figure 1 FA linkages with arabinoxylan and lignin in monocots (adapted from Monlau et al. 2013)

Sodium ferulate, found in the root *Angelica sinensis*, is used in the traditional Chinese medicine for treatment of cardiovascular and cerebrovascular diseases and for preventing thrombosis while Kraft Foods had patented the use of salts of hydroxycinnamic acids, such as FA, CA and chlorogenic acid, to mask the aftertaste of the artificial sweetener acesulfame potassium (Wang and Ou-Yang, 2005; Riemer 1994). FA can be used as a precursor for the biotechnological production of the artificial flavoring agent vanillin. Apart from its use for flavoring, vanillin is also a fundamental constituent for the synthesis of pharmaceuticals and is used extensively in the perfume industry and as brightener in the metal plating industry. Moreover, its herbicidal activity is useful as ripening agent for the achievement of higher sucrose yields in sugar canes (Fazary and Ju, 2008). Finally, although in the food industry FA is mainly produced from rice oil as γ-oryzanol, modern processes are focusing on the enzymatic release of FA from plant
Feruloyl esterases (FAEs, EC 3.1.1.73) are a subclass of carbohydrate esterases belonging to the CE1 family of the carbohydrate-active enzyme database (CAZy). They are a set of enzymes that is considered to be a biotechnological key in the plant cell wall hydrolysis and in the extraction of phenolics. Under normal conditions, they catalyze the hydrolysis of the ester bond between hydroxycinnamic acids and carbohydrates in plant biomass. FAEs are highly dependent on xylanases, belonging either to the GH10 or GH11 family of CAZy, and their synergistic effect with each family leads to either the release of FA or diFAs, respectively. The synergistic action between xylanases and FAEs seems to render biomass more vulnerable to glycoside hydrolases, all being secreted from microorganisms in order to break down the recalcitrant structure of plant cell walls (Yu et al. 2003).

**Figure 2** Hydroxycinnamic acids and diferulates
FAEs appear to be very diverse enzymes, with little unifying sequence and physicochemical characteristics linking them. One of the leading classification systems is based on the ability of the FAE to catalyze the hydrolysis of model synthetic substrates, such as methyl or ethyl esters of hydroxycinnamic acids (methyl/ethyl ferulate, MFA/EFA; methyl caffeate, MCA; methyl-p-coumarate, MpCA and methyl sinapate, MSA). Initially, FAEs were categorized into two subclasses, type A or type B, depending on their activity towards MSA or MCA, respectively. This was based on the substrate specificity of the two major FAEs from *Aspergillus niger*, AnFaeA and AnFaeB (De Vries et al. 1997, 2002; Faulds and Williamson, 1994; Kroon et al. 1996, 1997). Later, the classification was expanded into four functional subclasses, named types A,B,C and D, based on substrate utilization data regarding catalytic activities towards model, short alkyl chain esters of hydroxycinnamic acids and supported by primary sequence identity (Crepin et al. 2004). According to the classification, Types A FAEs prefer substrates containing methoxy substitution at C-3 and/or C-5 as found in MFA and MSA and are active towards MpCA, but not MCA. They are also capable of releasing 5,5’ and 8,4’-diFAs. Type B FAEs prefer substrates containing one or two hydroxyl substitutions, as found in MpCA and MCA, respectively. Hydrolytic rates of type B FAEs are significantly reduced when a methoxy group is present and they are not active against MSA or diFA. Type C and D FAEs have a broader specificity with activity towards all four synthetic substrates, however only type D is active against diFAs. A summary of the ABCD classification is presented in Table 1.

The type A AnFaeA and type C AnFaeB have received most attention to date. The enzymes differ strongly in biochemical properties. AnFaeA is a small protein (36 kDa) while AnFaeB is a larger protein (74 kDa). The presence of methoxy groups on the aromatic ring of methyl esters of hydroxycinnamic acids increases the activity of AnFaeA while it decreases the one of AnFaeB. Although both enzymes act on xylan and pectin (De Vries et al. 2002), AnFaeA hydrolyzes mainly bonds between FA and the O-5 hydroxyl group of L-arabinose but not those linked to O-2 of L-arabinose in xylan while it acts on FA linked to D-galactose in pectin. On the contrary, AnFaeB acts only on L-arabinose-linked FA, both from xylan and from pectin independently of the type of linkages (Ralet et al., 1994). It is obvious that the current classification system is based on small synthetic molecules and might not reflect the mechanisms of the enzymes when catalyzing hydrolytic reactions in natural environment. A few reports suggest that microorganisms produce several types of FAE that differ in their affinity towards 5-O- and 2-O-feruloylated α-L-arabinofuranosyl residues (Ralet et al. 1994; Williamson et al. 1998; Topakas et al. 2003a, 2003b). Interestingly, although AnFaeB was eventually classified as a type C FAE, it has the specificity profile of a type B FAE. Only one member of the type C FAEs, TsFaeC from *Talaromyces stipitatus*, has broad
substrate specificity as it corresponds to the type C group. The rest of them show a specificity profile of type B FAEs with weak or no activity against MSA, including AnFaeB (Kroon & Williamson et al. 1996), AoFaeB from Aspergillus oryzae (Suzuki et al. 2014) and FoFaeC from Fusarium oxysporum (Moukouli et al. 2008).

**Table 1** Classification based on activities towards synthetic substrates (Crepin et al. 2004)

<table>
<thead>
<tr>
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<th>FA</th>
<th>SA</th>
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</table>

As more FAEs were characterized, the availability of fungal genome sequences made possible to obtain an overview of the prevalence of FAEs in the fungal kingdom and provide a better basis for classification combining amino acid sequence comparison and substrate specificity. Benoit et al. (2008) introduced a classification system containing seven subfamilies (SF1-7) of putative FAEs based on amino acid sequence homology and phylogenetic analysis, demonstrating that FAEs evolved from highly divergent esterase families: tannases (SF1-4), acetyl xylan esterases (SF6) and lipases (SF7) even though they all contain a conserved Ser-His-Asp catalytic triad. More specifically, SF1 contained type C FAEs from A. niger (AnFaeB) and A. oryzae (AoFaeB, AoFaeC) which were closely related to tannases. SF2-SF4 only contained putative FAEs with similarity to SF1 and tannases. SF5 included type B FAEs from Aspergillus nidulans (AN5267) and Neurospora crassa (NcFaeD) and some members of the CE1 subfamily, all being closely related to acetyl xylan esterases. Interestingly, although FAEs are carbohydrate-active enzymes, only some FAEs from SF5 and SF6 subfamilies belong to the CE1 family of CAZy together with acetyl xylan esterases. SF7 was restricted to members of the genus Aspergillus containing type A AnFaeA which were closely related to lipases but were distant from all other SFs. The characterized members of the different subfamilies had different biochemical properties, suggesting that they may in fact describe different classes of FAEs.

Adilokpimol et al. (2016) constructed a novel phylogenetic tree using 20 sequences from characterized FAEs expanding the classification into 13 subfamilies. The characterized FoFaeC from F. oxysporum was included to SF2 while SF7 was expanded to cover other fungi than Aspergillus. The new subfamily SF8 contained FAEs from Auricularia auricula-judae (EstBC), Anaeromyces mucronatus (Fae1a) and Orpinomyces sp. (OrpFaeA), while SF9 was separated from SF4 which previously contained a putative FAE from A. oryzae (BAE66413). Three tannases were placed in SF11, indicating that
this subfamily may actually include only tannase activity or potentially enzymes with dual-activity. SF12 included FAEs from *Pleurotus sapidus* (Est1) and *Pleurotus eryngii* (PeFaeA) for which there were no homologs found before.

According to Olivares-Hernández et al. (2010) the four types of FAEs have different evolutionary origin, described by phylogenetic analysis resulting in five clades (I-V). More specifically, clade I included type A FAEs such as the characterized FAEs from *A. niger*, *Aspergillus awamori* and *Aspergillus tubingensis* that belong to Eurotiomycetes, except for one FAE sequence from *Laccaria bicolor* belonging to Agaricomycetes class of Basidiomycetes. Clade II contained type B FAE sequences from both Sordariomycetes and Eurotiomycetes without any taxonomic class-specific signatures. Clade III and IV contained characterized type B and type C FAEs from four taxonomic classes, with its basis consisting of esterases from *Magnaporthe*, *Pyrenophora*, *Phaeosphaeria* and *Fusarium* without having common phylogenetic origin. These findings indicate that information obtained from substrate specificity and biochemical characterization is not reflected in the primary sequence. Clade V contained 24 sequences of which none had been biochemically characterized to date, consisting of a mix of taxonomic classes similar to that of clade II and IV.

A descriptor-based computational analysis with pharmacophore modeling provided different approaches for the classification of FAEs regarding their functionality with the suggestion of 12 FAE families (FEF1-12) comprising of different subgroups (Gupta Udatha et al. 2011). All type A FAEs were classified in subfamily FEF12A while the FAE from *A. nidulans*, *Penicillium chrysogenum* and *A. niger*, characterized as type B, were classified into FEF4A. Other type B sequences from *Penicillium funiculosum*, *N. crassa* and *A. oryzae* were classified into subfamilies FEF5B, FEF6A and FEF12B, respectively. Type C FAEs were classified together in FEF4B. Subfamilies FEF3 and FEF7 contained not characterized sequences dominated by gram-negative bacteria and fungi, respectively. All the other families accommodated a mixture of sequences of fungi, bacteria and plantae, which signifies that FAE-related sequences might co-evolved together from a common ancestor into different families during evolution of the respective kingdoms.

To date, only six structures of FAEs have been solved (Figure 3). The crystal structures include the type A AnFaeA from *A. niger* (Hermoso et al. 2004; related PDB entries: 1UWC, 1USW, 1UZA, 2BJA, 2IX9, 2HL6, 2IX9, 2HL6), a FAE (Est1E) from *Butyrivibrio proteoplasticus* (Goldstone et al. 2010; PDB ID: 2WTM, 2WTN), XynY and XynZ (Prates et al. 2011; Schubot et al. 2001; related PDB entries: 1JJF, 1GKK, 1GKL, 1J72, 1WB4, 1WB5, 1WB6) of the cellulosome complex from *Clostridium thermocellum*, a cinnamoyl esterase (LJ0536) from *Lactobacillus johnsonii* (Lai et al. 2011; PDB IDs: 3PF8, 3PF9, 3PFB, 3PFC, 3QM1, 3S2Z) and AoFaeB from *A. oryzae* (Suzuki et al. 2011).
2014; PDB ID: 3WMT) allowing the elucidation of the first structure of type C FAEs. The aforementioned FAEs have a common α/β hydrolase fold that is well known in literature for diverse enzymes that belong to the superfamily such as hydrolases, lipases, cutinases, acetylxylan esterases etc. In addition, FAEs consist of a lid domain that covers the active site and a catalytic domain containing a conserved catalytic triad (Ser–His–Asp) with the serine residue being located at the center of a universally conserved pentapeptide with the consensus “nucleophilic elbow” i.e. (GXSXG where X is any aminoacid residue) (Gupta Udatha et al. 2011).

**Figure 3** The 3D structures of the fungal FAEs prepared in PyMOL (Katsimpouras et al. 2016) (a) AnFaeA from *A. niger* (b) AoFaeB from *A. oryzae* and the bacterial FAEs (c) Est1E from *B. proteoplasticus* (d) LJ0536 *L. johnsonii*, domains (e) XynY and (f) XynZ from *C. thermocellum* cellulose complex. The catalytic α/β hydrolase domain (green) and the lid domain (purple) are shown.

Chrysatallographic and mutagenesis studies on AnFaeA allowed identifying the catalytic triad Ser133-His247-Asp194 that forms the catalytic machinery of this enzyme (Hermoso et al. 2004). The active-site cavity in AnFaeA is confined by a lid that covers the active site (residues 68-80, 13 aa) with a high ratio of polar residues, on the analogy of lipases, and by a loop (residues 226-244) that confers plasticity to the substrate-binding site. The lid has a unique N-glycolation site that stabilizes it in an open conformation, conferring the esterase character to the enzyme. Analogously, Est1E’s catalytic triad was found to be Ser105–His225–Asp197 while the lid was small (46 aa) with no structural homologies in the Protein Data Bank (PDB). This newly discovered lid forms a flexible β-sheet structure around a small hydrophobic core underpinning the continuing diverting of insertions that decorate the common α/β fold of hydrolases (Goldstone et al. 2010). The catalytic triad of AoFaeB comprises of Ser203–Asp417–His457 and the serine and histidine residues are directly connected by a disulfide bond.
of the neighboring cysteine residues, Cys202 and Cys458, while the lid was significantly larger than AnFaeA and Est1E (residues 231-390, 159 aa). LJ0536 had the two classical serine esterase motifs (GXSXG) and the catalytic triad was formed by Ser106-His225-Asp197 (Lai et al. 2011). Although its structure resembled Est1E, it was revealed that the binding pocket also contained an unoccupied area that could accommodate larger ligands while a prominent inserted α/β subdomain of 54 aa (P131-Q184) could contact to the aromatic acyl groups of substrates. FAE domains from C. thermocellum do not possess a lid domain (Prates et al. 2011; Schubot et al. 2001).

1.3 The feruloyl esterases from Myceliophthora thermophila

*Myceliophthora thermophila* (Thermothelomyces thermophila; previously known as *Sporotrichum thermophile*) is a thermophilic filamentous fungus that expresses a powerful consortium of enzymes able to break down lignocellulosic biomass. The growth rate and cell density of this microorganism appear to be similar in media containing cellulose and glucose constituting it an attractive candidate in utilization of plant biomass (Bhat and Maheswari, 1987). One of the first FAE activities reported from thermophilic fungi was produced from *M. thermophila* ATCC 34628 under solid-state fermentation (SSF) conditions. The esterase with molecular weight and isoelectric point of 27 kDa and 5, respectively, was isolated and partially characterized for its ability to release FA from destarched wheat bran, optimally active at pH 8 and 60°C (Topakas et al. 2003c). Two FAEs, StFae-A and StFae-C were purified to homogeneity from *M. thermophila* ATCC 34628. Both enzymes were homodimeric with a subunit of 33 kDa and 23 kDa, respectively, and with isoelectric point equal to 3.1. StFae-A was characterized as Type B, hydrolyzing MpCA, MCA and MFA, while was active on substrates containing FA linked to the O-5 and O-2 hydroxyl groups of arabinofuranose showing higher catalytic efficiency to the O-5 linkage (Topakas et al. 2004). Optimal activity was at pH 6.0 and 55-60°C. StFae-C had a broad specificity towards the methyl esters of hydroxycinnamic acids and showed preference on the O-5 linkage of FA with arabinofuranose (Topakas et al. 2005; Vafiadi et al. 2005).

The genome of *M. thermophila* ATCC 42464 was entirely sequenced and annotated in 2011, revealing that it encodes over 200 secreted carbohydrate-active enzymes (CAZy) and other enzymes of industrial interest (Berka et al. 2011). The genome of *M. thermophila* possesses six genes encoding enzymes belonging to the CE1 family of the CAZy database, four of which are FAEs (Hinz et al. 2009; Karnaouri et al. 2014). *M. thermophila* was developed into a mature protein production platform named C1. The main features of C1 include low-viscosity morphology and high production levels (up to 100 g/L protein) in fed-batch fermentations providing an alternative to traditional
fungal protein production hosts for cost-effective industrial applications (Visser et al. 2011). The FAEs, FaeA1, FaeA2 and FaeB2, identical to three genes of *M. thermophila* ATCC 42646, have been over-expressed in the C1 platform and characterized (Kühnel et al. 2012). Sharing the same sequence with FaeB2, MtFae1a from ATCC 42646 has been heterologously expressed in *Pichia pastoris* and characterized (Topakas et al. 2012).

The four sequences from *Myceliophthora thermophila* (FaeA1, FaeA2, FaeB1 and FaeB2/MtFae1a) were analyzed for their similarity against non-redundant protein sequences, sequences of known structure belonging to the PDB, against each other and in more detail against FAEs with known structure using the Basic Local Alignment Search Tool (pBLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Confirming the intriguing diversity of this set of enzymes, it was found that Type A FAEs FaeA1 and FaeA2 have only 38% identity with each other (89% query coverage) while Type B FaeB1 and FaeB2 are very similar (66% identity, 99% query coverage) (Table 2). All enzymes appear to be secreted and bring several N- and/or O- glycosylation sites (Table 3). Comparison with non-redundant protein sequences showed that all FAEs have extremely high similarity with FAEs from *Chaetomium globosum* belonging to the same family with *M. thermophila* (Chaetomiaceae) (Table 4). BLAST against PDB showed very low similarities with only a handful of esterases of known structure (Table 5). In more detail, FAEs were compared against the six FAEs: AnFaeA, AoFaeB, EstE1, LJ0536, XynY and XynZ. Although FaeA1 has very low similarity with AnFaeA (query coverage 2%), a detected heptapeptide with 71% identity, (LQLPNNY and LQLDTNY, respectively) lies within the lid domain of AnFaeA. On the other hand, a part of the same oligopeptide is found on AoFaeB, without belonging to the lid domain, on XynY and XynZ.

**Table 2** Sequence comparison of FAEs from *M. thermophila*

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<td>Subfamily</td>
<td>$T_{\text{opt}}$ (°C)</td>
<td>$pH_{\text{opt}}$</td>
<td>Observed MW (kDa)</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------</td>
<td>------</td>
<td>-----------</td>
<td>------------------------</td>
<td>-----------------</td>
<td>------------------</td>
</tr>
<tr>
<td>FaeA1</td>
<td>JF826027</td>
<td>A</td>
<td>SF5</td>
<td>45</td>
<td>6.5</td>
<td>29</td>
</tr>
<tr>
<td>FaeA2</td>
<td>JF826028</td>
<td>A</td>
<td>SF5</td>
<td>40</td>
<td>n.d.</td>
<td>36</td>
</tr>
<tr>
<td>FaeB2</td>
<td>JF826029</td>
<td>B</td>
<td>SF6</td>
<td>45</td>
<td>7.0</td>
<td>33</td>
</tr>
<tr>
<td>MtFae1a</td>
<td>AEO62008</td>
<td>B</td>
<td>SF6</td>
<td>50</td>
<td>n.d.</td>
<td>39</td>
</tr>
</tbody>
</table>

Table 3 Characteristics of FAEs from *M. thermophila*

- **Type**: A or B
- **Subfamily**: SF5 or SF6
- **$T_{\text{opt}}$ (°C)**: 45 or 40
- **$pH_{\text{opt}}$**: 6.5
- **Observed MW (kDa)**: 29 or 36
- **Observed pl**: ~5.5 or 5.2
- **Source**: C1

Theoretical MW, *pI*, and other observations are predicted using the ProtParam tool of ExPASY, SignalP v 4.0 server, NetNGlyc 1.0 server, and NetOGlyc 3.1 server. Domains are predicted using InterPro v6.2 tool.
Table 4 Sequence comparison of FAEs from *M. thermophila* with non-redundant protein sequences (first two hits reported)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Alignments</th>
<th>Genbank accession number</th>
<th>Query coverage (%)</th>
<th>Identities (%)</th>
<th>Positives (%)</th>
<th>Gaps (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FaeA1</td>
<td><em>Chaetomium globosum</em> CBS 148.51 gene</td>
<td>EAQ89456</td>
<td>96</td>
<td>84</td>
<td>91</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Podospora anserina</em> S mat+ gene</td>
<td>CAP62092</td>
<td>96</td>
<td>82</td>
<td>89</td>
<td>0</td>
</tr>
<tr>
<td>FaeA2</td>
<td><em>Chaetomium globosum</em> CBS 148.51 gene</td>
<td>EAQ85663</td>
<td>99</td>
<td>71</td>
<td>82</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>Madurella mycetomatis</em> putative feruloyl esterase C</td>
<td>KXX73257</td>
<td>92</td>
<td>64</td>
<td>77</td>
<td>2</td>
</tr>
<tr>
<td>FaeB1</td>
<td><em>Chaetomium globosum</em> CBS 148.51 gene</td>
<td>EAQ85662</td>
<td>99</td>
<td>87</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Madurella mycetomatis</em> putative feruloyl esterase C</td>
<td>KXX77840</td>
<td>100</td>
<td>100</td>
<td>78</td>
<td>76</td>
</tr>
<tr>
<td>FaeB2/MtFae1a</td>
<td><em>Chaetomium</em> sp. CQ31 feruloyl esterase</td>
<td>AFU88756</td>
<td>100</td>
<td>78</td>
<td>87</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Madurella mycetomatis</em> feruloyl esterase B</td>
<td>KXX77840</td>
<td>100</td>
<td>76</td>
<td>85</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5 Sequence comparison of FAEs from *M. thermophila* with proteins of known structure belonging to the PDB

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Alignments</th>
<th>PDB ID</th>
<th>Query coverage (%)</th>
<th>Identities (%)</th>
<th>Positives (%)</th>
<th>Gaps (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FaeA1</td>
<td>Chain A, putative dipeptidyl aminopeptidase from <em>Bacteroides</em> Ovatus ATCC 8483</td>
<td>4Q1V_A</td>
<td>20</td>
<td>29</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Chain A, Extracellular metalloproteinase from <em>Aspergillus</em></td>
<td>4K90_A</td>
<td>15</td>
<td>33</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>FaeA2</td>
<td>Chain A, C-terminal Esterase Domain Of Lc-est1</td>
<td>3WYD_A</td>
<td>58</td>
<td>22</td>
<td>38</td>
<td>9</td>
</tr>
<tr>
<td>FaeB1</td>
<td>Chain A, C-terminal Esterase Domain Of Lc-est1</td>
<td>3WYD_A</td>
<td>44</td>
<td>27</td>
<td>44</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Chain A, Orthohombic structure of the acetyl esterase Mekb</td>
<td>5E4Y_A</td>
<td>18</td>
<td>41</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>FaeB2/MtFae1a</td>
<td>Chain A, Phb depolymerase (S39a) complexed with R3hb trimer</td>
<td>2D81_A</td>
<td>13</td>
<td>41</td>
<td>56</td>
<td>2</td>
</tr>
</tbody>
</table>
1.4 Applications based on the hydrolytic activity

The industrial utilization of FAEs’ hydrolytic activity has been mainly focused on the synergistic release of saccharides as accessory enzymes in enzymatic hydrolysis for biofuel production. Additionally, the release of hydroxycinnamates has been researched for many industries either for opening access for oxidoreductases during biobleaching in paper pulp making, or for their utilization as antioxidants, flavor precursors or functional food additives in the pharmaceutical, food and feed industries. Allowing the \textit{in-situ} digestibility of hemicellulose in animal feeds, FAEs can contribute to the improved feed utilization, control of body weight gain and higher milk yields in cattle and sheep (Howard et al. 2003). In the food industry, FAE preparations are widely used in baking along with glucanases and oxidases in order to solubilize arabinoxylan fractions of the dough resulting in increased bread volume and improved quality (Butt et al. 2008). Other applications include the enzymatic clarification of juice and the release of FA from biomass or food byproducts for use as antioxidant. A FAE from \textit{Streptomyces avermitilis} CECT 339 released FA from sugar beet pulp soluble feruloylated oligosaccharides (Ferreira et al. 1999).

In paper pulp processing, environment-friendly approaches include the replacement of chemicals with enzymes, resulting in the reduction of water pollution and associated clean-up costs (Koseki et al. 2009). Xylanases and laccases are mainly used for enzymatic delignification and biobleaching, respectively, leading to the reduction or even elimination of chlorine-based-chemicals (Valls et al. 2010; Thakur et al. 2012). The use of FAEs along with other accessory enzymes might enhance this process by removing substitutions and linkages between polymers, resulting to the detachment of hemicellulose walls and the release of lignin fragments. A recombinant FAEA from \textit{A. niger} has been used in combination with xylanase and laccase activities for the delignification of wheat straw, eucalyptus kraft, wheat and oilseed flax straw pulps (Record et al. 2003; Valls et al. 2010; Tapin et al. 2006). In addition, FAEA has been shown to enhance delignification in flax pulp resulting in very low kappa number and high pulp brightness (Sigoillot et al. 2005). The commercial lipase A “Amano” with significant FAE activity offered the first evidence that accessory enzymes from a commercial preparation such as FAE and arabinofuranosidase can result to direct bleaching effect in kraft pulps (Nguyen et al. 2008). Other minor applications include the removal of fine particles from the pulp facilitating water removal and the enhancement of chemical and mechanical paper-pulping methods allowing the easier solubilization of lignin–carbohydrate complexes (Fazary and Ju, 2008). A prerequisite is that enzyme preparations should be free of cellulases, since cellulose degradation would result in reduction of paper quality.
1.5 Applications as biosynthetic tools

A disadvantage of natural antioxidants such as FA and other hydroxycinnamic acids is their poor solubility in both oil and aqueous media limiting their application in formulations intended for food, cosmetic, cosmeceutical and pharmaceutical products. A common way to alter solubility is by esterification or transesterification, with the latter requiring a prior activation of FA into an esterified derivative. Generally, modification with lipophilic acceptors such as fatty alcohols leads to more lipophilic derivatives, while the modification with glycerol or sugars leads to more hydrophilic products. Additionally to solubility, lipophilization has been shown to enhance the antioxidant activity of alkyl ferulate derivatives (Vafiadi et al. 2008a). Classic methods of esterification involve use of strong acids or expensive and toxic reagents as catalysts, high temperatures (150-250°C), long reaction times, low yields and tedious operations (Li et al. 2009). Process limitations include the heat sensitivity and oxidation susceptibility of FA, safety concerns for human health and the environment and high-energy consumption for purification, deodorization and bleaching due to low selectivity (Kiran and Divakar 2001). Furthermore, the demand for greener processes and the consumers’ preference for natural products requires the development of biotechnological sustainable and competitive processes for the production of interesting compounds with biological activities such antioxidants. Enzyme-catalyzed (trans) esterification is an attractive alternative due to mild operating conditions, use of greener solvents and high selectivity, however, the choice of biocatalyst is a key step for the process development.

There are numerous reports on the enzymatic acylation of saccharides and alcohols catalyzed by lipases and proteases in low water content media such as organic solvents and ionic liquids (Chang and Shaw, 2009; Khan and Rathod, 2015). Nevertheless, lipase-catalyzed esterification of phenolics was found to be limited by lower yields due to electronic and/or steric effects (Vafiadi et al. 2008a). On the other hand, FAEs might be less stable in non-conventional media and low water content than lipases but have higher specificity towards hydroxycinnamic acids (Zeuner et al. 2011). FAE-catalyzed synthesis has been studied mainly in detergentless microemulsions and less in organic solvents and ionic liquids. Detergentless microemulsions consist of a hydrocarbon, a polar alcohol and water representing thermodynamically stable and optically transparent dispersions of aqueous microdroplets in the hydrocarbon solvent. The droplets are stabilized by alcohol molecules adsorbed at their surface and possess spherical symmetry (Khmelnitsky et al. 1988). Detergentless microemulsions are an ideal candidate for synthetic reactions as they have low water content, the enzyme is protected from inactivation in the microdroplet while products can be recovered by shifting the physicochemical equilibrium of the microemulsions.
The first synthetic reaction catalyzed by FAE was carried out in a water-in-oil microemulsion system for the synthesis of 1-pentyl-ferulate using a FAE from *A. niger* (Giuliani et al. 2001). Since then, novel FAEs from filamentous fungi such as *F. oxysporum, M. thermophila* and *T. stipitatus* have been employed for the transesterification of methyl donors to alkyl esters. StFae-A from *S. thermophile* along with FoFae-I from *F. oxysporum* synthesized various 1-butyl hydroxycinnamates exhibiting highest yield on the pCA derivative (up to 70%). On the other hand, FoFae-II esterified p-hydroxyphenyl acetic acid and p-hydroxyl-phenylpropionic acid with propanol (70-75% yield) (Topakas et al. 2003 a,b; Topakas et al. 2004; ). Multienzymatic preparations containing FAE activity such as Ultraflo L and Depol 740L from *Humicola insolens* have shown high yields (up to 97%) in the transesterification of MFA to butyl ferulate when immobilized with Cross Linked Enzyme Aggregates (CLEAs) methodology (Vafiadi et al. 2008b). Depol 740L immobilized on mesoporous silica MPS-90 supported significantly higher yields up to 90% comparing to the free enzyme using 1-butanol as reaction medium (Thörn et al. 2011).

Among many natural photoprotective agents, feruloylated lipids have gained attention due to their strong anti-oxidant, skin-whitening, anti-wrinkling and UV absorptive abilities (Radzi et al. 2014). Enzymatic synthesis of green sunscreens can offer stability and selectivity in contrast with chemical synthesis. Although esterification with fatty alcohols generally results in more lipophilic products, the glyceryl esters of hydroxycinnamic acids have been proved more hydrophilic than their donors due to the three hydroxyl groups of the acceptor that are responsible for the general hydroscopic nature and water solubility of glycerol. Fed-batch esterification of FA with diglycerin was catalyzed by a FAE from *A. niger* under reduced pressure yielding 69% feruloyl and 21% diferuloyl glycerols (Kikugawa et al. 2012). The major product (FA-DG1) showed higher water solubility while all products maintained their radical scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and their UV absorption properties. Diferuloyl diglycerols showed a two-fold increase in antioxidant activity comparing to feruloyl diglycerols and FA. Esterification of SA and pCA with glycerol yielded 70% glycerol sinapate and 60% glycerol-p-coumarate, respectively, with indication of the formation of minor dicinnamoyl glyceryl esters (Tsuchiyama et al. 2007). The ability of glycerol sinapate to scavenge DPPH radicals was higher than butylated hydroxytoluene (BHT) while it maintained its UV absorptive properties. Ionic liquids have been employed for the synthesis of glyceryl derivatives using AnFaeA from *A. niger*, AndFaeC from *A. nidulans* and Ultraflo L in varying yields (Zeuner et al. 2011).
Regarding the synthesis of saccharide esters, the type C FAE from *S. thermophile* (StFae-C) has been used for the transesterification of short chain alkyl ferulates with L-arabinose, D-arabinose and L-arabinobiose reaching a maximum yield of 40%, 45% and 24%, respectively, after 4-5 days when MFA was used as donor (Vafiadi et al. 2005, 2006a, 2007a). StFae-C had a broad specificity on saccharides having either a pyranose or furanose ring while it synthesized successfully four linear feruloyl arabino-saccharides containing from three to six L-arabinose units showing regioselectivity for the primary hydroxyl group of the non-reducing arabinofuranose (Topakas et al. 2005; Vafiadi et al. 2007b). The type C FAE from *T. stipitatus* catalyzed the conversion of MFA to L-arabinose ferulate at 21.2% yield after 4 days (Vafiadi et al. 2006b). Direct esterification of FA and transesterification of EFA with monomer sugars was catalyzed by FAE-PL, an enzyme purified from the preparation Pectinase PL “Amano” from *A. niger* (Tsuchiyama et al. 2006). Various multienzymatic preparations containing FAE activity have catalyzed the direct esterification of FA with mono-, di- and oligosaccharides in detergentless microemulsions and ionic liquids with maximum yield in the synthesis of D-galactose ferulate (61%) followed by D-arabinose ferulate (36.7%) (Couto et al. 2010, 2011).

Feruloyl esters are considered potent antioxidants thus the vast majority of the antioxidant activity of feruloyl carbohydrates is assessed with the DPPH assay. According to Couto et al. (2010), D-arabinose ferulate had almost half of the scavenging activity of free FA while at steady state the scavenging yield was 70%. Additionally, D-arabinose ferulate was found to be a potential anti-mycobacterial agent with minimal inhibitory concentration (MIC) against *Mycobacterium bovis* BCG of 25 μg mL\(^{-1}\) (Vafiadi et al. 2006a). The scavenging activity of feruloylated arabinobiose was equal to the one of FA while the yield was 83.2% and for FA 92.1% at steady state (Couto et al. 2011). In the same study, the acylation of FA with hexoses (galactobiose, sucrose, lactose, raffinose and FOS) resulted in higher scavenging activity as compared with pentoses (arabinobiose, xylobiose and XOS). These results could be explained by the effect of steric hindrance of the glycosidic substituents on the rotation degree of the phenolic moiety. Examples of transesterification reactions catalyzed by FAEs are shown in Table 6.
Table 6 Feruloyl esterase-catalyzed synthetic reactions in non-conventional media (Antonopoulou et al. 2016)

<table>
<thead>
<tr>
<th>Product</th>
<th>Donor</th>
<th>Acceptor</th>
<th>Enzyme</th>
<th>Solvent system</th>
<th>Yield (Time)</th>
<th>T (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Pentyl ferulate</td>
<td>FA</td>
<td>1-Pentanol</td>
<td>FAEA</td>
<td>CTAB: hexane; pentanol; buffer</td>
<td>60% (n.q.)</td>
<td>40</td>
<td>Giuliani et al. 2001</td>
</tr>
<tr>
<td>1-Butyl ferulate</td>
<td>MFA</td>
<td>1-Butanol</td>
<td>CLEAs Ultraflo L</td>
<td>Hexane: 1-butanol; buffer</td>
<td>97% (144 h)</td>
<td>37</td>
<td>Vafiadi et al. 2008a</td>
</tr>
<tr>
<td>1-Butyl sinapate</td>
<td>MSA</td>
<td>1-Butanol</td>
<td>AnFaeA</td>
<td>Hexane: 1-butanol; buffer</td>
<td>78% (120 h)</td>
<td>35</td>
<td>Vafiadi et al. 2008b</td>
</tr>
<tr>
<td>2-Butyl sinapate</td>
<td>MSA</td>
<td>2-Butanol</td>
<td>AnFaeA</td>
<td>Hexane: 2-butanol; buffer</td>
<td>9% (120 h)</td>
<td>37</td>
<td>Vafiadi et al. 2008a</td>
</tr>
<tr>
<td>1-Butyl caffeate</td>
<td>MCA</td>
<td>1-Butanol</td>
<td>StFae-A</td>
<td>Hexane: 1-butanol; buffer</td>
<td>up to 25% (144 h)</td>
<td>35</td>
<td>Topakas et al. 2004</td>
</tr>
<tr>
<td>1-Butyl-p-coumarate</td>
<td>MpCA</td>
<td>1-Butanol</td>
<td>FoFae-I</td>
<td>Hexane: 1-butanol; buffer</td>
<td>up to 70% (144 h)</td>
<td>35</td>
<td>Topakas et al. 2003a</td>
</tr>
<tr>
<td>1-Propyl-p-HPA</td>
<td>pHPA</td>
<td>1-Propanol</td>
<td>FoFae-II</td>
<td>Hexane: 1-propanol; buffer</td>
<td>75% (224 h)</td>
<td>30</td>
<td>Topakas et al. 2003b</td>
</tr>
<tr>
<td>1-Propyl-p-HPPA</td>
<td>pHPPA</td>
<td>1-Propanol</td>
<td>FoFae-II</td>
<td>Hexane: 1-propanol; buffer</td>
<td>70% (224 h)</td>
<td>30</td>
<td>Topakas et al. 2003b</td>
</tr>
<tr>
<td>Glycerol sinapate</td>
<td>SA</td>
<td>Glycerol</td>
<td>AnFaeA</td>
<td>[C5OHmim][PF6]; buffer</td>
<td>76.7% (24 h)</td>
<td>50</td>
<td>Tsuchiyama et al. 2009</td>
</tr>
<tr>
<td>Glycerol ferulate</td>
<td>FA</td>
<td>Glycerol</td>
<td>FAE-PL</td>
<td>Glycerol: DMSO; buffer</td>
<td>81% (n.q.)</td>
<td>50</td>
<td>Tsuchiyama et al. 2006</td>
</tr>
<tr>
<td>Diglycerol ferulates</td>
<td>FA</td>
<td>Diglycerin</td>
<td>FAE-PL</td>
<td>Diglycerin S: DMSO; buffer</td>
<td>95% (12 h)</td>
<td>50</td>
<td>Kikugawa et al. 2012</td>
</tr>
<tr>
<td>Glycerol p-coumarate</td>
<td>pCA</td>
<td>Glycerol</td>
<td>FAE-PL</td>
<td>Glycerol: DMSO; buffer</td>
<td>~60% (72 h)</td>
<td>50</td>
<td>Tsuchiyama et al. 2007</td>
</tr>
<tr>
<td>L-Arabinose ferulate</td>
<td>MFA</td>
<td>L-Arabinose</td>
<td>StFae-C</td>
<td>Hexane: t-butanol; buffer</td>
<td>up to 50% (120 h)</td>
<td>35</td>
<td>Vafiadi et al. 2005</td>
</tr>
<tr>
<td>D-Arabinose ferulate</td>
<td>MFA</td>
<td>D-Arabinose</td>
<td>Multifect P3000</td>
<td>Hexane: t-butanol; buffer</td>
<td>~6.3% (n.q.)</td>
<td>35</td>
<td>Couto et al. 2007a</td>
</tr>
<tr>
<td>D-Galactose ferulate</td>
<td>FA</td>
<td>D-Galactose</td>
<td>Depol 670</td>
<td>Hexane: 1-butanol; buffer</td>
<td>36.7% (144 h)</td>
<td>35</td>
<td>Couto et al. 2010</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>D-Xylose ferulate FA</th>
<th>D-Xylose</th>
<th>Hexane: 2-butanone:buffer</th>
<th>37.3% (144 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feruloyl raffinose FA</td>
<td>Raffinose</td>
<td>Hexane: 2-butanone:buffer</td>
<td>11.9% (7 d)</td>
</tr>
<tr>
<td>Feruloyl galactobiose FA</td>
<td>Galactobiose</td>
<td>Hexane: 1,4-dioxane:buffer</td>
<td>26.8% (144 h)</td>
</tr>
<tr>
<td>Feruloyl xylobiose FA</td>
<td>Xylobiose</td>
<td>Hexane: 2-butanone:buffer</td>
<td>9.4% (144 h)</td>
</tr>
<tr>
<td>Feruloyl arabinodiose FA</td>
<td>Arabinodiose</td>
<td></td>
<td>7.9% (144 h)</td>
</tr>
<tr>
<td>Feruloyl sucrose FA</td>
<td>Sucrose</td>
<td></td>
<td>13.2% (n.q.)</td>
</tr>
<tr>
<td>Feruloyl FOS FA</td>
<td>FOS</td>
<td></td>
<td>9.6% (n.q.)</td>
</tr>
</tbody>
</table>

n.q.: not quantified; pHPA: p-hydroxyphenylacetic acid; pHPPA: p-hydroxyphenylpropionic acid; FAEA: FAE from *A. niger*; CLEAs: cross-linked enzyme aggregates; AnFaeA: type A FAE from *A. niger*; StFae-A/StFae-C: FAE from *S. thermophile* ATCC 34628; FoFae-I/FoFae-II: FAE from *F. oxysporum*; FAE-PL: FAE from *A. niger* purified from Pectinase PL “Amano”; Multi-enzymatic preparations: UltraFlo L/Depol 740L: from *H. insolens*, Multifect P3000: from *Bacillus amyloliquefaciens*, Depol 670: from *Trichoderma resei*
1.6 Aim of the thesis

The thesis is focused on:

- Use of substrate engineering for the investigation of the basis of the classification of a well-characterized type A FAE of known structure, AnFaeA from *A. niger*, using methyl and arabinose esters of FA and CA.

- Use of medium engineering for the characterization and comparison of 5 novel FAEs from *M. thermophila* regarding the synthesis of two bioactive compounds: prenyl ferulate and L-arabinose ferulate. In order to optimize the reaction conditions, parameters such as the water content, the substrate concentration, the enzyme concentration, the pH, the temperature and agitation were investigated. The substrate affinity and catalytic efficiency on transesterification was determined and compared providing an insight into the synthetic potential of these enzymes.
2 SUMMARY OF RESULTS

2.1 Revisiting the classification of feruloyl esterases

In paper I, the basis of type A classification of the well-studied FAE, AnFaeA from *A. niger*, was investigated by determination of its specificity towards methyl and arabinose FA and CA esters. Although methyl esters (MFA and MCA) are commercially available, the lack of availability of natural substrates for assaying FAE activity (such as L-arabinose ferulate, AFA and L-arabinose caffeate, ACA) limits the in depth investigation of the hydrolytic mechanisms of FAEs. In this work, using substrate engineering techniques, the arabinose esters AFA and ACA were synthesized enzymatically using MtFae1a from *M. thermophilus* ATCC 42464 in the transesterification of 50 mM MFA with 700 mM L-arabinose in 100 mM MOPS-NaOH pH 6.0: dimethyl sulfoxide (DMSO) 70:30 v/v. Proton NMR characterization revealed that the synthesis was selective at the O-5 position of L-arabinose. Subsequently, AnFaeA was characterized by determining the hydrolytic activity at fixed conditions (45°C, 10 min) and by determining the Michaelis-Menten kinetic constants (*K_m*, *k_cat*) by studying the effect of substrate concentration (0-2 mM) on the hydrolysis rate (45°C, 30 min).

AnFaeA was active towards all methyl and arabinose esters showing clear preference on the arabinose-substituted substrates (Table 7). In terms of hydroxycinnamic acid substitution, there was a 63-fold increase in the specific activity of AnFaeA towards AFA comparing to MFA and a 12-fold increase towards ACA comparing to MCA. In terms of hydroxycinnamate linkage, ferulate esters were better substrates than caffeates with the activity towards caffeate being almost undetectable (72-fold lower). This is in agreement with the current classification system and the main characteristic of Type A FAE specificity (Crepin et al. 2004). However, the activity of AnFaeA towards ACA was only 6-fold lower than MFA showing that the enzyme can also hydrolyze caffeate esters at reasonable rates, which is actually a characteristic of type B, C and D FAE specificity. In studies where MFA or MSA activity is usually assessed, MCA activity would not normally get detected; in this work at enzyme loads suitable for assaying MFA activity, satisfactory activity could also be measured for ACA whereas no activity would be detected for MCA.

Studies on the effect of the substrate concentration demonstrated significant differences in the hydrolytic rate towards esters with short aliphatic alcohols like methanol or bulky sugar substituents like L-arabinose, connected to the same hydroxycinnamic acid moiety. AnFaeA had higher apparent affinity for arabinose esters than methyl esters, as
the estimated $K_m$ is approximately 5-fold lower for AFA than MFA and 3 times lower for ACA than MCA (Table 8). The affinity towards the equivalent sugar-linked pair, AFA and ACA, was almost the same (0.31 and 0.33 mM, respectively). On the other hand, AnFaeA showed lower affinity towards MFA comparing to MCA (1.42 and 0.87 mM, respectively). The catalytic efficiency of the enzyme was approximately 1600 times higher for AFA than MFA, and 6.5 higher for ACA than MCA. The extremely high $k_{cat}/K_m$ ratio for AFA is attributed to the very fast reaction (high $k_{cat}$) and high substrate affinity (low $K_m$).

Table 7 Specific activity of AnFaeA towards the hydrolysis of methyl and L-arabinose esters of FA and CA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (U mg FAE$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFA</td>
<td>17.3 (1.7)</td>
</tr>
<tr>
<td>AFA</td>
<td>1084.0 (151.3)</td>
</tr>
<tr>
<td>MCA</td>
<td>0.24 (0.06)</td>
</tr>
<tr>
<td>ACA</td>
<td>2.83 (0.42)</td>
</tr>
</tbody>
</table>

The biochemical characterization of AnFaeA was supported by docking studies of the tested substrates on the AnFaeA structure. The undetectable activity of MCA can be attributed to non-catalytic orientations of MCA or MpCA in the hydrophobic pocket of the enzyme which appear to happen more often than the catalytic ones. Interestingly, the hydrophobic pocket that accommodates the methoxy side group of FA and SA was occupied by the methyl group of MpCA or MCA leading to the conclusion that hydroxycinnamates lacking the methoxy substituents may be represented in orientations where the methyl group is a proxy for the methoxy group. On the other hand, docking of arabinose esters revealed that non-catalytic orientations are not favorable as the larger hydrophilic residues are unable to dock into the hydrophobic pocket and in the reversed orientation the sugar group occupies a large part of the binding cavity.

Table 8 Kinetic constants of AnFaeA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{max}$ (µmol mg$^{-1}$ FAE min$^{-1}$ L$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (mg FAE$^{-1}$ min$^{-1}$)</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFA</td>
<td>96.4 (9.0)</td>
<td>1.42 (0.26)</td>
<td>7.9</td>
<td>5.6</td>
</tr>
<tr>
<td>AFA</td>
<td>1027.9 (46.6)</td>
<td>0.31 (0.06)</td>
<td>2825.4</td>
<td>9099.4</td>
</tr>
<tr>
<td>MCA</td>
<td>0.55 (0.05)</td>
<td>0.87 (0.17)</td>
<td>0.003</td>
<td>0.004</td>
</tr>
<tr>
<td>ACA</td>
<td>2.14 (0.16)</td>
<td>0.33 (0.07)</td>
<td>0.009</td>
<td>0.026</td>
</tr>
</tbody>
</table>
From the characterization of AnFaeA it is clear that the substitution of the phenolic moiety can significantly affect the catalytic activity of the enzyme. This observation is supported by previous studies where the FAE efficiency against methyl to butyl esters of FA varied (Vafiadi et al. 2005; Vafiadi et al. 2006a; Topakas et al. 2012). On the other hand, there are only few reports on the activity of FAE towards arabinose substituted substrates showing that FAEs prefer L-arabinose substitutions compared to small synthetic methanol (Topakas et al. 2003; Topakas et al. 2004). Although some attention has been drawn towards the effect of substitution, its influence when combined with a range of hydroxycinnamic acids has been overlooked. From our findings, it is evident that the use of short alkyl chain hydroxycinnamates can lead to activity misclassification due to their potential to preferentially bind non-catalytically with the enzyme. Additionally, this new catalytic characterization using sugar esters give us a more detailed knowledge of protein structure and function which may greatly expand the abilities of protein engineering to improve the catalytic properties of these enzymes. The preference of FAE for the O-5 or O-2 ester linkage of FA with L-arabinose is yet to be investigated. A few reports suggest that microorganisms produce several types of FAE that differ in their affinity for O-5 and O-2 feruloylated α-L-arabinofuranosyl residues (Ralet et al. 1994; Williamson et al. 1998; Topakas et al. 2003b, 2003c; Topakas et al. 2004; Topakas et al. 2005).

2.2 Synthesis of bioactive esters in detergentless microemulsions

Paper II and III are focused on the synthesis of prenyl ferulate (PFA) and L-arabinose ferulate (AFA), respectively, using four FAEs from *M. thermophila* C1 (FaeA1, FaeA2, FaeB1 and FaeB2) and MtFae1a from *M. thermophila* ATCC 42464. Transesterification of vinyl ferulate (VFA) with prenol and L-arabinose was carried out in a detergentless microemulsion system comprising of *n*-hexane: t-butanol: buffer. Hydrolysis of VFA was observed as side reaction due to the presence of water (Figure 4). Detergentless microemulsions are an attractive system for synthesis, with vast use in (trans) esterification reactions catalyzed FAEs. As FAEs are generally less stable under low water content and inactivate in the presence of organic solvents, detergentless microemulsions are an ideal candidate offering protection from inactivation. In this system the enzyme is enclosed in the aqueous micro-droplets dispersed in the organic phase. In the same time, it allows easy product recovery through phase separation.

Parameters were optimized in the following order: water content, donor concentration (VFA), acceptor concentration (prenol or L-arabinose), enzyme concentration, pH and temperature, implementing obtained conditions in subsequent experiments. The effect of agitation (1000 rpm) and other donors (MFA, FA) was investigated. The kinetic
constants \((K_m, k_{cat})\) were calculated by fitting the Michaelis-Menten equation on the acquired data via non-linear regression.

Figure 4 Transesterification of VFA with a) prenol and b) L-arabinose. Partial hydrolysis of AFA is observed after its synthesis. Hydrolysis of VFA is observed in both cases as side-reaction

2.2.1 Optimization of reaction conditions

During PFA synthesis, Type B FAEs (FaeB1, FaeB2 and MtFae1a) preferred a reaction system with lower water content (2.0% and 3.2%, respectively) than type A FAEs (5.5%). Increase in prenol concentration and enzyme concentration affected positively the selectivity, as more prenol molecules were available near the interface between the organic and water phase of the microemulsion, allowing more frequent transesterification instead of hydrolysis. Having more enzyme molecules available near the interface of the organic and aqueous phase could allow better contact of enzyme with prenol and VFA. FaeB2 showed highest transesterification rate, yield and selectivity in every optimization step while optimal enzyme concentration was minimal (0.02 mg FAE mL\(^{-1}\)). Overall, it was observed that in every optimization step, the selectivity (PFA/FA ratio) was quite low for type A FAEs, without meaning that they are not active overall since the side hydrolysis of VFA was observed to be robust. After optimization, selectivity was <1 for FaeA1 and FaeA2 (0.782 and 0.239), respectively, while they appear to be more thermophilic enzymes with optimal temperature of 55 and 45\(^\circ\)C. On the contrary, type B FAEs are more mesophilic. At optimal conditions (60 mM VFA, 1 M prenol, 0.02 mg FAE mL\(^{-1}\), 30\(^\circ\)C, 24 h, 53.4:43.4:3.2 v/v/v n-hexane: t-butanol: 100 mM MOPS-NaOH pH 6.0), FaeB2 showed highest yield
(71.2%), rate (0.089 mol g FAE L⁻¹ h⁻¹) and selectivity (2.373) while VFA was 100% converted to products (Table 9).

During AFA synthesis, all tested FAEs preferred a reaction system with high water content (5.5%) and higher concentrations of VFA comparing to PFA synthesis. The increase in L-arabinose concentration was strongly limited due to its insolubility in organic solvents and limited water solubility. Selectivity (AFA/FA ratio) was mainly affected by the type of enzyme and the water content, L-arabinose concentration, enzyme concentration and reaction time. Increased water content and arabinose concentration could allow the enzyme to have better access to L-arabinose and VFA in the interface of the microemulsion. All enzymes showed highest selectivity at approximately 2-fold lower enzyme loads compared to the yield. Selectivity decreased after 8 h of incubation as the produced AFA was further hydrolyzed to small extent (Figure 4). FaeA1 showed highest transesterification yield and selectivity in every optimization step while FaeB1 showed highest transesterification rate and required minimal amount of enzyme after optimization (0.005 mg FAE mL⁻¹). Optimal temperature was similar to the synthesis of PFA for each enzyme. Overall, the selectivity was lower compared to PFA synthesis and <1 for all tested FAEs, except for FaeA1. At optimal conditions (80 mM VFA, 55 mM L-arabinose, 0.02 mg FAE mL⁻¹, 50°C, 8 h, 19.8: 74.7: 5.5 v/v/v n-hexane: t-butanol: 100 mM MOPS-NaOH pH 8.0), FaeA1 showed highest yield (35.9%) and selectivity (1.120). At optimal conditions, (80 mM VFA, 55 mM L-arabinose, 0.005 mg FAE mL⁻¹, 45°C, 8 h, 19.8: 74.7: 5.5 v/v/v n-hexane: t-butanol: 100 mM MOPS-NaOH pH 6.0), FaeB1 showed highest rate (0.333 mol g FAE⁻¹ L⁻¹ h⁻¹) (Table 10).

Agitation did not affect the yield in the synthesis of PFA using FaeB2 at optimal conditions although it offered higher selectivity during the first 2 hours of incubation. On the contrary, the initial rate was decreased 3-fold when agitation was applied during synthesis of AFA using FaeA1 at optimal conditions, while at 24 hours the yield was decreased by 16% comparing to no agitation. When no agitation was applied, the selectivity reached an optimum at 8 hours (1.120) and then decreased by 50% up to 24 hours, while agitation offered increasing selectivity until 8 hours (1.150) which remained constant thereafter. Results on the use of different donors for transesterification (MFA, FA) confirmed that VFA is a highly reactive donor offering high rates and reduced incubation times. Studies on transesterification of MFA with various alkyl and sugar acceptors report incubation times of 4 to 5 days (Vafiadi et al. 2006; Vafiadi et al. 2008).

When transesterification is carried out in detergentless microemulsions, the enzyme is enclosed and protected in the aqueous microdroplets ensuring its stability and protection from inactivation. The lipophilic VFA is present in the organic phase
(comprising of n-hexane, t-butanol, and prenol in the case of PFA synthesis) while the microdroplets are stabilized by t-butanol. When prenol is used, the acceptor is mainly present in the organic phase as it is less polar than t-butanol. When L-arabinose is used, the acceptor is introduced in the microdroplet along with the enzyme due to its insolubility in solvents (Figure 5). Generally, the reaction takes place in the interface of the microdroplet when the enzyme gets in contact with VFA and prenol or L-arabinose. We propose that the synthesized PFA is transferred in the organic phase immediately after its production due to its increased lipophilicity, which subsequently protects it from hydrolysis. However, this is not the case when AFA is synthesized, as it has similar hydrophilicity with FA remaining (partially) solubilized in the microdroplet, where it gets further hydrolyzed in small extent by the enzyme.

Table 9 Optimal conditions and obtained parameters for PFA synthesis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>FaeA1</th>
<th>FaeA2</th>
<th>FaeB1</th>
<th>FaeB2</th>
<th>MtFae1a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimized conditions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water content (% v/v)</td>
<td>5.5</td>
<td>5.5</td>
<td>2.0</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>VFA concentration (mM)</td>
<td>80</td>
<td>50</td>
<td>50</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>Prenol concentration (M)</td>
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<td>1</td>
<td>0.8</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Enzyme concentration (g FAE L⁻¹)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>pH</td>
<td>8</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>55</td>
<td>45</td>
<td>40</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Time (h)</td>
<td>24</td>
<td>48</td>
<td>24</td>
<td>24</td>
<td>24</td>
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<tr>
<td>Obtained parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFA concentration (mM)</td>
<td>32.9 (2.4)</td>
<td>7.6 (0.3)</td>
<td>24.1 (0.2)</td>
<td>42.9 (0.1)</td>
<td>42.7 (2.0)</td>
</tr>
<tr>
<td>PFA yield (% mM PFA/mM VFA_initial)</td>
<td>41.1 (3.0)</td>
<td>15.2 (0.5)</td>
<td>48.1 (0.4)</td>
<td>71.5 (0.2)</td>
<td>42.7 (2.0)</td>
</tr>
<tr>
<td>Overall yield (% mM products/mM VFA_initial)</td>
<td>93.9 (5.4)</td>
<td>82.2 (0.5)</td>
<td>83.1 (3.0)</td>
<td>102.0 (4.7)</td>
<td>63.2 (1.6)</td>
</tr>
<tr>
<td>Rate (mol PFA g FAE⁻¹ L⁻¹ h⁻¹)</td>
<td>0.014 (0.001)</td>
<td>0.0016 (0.0001)</td>
<td>0.010 (0.000)</td>
<td>0.089 (0.000)</td>
<td>0.0089 (0.0004)</td>
</tr>
<tr>
<td>Initial rate (mol PFA g FAE⁻¹ L⁻¹ h⁻¹)</td>
<td>0.053 (0.003)</td>
<td>0.0057 (0.001)</td>
<td>0.030 (0.002)</td>
<td>0.182 (0.008)</td>
<td>0.222 (0.004)</td>
</tr>
<tr>
<td>Selectivity (mM PFA/mM FA)</td>
<td>0.778 (0.021)</td>
<td>0.227 (0.011)</td>
<td>1.378 (0.093)</td>
<td>2.373 (0.362)</td>
<td>1.700 (0.041)</td>
</tr>
</tbody>
</table>

Transesterification in detergentless microemulsions offers attractive characteristics to the synthetic process such as easy product separation and recovery, solvent recycle and reuse and enzyme recovery and reuse. Shifting the physicochemical equilibrium of the microemulsions and causing the formation of two separate phases, the lipophilic product PFA is encountered in the upper organic phase, while the hydrophilic byproduct FA and the enzyme are found in the lower aqueous phase. At optimal conditions using FaeB2, 100% of the lipophilic donor (VFA) is converted to products within 24 hours allowing the isolation of PFA and FA by phase separation. In the same time, the recovered enzyme maintained 83% of its specific activity. Similar experiment was conducted in AFA synthesis using FaeA1 at optimal conditions. At the end of the
reaction, the phase separation resulted in the distribution of the vast majority of the lipophilic unconverted VFA in the upper organic phase, while AFA and by-product FA were found in the lower phase together with the enzyme.

Table 10 Optimal conditions and obtained parameters for AFA synthesis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>FaeA1</th>
<th>FaeA2</th>
<th>FaeB1</th>
<th>FaeB2</th>
<th>MtFae1a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimized conditions</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water content (%v/v)</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>VFA concentration (mM)</td>
<td>80</td>
<td>200</td>
<td>80</td>
<td>80</td>
<td>150</td>
</tr>
<tr>
<td>L-arabinose concentration (mM)</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>50</td>
</tr>
<tr>
<td>Enzyme concentration (g FAE L⁻¹)</td>
<td>0.02</td>
<td>0.02</td>
<td>0.005</td>
<td>0.02</td>
<td>0.1</td>
</tr>
<tr>
<td>pH</td>
<td>8</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Temperature (°C)</td>
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<td>30</td>
</tr>
<tr>
<td>Time (h)</td>
<td>8</td>
<td>48</td>
<td>8</td>
<td>24</td>
<td>24</td>
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<td>Obtained parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFA concentration (mM)</td>
<td>25.2 (3.8)</td>
<td>9.0 (0.2)</td>
<td>13.3 (0.9)</td>
<td>7.8 (0.5)</td>
<td>8.7 (1.8)</td>
</tr>
<tr>
<td>AFA yield (% mM)</td>
<td>35.9 (2.9)</td>
<td>4.5 (0.1)</td>
<td>16.7 (1.1)</td>
<td>9.8 (0.7)</td>
<td>10.4 (2.2)</td>
</tr>
<tr>
<td>AFA/mM VFA_initial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall yield (% mM products/mM VFA_initial)</td>
<td>68.5 (1.8)</td>
<td>84.7 (1.6)</td>
<td>70.9 (5.7)</td>
<td>85.1 (8.8)</td>
<td>52.7 (3.1)</td>
</tr>
<tr>
<td>Rate (mol PFA g FAE⁻¹ L⁻¹ h⁻¹)</td>
<td>0.180 (0.015)</td>
<td>0.009 (0.0002)</td>
<td>0.333 (0.021)</td>
<td>0.016 (0.001)</td>
<td>0.004 (0.001)</td>
</tr>
<tr>
<td>Initial rate (mol PFA g FAE⁻¹ L⁻¹ h⁻¹)</td>
<td>0.417 (0.008)</td>
<td>0.077 (0.049)</td>
<td>0.602 (0.049)</td>
<td>0.080 (0.012)</td>
<td>0.013 (0.001)</td>
</tr>
<tr>
<td>Selectivity (mM AFA/mM FA)</td>
<td>1.120 (0.0000)</td>
<td>0.056 (0.007)</td>
<td>0.308 (0.007)</td>
<td>0.131 (0.005)</td>
<td>0.137 (0.026)</td>
</tr>
</tbody>
</table>

Figure 5 Microemulsion representation when a) prenol and b) L-arabinose is used as acceptor
2.2.2 Enzyme kinetics

The apparent kinetic constants ($K_m$, $k_{cat}$) for each substrate for the transesterification reaction were determined by fitting the Michaelis-Menten equation on experimental data using non-linear regression. In the synthesis of lipophilic PFA, FaeB1 showed highest affinity for VFA and prenol, while FaeB2 had similar affinity towards VFA with FaeB1 and lower affinity towards prenol than FaeB1 and MtFae1a. FaeB2 catalyzed fastest the reaction (highest $v_{max}$) and was most efficient catalyst for both substrates (highest $k_{cat}/K_m$). It was observed that type A and type B FAEs from M. thermophila C1 had affinity and catalytic efficiency of the same magnitude with FAEs of the same type for each product. For instance, FaeA1 and FaeA2 had low affinity (54.5 and 46.5 mM) for VFA and prenol (831.4 and 504.3 mM, respectively) while FaeB1 and FaeB2 had higher affinity for VFA (30.6 and 31.6 mM) and prenol (113.2 and 228.6). In analogy, FaeA1 and FaeA2 had low catalytic efficiency for VFA (6.783 and 3.358) and for prenol (1.752 and 0.735) while FaeB1 and FaeB2 had higher catalytic efficiency for VFA (2245.1 and 3956.7) and prenol (492.2 and 619.9). MtFae1a, produced in P. pastoris and glycosylated, had the lowest affinity for VFA but good affinity for prenol. However, it catalyzed the transesterification reaction at very low rates (Table 11).

In the case of more hydrophilic AFA, type A FAEs had lower affinity for VFA than FaeB1 and FaeB2 while interestingly they showed highest affinity for L-arabinose (19.6 and 27.8 mM). This comes in agreement with previous reports on substrate specificity profiling of hydrolysis describing that type A FAEs have preference in more bulky natural substrates (Kroon et al. 1997; Williamson et al. 1998). Generally, Type B FAEs from M. thermophila C1 have highest catalytic efficiency while FaeB1 had highest maximum rate ($v_{max}$). MtFae1a had good affinity for L-arabinose but low for VFA while it catalyzed the transesterification reaction at very low rates (Table 12).

The comparison of the kinetic constants of each enzyme with respect to the different acceptor (prenol or L-arabinose) reveals that in general type B FAEs are more efficient enzymes than Type A FAEs, with the exception of glycosylated MtFae1a. Type B FAEs from M. thermophila C1 have higher affinity towards hydrophobic VFA and prenol while type A FAEs towards hydrophilic L-arabinose. Interestingly, all FAEs show a manifold increase of turnover rates ($k_{cat}$) and catalytic efficiency ($k_{cat}/K_m$) when L-arabinose is present underlining the preference of natural substrates as acceptors in synthesis. It is obvious that VFA adopts a catalytic orientation with requisite proximity to the catalytic nucleophilic serine when L-arabinose is present in contrast with prenol. A possible explanation can be that the hydrophobic pocket that accommodates the methoxy side group of FA is occupied by prenol during PFA synthesis, resulting in non-catalytic orientation of the synthetic hydrophobic substrate (VFA).
Table 11 Kinetic constants of FAEs from *M. thermophila* - PFA synthesis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>VFA</th>
<th>Prenol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$v_{max}$ (mol g FAE⁻¹ L⁻¹ h⁻¹)</td>
<td>$K_a$ (mM)</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
<td>--------</td>
</tr>
<tr>
<td>FaeA1</td>
<td>0.015 (0.002)</td>
<td>54.5 (11.7)</td>
</tr>
<tr>
<td>FaeA2</td>
<td>0.0052 (0.001)</td>
<td>46.5 (18.1)</td>
</tr>
<tr>
<td>FaeB1</td>
<td>0.284 (0.020)</td>
<td>30.6 (4.8)</td>
</tr>
<tr>
<td>FaeB2</td>
<td>0.455 (0.054)</td>
<td>31.6 (9.6)</td>
</tr>
<tr>
<td>MtFae1a</td>
<td>0.033 (0.005)</td>
<td>81.2 (22.3)</td>
</tr>
</tbody>
</table>

Table 12 Kinetic constants of FAEs from *M. thermophila* - AFA synthesis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>VFA</th>
<th>L-arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$v_{max}$ (mol g FAE⁻¹ L⁻¹ h⁻¹)</td>
<td>$K_a$ (mM)</td>
</tr>
<tr>
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<tr>
<td>FaeA1</td>
<td>0.285 (0.085)</td>
<td>131.2 (59.6)</td>
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<tr>
<td>FaeA2</td>
<td>0.047 (0.006)</td>
<td>153.6 (39.3)</td>
</tr>
<tr>
<td>FaeB1</td>
<td>0.600 (0.079)</td>
<td>56.1 (15.4)</td>
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<tr>
<td>FaeB2</td>
<td>0.312 (0.058)</td>
<td>32.7 (17.6)</td>
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<tr>
<td>MtFae1a</td>
<td>0.041 (0.008)</td>
<td>148.7 (51.8)</td>
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This is in accordance with small molecular docking studies within the active site of AnFaeA showing that hydrophobic synthetic substrates formed alternative non-catalytic docking conformations that were energetically favored and stabilized by active site residues. By contrast, binding energy calculations suggested that arabinose ferulate was unable to adopt the alternative conformation and preferred the catalytic orientation (Paper I).
3 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

In this work, the current FAE classification system was investigated by biochemical characterization of a well-studied FAE of known structure, AnFaeA from *A. niger*, towards small synthetic and natural substrates. It was found that AnFaeA shows higher activity towards the arabinose esters of FA and CA while the affinity towards AFA and ACA is similar, revealing that synthetic substrate assays might not reflect the behavior of FAEs in the natural environment. Furthermore, the profiling of the synthetic abilities of four FAEs from *M. thermophila* C1, FaeA1, FaeA2, FaeB1, FaeB2 and MtFae1a from *M. thermophila* ATCC 42464 was determined using the small lipophilic prenol and bulky L-arabinose as acceptor. It was found that type B show preference to prenol while type A prefer bulky L-arabinose, which is in accordance with findings regarding the hydrolytic specificity of type A and type B FAEs.

Future work includes the evaluation of a variety of novel FAEs and the well-studied AnFaeA regarding their ability to synthesize a broad spectrum of feruloyl derivatives and further characterization by optimizing the reaction conditions. A comparison between the hydrolytic and synthetic behavior of AnFaeA with other FAEs might offer an insight into the correlation between synthetic and hydrolytic mechanisms with enzyme structure. Furthermore, the effect of organic solvents on the free and immobilized FAEs from *M. thermophila* will be investigated providing information on the effect of the solvent polarity, substrate and water solubility and the effect of immobilization on the stability and selectivity of enzymes.
First of all, I would like to thank my supervisor Prof. Paul Christakopoulos who gave me the opportunity to start working in the field of Industrial Biotechnology and conduct my doctorate studies in Sweden, for his guidance and support all these years. I would also like to thank my co-supervisors Prof. Ulrika Rova and Prof. Evangelos Topakas for the critical reviewing of my work and all members of the Biochemical Process Engineering group for the friendly work environment.

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5 REFERENCES


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Paper I
# Revisiting the classification of ferulic acid esterases via arabinose hydroxycinnamate ester hydrolysis and molecular docking

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Revisiting the classification of ferulic acid esterases via arabinose hydroxycinnamate ester hydrolysis and molecular docking

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Running title: revision of ferulate esterase classification

Conflicts of Interest: None to declare
Abstract

Ferulic acid esterases (FAE, EC 3.1.1.73) cleave the arabinose hydroxycinnamate linkages in plant hemicellulose and a diverse range of related ester substrates. The enzymes are commonly categorised as type A, B, C or D based on catalytic activities towards model, short alkyl chain esters of hydroxycinnamates but this system correlates poorly with sequence and structural features of the enzymes therefore limited in terms of its predictive utility. In this study, we investigated the basis of the type A categorisation of an FAE from Aspergillus niger, AnFaeA, by comparing its activity toward methyl and arabinose hydroxycinnamate esters. $k_{cat}/K_m$ ratios revealed that AnFaeA hydrolyzed arabinose ferulate 1600-fold, and arabinose caffeate 6.5 times more efficiently than their methyl ester counterparts. Furthermore, small molecule docking studies within the active site of AnFaeA showed that while all substrates adopted a catalytic orientation with requisite proximity to the catalytic serine, methyl caffeate and methyl $p$-coumarate preferentially formed alternative non-catalytic docking conformations that were energetically favoured and stabilised by active site residues. By contrast, binding energy calculations suggested arabinose ferulate was unable to adopt the alternative conformation and arabinose caffeate preferred the catalytic orientation. Thus, docking studies were in agreement with activity measurements with AnFaeA. This study demonstrates that short alkyl chain hydroxycinnamate ester substrates can lead to activity misclassification due to their potential to preferentially bind non-catalytically with the enzyme. The findings of this study will aid in developing a robust classification system for FAE and form the basis of new sequence-function relationships for this class.

**Keywords:** substrate binding; enzyme kinetics; enzyme classification; feruloyl esterase; docking simulation
**Abbreviations:** FAE – ferulic acid esterase; MFA/EFA – methyl/ethyl ferulate; MpCA - methyl p-coumarate; MCA - methyl caffeate; MSA - methyl sinapate; AFA – arabinose ferulate; ACA - arabinose p-coumarate; MCA – arabinose caffeate; ASA – arabinose sinapate; SMD - small molecule docking; ADT – AutoDockTools; *AnFae* – *Aspergillus niger* ferulic acid esterase; *MtFae* - *Myceliophthora thermophila* ferulic acid esterase.

**Introduction**

Ferulic acid esterases (FAE, EC 3.1.1.73) cleave the ester linkage between hydroxycinnamate and the C(O)5 position of an arabinose residue in hemicellulose, a plant-derived sugar polymer. This linkage is referred to as FAX (2-O-[5-O-trans-feruloyl]-β-L-arabinofuranosyl]-D-xylpyranose) and it is the primary covalent interaction between lignin and hemicellulose (Iiyama et al., 1994) which, of themselves, are two of the three main polymers made by plants. FAE have received much recent attention for their potential use as industrial enzymes; the substrate promiscuity of some FAE has been employed for the deconstruction of plant biomass for biofuels and animal feed applications (Dilokpimol et al., 2016; Hunt et al., 2016; Topakas et al., 2007) and for transesterification reactions utilising hydroxycinnamic esters (Antonopoulou et al., 2016).

As the degree of reactivity of different FAE towards hydroxycinnamic ester linkages in biomass has become important in itself and valuable for biotechnological applications, classification systems based on substrate specificity have been developed for the enzyme family. The most common hydroxycinnamic acids found in plant biomass are ferulic, p-coumaric, caffeic and sinapinic (Smith and Hartley, 1983) which vary in the number of hydroxy and/or methoxy substituents in the meta-positions of the aromatic ring (Figure 1). One of the leading classification systems for FAE is based on the ability of a candidate to
hydrolyze model substrates, \textit{viz} the methyl- or ethyl esters of hydroxycinnamic acids such as ferulate (MFA/EFA), methyl \textit{p}-coumarate (MpqCA), methyl caffeate (MCA) and methyl sinapate (MSA). Initially, FAE candidates were divided into either type A or B depending on their activity towards MSA or MCA respectively (Crepin et al., 2003), and this was based on the activities of two classical FAEs, \textit{An Fa}e\textit{A} and \textit{An Fa}e\textit{B}, derived from \textit{Aspergillus niger}. The type A/B classification was later extended to include type C and D FAEs (Crepin et al., 2004). In general, type A FAEs show increased activity against model phenolic substrates containing a methoxy substitution at carbon-3 or -5, such as MSA and MFA. Type B FAE prefer small polar or no substitutions at carbon-3 or -5 and thus have strong activity towards MCA and MpqCA. Type C and D are active against all of these model short chain hydroxycinnamic esters but differ in their ability to hydrolyze esters of diferulic acid. The FAE type A-D classification system is summarised in Figure 1.

Genome sequencing programs have generated a large diversity of putative FAE sequences from bacteria and fungi and it would be very valuable to be able to build amino acid sequence-activity relationships for this expanding class of enzymes. However, attempts to link the type A-D activity classification system, which is based on hydrolysis of short chain esters of hydroxycinnamic acids, and the amino acid sequences of biochemically characterized FAE have hitherto been unsuccessful, resulting in groupings that contain mixed FAE activity types (Crepin et al., 2004; Faulds et al., 2005; Udatha et al., 2011). This may be due, in part, to the diversity of the FAE enzyme class, with some putative members having close sequence similarity to lipase, acetyl xylan esterase, xylanase and chlorogenate esterase enzymes. Regardless, our ability to predict the activity of a novel FAE based on sequence similarity to a characterized FAE is limited.
While classification can take many forms including structural or sequence similarity, substrate specificity and activity are important factors and thus the selection of substrates as a basis for classification should be robust. As described above, current FAE classification is based on enzyme activity toward model substrates that have similar linkages to the enzyme’s natural substrates of sugar-hydroxycinnamates but differ in the alcohol component of the ester. In the natural substrate, the alcohol is a pentose monosaccharide such as arabinose, while the model esters used for activity-based classification have short chain alkyl groups, especially methyl. In terms of the synthesis of more diverse substrates to assist in the classification of FAE by activity, FAE themselves have been used to synthesize a variety of sugar esters or similar via transesterification, in non-conventional media such as organic solvents (Kanelli et al., 2014; Vafiadi et al., 2009; Vafiadi et al., 2007).

Molecular modelling tools such as small molecule docking (SMD) have also been used to investigate the activities of FAE toward short chain ester substrates. In particular, research by Suzuki et al. 2014 determined the crystal structure of the FAE from Aspergillus oryzae, AoFaeB, and used AutoDockTools (ADT) to simulate docking of the 4 short chain ester model substrates into the active site. The researchers were able to compare the active centre and docking to that of the related A. niger enzyme, AnFaeA, concluding that the residue TYR356 was most likely responsible for the severely reduced activity of AoFaeB toward MFA and MSA through steric hindrance to the larger methoxy group (Suzuki et al., 2014). Furthermore, Goldstone et al. used GOLD to predict the binding mode of ferulic acid and a feruloyl arabinose ester to a previously unknown FAE, Est1E, identified from a ruminant bacterium (Goldstone et al., 2010). Correct binding orientations for hydrolytic activity were modelled for feruloyl arabinose ester and extrapolated for short chain model esters; enzyme assays verified the enzyme’s activity toward ethyl ferulate and ferulic-containing
hemicellulose fractions. Thus with both molecular modelling tools and more authentic enzyme substrates available, alternative FAE classification systems can be explored.

Here we compare the catalytic activities of the type A FAE from \textit{A. niger}, AnFaeA, toward the model hydroxycinnamic acid substrates, methyl ferulate and -caffeate, and their arabinose ester counterparts. The arabinose ester substrates were synthesized by transesterification and were fully characterized. While an enzyme from type A such as \textit{AnFaeA} would be expected to demonstrate good activity toward ferulate but negligible activity toward caffeate esters, we found this was only true for methyl esters; both arabinose hydroxycinnamic acid esters were good substrates for the enzyme. The basis for the stark contrast in enzyme activities measured for the methyl and arabinose-based esters was explored further by SMD and this provided new insights into substrate binding to FAE. The findings of this study will aid in developing a robust classification system for FAE and form the basis of a structure-function relationship for this enzyme class.

**Materials and methods**

**Enzyme production and purification**

Recombinant FAE type A from \textit{A. niger} (AnFaeA) was expressed in \textit{Pichia pastoris} as described previously (Vafiadi et al., 2007). For the synthesis of arabinose esters, a type B FAE from \textit{Myceliopithora thermophila} ATCC 42464 (MtFae1a) was expressed in \textit{P. pastoris}, as described previously (Topakas et al., 2012). The enzymatic preparations were concentrated and exchanged for 100 mM MOPS-NaOH pH 6.0. Protein concentration was determined by the Pierce™ BCA Protein Assay (ThermoFisher Scientific, USA). The FAE purity (%) of the enzyme preparations was determined by SDS-PAGE using a Novex Sharp pre-stained protein standard (Life Technologies, USA), followed by quantification using the JustTLC software (Sweday, Sweden).
Hydroxycinnamic ester substrates for enzyme reactions

MFA and MCA (99%) were purchased from Alfa Aesar (Germany). Arabinose ferulate and -caffeate (AFA and ACA, respectively) were synthesized through enzymatic transesterification at preparative scale (10 mL) in sealed flasks containing 70:30 v/v 100 mM MOPS-NaOH pH 6.0: dimethyl sulfoxide (DMSO). 50 mM of the donor (104 mg MFA or 97 mg MCA) was diluted in DMSO while 700 mM acceptor (L-arabinose, 1.051 g) and 0.08 mg MtFac1a mL$^{-1}$ were introduced in the form of concentrated stock solutions in buffer. Reactions were carried out at 35°C for 24 h without agitation.

Isolation and characterization of arabinose hydroxycinnamate substrates

Isolation of the arabinose ester from each flask was performed by preparative HPLC on a C18 Luna 5μ column (250 mm x 21.2 mm) (Phenomenex) according to Vafiadi et al. (2007). The reaction mixture after evaporation under vacuum and mild heating (50°C) was diluted in methanol (1 mL). Fractions containing AFA (3.2%, 3.3 mg) or ACA (12.9%, 12.5 mg) were pooled and evaporated. Proton NMR spectroscopy was done in DMSO-$d_6$ on a Bruker Ascend Acon WB 400 spectrometer.

$^1$H NMR (DMSO-$d_6$, 400 MHz) 5-O-(trans-feruloyl)-L-arabinofuranose: δ 9.60 (s, 1H, ArOH), 7.57 (dd, 1H, $J_1=4$Hz, $J_2=16$ Hz, -CHCHOOR), 7.34–7.32 (m, 1H, ArH), 7.12 (dd, 1H, $J_1=1.6$Hz, $J_2=8.4$ Hz, ArH), 6.79 (d, 1H, $J_1=8$Hz, ArH), 6.49 (dd, 1H, $J_1=4$Hz, $J_2=16$ Hz, -CHCHOOR), 6.26–6.24 (m, 1H, >CHOH), 5.32–5.28 (m, 2H, >CHOH), 4.96 (dd, 1H, $J_1=2.8$Hz, $J_2=5.2$Hz, >OCHOH), 4.30–4.25 (m, 1H, >CH$_2$), 4.12–4.08 (m, 1H, >CH$_2$), 4.00 (dt, 1H, $J_1=2.8$Hz, $J_2=6.8$Hz, >CH$_2$CH), 3.82 (s, 3H, -OCH$_3$), 3.74–3.72 (m, 1H, >CHOH), 3.68–3.64 (m, 1H, >CHOH)
1H NMR (DMSO-d$_6$, 400 MHz) 5-O-(trans-cafeoyl)-L-arabinofuranose: $\delta$ 7.48 (dd, 1H, $J_1$ = 4Hz, $J_2$ = 16 Hz, -CHCHCOR), 7.04 (d, 1H, $J_3$ = 2Hz ArH), 7.01–6.98 (m, 1H, ArH), 6.74 (d, 1H, $J_4$ = 8Hz, ArH), 6.24 (dd, 1H, $J_5$ = 4Hz, $J_6$ = 15.6 Hz, -CHCHCOR), 4.95 (d, 1H, $J_7$ = 2.8Hz, >OCHOH), 4.32–4.29 (m, 1H, >CH$_2$), 4.11–4.06 (m, 1H, >CH$_2$), 4.00 (dt, 1H, $J_8$ = 2.8Hz, $J_9$ = 7.2Hz, >CH$_3$CH), 3.74–3.72 (m, 1H, >CHOH), 3.67–3.64 (m, 1H, >CHOH)

**Biochemical characterization of AnFaeA**

For the characterization of AnFaeA, stock solutions of substrate (MFA, AFA, MCA, ACA) (5-10 mM) were prepared in DMSO. Each reaction was initialized by introducing enzyme in the reaction mixture in the form of concentrated stock solution in buffer. The AnFaeA activity was assayed on 0.5 mM substrate in MOPS-NaOH 100 mM pH 6.0 using different enzyme loads. Samples were incubated for 10 min at 45°C without agitation. All reactions were terminated by incubation at 100°C for 10 min. One unit (1 U) is defined as the amount of enzyme releasing 1 μmol of free acid per minute under the defined conditions. For determining the effect of substrate concentration on the reaction rate, the action of AnFaeA was studied at varying concentration of substrate (0.2 mM) in MOPS-NaOH 100 mM pH 6.0. Samples were incubated for 30 min at 45°C without agitation. The kinetic constants (Vmax, Km) were determined by fitting the Michaelis-Menten equation to the data using nonlinear regression ($R^2$>0.9784, p<0.0001). All assays were carried out in duplicate and concomitant with appropriate blanks. There was no hydrolysis observed in the absence of enzyme.

**Analysis of metabolites of enzyme reactions**

Quantitative analysis of samples was performed by HPLC on a C18 Nucleosil 100-5 column (250 mm x 4.6 mm) (Macherey Nagel, Germany). Elution was conducted with a linear gradient method using water (solvent A) and acetonitrile (solvent B) at 0.6 mL min-1 and ambient temperature. Total running time was 20 min during which the following proportions
of solvent B were used: 0-15 min 28-72% and 28% 15-20 min. Detection was achieved by a PerkinElmer Flexar UV/VIS detector at 300 nm based on calibration curves prepared using standard solutions of the detected compounds in 1:1 water : acetonitrile.

**Small molecule docking of hydroxycinnamic ester substrates**

Docking simulations were conducted as per the earlier report of Suzuki et. al. (2014) and consistent for all substrates. Here the rigid macromolecule was Chain A of the crystal structure of *AnFaeA* (PDB: 1UZA). Substrates used were the methyl esters (MSA, MFA, MCA and MpCA), the ethyl ester EFA, and the arabinose 5-O esters (ASA, AFA, ACA and ApCA). All were generated using Avogadro (Hanwell et al., 2012) and structure optimised using UFF.

Briefly, PDB entry 1UZA was first prepared by relaxing side group atoms using the amber suite (Case et al., 2005), keeping backbone atoms stationary. The PDB file was also cleaned for non-standard residues and water molecules were removed. AutoDockTools (ADT) (Morris et al., 2009) was used to add polar hydrogen and to assign Gasteiger charges to all atoms within Chain A. A grid box of 80x80x80, centred on the catalytic serine was used as the search space with a grid spacing of 0.375 Å.

AutoDock (AD) was used for docking via a Lamarckian genetic algorithm with 20 random starting conformations of the ligand with the maximum number of torsions available. Results were visualised using ADT or Chimera (Pettersen et al., 2004).
Results & Discussion

AnFaeA shows higher apparent affinity for arabinose than methyl esters of ferulic and caffeic acid

AnFaeA exhibited activity toward all methyl and arabinose esters of ferulic and caffeic acid as shown in Table 1, however, there was a 63-fold increase in the specific activity of AnFaeA towards AFA compared to MFA, and a 12-fold improvement towards ACA compared to MCA. Thus, there is a clear preference shown by AnFaeA towards arabinose- compared with methyl esters. In terms of hydroxyccinnamate linkage, ferulate esters were better substrates than caffèate for AnFaeA which is in agreement with the current classification system (Crepin et al., 2004), that is, if restricting the discussion to methyl esters, the activity toward caffèate was almost undetectable (72-fold lower) which is the main characteristic of type A FAE specificity. By contrast, the activity of AnFaeA against ACA was only 6-fold lower than MFA activity showing the enzyme can also hydrolyze caffèate esters at reasonable rates, which is usually characteristic of type B, C and D FAEs.

The enzyme velocity versus substrate concentration curves for AnFaeA towards the methyl and arabinose esters of hydroxyccinnamic acids are shown in Figure 2 and demonstrate the stark differences in velocity of the enzyme reaction towards esters with a short alcohol or sugar substituent attached to the same acid group. The kinetic parameters (kcat and Km) were determined from the fit of the Michalis-Menten reaction to the data (Table 2); AnFaeA shows an apparent higher affinity for arabinose esters than methyl esters, as the estimated Km is ~5-fold lower for AFA than MFA, and ~3 times lower for ACA than MCA. In general, AnFaeA shows higher affinity towards MFA than MCA, as expected under the current classification system. However, the affinity towards the equivalent sugar-linked pair, AFA and ACA, is almost the same (0.31 and 0.33 mM, respectively) revealing an important difference in
AnFaeA affinity towards sugar-linked hydroxycinnamate esters to that of model substrates. The $k_{cat}/K_m$ ratio revealed that AnFaeA hydrolyzes AFA ~1600 times more efficiently than MFA, and ACA 6.5 times more than MCA. The extremely high $k_{cat}/K_m$ ratio for AFA is attributed to the very fast reaction (high $k_{cat}$) and the high substrate affinity (low $K_m$) for this substrate.

The characterization of AnFaeA shows that the enzyme has greater activity on substrates containing a more bulky sugar linkage, such as AFA and ACA, than the respective short alkyl chain ester. Generally, type A FAEs show highest activity against phenolic substrates containing a methoxy substitution at C-3 and C-5 such as MFA and MSA while no activity has been detected for MCA according to previous reports (Faulds et al., 2005). Nevertheless, in the present study a low level of activity was detected with MCA which is attributed to the high enzyme load used for the MCA assays, approximately 100 times higher than the one used for MFA. The comparative protein concentrations for ester hydrolysis are shown in Figure 4 for the 4 substrates. In studies with type A enzymes where MFA or MSA activity is being assessed, activity on MCA would not normally be detected. Accordingly, at enzyme loads suitable for MFA, satisfactory activity could be also measured with ACA whereas no activity was detected with MCA (Table 2).

From the characterization of AnFaeA on both MFA and AFA it is clear that the moiety attached via the ester linkage can have a significant effect on the catalytic activity of the FAE. This observation is supported by studies conducted by Vifialdi et al. (2006) and Topakas et al. (2012), who used various alkyl ferulates to characterize FAE. In those reports, FAE activity was detected against ferulic acid esters ranging from methyl to butyl. Interestingly the $k_{cat}/K_m$ of MtFae1a towards ethyl ferulate was less than half of the same measure for either methyl ferulate or n-propyl ferulate. Other studies have shown FAE with
activity towards esters of p-nitrophenyl or alpha-naphthyl of up to C8 in length (Koseki et al., 2005; Rashamuse et al., 2013). However, while some attention has been drawn towards the effect of the alcohol-side constituent of the ester group on FAE activity, its influence when combined with a range of hydroxycinnamic acids has been overlooked. Thus an explanation for the large difference in activity demonstrated by AnFaeA towards MCA and ACA is not currently forthcoming.

Docking of small hydroxycinnamic esters to AnFaeA reveals an alternative energetically favourable binding orientation

Investigation of the AnFaeA protein structure by Faulds et al. (2005) revealed that the methoxy residue of ferulic acid occupies a hydrophobic hole in the binding cavity of the enzyme (PDB: 1UZA) which is made from the residues PRO161, PRO200, ILE199 and TYR80 as shown in Figure 4A. For sinapinic acid which has two opposing methoxy residues in positions 3- and 5- of the phenolic ring one methoxy residue occupies the hydrophobic hole in the binding cavity while the second extends outwards into the solvent with less interaction with AnFaeA (Figure 4B). These orientations of ferulic and sinapinic acids within the active site of AnFaeA were used in the current work to extrapolate the conformations of the other hydroxycinnamic acids in consideration such as p-coumarate and caffeate.

While AnFaeA is a type A FAE with strong activity towards MFA and MSA, there is also detectable activity toward MpCA but no MCA activity recorded (Faulds et al., 2005). Simulated docking of hydroxycinnamic esters was undertaken with the enzyme to investigate the basis for lack of activity of the closely related structures. The conventional reasoning has been that a polar hydroxyl side group of the phenolic ring of the hydroxycinnamic acid cannot occupy the hydrophobic hole (Faulds et al., 2005). However, with the knowledge that
one methoxy group of sinapate points outwards from the enzyme (Figure 4B), it is also possible for the equivalent group in caffeate, a hydroxyl, to adopt this same orientation with the hydroxyl group pointing outwards which would allow sufficient binding for detectable catalytic activity. In this instance, the phenol side of caffeate facing towards the binding cavity would have no additional side group on the aromatic ring, as is the case for p-coumarate, towards which \textit{AnFaeA} shows detectable catalytic activity.

The full range of solutions with factors such as orientation, mean binding energy (MBE) as well as the number of solutions within each clustered solution, the latter being a test of robustness of the derived docking, were considered in the simulations (Table 3). While the correct orientation in terms of the proximity to the catalytic serine was found for all five methyl and ethyl hydroxycinnamates (similar to that found by McAuley et al., 2004), for MCA and MpCA there were more favourable alternative docking conformations. This conformation occupied nearly the same space as the catalytic binding orientation but the placement of the aromatic ring was on the opposite side of the binding cavity. These reversed solutions for MCA and MpCA are unlikely to be catalytic as the estimated distance between the catalytic serine and the carbonyl carbon of the ester bond is 6.49 Å compared with 2.40 Å between the serine and carbonyl carbon in the catalytic orientation. Furthermore, the binding energy of MCA in the reversed orientation is 0.85 kcal/mol lower than for the catalytic orientation, and almost twice as represented in robustness (Table 3). Similarly, the reversed orientation for MpCA is 0.91 kcal/mol lower and over 3-fold more represented in the clustering of the docking results (Table 3). In fact, binding energies for the \textit{reversed} orientations of MpCA and MCA are similar to the binding energies of MSA and MFA in their \textit{catalytic} orientations. Assuming that the reversed orientations are in fact occurring for MpCA and MCA in the enzyme active site, this could explain the low apparent catalytic rates by \textit{AnFaeA} towards these substrates. That is, MpCA and MCA occupy non-catalytic
orientations at least as often, if not more frequently than catalytic orientations, leading to low apparent affinities for the substrates.

When visualised as docked substrates in the active site of AnFaeA, MpCA and MCA show an interesting phenomenon; the hydrophobic pocket that accommodates the methoxy side group of ferulate and sinapate is occupied by the methyl alcohol of MpCA or MCA. The residues that stabilise the 4'-OH of MCA in the catalytic orientation instead stabilise the carbonyl oxygen and residue TYR100 stabilises the ester bond (Fig 5 A and B). That is, the methyl ester bond is accommodated similarly to that of the methoxy residue in MFA or MSA and the distance between the 4'-OH of the phenol ring and the methoxy oxygen in MFA/MSA is a similar to the distance between the carbonyl oxygen and the ester oxygen in MCA and MpCA (i.e. 2.7 versus 2.4 Å). Thus, a hydroxycinnamate lacking the methoxy substituents may be represented in both orientations where the methyl ester is a proxy for the methoxy group and occupies this same position. This observation serves as an explanation for the undetectable activity of AnFaeA towards MCA: the substrate’s binding is stabilised in the reversed, non-catalytic orientation. MFA is also seen to dock in two orientations (Fig 5 C and D) similarly to MCA. In the catalytic orientation the methoxy side group on the phenol ring occupies the hydrophobic hole while for the reversed orientation the methyl ester group occupies a highly similar space to that observed for MCA in that orientation. The major difference in MFA compared with MCA is that the catalytic orientation is more energetically favoured than the alternative non-catalytic orientation, indicating that the docking seen in Figure 5 C would be preferable to that of D, compared with B being more preferable than A for MCA.

Docking of arabinose esters to AnFaeA is favourable in the catalytic orientation

To further investigate the effect of the residues in the ester linkage on substrate docking and compare these results to the catalytic activity profile measured with AnFaeA, the alkyl esters
were replaced with arabinose in the docking simulations. Arabinose is particularly relevant as enzyme characterization revealed that AnFaeA was significantly more active on the arabinose esters of ferulate and caffeate than methyl esters. Both AFA and ACA dock favourably in the forward, catalytic orientation (Figure 6 A and B) with binding energies of -5.81 and -5.90 kcal/mol respectively (Table 3). Here, the non-catalytic orientations are not favourable as the larger hydrophilic arabinose residues are unable to dock into the hydrophobic hole and in the reversed orientation the sugar group occupies a large proportion of the binding cavity (data not shown). This orientation diverts the docking position of the ester bond away from the catalytic serine and was the likely reason that no solutions were obtained for AFA in the reversed orientation (as well for ASA). A reversed orientation was identified for ACA but was highly unfavourable and less robust as it was stabilised through one hydrogen bond between THR68 and the hydroxyl on the phenolic ring (data not shown).

In this study we replaced a small alkyl ester with a larger, more polar sugar residue and this had a considerable effect on the simulated docking of the esters into the enzyme active site and catalytic activities. These findings support our proposition that the previously determined low activity of AnFaeA toward MCA, and by extension to MpCA, may be due them adopting the more favoured non-catalytic binding orientation in the active site. The docking simulation for MpCA, given in Figure 7 for the catalytic and non-catalytic orientations, show the molecule is reversed in such a way that the methyl ester occupies the hydrophobic hole that would be occupied by the methoxy residue (of substrates such as MFA or MSA) on the phenolic ring. Replacing the methyl group of MpCA with arabinose (ApCA) again shifts the simulation results favouring the catalytic orientation in MBE and robustness (Table 3).

The docking simulations provide a possible mechanism for the difference in AnFaeA activities toward MCA and ACA: by replacing the small alkyl with the arabinose ester, the
reversed orientation is much less favourable, with poorer MBE and clustering (Table 3). The larger arabinose group does not effectively occupy the pocket for the phenolic head preventing its docking and indicates the basis of the reversed, non-catalytic orientation can be heavily attributed to the methyl ester. In particular, the reversed orientation is significantly more poorly represented in the docking simulations when the ester is an arabinose residue, irrespective of which hydroxycinnamic acid is present. The methyl ester of CA binds in an energetically favourable, but non-catalytic manner to AnFaeA and this is supported by the very low measured catalytic activity and lower substrate affinity observed compared with ACA (Table 2). Similarly, an absence of a favourable reversed orientation for AFA in the AnFaeA active site correlates well with its higher affinity and much greater activity compared with MFA.

The commonly cited FAE classification system proposed by Crepin et al (2004) describes 4 types of FAE (A, B, C & D) determined by catalytic activity towards short alkyl chain esters of hydroxycinnamates but correlates poorly with sequence or structural features (Udatha et al., 2011). While previously classified as type A, catalytic characterization based on sugar hydroxycinnamate esters and SMD for AnFaeA indicate that this enzyme does indeed show activity towards caffeate-type esters that have 3’-hydroxy substitution on the phenolic ring and would now be considered type C or D FAE. However, on the basis of the enzyme activities and simulated docking studies presented here, the classification system for FAE could be simplified into two groups: enzymes that can accommodate hydroxycinnamic esters containing methoxy substituents in the active site and those that show preference for hydroxy or no substitutions on the ring, with both groups demonstrating activity against all hydroxycinnamic acids to some extent. In any case, short alkyl esters of hydroxycinnamates should be avoided as substrates for the enzyme family due to the propensity of some forms to
adopt non-catalytic but energetically favourable orientations in the active site and potentially causing misclassification.
Acknowledgements

Dr. Shubhankar Bhattacharyya (Luleå University of Technology, Luleå, Sweden) is acknowledged for his substantial help with the NMR measurements. Prof. Craig Faulds (Aix Marseille Université, Marseille, France) is gratefully acknowledged for providing AnFaeA. The Commonwealth Science and Industrial Research Organisation (CSIRO) Flagship Collaboration Fund with Monash University (fund number: 13–0182) is thanked for financial support of the project. The Monash - Luleå University of Technology collaboration, supported by the Swedish Foundation for International Cooperation in Research and Higher Education project Bioenergy Research-from seed to advanced fuels and chemicals, is acknowledged for the travel scholarship to CJH.

Author Contributions:

CH, IA, VH and PC planned experiments; CH and IA performed experiments and processed data; PC, AT and UR contributed reagents; CH, VH, IA and PC wrote the paper; all authors analysed data and critically reviewed the paper.
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### Table 1. Specific activity of *AnFaeA* towards the methyl and arabinose esters of ferulic and caffeic acid

<table>
<thead>
<tr>
<th>Ester substrates</th>
<th>Specific activity (μmol free acid mg FAE⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulate</td>
<td></td>
</tr>
<tr>
<td>MFA</td>
<td>17.3 ± 1.7</td>
</tr>
<tr>
<td>AFA</td>
<td>1084.0 ± 151.3</td>
</tr>
<tr>
<td>Caffeate</td>
<td></td>
</tr>
<tr>
<td>MCA</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>ACA</td>
<td>2.83 ± 0.42</td>
</tr>
</tbody>
</table>
Table 2. Kinetic parameters of *AnFaeA* determined against the methyl and arabinose esters of ferulic and caffeic acid

<table>
<thead>
<tr>
<th>Ester</th>
<th>$V_{\text{max}}$ (µmoles mg$^{-1}$)</th>
<th>$K_{\text{m}}$ (mM)</th>
<th>$k_{\text{cat}}$ (mg FAE$^{-1}$ min$^{-1}$)</th>
<th>$k_{\text{cat}}/K_{\text{m}}$ (FAE min$^{-1}$ L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MFA</td>
<td>96.4 ± 9.0</td>
<td>1.42 ± 0.26</td>
<td>7.9</td>
<td>5.6</td>
</tr>
<tr>
<td>AFA</td>
<td>1027.9 ± 46.6</td>
<td>0.31 ± 0.06</td>
<td>2825.4</td>
<td>9099.4</td>
</tr>
<tr>
<td>Caffeic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCA</td>
<td>0.55 ± 0.05</td>
<td>0.87 ± 0.17</td>
<td>0.003</td>
<td>0.004</td>
</tr>
<tr>
<td>ACA</td>
<td>2.14 ± 0.16</td>
<td>0.33 ± 0.07</td>
<td>0.009</td>
<td>0.026</td>
</tr>
</tbody>
</table>
Table 3. Mean Binding Energy (MBE) and number of solutions in each solution cluster (N Clusters) for small molecule docking of hydroxycinnamic alkyl and arabinose esters to *AnFacA* (PDB: 1UZA).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Catalytic Orientation</th>
<th>Reversed Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MBE (kcal/mol)</td>
<td>N Clusters</td>
</tr>
<tr>
<td>Alkyl esters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSA#</td>
<td>-6.46</td>
<td>15</td>
</tr>
<tr>
<td>MFA</td>
<td>-6.32</td>
<td>9</td>
</tr>
<tr>
<td>EFA</td>
<td>-6.36</td>
<td>13</td>
</tr>
<tr>
<td>MCA*</td>
<td>-5.40</td>
<td>5</td>
</tr>
<tr>
<td>MpCA *</td>
<td>-5.85</td>
<td>4</td>
</tr>
<tr>
<td>Arabinose esters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA#</td>
<td>-7.15</td>
<td>8</td>
</tr>
<tr>
<td>AFA#</td>
<td>-5.81</td>
<td>5</td>
</tr>
<tr>
<td>ACA</td>
<td>-5.90</td>
<td>6</td>
</tr>
<tr>
<td>ApCA</td>
<td>-5.69</td>
<td>5</td>
</tr>
</tbody>
</table>

The lower the MBE the greater the affinity for substrate binding while having a larger number of solutions in the solution cluster indicates that the orientation is robust.
*substrates have more favourable binding in a reversed orientation into the catalytic site of
AnFaeA.

#MSA (Faulds et al., 2005), ASA and AFA (this study) had no reversed orientations in
simulations and are substrates for which AnFaeA shows highest catalytic activity.
Figure legends

Figure 1. Classification of FAE according to the system developed by Crepin et al. 2003.

Figure 2. Velocity versus substrate concentration curves comparing the hydrolysis rates of arabinose and methyl esters of hydroxycinnamic acids (A) AFA (open circles) and MFA (solid circles) (B) ACA (open circles) and MCA (solid circles). Values are shown as the mean and s.d. for duplicate experiments.

Figure 3. Effect of AnFaeA protein concentration on hydrolysis of arabinose and methyl esters of hydroxycinnamates (A) AFA (B) MFA (C) ACA and (D) MCA.

Figure 4. (A) Binding of MFA into the active site of AnFaeA. The residues responsible for maintaining the hydrophobic hole are indicated as is the catalytic serine based on Faulds et al. (2005) and simulated using AutoDock tools. (B) Binding of MSA into the active site of AnFaeA in the only conformation that is catalytically active via docking simulations. The second methoxy group (indicated by the arrow) is pointed away from the binding site and into the solvent space and having less interaction with AnFaeA

Figure 5. Binding of MCA and MFA into the active site of AnFaeA. (A) represents the catalytic orientation of the MCA (B) shows an alternative non-catalytic orientation for MCA that is more energetically favourable as the methyl group occupies the hydrophobic hole however this more than doubles the distance from the catalytic serine. For reversed orientation the methyl group occupies the hydrophobic pocket that would normally be occupied by the methoxy side group of substrates like MFA. (C) represents the catalytic orientation for MFA while (D) represents an alternative non-catalytic orientation for MFA that is also energetically favourable.
Figure 6. Binding of arabinose hydroxycinnamic esters into the active site of AnFaeA. (A) docking of AFA into the active site (B) docking of ACA in a catalytic orientation; the arabinose molecule reduces the favourability of the reversed, non-catalytic binding.

Figure 7. Binding of MpCA into the active site of AnFaeA. (A) represents the correct catalytic orientation while (B) shows an alternative non-catalytic orientation that is more energetically favourable as the methyl group occupies the hydrophobic hole similar to that observed with MCA.
Fig 1.

<table>
<thead>
<tr>
<th>FAE type</th>
<th>p-Coumaric</th>
<th>Caffeic</th>
<th>Ferulic</th>
<th>Sinapinic</th>
<th>Diferulic</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>no</td>
<td>+++/+++</td>
<td>+++</td>
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</tr>
<tr>
<td>B</td>
<td>+++/+++</td>
<td>+++/+++</td>
<td>+</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>C</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>D</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>
Fig 2.
Fig 3.
Fig 4.
Fig 5.
Biotechnology & Bioengineering

Fig 6.
Fig 7.
Paper II
BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Optimized synthesis of novel prenyl ferulate performed by feruloyl esterases from Myceliophthora thermophila in microemulsions

Io Antonopoulou1 · Laura Leonov2 · Peter Jütten1 · Gabriella Cerullo4 · Vincenza Faraco4 · Adamantia Papadopoulou1 · Dimitris Kletsas5 · Marianna Ralli6 · Ulrika Rova1 · Paul Christakopoulos1

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Abstract Five feruloyl esterases (FAEs; EC 3.1.1.73), FaεA1, FaεA2, FaεB1, and FaεB2 from Myceliophthora thermophila C1 and MtFaε1a from M. thermophila ATCC 42464, were tested for their ability to catalyze the transesterification of vinyl ferulate (VFA) with prenol in detergentless microemulsions. Reaction conditions were optimized investigating parameters such as the medium composition, the substrate concentration, the enzyme load, the pH, the temperature, and agitation. FaεB2 offered the highest transesterification yield (71.5 ± 0.2%) after 24 h of incubation at 30 °C using 60 mM VFA, 1 M prenol, and 0.02 mg FAE/mL in a mixture comprising of 53.4:43.4:3.2 v/v/v n-hexane: t-butanol:100 mM MOPS-NaOH, pH 6.0. At these conditions, the competitive side hydrolysis of VFA was 4.7-fold minimized. The ability of prenyl ferulate (PFA) and its corresponding ferulic acid (FA) to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was significant and similar (IC50 423.39 μM for PFA, 329.9 μM for FA). PFA was not cytotoxic at 0.8–100 μM (IC50 220.23 μM) and reduced intracellular reactive oxygen species (ROS) in human skin fibroblasts at concentrations ranging between 4 and 20 μM as determined with the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay.

Keywords Feruloyl esterase · Transesterification · Myceliophthora thermophila · Prenyl ferulate · Antioxidant · DPPH · DCFH-DA

Introduction

Ferulic acid (FA), along with other hydroxycinnamic acids (p-coumaric, caffeic, sinapic), has a widespread industrial potential due to its strong antioxidant activity. It is ubiquitous in nature as a component of plant cell walls offering linkage with lignin; in arabinoxylans, FA is esterified to the C-5 of α-L-arabinofuranose; in pectins, it is esterified to the C-2 of α1➔5-linked arabinoxyranose or to the C-6 of β1➔4-linked galactopyranosyl, while in xyloglucans, it is found attached to the C-4 of α-D-xylopyranose (Kikugawa et al. 2012). FA is present in grains, fruits, and vegetables (Zhao and Moghadasian 2008). It has a broad spectrum of attractive biological properties including UV absorptive, antibacterial, antiviral, antinflammatory, antithrombosis, and antitumor effects, while during the recent years, it is revealed that it may have beneficial effects against Alzheimer’s disease (Huang et al. 1988; Graf 1992; Kanski et al. 2002; Suzuki et al. 2002; Ou and Kwok 2004; Sultana et al. 2005; Vafiadi et al. 2008a; Barone et al. 2009). It is also suggested to suppress melanin generation by antagonizing tyrosine, which makes it a potential skin-whitening agent (Briganti et al. 2003; Chandel...
Nevertheless, a major disadvantage of FA and other natural antioxidants is their poor solubility in both oil and aqueous media limiting their application in formulations intended for food, cosmetic, cosmeceutical, or pharmaceutical products. A common way to alter solubility is by esterification or transesterification, with the latter requiring a prior activation of FA into an esterified derivative. Modification with sugars or glycerol results to more hydrophilic products, whereas modification with fatty compounds results to more lipophilic products. Additionally to solubility, lipophilization has been shown to enhance the antioxidant activity of alkyl ferulate derivatives (Vafiadi et al. 2008b). Classic methods of esterification involve use of strong acids (concentrated sulfuric acid, hydrogen chloride) or expensive and toxic reagents as catalysts (boron trifluoride, aluminum chloride, trifluoroacetic anhydride, polyphosphate ester, neodymium oxide, dicyclohexylcarbodiimide, graphite bisulfate, etc.), high temperatures (150–250 °C), long reaction times, low yields, and tedious operations (Li et al. 2009). Process limitations include the heat sensitivity and oxidation susceptibility of FA; safety concerns for human health and the environment; and the high-energy consumption for purification, deodorization, and bleaching due to low selectivity (Kiran and Divakar 2001). The requirement for greener processes and the consumers’ preference for natural products demand the development of biotechnological sustainable and competitive processes for the production of interesting compounds with biological activities such as antioxidants. Enzymatic (trans)esterification is an attractive alternative as it offers mild conditions, use of greener solvents, and high selectivity. During the past 15 years, the potential of feruloyl esterases (FAEs; EC 3.1.1.73) as biosynthetic tools has been underlined. FAEs represent a subclass of carboxylic acid esterases that are generally known to catalyze the hydrolysis of the ester bond between hydroxycinnamic acids and sugars as accessory plant cell wall-degrading enzymes. Based on their specificity towards monoferulates and diferulates, for substrates on the phenolic ring and on their amino acid sequence identity, they have been classified into four types (A–D) (Crepin et al. 2004). The reported FAE-based modifications of hydroxycinnamic acids and their esters include their (trans)esterification with primary alcohols, e.g., 1-butanol, glycerol, or sugars in non-conventional media such as microemulsions of organic solvents characterized by low water content, solvent-free systems where the substrates function as reaction medium or single organic solvents. Specifically, lipophilization of FA has been performed by esterification with 1-pentanol using a type A FAE from Aspergillus niger in water-in-oil microemulsions (Giuliani et al. 2001). Transesterification of methyl ferulate (MFA) with 1-butanol has been reported in detergentless microemulsions of n-hexane:1-butanol:buffer using various FAEs such as StFae-A and StFae-C from Sporothrix thermophile ATCC 34628, FoFae-I from Fusarium oxysporum, AnFaeA from A. niger, multienzymatic preparations such as Ultraflo L or Depol 740 L from Humicola insolens, and Depol 670 L from Trichoderma reesei resulting in varying yields (3−97%) (Topakas et al. 2003, 2004, 2005; Vafiadi et al. 2008a, b). The same reaction reached a conversion up to 90% when performed in a single solvent system (1-butanol:buffer) by immobilized Depol 740 L (Thörn et al. 2011). FAEs are generally less stable in non-conventional media and low water content than lipases, whereas they are more selective having higher substrate specificity (Zeuner et al. 2011). (Trans)esterification can be carried out by lipases only if the aromatic ring is not para-hydroxylated and the lateral chain is saturated. Thus, enzymatic (trans)esterification of hydroxycinnamoyl substrates can be obtained efficiently only by FAEs (Vafiadi et al. 2008a).

Myceliotrichia thermophila (previously known as S. thermophile) is a thermophilic filamentous fungus that expresses a powerful consortium of enzymes able to break down lignocellulosic biomass (Karnouei et al. 2014; Kolbusz et al. 2014). Its genome, which was entirely sequenced and annotated in 2011, encodes over 200 secreted carbohydrate-active enzymes (CAZy) and other enzymes of industrial interest (Berka et al. 2011). M. thermophila was developed into a mature protein production platform named C1. The main features of C1 include a low-viscosity morphology and high production levels (up to 100 g/L protein) in fed-batch fermentations providing thus an alternative to traditional fungal protein production hosts for cost-effective industrial applications (Visser et al. 2011). The genome of M. thermophila possesses six genes encoding enzymes belonging to the CE1 family of the CAZy database; four of which are FAEs (Hinz et al. 2009; Karnouei et al. 2014). Three FAEs (FaeA1, FaeA2, and FaeB2) have been over-expressed in M. thermophila C1 and characterized (Kühlner et al. 2012). Sharing the same primary sequence with FaeB2, the type B FAE from M. thermophila ATCC 42464 (MfFaeA1) has been heterologously expressed in Pichia pastoris and characterized (Topakas et al. 2012).

In the present study, we synthesized a novel feruloylated derivative, namely, prenyl ferulate (PFA), by investigating different reaction parameters (medium composition, substrate concentration, enzyme concentration, pH, temperature, time, and agitation) in relation with the rate, the yield, and the product selectivity. Transesterification was performed using vinyl ferulate (VFA) as activated donor and prenol as acceptor, while a competitive side reaction of hydrolysis was observed with presence of water (Fig. 1). Prenol (3-methyl-2-buten-1-ol) is a natural occurring alcohol found in citrus fruits, berries, hops, tomato, grapes, passion fruit, and coffee, where it serves as a building block for terpenoids. In industry, it is used as a fragrance ingredient due to its fruity odor (Kabera et al. 2014). Polypropens are abundant in wood and needles, such as pine.
and birch. They have attractive pharmacological effects that are based on their substitutive effect in the case of dolichol deficits, which are observed with chronic inflammatory, degenerative, and oncological diseases (Khidyrova and Shakhidoyatov 2002). The use of prenol as acceptor offers synthesis of a highly lipophilic derivative utilizing substrates of natural origin. In the same time, the use of novel FAEs derived from the thermophilic fungus *M. thermophila* offers an insight into their potential for efficient transesterification. These FAEs have been proved to be excellent hydrolytic enzymes; however, this is the first time that they are evaluated for their transesterification efficiency.

**Materials and methods**

**Materials**

VFA was prepared in 52% overall yield after three steps starting from FA. The procedure was modified from Mastihubova and Mastihuba (2013), offering >95% purity after column chromatography on silica gel based on 1H NMR and LC-MS. Methyl ferulate (MFA) was purchased from Alfa Aesar (Karlsruhe, Germany), while FA, prenol (99%), n-hexane (<0.02% water), t-butanol (anhydrous, ≥99.5%), MOPS solution 1 M, and other materials were purchased from Sigma-Aldrich (Saint Louis, USA).

**Enzymes**

Feruloyl esterases FaeA1, FaeA2, FaeB1, and FaeB2 from *M. thermophila* C1 were over-expressed individually in low-background C1-expression hosts as reported previously (Kühnel et al. 2012; Visser et al. 2011). The C1-production strains were cultured aerobically in 2-L fermentors in a medium containing glucose, ammonium sulfate, and trace minerals. Protein production was done in a fed-batch system according to Verdoes et al. (2010). After fermentation, the enzyme-containing broth was separated from the biomass by centrifugation (15,000×g for 1 h at 4 °C) and filtration. The crude enzyme supernatant was concentrated eightfold, dialyzed against 10 mM potassium phosphate (pH 6.5), and was further lyophilized. MtFae1a from *M. thermophila* ATCC 42464 was recombinantly expressed in *P. pastoris* strain X33 as reported previously (Topakas et al. 2012). Samples of the culture broth were withdrawn after 3 days of incubation in 250-mL flasks at 20 °C; the biomass was removed by centrifugation (7000×g for 30 min at 4 °C); and the supernatant was 100-fold concentrated and dialyzed against 100 mM MOPS-NaOH, pH 6.0 using a tangential flow filtration system (10-kDa cutoff; PALL, NY, USA). Biochemical properties of the used FAEs are presented in Table 1.

**Enzyme and protein assays**

Protein concentration was determined by the Pierce™ BCA Protein Assay (ThermoFisher Scientific, Waltham, USA). The FAE content (w/w) of the enzymatic preparations was determined by SDS-PAGE using a Novex Sharp pre-stained protein standard (ThermoFisher Scientific, Waltham, USA), followed by quantification using the JustTLC software (Sweday, Lund, Sweden). For the assessment of hydrolytic activity, a stock solution of substrate was prepared in dimethyl sulfoxide (DMSO). The activity was assayed using 1 mM MFA or PFA in 100 mM MOPS-NaOH, pH 6.0 and 0.005 mg FAE/mL enzyme load. Samples were incubated for 10 min at 45 °C without agitation. Reaction was ended by incubating the reaction mixtures at 100 °C for 5–10 min. All reactions were performed in duplicate and concomitant with appropriate blanks. One unit (1 U) is defined as the amount of enzyme (mg) releasing 1 μmol of FA per minute under the defined conditions. No substrate consumption was observed in the absence of esterase.

**Transesterification reactions**

Transesterification reactions were performed in a ternary system of n-hexane:t-butanol:buffer forming detergentless microemulsions. Reactions were prepared by diluting adequate amount of donor in the mixture of n-hexane and t-butanol, followed by the addition of prenol and by vigorous shaking. Reaction was initiated by introducing the enzyme in the form of concentrated stock solution in buffer, followed by vigorous shaking until a stable one-phase solution was obtained. Transesterification was carried out.
in sealed glass vials at microscale (500 μL) in a temperature-controlled water bath. Medium composition, VFA concentration, prenol concentration, enzyme concentration, pH, and temperature were optimized. The effect of medium composition was studied at systems chosen according to previous reports (Topakas et al. 2005; Vafiadi et al. 2006b, 2008b) based on the phase diagram by Khmelnitsky et al. (1988). The effect of pH was studied using the following buffers at 100 mM concentration: sodium acetate (pH 4–6), MOPS-NaOH (pH 6–8), and Tris-HCl (pH 8–10). Optimal conditions obtained from each study were applied in subsequent experiments. Unless otherwise stated, reactions were performed at fixed conditions (50 mM VFA, 200 mM prenol, 0.02 mg FAE/mL FaeA1, FaeA2, 0.002 mg FAE/mL FaeB1, FaeB2, 0.04 mg FAE/mL MtFae1a, 40 °C, 100 mM MOPS-NaOH, pH 6.0, 8 h of incubation). Experiments that included agitation were performed in an Eppendorf thermomixer (Eppendorf, Hamburg, Germany). All reactions were performed in duplicate and concomitant with appropriate blanks. No donor consumption (<1%) was observed in the absence of esterase.

**Quantitative analysis**

Analysis was performed by HPLC on a 100–5 C18 Nucleosil column (250 × 4.6 mm) (Macherey Nagel, Düren, Germany). Reaction mixtures were diluted with acetonitrile before analysis. Elution was done with 7:3 v/v acetonitrile:water for 10 min at a flow rate of 0.6 mL/min and room temperature. Absorbance was measured at 300 nm with a PerkinElmer Flexar UV/Vis detector (Waltham, USA). Retention times for FA, MFA, VFA, and PFA were 4.5, 6.1, 7.4, and 8.7 min, respectively. Calibration curves were prepared using standard solutions of feruloyl compounds in acetonitrile (0.1–2 mM). The sum of molar amounts of the donor and products at the end of reaction was always within a 5% error margin compared to the starting molar amount of the donor. The transesterification yield (or PFA yield) was calculated as the molar amounts of generated PFA compared to the initial amount of donor, expressed as a percentage. The overall yield was calculated as the molar amounts of PFA and FA compared to the initial amount of donor, expressed as percentage. Product selectivity was defined by the PFA/FA ratio (the molar concentration of produced PFA divided by the molar concentration of produced FA).

**Isolation of products and enzyme recovery**

At the optimum conditions and after the reaction had been completed, the reaction mixture was diluted fivefold with buffer (100 mM MOPS-NaOH, pH 6.0), followed by an equal volume of n-hexane. After vigorous handshaking,
two liquid layers were produced. The upper layer (organic, containing the lipophilic compounds) was separated and evaporated under vacuum, whereas the lower phase (aqueous, containing the hydrophilic compounds and the enzyme) was collected, buffer exchanged, and subsequently analyzed for activity. All obtained fractions were analyzed by HPLC.

**Structural characterization of prenyl ferulate**

NMR spectroscopy was performed in DMSO-\(d_6\) with a Bruker Ascend Eon WB 400 spectrometer (Bruker BioSpin AG, Fällanden, Switzerland). \(^{1}H\) NMR (DMSO-\(d_6\), 400 MHz): \(\delta\) 9.59 (s, 1H, ArO\(\cdot\)), 7.53 (d, 1H, \(J=16\) Hz, \(-\text{CHCHCOOR}\)), 7.32 (d, 1H, \(J=4\) Hz, Ar\(\text{H1}\)), 7.11 (dd, 1H, \(J_1=2\) Hz, \(J_2=8.4\) Hz, Ar\(\text{H1}\)), 6.79 (d, 1H, \(J=8.4\) Hz, Ar\(\text{H1}\)), 6.46 (d, 1H, \(J=16\) Hz, \(-\text{CHCHCOOR}\)), 5.39–5.34 (m, 1H, \(-\text{OCH_2CH<}\)), 4.63 (d, 2H, \(J=7.2\) Hz, \(-\text{OCH_2CH<}\)), 3.81 (s, 3H, \(-\text{OCH}_3\)), and 1.72 (dd, 6H, \(J_1=1.2\) Hz, \(J_2=4\) Hz, Ar\(\text{H2}\)), \(\delta\) 7.53 (d, 1H, \(J=5.34\) Hz, 1H, \(-\text{OCH_2CH<}\)), 3.81 (s, 3H, \(-\text{OCH}_3\)), and 1.72 (dd, 6H, \(J_1=1.2\) Hz, \(J_2=14\) Hz, \(-\text{CHCH_2<}\)).

**Antioxidant activity and cytotoxicity**

The antioxidant activity of synthesized PFA as well as of its corresponding FA was monitored by the reduction in the optical density of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Serial dilutions of the test compounds were mixed with an equal volume of 1 mM DPPH in ethanol in U-bottomed 96-well plates and kept in the dark and at ambient temperature until measurement of ethanol in U-bottomed 96-well plates and kept in the dark and at ambient temperature until measurement of the optical density of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Serial dilutions of the test compounds were mixed with an equal volume of 1 mM DPPH in ethanol in U-bottomed 96-well plates and kept in the dark and at ambient temperature until measurement of absorbance at 520 nm at various time points. Human skin fibroblasts (HSFs; strain AG01523) were purchased from Coriell Institute for Medical Research (Camden, NJ, USA). Cells were cultured in monolayers and the possible cytotoxicity of PFA and FA was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Guldbrandsen et al. 2015). The antioxidant activity, expressed as the capacity to reduce the intracellular levels of reactive oxygen species (ROS), was assessed using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay. When HSFs were confluent, the medium was changed to serum-free Dulbecco’s modified Eagle medium (SF DMEM), and 18 h later, it was aspirated and renewed with phenol red and SF DMEM along with 10 \(\mu\)M of DCFH-DA. Following incubation with DCFH-DA for 1 h, serial dilutions of the test compounds were added and the fluorescence was measured in different time intervals for 480-nm excitation and 530-nm emission. The antioxidant activity of the compounds was visualized as reduction of DCF fluorescence and expressed as percent of control. Each experiment was conducted in triplicates.

**Results**

**Effect of medium composition**

Transmethylation was tested in four different compositions of the ternary system \(n\)-hexane/c-butanol:100 mM MOPS-NaOH, pH 6.0 monitoring the competitive hydrolysis of VFA as side reaction. Systems offering highest PFA concentration, rate, and yield were considered as optimal, system IV (19.8:74.5:5.7 v/v/v) for FaeA1 and FaeA2, system III (47.2:50.8:2.0 v/v/v) for FaeB1, and system II (53.4:43.4:3.2 v/v/v) for FaeB2 and MtFae1a (Fig. 2a).

Generally, the highest PFA/FA molar ratio was achieved by FaeB2, while it was <1 for all tested FAEs and systems (0.095 for FaeA1, 0.304 for FaeA2, 0.301 for FaeB1, 0.513 for FaeB2, and 0.297 for MtFae1a at optimum conditions). Product selectivity was highest at lowest water content (system III, 2% water) for all tested FAEs; however, it was shown that such low water content was detrimental to the yield (except in the case of FaeB1). FaeB2 offered highest rate (0.321 mol PFA/g FAE L h), followed by FaeB1 (0.187 mol PFA/g FAE L h).

**Effect of VFA concentration**

Under the optimal medium composition for each enzyme, the effect of VFA concentration on the transesterification was examined. Product selectivity was not affected by the variation in donor concentration (coefficient of variance ~2%), while higher concentrations resulted in higher yield but lower rate (Fig. 2b). The concentration of VFA that resulted in highest PFA/FA ratio (>1) was observed when VFA concentration was used as optimal donor concentration in subsequent experiments for each enzyme (80 mM for FaeA1, 50 mM for FaeA2 and FaeB1, 60 mM for FaeB2, and 100 mM for MtFae1a). Highest rate was 0.346 mol PFA/g FAE L h for FaeB2, followed by 0.183 mol PFA/g FAE L h for FaeB1 at optimal conditions.

**Effect of prenol concentration**

The rate and yield increased remarkably by increasing the acceptor concentration. FaeB2 transesterified at highest rate (0.412 mol/g FAE L h) when 1 M prenol was used, followed by FaeB1 at 0.8 M prenol (0.246 mol/g FAE L h) (Fig. 3a). Optimal prenol concentration was implemented in subsequent experiments for each enzyme (1 M for FaeA1, FaeA2, and FaeB2 and 0.8 M for FaeB1 and 0.6 M for MtFae1a). Increase in prenol concentration remarkably increased transesterification against the side hydrolytic reaction, as more prenol molecules were available near the interface between the organic and water phase of the microemulsion, allowing more frequent transesterification instead of hydrolysis (Fig. 3b). Highest PFA/FA ratio (>1) was observed when...
FaeB2 was used, followed by MtFae1a. Nevertheless, higher product selectivity is generally observed at acceptor concentrations higher than 1.5 M, where rates are generally reduced. For instance, in the case of FaeB2, highest rate and yield were observed at 1 M prenol, but highest PFA/FA ratio was found at 1.5 M, where the overall yield decreased by 23.8%. On the contrary, MtFae1a seemed to have higher tolerance for prenol as the rate was practically constant between 0.5 and 2 M, although optimal performance was observed at only 0.6 M prenol.

**Enzyme kinetics**

The apparent kinetic constants for each substrate were determined by fitting data regarding the effect of VFA and prenol concentration under standard reaction conditions (40 °C, pH 6.0) on the Michaelis-Menten equation using nonlinear regression \( (p < 0.0001) \) (Table 2). FaeB1 had highest affinity towards VFA and prenol (lowest \( K_m \)), while FaeB2 had similar affinity with FaeB1 towards VFA and lower affinity towards prenol comparing to FaeB1 and MtFae1a. However, FaeB2 catalyzed fastest the transesterification (highest \( v_{max} \)) and was most efficient catalyst for both substrates (highest \( K_m/ k_{cat} \)). MtFae1a had lower affinity for VFA and was approximately 300 times less efficient catalyst comparing to FaeB2. Type A FAEs had the lowest affinity towards prenol, showing low efficiency in the synthesis of PFA (Fig. 3).

**Effect of enzyme concentration**

In most industrial applications, cost of enzyme production and operational costs are usually the limiting factors. Improvement on the yield and reduction of reaction time occur when the enzyme concentration increases, leading to a reduction of operation costs; however, the process cost in terms of enzyme production increases significantly, too. With the addition of more enzyme, the rate (mol/g FAE L h) decreased exponentially (data not shown), but the PFA yield reached a peak at different enzyme concentration for each tested FAE (Fig. 4a). FaeB2 was able to synthesize PFA at highest yield (32.5%) and rate (0.122 mol/g FAE L h) at minimal enzyme load of 0.02 mg FAE/mL. FaeA1, FaeB1, and MtFae1a achieved similar PFA yields (~21%) at optimal FAE concentration of 0.1 mg/mL for FaeA1 and FaeB1 and 0.2 mg/ml for FaeB2.
Regarding product selectivity, only FaeB2 offered a ratio >1, while MtFae1a and FaeB1 achieved a value of 0.938 at optimal conditions (Fig. 4b). In low enzyme concentrations, an increase substantially affected the PFA/FA ratio, as more enzyme molecules were available near the interface of the organic and aqueous phase being able to catalyze the transesterification. However, for most FAEs, the yield and product selectivity stabilized or decreased over 0.1–0.2 mg FAE/mL. Optimal conditions were implemented in subsequent experiments.

**Effect of pH**

Although the water content in the reaction is low, the pH of the aqueous solution may influence the ionization state of the residues of the enzymes’ active site. Rate, yield, and product selectivity were observed in transesterification reactions performed at pH range of 4–10. Environment that offered highest PFA concentration, yield, and rate was considered as optimal and proved to be 100 mM MOPS-NaOH buffer at pH 6.0 for FaeB2 and FaeA2, pH 7.0 for FaeB1, and pH 8.0 for FaeA1 and MtFae1a. In these conditions, the PFA/FA ratio was equal to 0.692 for FaeA1, 0.295 for FaeA2, 1.119 for FaeB1, and 2.298 for FaeB2, and 1.670 for MtFae1a. Product selectivity was affected by pH, as highest PFA/FA ratio was observed at pH 4.0 for FaeA2, pH 5.0 for FaeB1, pH 6.0 for FaeB2, and pH 8.0 for FaeA1 and MtFae1a. However, in the cases of FaeA2 and FaeB1, low pH was detrimental to the yield.

**Effect of temperature**

Transesterification was monitored at different temperatures (25–60 °C) with respect to time (Fig. 5). Among all, FaeB2 had the highest PFA yield (71.5%), rate (0.211 mol PFA/g FAE L h), and product selectivity (2.373) at 30 °C after 24 h of incubation, achieving a 100% conversion of VFA to products. FaeA1 and FaeA2 appeared to be more thermophilic with optimal performance at 55 and 45 °C, respectively. Interestingly, although both FaeB2 and MtFae1a performed optimally at 30 °C, FaeB2 appeared to inactivate at 35 °C, while MtFae1a had the same profile as at 30 °C during transesterification. At optimal temperature, FaeA1, FaeB1, and MtFae1a reached a PFA yield of 40–48% out of 93.9, 83.1, and 63.2% overall yield after 24 h, respectively. Lowest yield was demonstrated by FaeA2 15.2% after 48 h out of 82.2% overall yield. A summary on the obtained parameters for each enzyme is presented in Table 3.

**Effect of other donors and agitation**

The effect of different donors and agitation was studied for 96 h at optimal conditions using FaeB2 (Fig. 6). As expected, VFA was a more reactive donor, as the by-product vinyl
alcohol tautomizes to acetaldehyde, shifting the equilibrium towards transesterification. Agitation offered higher selectivity towards synthesis during the first 2 h of incubation probably due to the better contact of the biocatalyst with substrates and better mass and heat transfer. At these conditions, the PFA/FA ratio decreased with respect to time, while it was observed that it is stabilized at ~2.36 after 48 h independently of agitation. The same value is reached when MFA was used as donor.
revealing that the synthetic specificity of FaeB2 is not affected significantly by the substitution on the feruloyl donor. Regarding the yield, VFA was fully converted to products fast (71.5% PFA after 24 h), while the reaction appeared much slower (25.8% PFA after 72 h) when MFA was used. Direct esterification of FA was negligible (1.4 after 96 h).

Isolation of products and recovery of enzyme at optimal conditions

At the end of 24 h, the reaction mixture (500 μL) was separated into two phases. The upper organic phase contained only PFA, as VFA was converted completely (100%) by FaeB2, while the lower aqueous phase contained FA and the enzyme. The organic phase was evaporated, and PFA was recovered successfully (98.5%, 5.542 mg) and was subjected to identification by 1H NMR (chemicals shifts are reported in materials and methods). FA was collected after buffer exchange, while FaeB2 maintained its initial specific activity by 83%. In that context, detergentless microemulsions are proved to be an attractive system with environmentally friendly prospects as the high lipophilicity of PFA combined with the total conversion of VFA allows product separation with low energy costs. Moreover, the recovery and potential reusability of FaeB2 could reduce the cost of enzyme production.

Antioxidant activity and cytotoxicity

The free radical scavenging activity of synthesized PFA as well as of its corresponding FA was investigated with the DPPH cell-free assay. Both compounds have significant scavenging activity, while FA was found slightly more potent. The half maximal inhibitory concentration (IC50), estimated after 3 h of incubation, was 329.9 μM for FA and 423.39 μM for

![Fig. 6 Effects of different donors and agitation on the a yield and b product selectivity. Reactions were performed by FaeB2 at optimal conditions. VFA (black circle), VFA and agitation (white circle) (1000 rpm), MFA (black square), and FA (white square).](image)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>FaeA1</th>
<th>FaeA2</th>
<th>FaeB1</th>
<th>FaeB2</th>
<th>MtFae1a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimized conditions*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water content (%)</td>
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<td>5.5</td>
<td>2.0</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>VFA concentration (mM)</td>
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<td>50</td>
<td>50</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>Prenol concentration (M)</td>
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<td>0.8</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Enzyme concentration (g FAE/L)</td>
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<td>0.1</td>
<td>0.1</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>pH</td>
<td>8</td>
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<td>7</td>
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<td>8</td>
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<td>Temperature (°C)</td>
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<td>40</td>
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<td>30</td>
</tr>
<tr>
<td>Time (h)</td>
<td>24</td>
<td>48</td>
<td>24</td>
<td>24</td>
<td>24</td>
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<tr>
<td>Obtained parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFA concentration (mM)</td>
<td>32.9 (2.4)</td>
<td>7.6 (0.3)</td>
<td>24.1 (0.2)</td>
<td>42.9 (0.1)</td>
<td>42.7 (2.9)</td>
</tr>
<tr>
<td>PFA yield (%)</td>
<td>41.1 (3.0)</td>
<td>15.2 (0.5)</td>
<td>48.1 (0.4)</td>
<td>71.5 (0.2)</td>
<td>42.7 (2.0)</td>
</tr>
<tr>
<td>Overall yield (%)</td>
<td>93.9 (5.4)</td>
<td>82.2 (0.5)</td>
<td>83.1 (3.0)</td>
<td>102.0 (4.7)</td>
<td>63.2 (1.6)</td>
</tr>
<tr>
<td>Rate (mol PFA/g FAE L h)</td>
<td>0.014 (0.001)</td>
<td>0.0016 (0.0001)</td>
<td>0.010 (0.000)</td>
<td>0.089 (0.000)</td>
<td>0.0089 (0.0004)</td>
</tr>
<tr>
<td>Initial rate (mol PFA/g FAE L h)</td>
<td>0.053 (0.003)</td>
<td>0.0057 (0.001)</td>
<td>0.030 (0.002)</td>
<td>0.182 (0.008)</td>
<td>0.022 (0.004)</td>
</tr>
<tr>
<td>PFA/FA ratio</td>
<td>0.778 (0.021)</td>
<td>0.227 (0.031)</td>
<td>1.378 (0.093)</td>
<td>2.373 (0.362)</td>
<td>1.700 (0.041)</td>
</tr>
</tbody>
</table>

Numbers in the parentheses are the estimates of standard errors
* Concentrations are expressed as in total volume of reaction (500 μL)
PFA. Our observations are in accordance with previous studies reporting the scavenging ability of FA and its related esters (Kikuzaki et al. 2002). Following, the study of the antioxidant activity was aimed in a cell system based on HSFs. To this end, cytotoxic activity was determined by the MTT assay. Data indicate that both PFA and FA at concentrations ranging from 0.8 to 100 μM do not affect HSFs’ viability. At higher concentrations, PFA displayed a cytotoxic action (IC50 220.23 μM), while FA was non-cytotoxic even at 500 μM, the highest concentration tested. These results are in agreement with previous reports on the effect of FA on the viability of human skin fibroblasts (Cos et al. 2002) and human embryonic kidney 293 (HEK293) cells (Bian et al. 2015). The DCFH-DA assay was employed at non-cytotoxic concentrations in HSFs. As shown in Fig. 7, FA reduced significantly the intracellular levels of ROS in a concentration-dependent manner, in accordance with previous data from a murine macrophage cell line (Nadal et al. 2016). PFA also provoked a reduction of intracellular ROS at concentrations ranging from 4 to 20 μM, while at 100 μM, no significant antioxidant effect was observed.

Discussion

In this work, we demonstrated the potential of five FAEs derived from M. thermophila to synthesize the novel compound prenyl ferulate. Among them, FaeB2 proved to be the most efficient biocatalyst with attractive properties including high product selectivity (2.373), high yield (71.5% PFA), high overall yield (100%), low enzyme load (0.02 mg FAE/mL), reduced reaction time (24 h), and low operation temperature (30 °C). Results on the effect of solvent composition revealed that type A FAEs (FaeA1 and FaeA2) prefer an environment with higher water content (5.5%; system IV) than type B FAEs. Moreover, it was observed that in every optimization step, the product selectivity (PFA/FA ratio) was quite low for type A FAEs, without meaning that they are not active overall since the side hydrolysis was observed to be very robust for these enzymes. Subsequently, they showed low affinity towards lipophilic prenol comparing to type B FAEs and proved to be less efficient biocatalysts for the synthesis of PFA. Interestingly, each type B FAE demonstrated similar turnover rates (kcat) for both substrates, while each type A FAE had higher turnover rate for prenol comparing to VFA (Table 2). After optimization, the PFA/FA ratio was <1 only for FaeA1 and FaeA2 (0.782 and 0.239, respectively), while they appear to be more thermophilic enzymes with optimal temperature of 55 and 45 °C. On the contrary, type B FAEs are more mesophilic.

The synthetic activity and potential of tested FAEs are not directly correlated with their hydrolytic activity. All FAEs showed specific hydrolytic activities of the same magnitude (Table 1), while the transesterification potential of type B FAEs performed in detergentless microemulsions was generally twofold to threefold higher than the one of type A. This substantial difference could be attributed to structural differences. In most lipases, a mobile hydrophobic lid covers the substrate-binding site, and in this closed structure, the lipase is assumed to be inactive. Upon activation of the lipase by contact with a hydrophobic solvent or at a hydrophobic interface, the lid opens offering accessibility to substrate binding (Rehm et al. 2010). Although very few structures are available for FAEs, it is proved that the catalytic triad of the type A AnFaeA and the active site cavity is confined by a lid and a loop that confers plasticity to the substrate-binding site in analogy with lipases (Hermoso et al. 2004). What is surprising is the fact that FAE’s lid exhibited a high ratio of hydrophilic residues, keeping it in an open conformation that gives the typical preference of catalyzing the hydrolysis of hydrophilic substrates compared to lipases. Accordingly to this, the low product selectivity (PFA/FA ratio) of type A FAEs and the preference for environments with higher water content could be attributed to the presence of a higher percentage of hydrophilic amino acids in their lid comparing to the type B FAEs. Glycosylation can affect the transesterification along with the enzyme properties. Among tested enzymes, FaeB2 and MtFaeA1 share the same primary sequence, while the latter
has undergone glycosylation resulting to an observed MW of 39 kDa, instead of 33 kDa. The difference of the post-translational modification in their respective production hosts results in two biocatalysts with common origin but different performance. Specifically, both FAEs showed preference in the same media composition and water content (3.2%), while MtFaeA1 had 2.6-fold lower affinity towards VFA, is required at a 10-fold higher concentration in the reaction mixture, and operates optimally at alkaline pH. Interestingly, its affinity towards prenol is slightly higher (1.22-fold), and it appears more stable at very high acceptor concentrations (1.5–3 M). Finally, although both enzymes perform optimally at 30 °C, Faeb2 appears to inactivate at 35 °C, while MtFaenA has the same profile as at 30 °C. Overall, glycosylation offered increased stability regarding prenol, pH, and temperature but influenced negatively the process in terms of efficiency and cost, as the transesterification was characterized by lower substrate affinity for VFA, lower biocatalytic efficiency (260–560-fold decrease), limited yields (1.7-fold), and product selectivity (1.4-fold decrease).

In transesterification reactions catalyzed by FAEs, only the use of methyl activated donors is reported (Antonopoulou et al. 2016). In this work, for the first time, a vinyl donor was used in a FAE-catalyzed reaction. The use of vinyl activated donors is a common practice as they are more reactive offering high rates and reduced reaction times. Moreover, under normal conditions, the by-product vinyl alcohol tautomerizes to acetaldehyde and is easier to be removed comparing to methanol when MFA is used as donor. Faeb2 was the only enzyme that fully converted VFA to products after 24 h of incubation, while other enzymes such as FaeA1 reached very high overall yield (93.9%) at given time. As VFA and the transesterification product PFA have high and similar lipophilicity, a total conversion of donor relieves the process from laborious purification steps. On the other hand, it is evident that when MFA is used as donor, only 25.8% are converted to PFA after 72 h, indicating that separation, recovery, and reuse of substrate are essential additionally to increased operational costs (Fig. 6). There are numerous reports on the use of vinyl donors in lipase-catalyzed reactions (Chigorimbo-Murefu et al. 2009; Yang et al. 2010; Yu et al. 2010; Schär and Nyström 2016). Recently, a cutinase from F. oxysporum catalyzed the acylation of tyrosol using various vinyl esters with good yields (up to 60.7%) (Nikolaivits et al. 2016). However, the cost of the synthesis of an activated donor such as VFA is an issue to be addressed.

Using VFA as donor and at optimal conditions, the obtained yield is comparable with other reports on the synthesis of lipophilic feruloyl or other hydroxycinnamic acid derivatives catalyzed by FAEs. Very high yield (95% after 12 h) has been reported regarding the synthesis of diglycerol ferulates catalyzed by a FAE from A. niger (purified from the commercial preparation Pectinase PL “Amano”) (Kikugawa et al. 2012). The esterification of FA with glycerol yielded 81%, while esterification of p-coumaric acid yielded approximately 60% after 72 h using the same enzyme (Tsai-chi yam et al. 2006, 2007). The transesterification of methyl sinapate with 1-butanol using AnFaeA resulted to 78% conversion after 120 h (Vafiadi et al. 2005b). All aforementioned modifications though were employed in systems where the acceptor is used in very high concentrations as solvent component. Although high concentrations of prenol lead to increased product selectivity, a limiting factor in the reaction is the inactivation of Faeb2 and other enzymes at concentrations higher than 1 M. Given this, a 71.5% yield of PFA after optimization is quite promising.

Detergentless microemulsions are a reaction system used in the vast majority of synthetic reactions based on FAEs as they do not affect enzyme activity (Topakas et al. 2003, 2004, 2005; Vafiadi et al. 2005, 2006a, b, 2008a, b; Couto et al. 2010, 2011). They form in ternary systems consisting of a hydrocarbon, a polar alcohol, and water representing thermodynamically stable and optically transparent dispersions of aqueous microdroplets in the hydrocarbon solvent. The droplets are stabilized by alcohol molecules adsorbed at their surface and possess spherical symmetry (Khmelnitsky et al. 1988). In the present reaction, we propose that the enzyme is enclosed and protected in the microdroplet, while the lipophilic donor VFA is present in the organic phase (comprising of r-hexane, t-butanol, and prenol). Microdroplets are stabilized by t-butanol, while the acceptor prenol (logP equal to 0.91) is mostly present in the organic phase as it is less polar than t-butanol (log P equal to 0.584). Given that, the target reaction takes places in the interface of the microdroplet when the enzyme gets in contact with VFA and prenol leading to transesterification. The side reaction of hydrolysis occurs when the enzyme gets in contact with VFA and water molecules. We propose that the transesterification product (PFA) is transferred in the organic phase immediately after its production due to its increased lipophilicity, which subsequently protects it from further hydrolysis. This is the main reason why this reaction keeps moving towards synthesis until equilibrium is reached.

An ideal solvent should offer attractive characteristics to the process such as low toxicity, easy product recovery, aiding substrate solubility, aiding the targeted reaction, and not affecting enzyme activity (Wei et al. 2003). The transesterification of VFA with prenol in detergentless microemulsions offers easy product separation and recovery, recycling of solvents, enzyme recovery, and reuse. Shifting the physicochemical equilibrium of the microemulsion and causing the formation of two separate phases, the lipophilic product PFA is encountered in the upper organic phase, while the hydrophilic FA and enzyme are found in the lower aqueous phase. Enzyme can be recovered and reused by ultrafiltration, while PFA and FA can be recovered by vacuum evaporation from their respective phase. A drawback of the
composition of microemulsions is the lower solubility of VFA at room temperature, while they appear to enable a robust hydrolytic side reaction additionally to the target reaction. Nevertheless, the presence of water is essential for FAEs as they tend to be inactive in pure hydrophobic media contrary to lipases. In enzymatic (trans)esterification, an amount of donor inevitably will get hydrolyzed due to the presence of water constituting product separation and purification essential.

A methodological dilemma in transesterification is whether as optimum parameter should be considered the one that offers higher concentration of desired product (or yield or rate) or the one that offers highest product selectivity. In the present optimization study, conditions that offered highest concentration of PFA were chosen as optimal, as parameter values that offered highest product selectivity (PFA/FA ratio) in most cases were detrimental to the yield. For instance, it was observed that lower water content (2%) offered higher PFA/FA ratio but decreased the rate and yield up to 50% for all FAEs except for FaEB1. Donor concentration did not affect product selectivity, probably because the presence of more VFA molecules close to the interface of the microemulsions has the same chance for transesterification and hydrolysis. Prenol concentration and enzyme load were factors that affected product selectivity the most. When prenol concentration is increased, more molecules are available near the interface of the organic and water phase of the microemulsion, allowing more frequent transesterification instead of hydrolysis. However, very high prenol concentration (>1 M) seemed to cause enzyme inactivation. In the case of enzyme concentration, optimal values for yield and product selectivity coincided. Finally, pH values offering high PFA/FA ratio resulted in lower yields for FaE2 and FaEB1, while temperature, time, and agitation did not affect significantly the selectivity. Although FA production could be further minimized in an optimization study focused on highest selectivity, still some hydrolysis would be observed due to the inevitable presence of water sustaining the need of a purification step. In the same time, higher enzyme loads would be needed increasing the cost. Using FaEB2 at optimal conditions, hydrolysis was reduced 4.7-fold compared to the initial conditions. Moreover, detergentless microemulsions offer easy separation between PFA and FA, allowing the utilization of the latter for its beneficial effects as food additive, dietary supplement, etc.

Overall, this novel reaction can prove attractive for industrial utilization, as it offers an environmentally friendly substitute for existing synthetic reactions. The novel compound prenyl ferulate is a potential alternative to FA, showing high lipophilicity, similar antioxidant activity, and non-toxic effects at low concentrations.

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Compliance with ethical standards
Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Paper III
Optimization of chemoenzymatic synthesis of L-arabinose ferulate catalyzed by feruloyl esterases from *Myceliophthora thermophila* in detergentless microemulsions

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Abstract
The feruloyl esterases FaeA1, FaeA2, FaeB1, FaeB2 from *Myceliophthora thermophila* C1 and MtFae1a from *M. thermophila* ATCC 42464 were used in the transesterification of vinyl ferulate (VFA) with L-arabinose. The effect of parameters such as the microemulsion composition, the substrate concentration, the enzyme load, the pH, the temperature and the agitation was investigated. FaeA1 offered the highest transesterification yield (35.9±2.9%) after 8 h of incubation at 50°C using 80 mM VFA, 55 mM L-arabinose and 0.02 mg FAE/mL in a mixture comprising of 19.8: 74.7: 5.5 v/v/v n-hexane: t-butanol: 100 mM MOPS-NaOH pH 8.0. The ability of L-arabinose ferulate (AFA) to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was significant (IC₅₀ 386.5 μM). AFA was not cytotoxic even at high concentrations (1000 μM). However AFA was found to be pro-oxidant at concentrations higher than 20 μM when the antioxidant activity was determined with the dichloro-dihydrofluorescein diacetate (DCFH-DA) assay in human skin fibroblasts.

Graphical abstract
Highlights
Type A FAEs from *M. thermophila* C1 have higher affinity for L-arabinose
FaeA1 offered highest yield (35.9%) at 8 h after optimization of reaction conditions
FaeB1 offered highest rate (0.602 mol·g⁻¹ FAE ·L⁻¹ ·h⁻¹) at 4 h after optimization of reaction conditions
AFA is not cytotoxic and has significant scavenging activity against the DPPH radical

Keywords
Transesterification, feruloyl esterase, L-arabinose ferulate, antioxidants, *Myceliophthora thermophila*

1. Introduction

Ferulic acid (FA) is the most abundant hydroxycinnamic acid in the plant cell walls with exceptional properties, such as antioxidant, antibacterial, antitumor, anti-inflammatory, skin-whitening and UV-absorptive activities, among others [1]-[6]. In graminaceous monocots such as maize, wheat and barley, FA is esterified to the C-5 of a-L-arabinose substituents of the xylan backbone, while in few dicots, such as sugar beet and spinach, FA is esterified to the C-2 of a-L-arabinose or to the C-6 of β-D-galactose in the neutral side chains of pectin [7]. Diferulates occurring in high-arabinose substitutions of arabinoxylan offer cross-linkages with lignin leading to a dramatic increase of mechanical strength of the cells and a decrease of their digestibility by microorganisms [8], [9].

In order to be able to digest the recalcitrant cell walls, microorganisms are equipped with a consortium of enzymes such as cellulases, hemicellulases, and pectinases. In particular, feruloyl esterases (FAEs, EC 3.1.1.73) are a subclass of carboxylic acid esterases that are generally known to catalyze the hydrolysis of ester bonds between FA (or other hydroxycinnamic acids) and sugars in plant cell walls. Being very diverse enzymes, FAEs have been classified into four types (A-D) depending on their specificity towards monofurulates and diferulates, for substitutions on the phenolic ring and on their amino acid sequence identity [10]. In recent years, they have received increased attention mainly for their ability to deconstruct plant biomass along with xylanases and other lignocellulolytic enzymes for its utilization in biofuel, paper and pulp and animal feed industries or for the release of hydroxycinnamates that can be used as antioxidants, flavor precursors and functional food additives in the food and pharmaceutical industry [11] - [18]. Less attention has been paid to their synthetic ability as they are able to catalyze the esterification or transesterification of hydroxycinnamic acids and their esters with alcohols and sugars under low water content, resulting in products with tailor-made lipophilicity and modified biological properties [19].

Finding the most appropriate biocatalyst for (trans) esterification is a key step. There are numerous reports on the enzymatic acylation of saccharides catalyzed by lipases and proteases [20], [21]. The products have many promising properties such as surfactant, antitumor and plant-growth inhibiting activities; they are amphiphilic, non-toxic and biodegradable [22]. However, lipase-catalyzed esterification of phenolic acids is found to be limited by low yields due to electronic and/or steric effects [23]. The enzymatic acylation of hydroxycinnamic acids with sugars catalyzed by FAEs has been focused on
the use of mono- and di-saccharides in detergentless microemulsions resulting in varying yields (1-60%).

A type C FAE from *Sporotrichum thermophile* (StFaeC) has been used for the transesterification of short chain alkyl ferulates with L-arabinose, D-arabinose and L-arabinobiose reaching a maximum yield of 40\%, 45\% and 24\%, respectively, after 4-5 days when methyl ferulate (MFA) was used as a donor [23]-[25]. StFaeC had a broad specificity on saccharides having either a pyranose or furanose ring, while it synthesized successfully four linear feruloyl arabinino-saccharides, containing from three to six L-arabinose units [26], [27]. The type C FAE from *Talaromyces stipitatus* (TsFaeC) catalyzed the conversion of MFA to L-arabinose ferulate at 21.2\% yield after 4 days [28]. Direct esterification of FA and transesterification of ethyl ferulate (EFA) with monomeric sugars were carried out using FAE-PL, an enzyme purified from the preparation Pectinase PL “Amano” from *Aspergillus niger* [29]. Various multi-enzyme preparations containing FAE activity have catalyzed the direct esterification of FA with mono-, di- and oligosaccharides in microemulsions and ionic liquids with maximum yield in the synthesis of feruloyl D-galactose (61\%) followed by feruloyl D-arabinose (36.7\%) [30], [31].

The potential of FAEs for the synthesis of feruloylated carbohydrates opens the door to design prebiotics and modified biopolymers with altered properties and bioactivities such as delivering phenolics to the colon and thereby reduce the risk of chronic diseases in the distal intestinal region. For instance, the feruloylated oligosaccharides from plant cell walls have shown complementary functional properties, both stimulating the growth of *Bifidobacterium* and protecting against oxidative damage [31]. D-arabinose ferulate was found to be a potential anti-mycobacterial agent with an MIC value against *Mycobacterium bovis* BCG of 25 \( \mu \text{g mL}^{-1} \) [24]. On the other hand, the development of competitive synthetic processes for the production of feruloylated sugars, such as L-arabinose ferulate and D-galactose ferulate, could be a tool for further investigating the natural specificity of FAEs. Until now, the FAE classification is based on synthetic substrates comprising of methyl esters of hydroxycinnamic acids, however in plant cell walls FAEs cleave the ester bond between FA and arabinose or galactose.

The aim of the present work is the evaluation of five feruloyl esterases (FaeA1, FaeA1, FaeB1, FaeB2 and MtFae1a) from *Myceliophthora thermophila* regarding their ability to synthesize L-arabinose ferulate and to optimize various reaction conditions such as water content, substrate and enzyme concentration, pH and temperature. The transesterification reaction was performed using the activated donor, vinyl ferulate (VFA), and L-arabinose as acceptor, while a competitive side hydrolysis was observed (Fig. 1). *M. thermophila* is a thermophilic filamentous fungus which expresses four FAEs. Three FAEs (FaeA1, FaeA2, FaeB2) have been over-expressed in *M. thermophila* C1 and characterized [32], while MtFae1a, sharing the same sequence with FaeB2, has been heterologically expressed in *Pichia pastoris* and characterized [33]. The five enzymes have been previously optimized for the synthesis of a highly lipophilic feruloyl derivative, prenyl ferulate (PFA) in detergentless microemulsions [34].
2. Results and discussion

2.1. Synthetic activity
The FAE activity towards the synthesis of AFA using VFA as donor was determined at fixed conditions (45°C, 10 min of incubation) (Table 1). FaeB1 showed highest transesterification activity followed by FaeA1.

2.2. Effect of medium composition
Transesterification was performed in four different compositions of n-hexane: t-butanol: 100 mM-NaOH pH 6.0 monitoring the competitive hydrolytic reaction. System IV which contained the highest water content and highest concentration in polar t-butanol (19.8: 74.7: 5.5 v/v/v n-hexane: t-butanol: buffer) was considered optimal (Fig. 2). Highest yield (25.9%) and selectivity (0.832) was observed when FaeA1 was used as biocatalyst. Highest rate was observed when FaeB1 was used (0.316 mol g⁻¹ FAE L⁻¹ h⁻¹).

2.3. Effect of substrate concentration
Under the optimal medium composition, the effect of VFA concentration was examined at fixed conditions. By increasing the VFA concentration, the yield was decreased as expected. The optimal donor concentration offering highest rate was applied in subsequent experiments (80 mM for FaeA1, 200 mM for FaeA2, 80 mM for FaeB1, 80 mM for FaeB2 and 150 mM for MtFae1a) (Fig. 3a). Highest yield was 24.5% for FaeA1 at 80 mM while highest rate was observed for FaeB1 at 80 mM (0.372 mol g⁻¹ FAE L⁻¹ h⁻¹). Selectivity was not increased significantly except in the case of FaeA1 (Fig. 3b).

The effect of L-arabinose concentration was examined at optimal medium composition and VFA concentration. The optimal acceptor concentration offering highest rate, yield and selectivity was equal to 55 mM for FaeA1, FaeA2, FaeB1, FaeB2 and 50 mM for MtFae1a (Fig. 3c, Fig. 3d). Highest yield and selectivity was observed for FaeA1 (30.9% and 1.567, respectively) while highest rate for FaeB1 (0.602 mol g⁻¹ FAE L⁻¹ h⁻¹). Limiting factors in increasing the acceptor concentration was the insolubility of arabinose in organic solvents and its low solubility in water (1 M at 20°C). In order to overcome the solvent insolubility, L-arabinose was entrapped in the water phase of the microemulsions and was prepared as a stock concentrated solution in buffer containing the enzyme. At concentrations higher than 55 mM, which equals to approximately 1 M of stock solution at 5.5% water (system IV), sugar precipitation was evident after the addition of stock solution into the organic mixture. The solubility limitation of L-arabinose was reflected in the transesterification yield and rate as at concentration higher than 55 mM, the production of L-arabinose remained constant or was reduced.

The apparent kinetic constants for each substrate were determined by fitting the data on the Michaelis-Menten equation using non-linear regression (p<0.0001) (Table 2). Type B FAEs from M. thermophila C1 (FaeB2 followed by FaeB1) had the highest affinity towards VFA (lowest Kₘ) while type A FAEs (FaeA1 and FaeA2) had approximately a 3-fold lower affinity towards VFA. On the other hand, type A FAEs had the highest affinity towards L-arabinose. This finding comes in agreement with previous reports on substrate specificity profiling showing that type A FAEs have preference on the
hydrolysis of more bulky natural substrates, such as feruloyl saccharides rather than small synthetic ones [35]- [38]. FaeB1 catalyzed fastest the transesterification (highest $v_{\text{max}}$) and was the most efficient catalyst for both substrates (highest $K_m/k_{\text{cat}}$). MtFae1a from M. thermophila ATCC 42464 had a 4.5-fold lower affinity towards VFA and 1.5-fold higher affinity towards L-arabinose than FaeB2, revealing that glycosylation and the production in different hosts can affect the specificity of an enzyme. Nevertheless, MtFae1a was 128 times less efficient biocatalyst than FaeB2.

In comparison with our previous work [34], it is observed that in general FAEs synthesize more efficiently the bulky substituted AFA (higher $K_m/k_{\text{cat}}$, highest $v_{\text{max}}$) than the lipophilic derivative PFA, except for FaeB2. Moreover, the specificity towards L-arabinose is higher for all tested FAEs independently of the solubility limitations of the acceptor. Finally, the specificity towards VFA is lower when AFA is synthesized, except in the case of FaeB2.

### 2.4. Effect of enzyme concentration

Enzyme concentration is usually the limiting factor in bioconversion applications. In the synthesis of AFA, enzyme concentration affected yield and selectivity in different manners (Fig. 4). Generally, the AFA/FA ratio had an optimum at 2-fold lower enzyme concentrations comparing to the yield. FaeA1, FaeA2 and FaeB2 offered highest yield at 0.02 mg FAE mL$^{-1}$, while FaeB1 at 0.005 mg FAE mL$^{-1}$ and MtFae1a at 0.1 mg FAE mL$^{-1}$. On the contrary, the AFA/FA ratio was highest at only 0.005 mg FAE mL$^{-1}$ for FaeA1, FaeB2 and MtFae1a and 0.0002 mg FAE mL$^{-1}$ for FaeA2 and FaeB1 following a linear decrease by the increase in enzyme concentration. The rate decreased exponentially with the addition of more enzyme (data not known). Highest yield was observed for FaeA1 (30.7%) and highest rate for FaeB1 (0.376 mol g$^{-1}$ FAE L$^{-1}$ h$^{-1}$).

### 2.5. Effect of pH

Although the water content of the reaction mixture is low (5.5%), the pH of the aqueous phase in the microemulsion may influence the ionization state of the residues of the enzymes’ active site. Optimal yield and rate was observed at pH 8 for FaeA1 and MtFae1a, pH 6 for FaeA2 and FaeB1 and pH 7 for FaeB2. Optimal selectivity was observed at the same conditions except for FaeB1 that had an optimum at pH 5. Highest yield was 32.8% for FaeA1 while highest rate was 0.398 mol g$^{-1}$ FAE L$^{-1}$ h$^{-1}$ for FaeB1.

### 2.6. Effect of temperature and time

Transesterification was monitored at different temperatures up to 72 h at optimum conditions for each enzyme (Fig. 5). Among all tested FAEs, FaeA1 had the highest AFA yield (35.9%) at 8 h of incubation at 50°C. The yield decreased at 31.2% until 24 h and then remained constant up to 72 h. The same profile was observed for selectivity, where the AFA/FA ratio reached a value of 1.120 at 8 h, decreased at 0.592 until 24 h and remained constant. The overall yield was 68.5% at 8 h and increased to 84.7% at 24 h. Accordingly, FaeB1 offered 16.7% transesterification yield at 8 h of incubation at 45°C while it decreased at 15.2% at 24 h. The overall yield was 70.9% and 82.5% at 8 h and 24 h of incubation, respectively, remaining constant therefrom. The selectivity increased to 1.100 at 6 h and then decreased to 0.301 and further to 0.225 at 8 and 24 h.
of incubation remaining constant thereof. As AFA is an amphiphilic compound, we propose that after its synthesis it is transferred partially in the organic phase of the microemulsions that subsequently protects it from further hydrolysis. However, an amount of AFA remains inside the microdroplets where it is further hydrolyzed in small extent until equilibrium is reached which can explained the decrease in transesterification yield with respect to time. FaeB2 and MtFae1a had similar yield (10%) at 30°C after 24 h of incubation. Lowest yield was observed for FaeA2 (4.5%) after 48 h out of 84.7% overall yield. A summary of the optimal conditions and the obtained parameters is presented in Table 3. Interestingly, at optimal conditions the obtained rate comes in agreement with the determined synthetic assay (Table 1), while highest yield is attributed to FaeA1 since the enzyme might not catalyze the reaction with high rate but has high specificity towards L-arabinose.

2.7. Effect of other donors and agitation
The possibility of substituting VFA with other donors (MFA or FA) and the effect of agitation were investigated at optimal conditions for 24 h using FaeA1. As expected, VFA was a more reactive donor while transesterification of MFA was negligible (3.53% at 8 h) (Fig.6a). Direct esterification of FA was not observed. Agitation (1000 rpm) affected negatively the initial rate of reaction (3-fold decrease) while at 24 h the yield was comparable to samples where no agitation was applied (26.13% and 31.2%, respectively). Without agitation, the selectivity reached an optimum at 8 h and then decreased by 50% up to 24 h, while when agitation was applied selectivity increased until 8 h and remained constant up to 24 h (Fig. 6b).

2.8. Cytotoxicity and antioxidant activity
AFA had significant antioxidant activity with a half maximal inhibitory (IC₅₀) equal to 386.5 μM, assessed after 3 h of incubation (Fig. 7a). In our previous work [34], the IC₅₀ value of FA was estimated to be similar (329.9 μM) while the scavenging yield of AFA was found 70% at steady state at concentrations of 1–4 mM. Interestingly, AFA was found not cytotoxic even at high concentrations (up to 1000 μM) (Fig. 7b) but when the antioxidant activity was assessed with the DCFH-DA assay using human skin fibroblasts, AFA had no anti-oxidant activity at 4 μM, while at concentrations higher than 20 μM it had pro-oxidant activity (Fig. 7c). The antioxidant activity of phenolic esters is mainly assessed with the DPPH assay where the effect of acylation of the carboxyl group of phenolic acid was ascribed mainly to the steric hindrance and the H-donating effect of the acyl groups on the interaction with the DPPH free radical and the subsequent resonance stabilization and the formation of quinone [31]. However, there are still some controversies regarding the effect of the molecular structure of phenolic compounds on their reactivity with reactive oxygen species (ROS) in actual living cells. Generally, phenolic carbohydrates are found to be more effective antioxidants towards low-density lipoprotein oxidation than free phenolic acids while isolated feruloylated arabinomucro-arabinosyl-NDOs exhibited a reduced scavenger activity towards DPPH [39]. According to Couto et al. [30], the scavenging yield of D-arabinose ferulate was 70% at steady state comparing to FA which had 93%, which comes in agreement with our findings. The scavenging activity of feruloylated arabinobiose was equal to the one of FA while the scavenging yield was 83.7% and 92.1% for FA at steady state. The scavenging activity of ferulates towards the DPPH
radical seems to be dependent on the chemical structure of the glycosides. The acylation of FA with hexoses (galactobiose, sucrose, lactose, raffinose, and FOS) results in higher scavenging activity as compared with pentoses (arabinobiose, xylobioses and XOS). These results could be explained by the effect of steric hindrance of the glycosidic substituents on the rotation degree of the phenyl moiety [31].

3. Conclusions

In this work, we evaluated the potential of five FAEs derived from *M. thermophila* to synthesize L-arabinose ferulate in detergentless microemulsions. All tested FAEs preferred a system with higher water content (5.5%) and higher concentration of the polar component (t-butanol). Type A FAEs (FaeA1 and FaeA2) from *M. thermophila* C1 had highest affinity towards L-arabinose but lower affinity towards VFA comparing to the Type B FAEs (FaeB1 and FaeB2). At optimal conditions FaeA1 had the highest yield (35.9%) at 50°C, pH 8.0 after 8 h of incubation using 80 mM VFA and 55 mM L-arabinose. Although the hydrolytic and synthetic activity of the tested FAEs is not correlated, it was observed that FaeB1 is an enzyme with unique ability to catalyze the synthesis of AFA in high rates. The efficient chemoenzymatic synthesis of feruloylated sugars such as AFA may open the pathway to the utilization of natural bioactive compounds in amphiphilic preparations, as delivering agents and for the revisitation of classification of FAEs based on natural substrates.

4. Experimental

4.1. Enzymes and materials

The feruloyl esterases FaeA1, FaeA2, FaeB1 and FaeB2 from *M. thermophila* C1 were over-expressed individually in low-background C1-expression hosts while MtFae1a from *M. thermophila* ATCC 42464 was recombinantly expressed in *P. pastoris* strain X33 as reported previously [32],[33],[40]. VFA was prepared as described previously [34]. MFA was purchased from Alfa-Aesar (Karlsruhe, Germany). L-arabinose (≥99%), FA, n-hexane (<0.02% water), t-butanol (anhydrous, ≥99.5%), MOPS solution 1 M and other materials were purchased from Sigma-Aldrich (Saint Louis, USA).

4.2. Protein and enzyme assays

Protein concentration was determined by the Pierce™ BCA Protein Assay (ThermoFisher Scientific, Waltham, USA). The FAE content (w/w) of the enzymatic preparations was determined by SDS-PAGE and subsequent analysis with the JustTLC software (Sweday, Lund, Sweden). The hydrolytic activity was assayed in 100 mM MOPS-NaOH pH 6.0 at 45°C for 10 min using 1 mM AFA and different enzyme loads (0.005-1 μg FAE/mL). Reaction was ended by incubation at 100°C for 5-10 min. The synthetic activity was assayed in 19.8: 74.7: 5.5 v/v/v n-hexane: t-butanol: 100 mM MOPS-NaOH pH 6.0 at 45°C for 10 min using 5 mM VFA, 55 mM L-arabinose and different enzyme loads (0.005-0.02 mg FAE/mL). Reaction was ended by dilution in acetonitrile. One unit is defined as the amount of enzyme (mg) releasing 1 μmol product per minute under the defined conditions.
4.3. Transesterification reactions

Enzymatic transesterification was carried out at 500 µL in a ternary system of n-hexane: t-butanol: buffer forming detergentless microemulsions. Reaction mixtures were prepared by diluting amount of donor in a mixture of n-hexane and t-butanol followed by vigorous shaking. Reaction was initiated by introducing enzyme and L-arabinose in the form of concentrated stock solution in buffer followed by vigorous shaking until a stable one-phase solution was obtained. Parameters such as the medium composition, the substrate concentration (VFA, L-arabinose), the enzyme concentration, the pH and the temperature were optimized. Optimal conditions obtained from each study, offering the highest AFA concentration in the mixture, were applied in subsequent experiments.

Unless otherwise stated, reactions were carried out at 50 mM VFA, 30 mM L-arabinose, 0.02 mg FAE/mL FaeA1, FaeA2, 0.002 mg FAE/mL FaeB1, FaeB2, 0.04 mg FAE/mL MtFae1a, 40°C, 100 mM MOPS-NaOH pH 6.0, 8 h of incubation and no agitation. The effect of medium composition was studied in four different systems (n-hexane: t-butanol: buffer v/v/v) according to previous reports [26], [24]: system I (37.8: 57.2: 5.0 v/v/v), system II (53.4: 43.4: 3.2 v/v/v), system III (47.2: 50.8: 2.0 v/v/v) and system IV (19.8: 74.7: 5.5 v/v/v). The effect of substrate concentration was studied in a range of 10-300 mM VFA and 10-80 mM L-arabinose while enzyme concentration was varied up to 0.2 mg FAE/mL. The effect of pH was studied using the following buffers (100 mM): sodium acetate (pH 4-6), MOPS-NaOH (pH 6-8) and Tris-HCl (pH 8-10). The effect of temperature was studied at a range of 20-60°C. The effect of different donors in synthesis was studied using MFA and FA. Reactions took place in a water controlled-water bath without agitation. Experiments that included agitation were carried out in an Eppendorf thermomixer (Eppendorf, Hamburg, Germany). All reactions were prepared in duplicate and concomitant with appropriate blanks. No donor consumption (<1%) was observed in the absence of esterase.

4.4. Analysis of feruloyl compounds

Quantitative analysis was made by HPLC on a 100-5 C18 Nucleosil column (250 mm x 4.6 mm) (Macherey Nagel, Düren, Germany) and detection of feruloyl compounds by a PerkinElmer Flexar UV/VIS detector (Waltham, USA) at 300 nm. The reaction mixtures were diluted with acetonitrile before analysis. Elution was done with 70:30 v/v acetonitrile: water for 10 min at flow rate of 0.6 mL/min and room temperature. Retention times for FA, AFA, MFA and VFA were 4.1, 4.4, 6.1 and 7.4 min, respectively. Calibration curves were prepared using standard solutions of feruloyl compounds in acetonitrile (0.1-2 mM). The sum of molar amounts of the donor and products at the end of reaction was within a 5% error margin compared to the starting molar amount of the donor. The transesterification yield (AFA yield) is defined as the molar amount of generated AFA compared to the initial amount of donor, expressed as a percentage. The overall yield was defined as the molar amounts of AFA and FA compared to the initial amount of donor, expressed as percentage. Product selectivity (AFA/FA molar ratio) was defined as the molar concentration of produced AFA divided by the concentration of produced FA.
4.5. Isolation of products
At the end of reaction at optimal conditions, separation of products and substrates was
done by HPLC and UV detection using an acetonitrile (solvent A): water (solvent B)
linear gradient at 0.6 mL min\(^{-1}\) and ambient temperature. Total running time was 43
min during which the following proportions of solvent A were used: 0–35 min 0–
100\%, 35–38 min 100\% and 38–43 min 0\%. Retention times for FA, AFA and VFA
were 16.5, 19.6 and 29.6 min, respectively. Fractions were pooled and evaporated
under vacuum.

In order to estimate the distribution of the feruloyl compounds in the lipophilic and
hydrophilic phase of the microemulsions, the physicochemical separation of the
microemulsion system into two phases was attempted. More specifically, the reaction
mixture was diluted fivefold with 100 mM MOPS-NaOH pH 6.0 followed by an
equal amount of n-hexane. After vigorous shaking, two layers were produced: the
upper layer (hexane rich) and the lower phase (water rich). Samples from each layer
were withdrawn and analyzed by HPLC. 89\% of the unconverted VFA was found in
the organic phase while 100\% of AFA and FA were found in the lower water phase.

4.6. Structural characterization of L-arabinose ferulate
NMR spectroscopy was performed in DMSO-d\(_6\) with a Bruker Ascend Eon WB 400
spectrometer (Bruker BioSpin AG, Fällanden, Switzerland). \(^1\)H NMR (DMSO-d\(_6\), 400
MHz):
\[ \delta 9.60 (s, 1H, Ar\text{-}OH), 7.57 (dd, 1H, J_1= 4Hz, J_2= 16 Hz, -\text{CHCHCOOR}), 7.34–7.32
(m, 1H, Ar\text{-}H), 7.12 (dd, 1H, J_1= 1.6Hz, J_2= 8.4 Hz, Ar\text{-}H), 6.79 (d, 1H, J_1= 8Hz,
Ar\text{-}H), 6.49 (dd, 1H, J_1= 4Hz, J_2= 16 Hz, -\text{CHCHCOOR}), 6.26–6.24 (m, 1H, 
>\text{OCHOH}), 5.32–5.28 (m, 2H, >\text{CHOH}), 4.96 (dd, 1H, J_1= 2.8Hz, J_2= 5.2Hz,
>\text{OCHOH}), 4.30–4.25 (m, 1H, >\text{CH}_2), 4.12–4.08 (m, 1H, >\text{CH}_2), 4.00 (dd, 1H, J_1= 
2.8Hz, J_2= 6.8Hz, >\text{CH}_2\text{CH}), 3.82 (s, 3H, -\text{OCH}_3), 3.74–3.72 (m, 1H, >\text{CHOH}),
3.68–3.64 (m, 1H, >\text{CHOH}).

4.7. Antioxidant activity in a cell-free system
The antioxidant activity of AFA was determined in a cell-free system based on the 2,2-
diphenyl-1-picrylhydrazyl (DPPH) assay. In U-bottomed 96-well plates, serial dilutions
of the compound were mixed with an equal volume of 1 mM DPPH in ethanol.
Plates were kept in dark and at ambient temperature until measurement of absorbance
at 520 nm at various time points. The corresponding dilutions of DMSO served as
negative controls. The capacity of the compounds to scavenge free radicals was
visualized as reduction of DPPH absorbance.

4.8. Cytotoxicity and antioxidant activity in a cell-based system
Cell-based assays were done using human skin fibroblasts strain AG01523 (Coriell
Institute for Medical Research, Camden, NJ, USA). Cells were plated in flat-bottomed,
tissue culture-treated 96-well plates at a density of 5,000 cells/well in Dulbecco’s
modified Eagle medium (DMEM) supplemented with penicillin (100 IU/mL),
streptomycin (100 \(\mu\)g/mL) (Biochrom AG, Berlin, Germany) and 15\% (v/v) FBS
(Gibco BRL, Invitrogen, Paisley, UK). For the determination of cell viability, the
medium was changed to serum-free (SF) DMEM when cultures were confluent and
after 18 h, serial dilutions of AFA were added followed by 72 h incubation. The
corresponding dilutions of dimethyl sulfoxide (DMSO) served as negative controls. Then, the medium was changed to serum-free, phenol red-free DMEM containing 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, USA). After incubation with MTT for 4 h, the medium was discarded, and the MTT-formazan crystals were dissolved in 100 μL isopropanol. Absorbance was measured at 550 nm.

The antioxidant activity, expressed as the capacity for reduction of intracellular levels of reactive oxygen species (ROS) was determined with the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay. When cultures were confluent, the serum-free medium was aspirated and renewed with phenol red and serum-free DMEM along with 10 μM DCFH-DA. Following incubation with DCFH-DA for 1 h, serial dilutions of AFA were added and the fluorescence was measured different time intervals. Fluorescence was measured for 480-nm excitation and 530-nm emission. The antioxidant activity of the compounds was visualized as reduction of DCF fluorescence and expressed as % of control. Each experiment was conducted in triplicate.

Acknowledgements
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References
### TABLES:

**Table 1** Biochemical characteristics of FAEs from *M. thermophila*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ID</th>
<th>Type</th>
<th>Subfamily</th>
<th>Specific synthetic activity (U/mg FAE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FaeA1</td>
<td>JF826027.1a</td>
<td>A</td>
<td>SF5</td>
<td>1.87 (0.28)</td>
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<tr>
<td>FaeA2</td>
<td>JF826028.1a</td>
<td>A</td>
<td>SF5</td>
<td>0.25 (0.01)</td>
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<tr>
<td>FaeB1</td>
<td>Sequence ID 71b</td>
<td>B</td>
<td>SF6</td>
<td>4.80 (0.37)</td>
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<td>FaeB2</td>
<td>JF826029.1a</td>
<td>B</td>
<td>SF6</td>
<td>0.69 (0.06)</td>
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<tr>
<td>MtFae1a</td>
<td>AEO62008.1a</td>
<td>B</td>
<td>SF6</td>
<td>0.67 (0.21)</td>
</tr>
</tbody>
</table>

Numbers in the parentheses are the estimates of standard deviation

* GenBank ID
* Patent ID
Table 2 Apparent kinetic constants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>VFA</th>
<th>L-arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$v_{\text{max}}$ (mol/g FAE L h)</td>
<td>$K_m$ (mM)</td>
</tr>
<tr>
<td>FaeA1</td>
<td>0.285 (0.085)</td>
<td>131.2 (59.6)</td>
</tr>
<tr>
<td>FaeA2</td>
<td>0.047 (0.006)</td>
<td>153.6 (39.3)</td>
</tr>
<tr>
<td>FaeB1</td>
<td>0.600 (0.079)</td>
<td>56.1 (15.4)</td>
</tr>
<tr>
<td>FaeB2</td>
<td>0.312 (0.058)</td>
<td>32.7 (17.6)</td>
</tr>
<tr>
<td>MtFaeA1a</td>
<td>0.041 (0.008)</td>
<td>148.7 (51.8)</td>
</tr>
</tbody>
</table>

Numbers in the parentheses are the estimates of standard deviation
### Table 3  Summary of optimal conditions and obtained parameters

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>FaeA1</th>
<th>FaeA2</th>
<th>FaeB1</th>
<th>FaeB2</th>
<th>MtFae1a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Optimized conditions</strong></td>
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<td></td>
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<tr>
<td>Water content (%v/v)</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>VFA concentration (mM)</td>
<td>80</td>
<td>200</td>
<td>80</td>
<td>80</td>
<td>150</td>
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<tr>
<td>L-arabinose concentration (mM)</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>55</td>
<td>50</td>
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<tr>
<td>Enzyme concentration (g FAE/L)</td>
<td>0.02</td>
<td>0.02</td>
<td>0.005</td>
<td>0.02</td>
<td>0.1</td>
</tr>
<tr>
<td>pH</td>
<td>8</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Temperature (°C)</td>
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<td>45</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Time (h)</td>
<td>8</td>
<td>48</td>
<td>8</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td><strong>Obtained parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFA concentration (mM)</td>
<td>25.2 (3.8)</td>
<td>9.0 (0.2)</td>
<td>13.3 (0.9)</td>
<td>7.8 (0.5)</td>
<td>8.7 (1.8)</td>
</tr>
<tr>
<td>AFA yield (% mM AFA/mM VFA&lt;sub&gt;initial&lt;/sub&gt;)</td>
<td>35.9 (2.9)</td>
<td>4.5 (0.1)</td>
<td>16.7 (1.1)</td>
<td>9.8 (0.7)</td>
<td>10.4 (2.2)</td>
</tr>
<tr>
<td>Overall yield (mM products/mM VFA&lt;sub&gt;initial&lt;/sub&gt;%)</td>
<td>68.5 (1.8)</td>
<td>84.7 (1.6)</td>
<td>70.9 (5.7)</td>
<td>85.1 (8.8)</td>
<td>52.7 (3.1)</td>
</tr>
<tr>
<td>Rate (mol AFA/g FAE L h)</td>
<td>0.180 (0.015)</td>
<td>0.009 (0.0002)</td>
<td>0.333 (0.021)</td>
<td>0.016 (0.001)</td>
<td>0.004 (0.001)</td>
</tr>
<tr>
<td>Initial rate (mol AFA/g FAE L h)</td>
<td>0.417 (0.026)</td>
<td>0.077 (0.008)</td>
<td>0.602 (0.049)</td>
<td>0.080 (0.012)</td>
<td>0.013 (0.001)</td>
</tr>
<tr>
<td>Selectivity (mM AFA/mM FA)</td>
<td>1.120 (0.254)</td>
<td>0.056 (0.0000)</td>
<td>0.308 (0.007)</td>
<td>0.131 (0.005)</td>
<td>0.137 (0.026)</td>
</tr>
</tbody>
</table>

Numbers in the parentheses are the estimates of standard deviation
FIGURES:

Figure 1  a) Scheme of transesterification of VFA (donor) with L-arabinose (acceptor). b) Hydrolysis of VFA (competitive side-reaction) c) Hydrolysis of L-arabinose ferulate (AFA) (competitive-side reaction). Under normal conditions vinyl alcohol tautomerizes to acetaldehyde.

Figure 2 Effect of medium composition on the a) rate and b) selectivity. Reactions were performed in n-hexane: t-butanol: 100 mM MOPS-NaOH pH 6.0 using 50 mM VFA, 30 mM L-arabinose at 40°C for 8 h. Black system I (37.8: 57.2: 5.0 v/v/v), gray system II (53.4: 43.4: 3.2 v/v/v), striped system III (47.2: 50.8: 2.0 v/v/v), and white system IV (19.8: 74.7: 5.5 v/v/v).
Figure 3 Effect of donor concentration on the a) rate and b) selectivity. Reactions were performed in system IV, 30 mM L-arabinose, 40°C, 100 mM MOPS-NaOH pH 6.0 for 8 h. Effect of L-arabinose concentration on the c) rate and d) selectivity. Reactions were performed in system IV at optimal VFA concentration for each enzyme, 40°C, 100 mM MOPS-NaOH pH 6.0 for 8 h. Black circle: FaeA1, White circle: FaeA2, Black square: FaeB1, White square: FaeB2, Black rectangle: MtFae1a

Figure 4 Effect of enzyme concentration on the a) yield and b) selectivity. Reactions were performed at the optimum medium composition and substrate concentration for each enzyme, 40°C, 100 mM MOPS-NaOH pH 6.0 for 8 h. Black circle: FaeA1, White circle: FaeA2, Black square: FaeB1, White square: FaeB2, Black rectangle: MtFae1a
Figure 5 Effect of temperature on the yield. Reactions were carried out at optimal medium composition, substrate concentration, enzyme concentration and pH for each enzyme. a) FaeA1 b) FaeA2 c) FaeB1 d) FaeB2 e) MtFae1a
Figure 6 Effect of donor and agitation on the a) yield and selectivity b). Reactions were performed by FacA1 at optimal conditions. Black circle: VFA, no agitation White circle: VFA, 1000 rpm Black square: FA, no agitation White square: MFA, no agitation
Figure 7  a) Antioxidant activity determined with the cell-free DPPH assay depending on AFA concentration and time  b) Cell viability of human skin fibroblasts assessed with MTT assay following incubation with AFA for 72 h in the presence (FBS) or absence (serum free-SF) of fetal bovine serum  c) Antioxidant activity of AFA determined with the cell-based DCFH-DA assay